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Event

Differentiation

Budding

Specification

Human liver development

Liver bud

Maturation continues
in the neonate

Hepatocyte
and biliary cell
differentiation

Liver bud
growth

Foregut

Liver bud
formation

Liver
diverticulum

Blastocyst
implantation

Endoderm
formation

Oosperm

1   2   3

Birth

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2010-2013

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Contents

Contents

Contents

AIM AND SCOPE   *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

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EDITORIAL

Idiopathic sclerosing encapsulating peritonitis: Abdominal cocoon

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Abstract

Abdominal cocoon, the idiopathic form of sclerosing encapsulating peritonitis, is a rare condition of unknown etiology that results in an intestinal obstruction due to total or partial encapsulation of the small bowel by a fibrocollagenous membrane. Preoperative diagnosis requires a high index of clinical suspicion. The early clinical features are nonspecific, are often not recognized and it is difficult to make a definite pre-operative diagnosis. Clinical suspicion may be generated by the recurrent episodes of small intestinal obstruction combined with relevant imaging findings and lack of other plausible etiologies. The radiological diagnosis of abdominal cocoon may now be confidently made on computed tomography scan. Surgery is important in the management of this disease. Careful dissection and excision of the thick sac with the release of the small intestine leads to complete recovery in the vast majority of cases.

Key words: Peritonitis; Sclerosis; Encapsulate; Intestinal obstruction; Computed tomography scan; Surgery

INTRODUCTION

Sclerosing encapsulating peritonitis (SEP) is a rare condition of unknown etiology. It is characterized by a thick grayish-white fibrotic membrane, partially or totally encasing the small bowel, and can extend to involve other organs like the large intestine, liver and stomach. It was first observed by Owtschinnikow in 1907 and was called peritonitis chronica fibrosa incapsulata[1-5]. SEP can be classified as idiopathic or secondary. The idiopathic form is also known as abdominal cocoon, was first described by Foo *et al* in 1978. Abdominal cocoon is a relatively rare cause of intestinal obstruction[6-21]. Postoperative adhesions account for about 60% of patients with small bowel obstruction. Unusual cases are encountered in only 6% of patients. Abdominal cocoon is one such unusual case of small bowel obstruction[9]. Based on a review of the literature (case series and case reports), we discuss in this paper, etiology, clinical presentation, radiological appearances, diagnosis, treatment, prognosis, and histopathology of abdominal cocoon.

ETIOLOGY

The etiology of this entity has remained relatively unknown. The abdominal cocoon has been classically

described in young adolescent females from the tropical and subtropical countries, but adult case reports from temperate zones can be encountered in literature[1,22-27]. To explain the etiology, a number of hypotheses have been proposed. These include retrograde menstruation with a superimposed viral infection, retrograde peritonitis and cell-mediated immunological tissue damage incited by gynecological infection. However, since this condition has also been seen to affect males, premenopausal females and children, there seems to be little support for these theories[1,24-28]. Further hypotheses are therefore needed to explain the cause of idiopathic SEP. Since abdominal cocoon is often accompanied by other embryologic abnormalities such as greater omentum hypoplasia, and developmental abnormality may be a probable etiology[1]. Greater omentum hypoplasia and mesenteric vessel malformation was demonstrated in some cases. To elucidate the precise etiology of idiopathic SEP, further studies of cases are necessary.

The secondary form of SEP has been reported in association with continuous ambulatory chronic peritoneal dialysis (PD)[29-38]. SEP is a serious complication of PD which leads to decrease ultrafiltration and ultimately intestinal obstruction. For some authors[37], the incidence of SEP was 1.2%, but rose to 15% after 6 years, and 38% after 9 years on PD. The risk of SEP is low early in the course of PD, but increases progressively at 6 years and beyond. For others, the respective cumulative incidences of peritoneal sclerosis at 3, 5 and 8 years were 0.3%, 0.8% and 3.9%. This condition was independently predicted by younger age and the duration of PD, but not the rate of peritonitis[33]. Other rare causes of secondary form of SEP[39-52] include, prior abdominal surgery, subclinical primary viral peritonitis, recurrent peritonitis, beta-blocker treatment (practolol), peritoneovenous shunting, pertioneoventricular shunting and, more rarely, abdominal tuberculosis, sarcoidosis, familial Mediterranean fever, intraperitoneal chemotherapy, cirrhosis, liver transplantation, gastrointestinal malignancy, luteinized ovarian thecomas, endometriosis, protein S deficiency, dermoid cyst rupture, and fibrogenic foreign material.

CLINICAL PRESENTATION

Preoperative diagnosis requires a high index of clinical suspicion. The early clinical features of SEP are nonspecific and are often not recognized[1-12]. Clinically, it presents with recurrent abdominal pain, nausea, vomiting, anorexia, weight loss, malnutrition, recurrent episodes of acute, subacute or chronic small bowel incomplete or complete obstruction, and at times with a palpable soft non tender abdominal mass, but some patients may be asymptomatic. In some cases, abdominal distension was secondary to ascites. A high index of clinical suspicion may be generated by the recurrent attacks of non-strangulating obstruction in the same individual combined with relevant imaging findings and lack of other etiolo-

gies. The preoperative diagnosis of this entity may be helpful for proper treatment of these patients[2-5].

Less than 1% of PD patients develop overt SEP as manifested by combinations of weight loss, ultrafiltration failure, and intestinal obstruction[33-35].

Clinicians must rigorously pursue a preoperative diagnosis, as it may prevent a "surprise" upon laparotomy and unnecessary procedures for the patient, such as bowel resection. Although it is difficult to make a definite preoperative diagnosis, most cases are diagnosed incidentally at laparotomy. A better awareness of this entity and the imaging techniques may facilitate preoperatively diagnosis[1,6-9].

RADIOLOGY APPEARANCES

Conventional radiographs may show dilated bowel loops and air fluid level. Contrast study of the small intestine in SEP shows varying lengths of small bowel tightly enclosed in a "cocoon" of thickened peritoneum, proximal small bowel dilatation, and increased transit time. It may show a fixed cluster of dilated small bowel loops lying in a concertina like fashion, giving a cauliflower-like appearance ("cauliflower sign")[10,53].

Ultrasound findings described in SEP include a trilaminar appearance of the bowel wall, tethering of the bowel to the posterior abdominal wall, dilatation and fixation of small bowel loops, ascites, and membrane formation. Ultrasonography may show a thick-walled mass containing bowel loops, loculated ascites and fibrous adhesions. Sonography shows the small bowel loops encased in a thick membrane made best visible only in the presence of ascites, and may show small bowel loops arranged in concertina shape with a narrow posterior base, having overall appearance of cauliflower[10,53-55].

The radiological diagnosis of SEP may now be confidently made on computed tomography (CT) scan[10,53-60]. CT of the abdomen may help in obtaining an early, reliable, and noninvasive diagnosis of SEP for which optimal management can be planned (Figure 1A and B). CT gives complete picture of the entity and associated complications with exclusion of other causes of intestinal obstruction. The exact diagnosis of this entity is made by computed tomography of the abdomen demonstrating small bowel loops congregated to the center of abdomen encased by a thick membrane[58]. CT features of peritoneal calcification, peritoneal thickening, marked enhancement of the peritoneum, loculated fluid collections, gross ascites with small-bowel intestine loops congregated in a single area in the peritoneal cavity, clustered small-bowel loops encased by a thin membrane-like sac, tethering or matting of the small bowel loops, thickening of the bowel wall, soft tissue density mantle, serosal bowel wall calcification, and calcification over liver capsule, spleen, posterior peritoneal wall may be diagnostic of SEP in the appropriate clinical setting. Tethering or matting of the small bowel is usually posterior to the loculated fluid collection, although bowel is sometimes

Figure 1 Computed tomography scan. A: Axial contrast enhanced computed tomography (CT) scan of the abdomen showing bowel loop mass encased in a membrane; B: Anterio-posterior CT scan of the abdomen showing thin membrane around bowel loops.

seen to be floating within these collections[57]. Fibrosis results in retraction of the root of the mesentery causing the bowel to clump together leading to obstruction and dysfunction. Retraction of the mesentery can lead to a characteristic appearance of the tethered small bowel loops that we have dubbed the "gingerbread man" sign. In some series, diagnosis of abdominal cocoon was made by a combination of abdominal CT and clinical presentations.

DIAGNOSIS

A high index of clinical suspicion may be generated by the recurrent attacks of non-strangulating obstruction in the same individual combined with relevant imaging findings and lack of other etiologies. The preoperative diagnosis of this entity may be helpful for proper treatment of these patients. Most cases are diagnosed incidentally at laparotomy, although a preoperative diagnosis is purported feasible by a combination of barium follow-through (concertina pattern or cauliflower sign and delayed transit of contrast medium), ultrasound, and computed tomography of the abdomen (small bowel loops congregated to the center of the abdomen encased by a soft-tissue density mantle)[3-5,61].

There are many causes of intestinal obstruction but differential diagnosis of this condition is mainly from internal hernias[11], voluminous intussusception[62], simple localized peritoneal adhesions, and chronic idiopathic

intestinal pseudo-obstruction[63]. The main CT features of an internal hernia are: (1) Central location of the small bowel; (2) Evidence of small bowel obstruction; (3) Clustering of the small bowel; (4) Displacement of and mass effect on adjacent organs; and (5) Stretched, displaced, crowded, and engorged mesenteric vessels. No membrane-like sac can be detected in patients with internal hernias as seen in abdominal cocoon[11]. In chronic idiopathic intestinal pseudo-obstruction, CT scan showed distention of small ad large bowels and no membrane-like sac.

TREATMENT AND PROGNOSIS

Management of SEP is debated. Most authors agreed that surgical treatment is required[64-66]. In some cases, the diagnosis is established at a late stage of the disease at laparotomy when the patient develops partial or complete small bowel obstruction. Laparotomy reveals characteristic gross thickening of the peritoneum, which encloses some or all of the small intestine in a cocoon of opaque tissue (Figure 2). The root of the mesentery may also be sclerotic and retracted. Fibrous bands form between the loops of bowel, and when the mass of bowel is sectioned, many small loculated abscesses due to local perforations are found. The entity was categorized into 3 types according to the extent of the encasing membrane: (1) Type Ⅰ - the membrane encapsulated partial intestine; (2) Type Ⅱ - the entire intestine was encapsulated by the membrane; and (3) Type Ⅲ - the entire intestine and other organs (e.g., appendix, cecum, ascending colon, ovary, *etc.*) were encapsulated by the membrane[4]. Various treatment options are adopted, such as subtotal excision of the membrane, enterolysis, small bowel intubation, bowel resection, and exploratory laparotomy with postoperative medical treatment in patients with high perforative risk. When feasible, a stripping of the membrane with intestinal releasing without intestinal resection is the treatment of choice. A simple surgical release of the entrapped bowel *via* removal of the fibrotic membrane is all that is required to free the bowel if no other cause of obstruction, such as a stricture, is found. In order to avoid complications of postoperative intestinal leakage and short-intestine syndrome, resection of the bowel is indicated only if it is nonviable because resection of the bowel is unnecessary and it increases morbidity and mortality. In some patients, repeated adhesiolysis was required. For some authors, laparoscopic approach was possible to diagnosis and management of abdominal cocoon[67-70]. An excellent long-term postoperative prognosis is most of the times guaranteed with a little risk of recurrence in long term follow-up. No surgical treatment is required in asymptomatic SEP. Surgical complications were reported including intra-abdominal infections, enterocutaneous fistula and perforated bowel[66].

Treatment for secondary SEP in dialysis patients is cessation of PD, nutritional support, and surgery for in-

Figure 2 Laparotomy showing an encapsulating thick, white adherent membrane encasing the small bowel.

testinal obstruction, if required. Treatment was variable, but in recent years, steroids and tamoxifen were generally used when SEP was recognized. Preliminary results suggest that steroids and tamoxifen[37] or Angiotensin II inhibitors[71] are beneficial. Transfer to haemodialysis is necessary. Prognosis of SEP is poor, with death usually occurring within a few weeks or months after surgery as it carries a high mortality (20%-80%). This is the result of diagnosis in the latter stages of disease when patients have already developed bowel obstruction. Earlier diagnosis, biocompatible dialysates, and immunosuppressive therapy may improve the outcome for such patients in the future[30-37].

HISTOPATHOLOGY OF SEP

Histopathology is now seldom required as CT imaging appearances along with the clinical features allow a confident diagnosis of SEP. Histologically, the peritoneum shows a proliferation of fibro-connective tissue, inflammatory infiltrates, and dilated lymphatics, with no evidence of foreign body granulomas, giant cells, or birefringent material[72-74]. "Sclerosing" refers to the progressive formation of sheets of dense collagenous tissue; "encapsulating" describes the sheath of new fibrous tissue that covers and constricts the small bowel and restricts its motility; and "peritonitis" implies an ongoing inflammatory process and the presence of a mononuclear inflammatory infiltrate within the new fibrosing tissue[73].

CONCLUSION

Abdominal cocoon, or idiopathic sclerosing encapsulating peritonitis, is a rare condition of unknown cause characterized by total or partial encasement of the small bowel by a fibrocollagenous cocoon-like sac. Although it was first described in tropical and subtropical adolescent girls, it can occur in all age groups, both genders, and in several regions of the world. The preoperative diagnosis of abdominal cocoon is difficult and the diagnosis should always be considered whenever a patient reports episodes of abdominal pain, nausea and vomiting as-

sociated with weight loss. Combination of diagnostic modalities like sonography and CT scan can help in making preoperative diagnosis of this entity and prevent unnecessary bowel resection. This condition should be managed in specialized centers. Surgery is important in the management of this disease. Careful dissection and excision of the thick sac with the release of the small intestine leads to complete recovery.

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TOPIC HIGHLIGHT

Toru Ishikawa, MD, *Series Editor*

Branched-chain amino acids to tyrosine ratio value as a potential prognostic factor for hepatocellular carcinoma

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Abstract

The prognosis of hepatocellular carcinoma (HCC) depends on tumor extension as well as hepatic function. Hepatic functional reserve is recognized as a factor affecting survival in the treatment of HCC; the Child-Pugh classification system is the most extensively used method for assessing hepatic functional reserve in patients with chronic liver disease, using serum albumin level to achieve accurate assessment of the status of protein metabolism. However, insufficient attention has been given to the status of amino acid (AA) metabolism in chronic liver disease and HCC. Fischer's ratio is the molar ratio of branched-chain AAs (BCAAs: leucine, valine, isoleucine) to aromatic AAs (phenylalanine, tyrosine) and is important for assessing liver metabolism, hepatic functional reserve and the severity of liver dysfunction. Although this ratio is difficult to determine in clinical situations, BCAAs/tyrosine molar concentration ratio (BTR) has been proposed as a simpler substitute. BTR correlates with various liver function examinations, including markers of hepatic fibrosis, hepatic blood flow and hepatocyte function, and can thus be considered as reflecting the degree of hepatic impairment. This manuscript examines the literature to clarify whether BTR can serve as a prognostic factor for treatment of HCC.

TREATMENT OF HEPATOCELLULAR CARCINOMA REGARDING RECURRENCE

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide[1]. With advances in imaging diagnostics, together with the understanding of high-risk patients, HCC can now often be detected at an early stage[2]. Furthermore, HCC is associated with severe complications in patients with cirrhosis or chronic hepatitis with severe fibrosis.

In addition to surgical resection as a treatment for HCC, techniques that can be used alone or in combination include transcatheter arterial embolization, transcatheter arterial chemoembolization, percutaneous ethanol injection therapy, percutaneous microwave coagulation therapy, and percutaneous radiofrequency ablation. Thus, local control of HCC can now be achieved in consideration of the location of the tumors, the area occupied and hepatic functional reserve.

Recently, the prognosis of HCC has improved dra-

matically with the identification of high-risk populations and the advancement of diagnostic imaging and treatment. However, recurrence of HCC is frequent in the early post-treatment period even in patients who have undergone radical hepatectomy or radical local treatment including percutaneous treatment, because HCC arises from chronic liver disease. The recurrence rate after treatment of HCC is higher than that of cancer in other organs.

Therefore, despite initial remission of HCC after surgical and interventional treatments, limits are seen on the prolongation of survival. In other words, the therapeutic options available to deal with recurrence determine survival of patients, because risk of recurrence is high even if radical therapy is undertaken.

Treatment tactics may be selected depending on the tumor stage and severity of underlying liver disease.

The reasons for poor survival are that intrahepatic distant recurrence is common and, even more importantly, decompensation occurs due to a decrease in hepatic functional reserve that accompanies progression of chronic liver disease. Therefore, death due to liver failure represents a major problem. In other words, hepatic functional reserve is recognized as a factor affecting survival. However, sufficient research into the effects of the reserve liver function has not been carried out.

BRANCHED-CHAIN AMINO ACIDS TO TYROSINE RATIO AS STATUS OF AMINO ACID METABOLISM

When treating HCC, the Child-Pugh classification system is the most extensively used method worldwide for assessing the hepatic function in patients with chronic liver disease, and represents an important assessment factor. The Child-Pugh classification has been widely used to evaluate hepatic functional reserve in cirrhotic patients, and has a good correlation with prognosis[3], but cannot be used to predict survival in patients with HCC.

In the Child-Pugh classification, the serum albumin level is used to achieve accurate assessment of the status of protein metabolism. However, to date, no attention has been given to the status of amino acid (AA) metabolism in chronic liver disease and HCC.

Amino acid abnormalities are reportedly common even in patients who have liver cirrhosis but no hepatic encephalopathy and in patients with chronic hepatitis[4]. The amino acid molar ratio called Fischer's ratio [branched chain amino acids (BCAAs): leucine, valine, isoleucine/aromatic amino acids (AAAs): phenylalanine, tyrosine] is important for assessing liver metabolism, hepatic functional reserve and the severity of liver dysfunction[5]. Protein malnutrition is a result of amino acid imbalance. Accordingly, to accurately assess the status of protein metabolism in HCC patients with a background of chronic liver disease, determining not only the serum albumin level but also the status of amino acid metabolism is essential.

Proteins contained in biological cells are broken down into amino acids, while at the same time proteins are newly synthesized from free amino acids. Metabolic turnover is achieved when the breakdown and synthetic processes are in balance. The liver is the main organ involved in protein and amino acid metabolism. Hypoalbuminemia and fluctuations in plasma free-amino acid concentrations are usually seen in patients with chronic liver disease. Serum albumin is a protein that is synthesized and secreted by hepatocytes, and is used as an index of hepatic synthetic capacity for protein. This parameter is particularly important for evaluating the severity and prognosis of cirrhosis.

Fluctuations in plasma free-amino acid concentrations are particularly observed in cirrhosis. These changes include marked decreases in BCAAs and increases in AAAs, methionine, and other amino acids. The molar concentration ratio of BCAAs/AAAs (Fischer's ratio) and the BCAAs/tyrosine molar concentration ratio (BTR) decrease with increasing severity of hepatic damage. The Fischer's ratio has long been used for analysis of plasma free-amino acid concentrations, while BTR represents a simplified version of Fischer's ratio[6]. Azuma et al[6] proposed the BTR as a substitute for Fischer's ratio as an index of hepatic damage, and later reported that BTR reflected the progression of chronic liver disease.

Fluctuations in plasma free-amino acid concentrations are also seen in compensatory cirrhosis. For that reason, amino acid metabolic abnormalities in the liver become more severe as the state of chronic liver disease worsens.

On the other hand, assessing hepatic functional reserve from the perspective of amino acid metabolism can prove useful in different ways compared with investigations of the degree of hepatic fibrosis, hepatic blood flow and hepatocyte function. BTR correlates with each of the various liver function examinations, including fibrosis markers, which indicate the degree of hepatic fibrosis; indocyanine green retention rate 15 min (ICG R15), which primarily indicates hepatic blood flow; and asialo-scintigraphy, which reflects hepatocyte function. BTR also reportedly shows significant correlations with albumin value and cholinesterase (Ch-E) levels[7]. As a result, BTR can be thought to reflect the degree of hepatic impairment.

BTR offers a significant indicator of reserve liver function. However, to date, no reports have clarified the potential of BTR as a prognostic factor at the time of treating HCC.

RELATIONSHIP BETWEEN BTR AND TREATMENT OF HCC

The significance of amino acid analysis for assessing hepatic functional reserve has not been elucidated in patients with HCC.

In the case of poor nutritional status, BTR decreases in advance of decreases in serum albumin level. For

that reason, early identification of patients at risk of hypoalbuminemia is possible; specifically, determination of BTR enables prediction of changes in the serum albumin level[8], in turn allowing prediction of the need for administration of BCAAs. Moreover, because of the existence of that time-lag, monitoring of BTR separately from albumin is necessary when considering prognostic factors for HCC. A large-scale clinical study has demonstrated the usefulness of administering oral BCAA preparations to patients showing decreased BTR[9]. In other words, there is a strong possibility that determining BTR provides a prognostic factor for HCC.

In this paper, I have undertaken a review of the published literature with regard to whether BTR can serve as a prognostic factor for HCC.

A small number of experimental and clinical studies have examined BTR in terms of amino acid fluctuations following hepatectomy[10-12]. In experimental models, BTR is correlated with the extent of hepatectomy, with the post-operative interval time and with the liver weight when animals are sacrificed. In clinical studies, BTR has been determined on the immediate post-operative day and every day during the first post-operative week[10-12]. In addition, BTR reportedly decreased following hepatectomy, but then recovered on post-operative day 3 with administration of a BCAA-rich amino acid transfusion[13]. In that report, BTR on day 14 was lower than immediately before the hepatectomy, and the significance of improvements in BTR due to administration of a BCAA-rich amino acid transfusion for several days following the surgery was unclear.

In general, total bilirubin level is used as an indicator of hepatic functional reserve following hepatectomy, but in some cases hepatic functional reserve cannot be fully understood on the basis of total bilirubin level alone. For that reason, determination of arterial blood ketone bodies has been recommended[14]. However, no association exists between arterial blood ketone body values and the actual disease state, and thus this measurement cannot be claimed to offer a superior index compared with total bilirubin level. Moreover, substances such as serum albumin, fibrinogen and cholinesterase, which are synthesized in the liver, show high levels of specificity as indicators of hepatic functional reserve. However, levels are modified by aggressive replacement therapy, and thus are not useful in the clinic. A simple indicator that will permit objective assessment of recovery of liver function following hepatectomy is therefore needed. On the other hand, Fischer's ratio (BCAAs/AAAs), which decreases in various diseases such as cirrhosis that are characterized by a decrease in liver function, has been reported to be useful for understanding the status of liver function[15]. However, Fischer's ratio is difficult to determine, and is not commonly used as a test value following procedures such as hepatectomy. Furthermore, BTR has been developed as a simpler assay method and costs less, and is starting to be used clinically instead of Fischer's ratio in recent times[6,16].

With regard to assessing liver function from the perspective of amino acid metabolism, BTR (BCAAs/tyrosine) has been shown to be useful in different ways compared with such liver function investigations as determining the degree of hepatic fibrosis, hepatic blood flow or hepatocyte function. However, our preliminary experience suggests that BTR can be considered a survival factor in Stage I / II HCC (data not shown). Thus, overall survival in the high BTR group (4.5 or higher) was significantly longer than in the low BTR groups (4.4 or lower) regardless of serum albumin value, respectively. BTR may represent a contributing factor for hepatic functional reserve and survival at the time of HCC treatment. In the future, closer investigations of this issue in a large number of HCC cases will be needed.

In addition, prospective studies will be required to investigate various aspects, including whether maintenance of hepatic functional reserve by administration of BCAA preparations, as indicated by the BTR, is useful in improving the prognosis of HCC.

CONCLUSION

Nutritional management plays an important role in the treatment of HCC, particularly in patients with chronic liver disease. With the objective of improving protein metabolism in patients with cirrhosis, supplemental therapy using oral BCAA preparations is administered to patients with decreased BTR. We can hope that this approach will be found to improve the prognosis of HCC, and that BTR will be thought to be useful as an indicator of such improvement. In the future, it will be necessary to carry out a large-scale prospective study designed to elucidate these points.

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REVIEW

Hemorrhoids: From basic pathophysiology to clinical management

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Abstract

This review discusses the pathophysiology, epidemiology, risk factors, classification, clinical evaluation, and current non-operative and operative treatment of hemorrhoids. Hemorrhoids are defined as the symptomatic enlargement and distal displacement of the normal anal cushions. The most common symptom of hemorrhoids is rectal bleeding associated with bowel movement. The abnormal dilatation and distortion of the vascular channel, together with destructive changes in the supporting connective tissue within the anal cushion, is a paramount finding of hemorrhoids. It appears that the dysregulation of the vascular tone and vascular hyperplasia might play an important role in hemorrhoidal development, and could be a potential target for medical treatment. In most instances, hemorrhoids are treated conservatively, using many methods such as lifestyle modification, fiber supplement, suppository-delivered anti-inflammatory drugs, and administration of venotonic drugs. Non-operative approaches include sclerotherapy and, preferably, rubber band ligation. An operation is indicated when non-operative approaches have failed or complications have occurred.

Several surgical approaches for treating hemorrhoids have been introduced including hemorrhoidectomy and stapled hemorrhoidopexy, but postoperative pain is invariable. Some of the surgical treatments potentially cause appreciable morbidity such as anal stricture and incontinence. The applications and outcomes of each treatment are thoroughly discussed.

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Key words: Hemorrhoids; Pathophysiology; Treatment; Management; Outcome

INTRODUCTION

Hemorrhoids are a very common anorectal condition defined as the symptomatic enlargement and distal displacement of the normal anal cushions. They affect millions of people around the world, and represent a major medical and socioeconomic problem. Multiple factors have been claimed to be the etiologies of hemorrhoidal development, including constipation and prolonged straining. The abnormal dilatation and distortion of the vascular channel, together with destructive changes in the supporting connective tissue within the anal cushion, is a paramount finding of hemorrhoidal disease[1]. An inflammatory reaction[2] and vascular hyperplasia[3,4] may be evident in hemorrhoids. This article firstly reviewed the pathophysiology and other clinical backgrounds of hemorrhoidal disease, followed by the current approaches to

non-operative and operative management.

PATHOPHYSIOLOGY OF HEMORRHOIDAL DISEASE

The exact pathophysiology of hemorrhoidal development is poorly understood. For years the theory of varicose veins, which postulated that hemorrhoids were caused by varicose veins in the anal canal, had been popular but now it is obsolete because hemorrhoids and anorectal varices are proven to be distinct entities. In fact, patients with portal hypertension and varices do not have an increased incidence of hemorrhoids[5].

Today, the theory of sliding anal canal lining is widely accepted[6]. This proposes that hemorrhoids develop when the supporting tissues of the anal cushions disintegrate or deteriorate. Hemorrhoids are therefore the pathological term to describe the abnormal downward displacement of the anal cushions causing venous dilatation. There are typically three major anal cushions, located in the right anterior, right posterior and left lateral aspect of the anal canal, and various numbers of minor cushions lying between them[7] (Figure 1). The anal cushions of patients with hemorrhoids show significant pathological changes. These changes include abnormal venous dilatation, vascular thrombosis, degenerative process in the collagen fibers and fibroelastic tissues, distortion and rupture of the anal subepithelial muscle (Figure 2). In addition to the above findings, a severe inflammatory reaction involving the vascular wall and surrounding connective tissue has been demonstrated in hemorrhoidal specimens, with associated mucosal ulceration, ischemia and thrombosis[2].

Several enzymes or mediators involving the degradation of supporting tissues in the anal cushions have been studied. Among these, matrix metalloproteinase (MMP), a zinc-dependent proteinase, is one of the most potent enzymes, being capable of degrading extracellular proteins such as elastin, fibronectin, and collagen. MMP-9 was found to be over-expressed in hemorrhoids, in association with the breakdown of elastic fibers[8]. Activation of MMP-2 and MMP-9 by thrombin, plasmin or other proteinases resulted in the disruption of the capillary bed and promotion of angioproliferative activity of transforming growth factor β (TGF-β)[9].

Recently, increased microvascular density was found in hemorrhoidal tissue, suggesting that neovascularization might be another important phenomenon of hemorrhoidal disease. In 2004, Chung et al[4] reported that endoglin (CD105), which is one of the binding sites of TGF-β and is a proliferative marker for neovascularization, was expressed in more than half of hemorrhoidal tissue specimens compared to none taken from the normal anorectal mucosa. This marker was prominently found in venules larger than 100 μm. Moreover, these workers found that microvascular density increased in hemorrhoidal tissue especially when thrombosis and stromal vascular endothelial growth factors (VEGF)

Figure 1 Diagram of common sites of major anal and internal hemorrhoids. A: Diagram of common sites of major anal cushions; B: Common sites of internal hemorrhoids.

Figure 2 Pathological changes in hemorrhoids. *: Marked dilatation of hemorrhoidal venous plexus; #: Fragmented anal subepithelial muscle (the Treitz's muscle or mucosal suspensory ligament) (Scale bar = 1 mm).

were present. Han et al[8] also demonstrated that there was a higher expression of angiogenesis-related protein such as VEGF in hemorrhoids.

Regarding the study of morphology and hemodynamics of the anal cushions and hemorrhoids, Aigner et al[3,10] found that the terminal branches of the superior rectal artery supplying the anal cushion in patients with hemorrhoids had a significantly larger diameter, greater blood flow, higher peak velocity and acceleration velocity, compared to those of healthy volunteers. Moreover, an increase in arterial caliber and flow was well correlated with the grades of hemorrhoids. These abnormal findings still remained after surgical removal of the hemorrhoids, confirming the association between hypervascularization and the development of hemorrhoids.

Using an immunohistochemical approach, Aigner et al[3] also identified a sphincter-like structure, formed by a thickened tunica media containing 5-15 layers of smooth muscle cells, between the vascular plexus within the subepithelial space of the anal transitional zone in normal anorectal specimens. Unlike the normal specimens, hemorrhoids contained remarkably dilated, thin-walled vessels within the submucosal arteriovenous plexus, with absent or nearly-flat sphincter-like constriction on the vessels. These investigators concluded that a smooth muscle sphincter in the arteriovenous plexus helps in reducing the arterial inflow, thus facilitating an effective venous drainage. Aigner et al[3] then proposed that, if this mechanism is impaired, hyperperfusion of the arteriovenous plexus will lead to the formation of hemorrhoids.

Based on the histological findings of abnormal

venous dilatation and distortion in hemorrhoids, dys-regulation of the vascular tone might play a role in hemorrhoidal development. Basically, vascular smooth muscle is regulated by the autonomic nervous system, hormones, cytokines and overlying endothelium. Imbalance between endothelium-derived relaxing factors (such as nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factor) and endothelium-derived vasoconstricting factors (such as reactive oxygen radicals and endothelin) causes several vascular disorders[11]. In hemorrhoids, nitric oxide synthase, an enzyme which synthesizes nitric oxide from L-arginine, was reported to increase significantly[8].

Several physiological changes in the anal canal of patients with hemorrhoids have been observed. Sun et al[12] revealed that resting anal pressure in patients with non-prolapsing or prolapsing hemorrhoids was much higher than in normal subjects, whereas there was no significant change in the internal sphincter thickness. Ho et al[13] performed anorectal physiological studies in 24 patients with prolapsed hemorrhoids and compared with results in 13 sex- and age-matched normal subjects. Before operation, those with hemorrhoids had significantly higher resting anal pressures, lower rectal compliance, and more perineal descent. The abnormalities found reverted to the normal range within 3 mo after hemorrhoidectomy, suggesting that these physiological changes are more likely to be an effect, rather than the cause, of hemorrhoidal disease.

EPIDEMIOLOGY AND RISK FACTORS OF HEMORRHOIDS

Although hemorrhoids are recognized as a very common cause of rectal bleeding and anal discomfort, the true epidemiology of this disease is unknown because patients have a tendency to use self-medication rather than to seek proper medical attention. An epidemiologic study by Johanson et al[14] in 1990 showed that 10 million people in the United States complained of hemorrhoids, corresponding to a prevalence rate of 4.4%. In both sexes, peak prevalence occurred between age 45-65 years and the development of hemorrhoids before the age of 20 years was unusual. Whites and higher socioeconomic status individuals were affected more frequently than blacks and those of lower socioeconomic status. However, this association may reflect differences in health-seeking behavior rather than true prevalence. In the United Kingdom, hemorrhoids were reported to affect 13%-36% of the general population[1,15]. However, this estimation may be higher than actual prevalence because the community-based studies mainly relied on self-reporting and patients may attribute any anorectal symptoms to hemorrhoids.

Constipation and prolonged straining are widely believed to cause hemorrhoids because hard stool and increased intraabdominal pressure could cause obstruction of venous return, resulting in engorgement of the hemorrhoidal plexus[1]. Defecation of hard fecal material increases shearing force on the anal cushions. However, recent evidence questions the importance of constipation in the development of this common disorder[14,16,17]. Many investigators have failed to demonstrate any significant association between hemorrhoids and constipation, whereas some reports suggested that diarrhea is a risk factor for the development of hemorrhoids[16]. Increase in straining for defecation may precipitate the development of symptoms such as bleeding and prolapse in patients with a history of hemorrhoidal disease. Pregnancy can predispose to congestion of the anal cushion and symptomatic hemorrhoids, which will resolve spontaneously soon after birth. Many dietary factors including low fiber diet, spicy foods and alcohol intake have been implicated, but reported data are inconsistent[1].

CLASSIFICATION AND GRADING OF HEMORRHOIDS

A hemorrhoid classification system is useful not only to help in choosing between treatments, but also to allow the comparison of therapeutic outcomes among them. Hemorrhoids are generally classified on the basis of their location and degree of prolapse. Internal hemorrhoids originate from the inferior hemorrhoidal venous plexus above the dentate line and are covered by mucosa, while external hemorrhoids are dilated venules of this plexus located below the dentate line and are covered with squamous epithelium. Mixed (interno-external) hemorrhoids arise both above and below the dentate line. For practical purposes, internal hemorrhoids are further graded based on their appearance and degree of prolapse, known as Goligher's classification: (1) First-degree hemorrhoids (grade I): The anal cushions bleed but do not prolapse; (2) Second-degree hemorrhoids (grade II): The anal cushions prolapse through the anus on straining but reduce spontaneously; (3) Third-degree hemorrhoids (grade III): The anal cushions prolapse through the anus on straining or exertion and require manual replacement into the anal canal; and (4) Fourth-degree hemorrhoids (grade IV): The prolapse stays out at all times and is irreducible. Acutely thrombosed, incarcerated internal hemorrhoids and incarcerated, thrombosed hemorrhoids involving circumferential rectal mucosal prolapse are also fourth-degree hemorrhoids[18].

Some authors proposed classifications based on anatomical findings of hemorrhoidal position, described as primary (at the typical three sites of the anal cushions), secondary (between the anal cushions), or circumferential, and based on symptoms described as prolapsing and non-prolapsing[19]. However, these classifications are in less widespread use.

CLINICAL EVALUATION OF HEMORRHOIDS

The most common manifestation of hemorrhoids is painless rectal bleeding associated with bowel move-

Table 1 Current management of internal hemorrhoids by grade

Treatments	Grade I	Grade II	Grade III	Grade IV	Acute thrombosis or strangulation
Dietary and lifestyle modification	×	×	×	×	×
Medical treatment	×	×	×-selected		
Non-operative treatment					
Sclerotherapy	×	×			
Infrared coagulation	×	×			
Radiofrequency ablation	×	×			
Rubber band ligation	×	×	×-selected		
Operative treatment					
Plication		×	×		
DGHAL		×	×		
Hemorrhoidectomy		×-selected	×	×	×-emergency
Stapled hemorrhoidopexy			×	×	

DGHAL: Doppler-guided hemorrhoidal artery ligation; ×: Applicable.

ment, described by patients as blood drips into toilet bowl. The blood is typically bright red as hemorrhoidal tissue has direct arteriovenous communication[3]. Positive fecal occult blood or anemia should not be attributed to hemorrhoids until the colon is adequately evaluated especially when the bleeding is atypical for hemorrhoids, when no source of bleeding is evident on anorectal examination, or when the patient has significant risk factors for colorectal neoplasia[18].

Prolapsing hemorrhoids may cause perineal irritation or anal itching due to mucous secretion or fecal soiling. A feeling of incomplete evacuation or rectal fullness is also reported in patients with large hemorrhoids. Pain is not usually caused by the hemorrhoids themselves unless thrombosis has occurred, particularly in an external hemorrhoid or if a fourth-degree internal hemorrhoid becomes strangulated. Anal fissure and perianal abscess are more common causes of anal pain in hemorrhoidal patients.

The definite diagnosis of hemorrhoidal disease is based on a precise patient history and careful clinical examination. Assessment should include a digital examination and anoscopy in the left lateral position. The perianal area should be inspected for anal skin tags, external hemorrhoid, perianal dermatitis from anal discharge or fecal soiling, fistula-in-ano and anal fissure. Some physicians prefer patients sitting and straining in the squatting position to watch for the prolapse. Although internal hemorrhoids cannot be palpated, digital examination will detect abnormal anorectal mass, anal stenosis and scar, evaluate anal sphincter tone, and determine the status of prostatic hypertrophy which may be the reason for straining as this aggravates descent of the anal cushions during micturition. Hemorrhoidal size, location, severity of inflammation and bleeding should be noted during anoscopy. Intrarectal retroflexion of the colonoscope or transparent anoscope with flexible endoscope also allow excellent visualization of the anal canal and hemorrhoid, and permit recording pictures[20].

MANAGEMENT OF HEMORRHOIDAL DISEASE

Therapeutic treatment of hemorrhoids ranges from die-tary and lifestyle modification to radical surgery, depending on degree and severity of symptoms[21,22]. The current management of internal hemorrhoids is illustrated in Table 1. In addition, selected meta-analyses showing various treatment options of hemorrhoidal disease are shown in Table 2[23-32].

Dietary and lifestyle modification
Since shearing action of passing hard stool on the anal mucosa may cause damage to the anal cushions and lead to symptomatic hemorrhoids, increasing intake of fiber or providing added bulk in the diet might help eliminate straining during defecation. In clinical studies of hemorrhoids, fiber supplement reduced the risk of persisting symptoms and bleeding by approximately 50%, but did not improve the symptoms of prolapse, pain, and itching[26]. Fiber supplement is therefore regarded as an effective treatment in non-prolapsing hemorrhoids; however, it could take up to 6 wk for a significant improvement to be manifest[33]. As fiber supplements are safe and cheap, they remain an integral part of both initial treatment and of a regimen following other therapeutic modalities of hemorrhoids.

Lifestyle modification should also be advised to any patients with any degree of hemorrhoids as a part of treatment and as a preventive measure. These changes include increasing the intake of dietary fiber and oral fluids, reducing consumption of fat, having regular exercise, improving anal hygiene, abstaining from both straining and reading on the toilet, and avoiding medication that causes constipation or diarrhea.

Medical treatment
Oral flavonoids: These venotonic agents were first described in the treatment of chronic venous insufficiency and edema. They appeared to be capable of increasing vascular tone, reducing venous capacity, decreasing capillary permeability[34], and facilitating lymphatic drainage[35] as well as having anti-inflammatory effects[36]. Although their precise mechanism of action remains unclear, they are used as an oral medication for hemorrhoidal treatment, particularly in Europe and Asia. Micronized purified flavonoid fraction (MPFF), consisting of 90% dios-

Table 2 Selected meta-analyses showing various treatment options for hemorrhoidal disease (in order of publication year)

Authors	Characteristics of comparative studies	Number of trials (total cases)	Results
Johanson et al[23]	IC, IS and RBL	5 (863)	RBL had greater long-term efficacy, but led to a higher incidence of post-treatment pain. IC was associated with both fewer and less severe complications
MacRae et al[24]	IC, IS, RBL, manual anal dilation and hemorrhoidectomy	18 (1952)[1]	Hemorrhoidectomy was more effective than manual anal dilation and RBL, but more pain and complications. RBL had greater efficacy than IS for treating grade Ⅰ-Ⅲ hemorrhoids, with no difference in the complication rate. Patients treated with IC or IS were more likely to require further therapy
Shanmugam et al[25]	RBL vs hemorrhoidectomy	3 (202)	Hemorrhoidectomy was superior to RBL for the long-term treatment of grade Ⅲ, not grade Ⅱ, hemorrhoids. Although hemorrhoidectomy had more pain, higher complications and more time off work, patient satisfaction and acceptance of the two treatment modalities seems to be similar
Alonso-Coello et al[26]	Fiber vs no therapy	7 (378)	Fiber reduced the risk of bleeding and persisting by 50% and 47%, respectively, but it had no significant effect on pain and prolapse
Alonso-Coello et al[27]	Oral flavonoids vs placebo or no therapy	14 (1514)	Flavonoids reduced the risk of bleeding, pain, persisting symptoms and recurrence by 67%, 65%, 58% and 47%, respectively
Ho et al[28]	Closed vs open hemorrhoidectomy	6 (686)	Closed hemorrhoidectomy had faster wound healing but longer operating time. There was no difference in treatment efficacy, pain, complication and hospital stay between the two operations
Nienhuijs et al[29]	Conventional vs ligasure hemorrhoidectomy	12 (1142)	Ligasure hemorrhoidectomy resulted in significantly shorter operative time, less early postoperative pain, earlier recovery, without any difference in recurrent bleeding or incontinence
Burch et al[30]	Hemorrhoidectomy vs SH	27 (2279)	SH had less postoperative pain, shorter operative time, shorter hospital stay, and shorter convalescence, but a higher rate of prolapse and reintervention for prolapse
Giordano et al[31]	Hemorrhoidectomy vs SH (minimum follow-up of 1 yr)	15 (1201)	SH had a significantly higher incidence of recurrences and additional operations
Gan et al[32]	Various TCMH vs another TCMH or Western medicines	9 (1822)	TCMHs significantly improved overall symptoms and bleeding as well as decreased the inflammation of perianal mucosa

[1]With available detailed data on the patients enrolled. IC: Infrared coagulation; IS: Injection sclerotherapy; RBL: Rubber band ligation; SH: Stapled hemorrhoidopexy; TCMH: Traditional Chinese medicinal herbs.

min and 10% hesperidin, is the most common flavonoid used in clinical treatment[27]. The micronization of the drug to particles of less than 2 μm not only improved its solubility and absorption, but also shortened the onset of action. A recent meta-analysis of flavonoids for hemorrhoidal treatment, including 14 randomized trials and 1514 patients, suggested that flavonoids decreased risk of bleeding by 67%, persistent pain by 65% and itching by 35%, and also reduced the recurrence rate by 47%[27]. Some investigators reported that MPFF can reduce rectal discomfort, pain and secondary hemorrhage following hemorrhoidectomy[37].

Oral calcium dobesilate: This is another venotonic drug commonly used in diabetic retinopathy and chronic venous insufficiency as well as in the treatment of acute symptoms of hemorrhoids[38]. It was demonstrated that calcium dobesilate decreased capillary permeability, inhibited platelet aggregation and improved blood viscosity; thus resulting in reduction of tissue edema[39]. A clinical trial of hemorrhoid treatment showed that calcium dobesilate, in conjunction with fiber supplement, provided an effective symptomatic relief from acute bleeding, and it was associated with a significant improvement in the inflammation of hemorrhoids[40].

Topical treatment: The primary objective of most topical treatment aims to control the symptoms rather than to cure the disease. Thus, other therapeutic treatments could be subsequently required. A number of topical preparations are available including creams and suppositories, and most of them can be bought without a prescription. Strong evidence supporting the true efficacy of these drugs is lacking. These topical medications can contain various ingredients such as local anesthesia, corticosteroids, antibiotics and anti-inflammatory drugs[41].

Topical treatment may be effective in selected groups of hemorrhoidal patients. For instance, Tjandra et al[42] showed a good result with topical glyceryl trinitrate 0.2% ointment for relieving hemorrhoidal symptoms in patients with low-grade hemorrhoids and high resting anal canal pressures. However, 43% of the patients experienced headache during the treatment. Perrotti et al[43] reported the good efficacy of local application of nifedipine ointment in treatment of acute thrombosed external hemorrhoids. It is worth noting that the effect of topical application of nitrite and calcium channel blocker on the symptomatic relief of hemorrhoids may be a consequence of their relaxation effect on the internal anal sphincter, rather than on the hemorrhoid tissue *per se* where one might anticipate a predominantly vasodilator effect.

Apart from topical medication influencing tone of the internal anal sphincter, some topical treatment targets vasoconstriction of the vascular channels within hemorrhoids such as Preparation-H® (Pfizer, United States), which contains 0.25% phenylephrine, petrola-

tum, light mineral oil, and shark liver oil. Phenylephrine is a vasoconstrictor having preferential vasopressor effect on the arterial site of circulation, whereas the other ingredients are considered protectants. Preparation-H is available in many forms, including ointment, cream, gel, suppositories, and medicated and portable wipes[44]. It provides temporary relief of acute symptoms of hemorrhoids, such as bleeding and pain on defecation.

Non-operative treatment

Sclerotherapy: This is currently recommended as a treatment option for first- and second-degree hemorrhoids. The rationale of injecting chemical agents is to create a fixation of mucosa to the underlying muscle by fibrosis. The solutions used are 5% phenol in oil, vegetable oil, quinine, and urea hydrochloride or hypertonic salt solution[22]. It is important that the injection be made into submucosa at the base of the hemorrhoidal tissue and not into the hemorrhoids themselves; otherwise, it can cause immediate transient precordial and upper abdominal pain[45]. Misplacement of the injection may also result in mucosal ulceration or necrosis, and rare septic complications such as prostatic abscess and retroperitoneal sepsis[46]. Antibiotic prophylaxis is indicated for patients with predisposing valvular heart disease or immunodeficiency because of the possibility of bacteremia after sclerotherapy[47].

Rubber band ligation: Rubber band ligation (RBL) is a simple, quick, and effective means of treating first- and second-degree hemorrhoids and selected patients with third-degree hemorrhoids. Ligation of the hemorrhoidal tissue with a rubber band causes ischemic necrosis and scarring, leading to fixation of the connective tissue to the rectal wall. Placement of rubber band too close to the dentate line may cause severe pain due to the presence of somatic nerve afferents and requires immediate removal. RBL is safely performed in one or more than one place in a single session[48] with one of several commercially available instruments, including hemorrhoid ligator rectoscope[49] and endoscopic ligator[50] which use suction to draw the redundant tissue in to the applicator to make the procedure a one-person effort.

The most common complication of RBL is pain or rectal discomfort, which is usually relieved by warm sitz baths, mild analgesics and avoidance of hard stool by taking mild laxatives or bulk-forming agents. Other complications include minor bleeding from mucosal ulceration, urinary retention, thrombosed external hemorrhoids, and extremely rarely, pelvic sepsis. The patients should stop taking anticoagulants for one week before and two weeks after RBL.

Infrared coagulation: The infrared coagulator produces infrared radiation which coagulates tissue and evaporizes water in the cell, causing shrinkage of the hemorrhoid mass. A probe is applied to the base of the hemorrhoid through the anoscope and the recommended contact time is between 1.0-1.5 s, depending on the intensity and wavelength of the coagulator[51]. The necrotic tissue is seen as a white spot after the procedure and eventually heals with fibrosis. Compared with sclerotherapy, infrared coagulation (IRC) is less technique-dependent and avoids the potential complications of misplaced sclerosing injection[22]. Although IRC is a safe and rapid procedure, it may not be suitable for large, prolapsing hemorrhoids.

Radiofrequency ablation: Radiofrequency ablation (RFA) is a relatively new modality of hemorrhoidal treatment. A ball electrode connected to a radiofrequency generator is placed on the hemorrhoidal tissue and causes the contacting tissue to be coagulated and evaporized[52]. By this method, vascular components of hemorrhoids are reduced and hemorrhoidal mass will be fixed to the underlying tissue by subsequent fibrosis. RFA can be performed on an outpatient basis and *via* an anoscope similar to sclerotherapy. Its complications include acute urinary retention, wound infection, and perianal thrombosis. Although RFA is a virtually painless procedure, it is associated with a higher rate of recurrent bleeding and prolapse[53].

Cryotherapy: Cryotherapy ablates the hemorrhoidal tissue with a freezing cryoprobe. It has been claimed to cause less pain because sensory nerve endings are destroyed at very low temperature. However, several clinical trials revealed that it was associated with prolonged pain, foul-smelling discharge and a high rate of persistent hemorrhoidal mass[54]. It is therefore rarely used.

There are two meta-analyses comparing outcomes among the three common non-operative treatments of hemorrhoids (sclerotherapy, RBL and IRC)[23,24]. These two studies demonstrated that RBL resulted in the fewest recurrent symptoms of hemorrhoids and the lowest rate of retreatment, but that it led to a significantly higher incidence of pain following the procedure. Hence, RBL could be recommended as the initial non-operative modality for treatment of grade I - III hemorrhoids. In a British survey of almost 900 general and colorectal surgeons[55], RBL was the most common procedure performed, following by sclerotherapy and hemorrhoidectomy.

Operative treatment

An operation is indicated when non-operative approaches have failed or complications have occurred. Different philosophies regarding the pathogenesis of hemorrhoidal disease creates different surgical approaches (Table 3).

Hemorrhoidectomy: Excisional hemorrhoidectomy is the most effective treatment for hemorrhoids with the lowest rate of recurrence compared to other modalities[24]. It can be performed using scissors, diathermy[56,57], or vascular-sealing device such as Ligasure (Covidien, United States)[29,58] and Harmonic scalpel (Ethicon Endosurgery, United States)[59,60]. Excisional hemorrhoidectomy can be performed safely under perianal anesthetic infiltration as an ambulatory surgery[61,62]. Indications for hemorrhoidectomy include failure of non-operative management,

Table 3 Summary of different philosophies regarding the pathogenesis of hemorrhoids and related surgical approaches

Theory	Short description	Surgical approach
Sliding anal cushions	Hemorrhoids develop when the supporting tissues of the anal cushions disintegrate or deteriorate	Hemorrhoidectomy, plication
Rectal redundancy	Hemorrhoidal prolapse is associated with an internal rectal prolapse	Stapled hemorrhoidopexy
Vascular abnormality	Hyperperfusion of arteriovenous plexus within anal cushion results in the formation of hemorrhoids	Doppler-guided hemorrhoidal artery ligation

acute complicated hemorrhoids such as strangulation or thrombosis, patient preference, and concomitant anorectal conditions such as anal fissure or fistula-in-ano which require surgery[18]. In clinical practice, the third-degree or fourth-degree internal hemorrhoids are the main indication for hemorrhoidectomy.

A major drawback of hemorrhoidectomy is postoperative pain[62]. There has been evidence that Ligasure hemorrhoidectomy results in less postoperative pain, shorter hospitalization, faster wound healing and convalescence compared to scissors or diathermy hemorrhoidectomy[63-65]. Other postoperative complications include acute urinary retention (2%-36%), postoperative bleeding (0.03%-6%), bacteremia and septic complications (0.5%-5.5%), wound breakdown, unhealed wound, loss of anal sensation, mucosa prolapse, anal stricture (0%-6%), and even fecal incontinence (2%-12%)[66-69]. Recent evidence has suggested that hemorrhoidal specimens can be exempt from pathological examination if no malignancy is suspected[70].

Plication: Plication is capable of restoring anal cushions to their normal position without excision. This procedure involves oversewing of hemorrhoidal mass and tying a knot at the uppermost vascular pedicle. However, there are still a number of potential complications following this procedure such as bleeding and pelvic pain[21].

Doppler-guided hemorrhoidal artery ligation: A new technique based on doppler-guided ligation of the terminal branches of the superior hemorrhoidal artery was introduced in 1995 as an alternative to hemorrhoidectomy[71]. Doppler-guided hemorrhoidal artery ligation (DGHAL) has become increasingly popular in Europe. The rationale of this treatment was later supported by the findings from vascular studies[3,10], which demonstrated that patients with hemorrhoids had increased caliber and arterial blood flow of the terminal branch of the superior rectal arteries. Therefore, ligating the arterial supply to hemorrhoidal tissue by suture ligation may improve hemorrhoidal symptoms. DGHAL is most effective for second- or third-degree hemorrhoids. Notably, DGHAL may not improve prolapsing symptoms in advanced hemorrhoids. Short-term outcomes and 1-year recurrence rates of DGHAL did not differ from those of conventional hemorrhoidectomy[72]. Given the fact that there is the possibility of revascularization and recurrence of symptomatic hemorrhoids, further studies on the long-term outcomes of DGHAL are still required[73].

Stapled hemorrhoidopexy: Stapled hemorrhoidopexy (SH) has been introduced since 1998[74]. A circular stapling device is used to excise a ring of redundant rectal mucosa proximal to hemorrhoids and resuspend the hemorrhoids back within the anal canal. Apart from lifting the prolapsing hemorrhoids, blood supply to hemorrhoidal tissue is also interrupted. A recent meta-analysis comparing surgical outcomes between SH and hemorrhoidectomy, which included 27 randomized, controlled trials with 2279 procedures, showed that SH was associated with less pain, earlier return of bowel function, shorter hospital stay, earlier return to normal activities, and better wound healing, as well as higher degree of patient satisfaction[30]. However, in the longer term, SH was associated with a higher rate of prolapse[30,31,75]. Considering the recurrence rate, cost of stapling device and potential serious complications including rectovaginal fistula[76] and rectal stricture[77,78], SH is generally reserved for patients with circumferential prolapsing hemorrhoids and having ≥ 3 lesions of advanced internal hemorrhoids.

These two recent surgical options, DGHAL and SH, aim to correct the pathophysiology of hemorrhoids by reducing blood flow to the anal canal (dearterialization) and eliminating anorectal mucosal prolapse (reposition), respectively. A recent retrospective study of 18-mo outcomes of DGHAL ($n = 51$) and SH ($n = 63$) for grade Ⅲ hemorrhoids revealed that both procedures were safe and effective. DGHAL had less pain, shorter hospital stay, and faster functional recovery; however, it was associated with higher recurrence rate and lower patient satisfaction rating[79]. Lately, a smaller prospective trial comparing DGHAL to SH for grade Ⅱ-Ⅲ hemorrhoids showed similar short-term and long-term outcomes of the two procedures[80]. Nevertheless, patients undergoing DGHAL returned to work quicker, and had fewer complication rates than those receiving SH.

CONCLUSION

Therapeutic treatment of hemorrhoids ranges from dietary and lifestyle modification to radical surgery, depending on degree and severity of symptoms. Although surgery is an effective treatment of hemorrhoids, it is reserved for advanced disease and it can be associated with appreciable complications. Meanwhile, non-operative treatments are not fully effective, in particular those of topical or pharmacological approach. Hence, improvements in our understanding of the pathophysiology of hemorrhoids are needed to prompt the development of novel and innova-

tive methods for the treatment of hemorrhoids.

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REVIEW

Stem cell differentiation and human liver disease

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Abstract

Human stem cells are scalable cell populations capable of cellular differentiation. This makes them a very attractive *in vitro* cellular resource and in theory provides unlimited amounts of primary cells. Such an approach has the potential to improve our understanding of human biology and treating disease. In the future it may be possible to deploy novel stem cell-based approaches to treat human liver diseases. In recent years, efficient hepatic differentiation from human stem cells has been achieved by several research groups including our own. In this review we provide an overview of the field and discuss the future potential and limitations of stem cell technology.

INTRODUCTION

End stage liver disease (ESLD) is an irreversible condition that leads to the eventual failure of the liver. It may be the final stage of many liver diseases, for example, viral hepatitis, autoimmune hepatic disorders, fatty liver disease, drug induced liver injury, and hepatocellular carcinoma, with extremely poor prognosis. The incidence of ESLD is increasing worldwide[1], and current optimal treatment for ESLD is orthotopic liver transplantation[2]. However limited availability of donor livers and immunological incompatibilities are two major obstacles to its routine deployment[3]. This highlights the important need for alternative therapeutic strategies. Researchers have proposed that stem cell biology could provide a scalable answer for the treatment of ESLD, providing cells for transplant and/or cell sources for studying liver disorders and identifying novel treatments.

Cell-based therapy requires the use of cells to replace or facilitate the repair of damaged tissue. Candidate cells for this approach include bipotential, multipotent, pluripotent cells, and primary hepatocytes. Pluripotent stem cells (PSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), possess the ability to self renew and differentiate into all somatic cells, offering unlimited potential and are not restricted by donor tissue supply.

hESCs are derived from the inner cell mass of the human blastocyst, and can differentiate into all three primary germ layers[4]. Human iPSCs are produced by forced

expression of specific stem cell genes[5]. In recent years, researchers have developed robust procedures to generate functional hepatocyte-like cells (HLCs) from both PSC populations[6-8]. However, it is notable that PSC strategies have not yielded, as yet, a cell type that appropriately contributes to tissue homeostasis as cell transplantation frequently results in tumour formation[9,10]. As a result, scalable cell-based therapies from PSCs are likely to be longer-term strategies which require significant refinement.

Extra-corporeal support has been proposed as a mid-term strategy, in particular bio-artificial livers, to treat human liver disease. Bio-artificial livers (BALs) are designed to filter and biotransform toxic substances, and have been used successfully to bridge patients to transplant or treat acute liver failure. Research demonstrates that BALs can reduce mortality in acute liver failure compared with traditional standard medical therapy[11,12], but the application has been severely limited by the poor availability of functional human hepatocytes. By employing PSC technology, it may now be possible to produce humanised BAL devices at reasonable cost.

In addition to their important role in the clinic, human hepatocytes have a critical part to play in the drug discovery process. Many candidate compounds fail at late stage or even after approval due to unanticipated toxicity. In routine drug discovery, the pharmaceutical industry deploys the tumor-derived cells and primary hepatocytes to screen compounds. While these models are useful, they do not always extrapolate to human biology and exhibit poor lifespan and variable metabolic activity. PSCs-derived hepatocytes have the potential to overcome these problems. Advances in stem cell biology and reprograming have allowed the development of novel models which have the potential to provide another level of understanding behind the pathophysiology of liver diseases. iPSC modelling also provides us with potential system to better understand the influence of gene polymorphisms.

Human PSCs derived HLCs show great promise for research and clinical applications including cell-based therapies, drug development, and disease modeling. This review gives an overview of human hepatic differentiation from PSCs and their potential application in modern medicine.

CURRENT CELL SOURCES USED IN HEPATOLOGY

Primary human hepatocytes and liver cancer cell lines

Hepatocytes are the principle cell type found in the liver and perform the majority of the liver functions. Primary human hepatocytes (PHHs) are therefore a useful tool for medical applications such as cell-based therapy and drug discovery. However, PHHs are mainly obtained from scarce and low quality resected surgical specimens[13]. The scarcity and variability in these preparations restricts their widespread application *in vitro*[14]. Therefore liver cell lines have been employed routinely as they dem-

onstrate long lifespan and are easy to maintain. HepG2 is a liver cell line derived from fetal tissue which exhibits poor metabolic function and secrete a variety of soluble serum proteins[15]. They have been used as a model system for cytochrome P450 (CYP) metabolism and toxicity. And additionally they have been used in clinical trials with bioartificial liver devices[16,17]. Interestingly, a clonal derivative of the HepG2 line, C3A, demonstrates marked reduction in α-fetoprotein (AFP) and increased albumin (ALB) secretion, indicating a more mature status *in vitro*. More recently, a new human hepatoma cell line has been derived. HepaRG demonstrates a number of liver-specific functions including the expression of CYP 1A2, 2B6, 2C9, 2E1, 3A4[18,19] and better overall performance than existing liver cell lines. Although informative and scalable, liver cancer cell lines show lower drug-metabolizing activity than their adult counterparts and do not accurately predict human drug toxicity[20] and therefore do not constitute a real alternative to the gold standard primary hepatocyte. Moreover, such cells may provide interesting *in vitro* models or the bio-component of the BAL, but they could not be used for cell transplantation *in vivo*.

Oval cells and hepatoblasts

Oval cells are an adult liver cell population that emerges from the biliary tree following chronic liver injury. Several studies have investigated the transplantation of oval cells showing that these bipotential cells could proliferate and contribute to both parenchyma and biliary epithelia *in vivo*[21-28]. Oval cells express the stem cell markers Thy-1 (CD90), CD34 and Sca-1, along with liver-specific markers, including AFP, Gamma-glutamyltransferase, laminin and cytokeratin 19 (CK 19)[23,24].

The tissue microenvironment plays an essential role in orchestrating oval cell-mediated liver regeneration. Laminin contributes to the maintenance of undifferentiated progenitor cells and progenitor cell-mediated tissue repair[29]. Moreover, Kallis *et al*[30] demonstrated that extracellular matrix (ECM) remodelling during resolution and laminin deposition was likely to be important prerequisite to hepatic progenitor cell activation, expansion and repair.

Similar to oval cells, hepatoblasts from fetal liver could also represent a potential source of hepatocytes and biliary epithelial cells[24,31]. The bipotential nature of this cell type also makes it an attractive target for therapy. Transplant studies demonstrate that hepatoblasts may be a potential therapeutic strategy for ESLDs or hepatic failure. Although great progress in the fundamental research and clinical application have been made, there are still limitations to widespread use of these cells, such as low cell number *in vivo*, no specific biomarker for purification and poor expansion *in vitro*.

Bone marrow stem cells and mesenchymal stem cell

The bone marrow (BM) contains stem cells populations *in vivo*. They can be roughly divided into of hematopoietic (HSCs) and nonhematopoietic stem cells usually referred

to as mesenchymal stem cells (MSCs). The great success of BM stem cell for treatment of leukaemia has attracted scientists to use these cells for other serious diseases such as ESLD. Analysis of BM transplant into mouse models and patients have demonstrated that transplanted BM could contribute to partial correction of hepatic function[32-34]. However, the role of BM is controversial; some researchers found that BM didn't contribute to hepatocyte or biliary cell differentiation and liver regeneration, but actually contributed to liver fibrosis[35,36], which raises serious safety concerns.

Similar to BM, MSCs have been successfully transplanted[37]. They are multipotent stem cells capable of mesodermal, neuro-ectodermal and endodermal differentiation depending on surrounding microenvironment[38-41]. In addition, MSCs have anti-fibrotic properties inhibiting activated fibrogenic cells such as hepatic stellate cells[42]. The role of MSCs in liver regeneration and disease has been evidenced in animal models. Moreover MSC based therapies for patients with ESLDs have shown promise in phase I and II clinical trials[37,43,44]. Treatment was well tolerated by all patients with liver fibrosis and heptic function improved following MSCs transplantation[37] and during the follow-up[43]. Peng *et al*[45] reported that the biochemical hepatic index and MELD score were markedly improved from 2-3 wk post transplantation. However, long-term hepatic function were not significantly enhanced in 527 patients with liver failure caused by hepatitis B. Although MSC transplantation confers benefit to patients with liver cirrhosis, it may not be applicable to all kind of ESLDs.

HEPATIC DIFFERENTIATION FROM PLURIPOTENT STEM CELLS

Human ESCs are derived from the inner cell mass of blastocyst stage embryos and are highly primitive cells which exhibit pluripotency and the ability to self-renew[4,46]. Rambhatla *et al*[47] reported the directed differentiation of human ESCs to HLCs for the first time in 2003, which could express some hepatocyte markers. Since then labs have established more robust and efficient procedures to derive better functioning HLCs. Embryoid body (EB) formation has been one method to differentiate ESCs into hepatocytes. However, this approach exhibits limitations to scale and culture definition. Therefore, monolayer adherent culture systems have been developed to direct ESC hepatic differentiation into hepatocytes, which bypass these limitations[6,48-51]. We developed a simple 3-stage procedure by which hESCs can be directly differentiated to HLCs at an efficiency of about 90%[6,8]. Our research demonstrated that Wnt3a signaling was important in this process, improving hepatocellular function both *in vitro* and *in vivo*[6,52]. Most recently we identified a novel polyurethane extra cellular support which delivers long-term and stable HLC function which is drug inducible[53].

In 2006, it was demonstrated that murine fibroblasts

could be reprogrammed into a pluripotent state similar to that observed in ESCs[54]. Subsequently Takahashi *et al*[5] and Park *et al*[55] successfully reprogrammed human somatic cells into iPSCs. They generated PSCs from human skin through ectopic expression of four genes (*Oct3/4, Sox2, c-Myc,* and *Klf4*), which were known to be involved in the induction of murine pluripotency. Since these experiments researchers continue to refine and simplify the reprogramming process.

Human iPSCs and ESCs display similar morphologies, proliferation rates and expression of a number of stem cell biomarkers. However, specific differences between ESCs and iPSCs exist. Obviously the biggest difference is that iPSCs are derived from adult tissues. In addition, some comparative genomic analyses shows that hundreds of genes are differentially expressed in these two cell types[56]. Given their adult origin, iPSCs can contain an epigenetic "memory" of the donor tissue[57,58], which can restrict their differentiation potential and therefore utility.

iPSCs have been differentiated to numerous cell types[59], including hepatocytes. We and others have devised efficient methods to generate hepatocytes *in vitro*[7,60,61]. The derivative HLCs from both hESC and iPSC models demonstrate a similar expression of genes important for normal liver physiology. Jozefczuk *et al*[62] demonstrated 80% similarity of gene expression between HLCs derived from hESCs or iPSCs. Additionally, there were specific differences between the types of HLCs derived from ESCs and iPSCs in particular the *CYP* genes.

Most recently a study reported the direct conversion of murine fibroblasts to HLCs without the need for cellular pluripotency. In two studies HLC differentiation was conferred using either Gata4, Hnf1α and Foxa3, or HNF4a in combination with Foxa1, Foxa2 or Foxa3[63,64]. HLCs exhibited hepatic gene expression and function *in vitro* and rescued fumarylacetoacetate-hydrolase-deficient (Fah-/-) mice *in vivo*[63,64]. These studies provide another alternative method of hepatic conversion, which offer potential for liver research and therapy.

HEPATIC DIFFERENTIATION IN CELL-BASED THERAPIES AND TOOLS

Hepatic differentiation for cell-based therapy

PSCs offer a possible source to treat liver disease. Cell therapy for liver disease includes transplantation (including genome edited cells to correct metabolic defects[65]) and bio-artificial liver devices. The cell-based approaches are very encouraging, but further studies are required to demonstrate long-term safety of cell-based transplantation[9,10]. In the interim BALs containing hepatocytes could provide alternative support for patients with acute hepatic failure or awaiting liver transplantation. Efforts to generate long-lived functional HLCs may allow the development of more highly effective BALs. The potential application of human stem cells in cell-based therapy for liver diseases is summarised in Figure 1.

Figure 1 Potential application of human stem cells in cell based therapy for liver disease. Pluripotent and multipotent stem cells can be reprogrammed or purified from human material that was been ethically sourced. Following expansion and differentiation the derivative hepatocyte like cells can be used for transplantation or bio-artificial liver construction.

Figure 2 Human liver cells in drug discovery[7,53,66,67]. Drug development process is a lengthy and expensive process. The derivation of hepatocyte-like cells from different human genotypes may provide novel *in-vitro* models for the screening of new compounds in the drug discovery process.

Hepatic differentiation for drug discovery

The drug development process is a hugely expensive process, due to its length and high levels of compound attrition. Drug development proceeds through several stages in order to produce a drug that is safe, efficacious, and meets regulatory requirements (Figure 2). The liver plays a central role in the metabolism of a majority of drugs. Therefore, a standardized screening model with human hepatocytes for new drug compounds could help to reduce drug attrition and costs. Traditional cell models for drug discovery include primary human hepatocytes, immortalised cell lines and animal tissues; however, these cell sources possess a number of limitations including poor function, species variability and instability in culture[14,20]. Advances in PSCs research and liver engineering

have provided models that may overcome some of the problems associated with existing technology. Moreover, in parallel with extracorporeal device development, stem-cell-derived HLCs in three dimensional (3D) are more likely to mimic human liver properties *in vitro*.

Hepatic differentiation for disease modelling

PSCs have provided scientists with novel models to study human liver disease. Rashid *et al*[60] reported an effective procedure for hepatocyte generation from iPSCs exhibiting disease mutations. Using these cells, they modeled inherited metabolic disorders that affect the liver; alpha1-antitrypsin deficiency, familial hypercholesterolemia, and glycogen storage disease type 1a. These models accurately reflected elements of the disease process. More recently research iPSCs, obtained from patients with tyrosinemia, glycogen storage disease, progressive familial hereditary cholestasis, and Crigler-Najjar syndrome, were differentiated into functioning HLCs[68]. These inherited liver diseases that mainly arise as a result of loss of function mutation, therefore these studies offers a unique opportunity to study the effects of specific gene defects on human liver biology and to better understand liver pathogenesis in disease.

Improving hepatic differentiation

PSC technologies have the potential to produce unlimited amounts of human liver cells. As discussed above, human hepatocytes from PSCs could be utilized for cell-based therapy, assessment of drug toxicity and disease modelling. Therefore, the PSC-derived HLCs should be reliable, stable in character and display high levels of metabolic activity. A better understanding of human liver development and optimal tissue microenvironments are likely to play an important role in this process.

HUMAN LIVER DEVELOPMENT

Liver development occurs through a series of reciprocal tissue interactions between the embryonic endoderm and

Figure 3 Human fetal liver development[31,74]. The key stages of human liver development are shown in pink and blue. Endoderm formation occurs in the 2nd-3rd wk of fetal development. The liver bud forms between week 3-4 and expands rapidly. Hepatocytes and biliary epithelia differentiate and mature from 7 wk post fertilisation and this process continues in the neo-nate.

nearby mesoderm. Endoderm contributes to the digestive tract and has a principal role in the development of the liver (Figure 3). The secretions of fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) from the cardiac mesoderm and septum transversum mesenchyme (STM) help orchestrate human liver development from foregut endoderm in concert[69] with canonical Wnt signalling[6,70,71]. Three to 4 wk post fertilisation cells called hepatoblasts, positive for CK19 and HepPar1, are detected for the first time[31]. The hepatoblasts proliferate and form the liver bud. The hepatic endoderm thickens into a columnar epithelium, and hepatoblasts delaminate and invade the STM and undergo cellular proliferation and differentiation. Experiments have shown that a number of factors such as FGF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor (TGF), tumor necrosis factors (TNF), and interleukin-6 contribute to the hepatocytes proliferation and differen-

tiation[72,73]. Between 6-8 wk gestation, the bile duct and hepatic structure are easily identified[31]. Maturation of hepatocytes and bile epithelial cells continues after birth. An overview of embryonic liver development is summarized in Figure 3.

IMPROVING CELL CULTURE MICROENVIRONMENT

The tissue microenvironment also plays an essential role in liver development and hepatic differentiation. Two dimensional (2D) hepatic differentiation is probably the most widely used system in laboratories. While this technology is efficient and scalable, there are several drawbacks related to 2D culture, including poor drug inducibility and rapid cell dedifferentiation. During human liver development, hepatocytes mature in a 3D environment with a number of cell types providing support.

In light of the increasing need for better-differentiated hepatocytes from PSCs, we and others have developed 3D systems to improve and stabilize hepato-cellular phenotype[53,75,76].

Undoubtedly 3D culture leads to improvements in hepatic function. In the future modulation of oxygenation and physiological delivery of nutrients in 3D environment have great potential to improve cell phenotype and therefore utility.

CONCLUSION

The development of hESC and iPSC technology has led to a new era of discovery in liver medicine. Advances in PSC technology offer the promise of scalable human hepatocytes for cell-based therapies, assessment of drug efficacy and toxicity, and disease modelling. The challenge remains to cost effectively scale up this technology for industrial manufacture. A better knowledge of liver development and the use of novel supportive culture systems will help to improve the manner in which we derive mature human hepatocytes.

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ORIGINAL ARTICLE

Antifibrotic effect of aloe vera in viral infection-induced hepatic periportal fibrosis

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Abstract

AIM: To investigate the anti-oxidative and anti-fibrotic effects of aloe vera in patients with liver fibrosis.

METHODS: Aloe vera high molecular weight fractions (AHM) were processed by patented hyper-dry system in combination of freeze-dry technique with microwave and far infrared-ray radiation. Fifteen healthy volunteers as the control group and 40 patients were included. The patients were randomly subdivided into two equal groups: the conventional group was treated with placebo (starch), and AHM group was treated with 0.15 gm/d AHM, both for 12 consecutive weeks. The patients were investigated before and after treatment. Serum activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), hyaluronic acid (HA), transforming growth factor-β (TGF-β) and matrixmetalloproteinase-2 (MMP-2) were determined. The reduced glutathione (GSH) and malondialdehyde (MDA) levels in liver were assayed and the expression of hepatic α-smooth muscle actin (α-SMA) was identified by immunohistochemistry.

RESULTS: At the start of the study, the hematoxylin and eosin staining revealed fibro-proliferated bile ductules, thick fibrous septa and dense inflammatory cellular infiltration in the patients before treatment. The use of AHM for 12 wk significantly ameliorated the fibrosis, inhibited the inflammation, and resulted in minimal infiltration and minimal fibrosis compared to the conventional group. The enzyme activities of the liver (ALT, AST and ALP) were attenuated after treatment in both groups, and the decrease in the AHM group was more significant as compared with the conventional group. Similar to the AST, the MDA levels were significantly higher before treatment, and were attenuated after treatment in both groups. In contrast, the hepatic glutathione content in the patients were decreased significantly in the AHM group compared to the controls. The serum levels of the fibrosis markers (HA, TGF-β and MMP-2) were also reduced significantly after treatment. The expression of α-SMA was modified in patients before and after treatment as compared with the normal controls. In the conventional group, there was only thin and incomplete parenchymal α-SMA positive septum joining the thickened centrilobular veins, while in the AHM group, few α-SMA positive cells were present in sinusoid and lobule after treatment.

CONCLUSION: Oral supplementation with AHM could be helpful in alleviating the fibrosis and inflammation of hepatic fibrosis patients.

INTRODUCTION

Hepatic fine periportal fibrosis is a common response to liver injury caused by viral hepatitis, hepatitis B virus (HBV), and hepatitis C virus (HCV) infection, and other factors. The pathophysiological events leading to periportal fibrosis provoke excessive hepatocytes apoptosis and necrosis[1,2]. The damaged hepatocytes are the activators of Kupffer cells. The activated Kupffer cells release a number of soluble agents, including cytokines, reactive oxygen species (ROS), and other factors. These factors act on the hepatic stellate cells (HSCs), which undergo morphological transition to myofibroblast-like cells and proliferate. This transition is characterized by an accelerated production of large amounts of extracellular matrix (ECM) involving molecular and histological re-arrangement of various types of collagens, proteoglycans, structural glycoprotein and hyaloronic acid[3].

Oxidative stress has been recognized as a fundamental factor in the pathological changes observed in various liver diseases[4,5]. It can cause excessive damage to hepatocytes through lipid peroxidation and protein alkylation[6].

Acute and chronic liver diseases constitute a global concern, and treatment for these diseases is difficult and have limited efficacy. Therefore, considerable efforts are being made to obtain useful herbal medicine from documented medicinal plants for a wide variety of clinical conditions. Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically.

Aloe vera is a cactus-like plant that grows in hot, dry climates. Two distinct preparations of aloe plants are most frequently used. The leaf exudate (aloe) is used as a laxative, and the mucilaginous gel (aloe vera) extracted from the leaf parenchyma is used as a remedy against a variety of skin disorders. Aloe vera gel has been demonstrated to have liver protective effect in rats[7-9], and many toxicity studies have been conducted to determine the LD50 of aloe vera[10-13]. However, the antifibrotic effect of aloe vera on liver fibrosis has not yet been reported. The aim of this study was to investigate the anti-oxidative, and anti-fibrotic effects of aloe vera in patients with acute liver fibrosis.

MATERIALS AND METHODS

Extraction and preparation

Aloe vera high molecular weight fractions (AHM) were obtained from water-washed gel of aloe vera leaves cultivated in Okinawa, Japan. Voucher specimens of aloe vera collected in Okinawa, were compared and de-termined to be *aloe vera L.* plant (syn. Aloe barbadensis Miller) (Herbarium number 54-3 in Medicinal garden, Fukuyama University by Emeritus Prof. A Yagi). AHMs were processed by patented hyper-dry system in combination of freeze-dry technique with microwave and far infrared-ray radiation. AHM contains the following chemical and physical properties: molecular weight (MW): 1119500 D by high performance liquid chromatography (HPLC) analysis; TSK gel GMPW column: two columns in series, 10 μm, 7.8 mm × 30 cm; eluent 0.2 mol $NaNO_3$; flow rate: 1.0 mL/min; temperature: 40 ℃; and use of refractive index detector.

Sample treatment

AHM sample (1 g) was homogenized in 2 mL of 0.2 mol $NaNO_3$. Homogenate was centrifuged at 2000 × g for 1 min. Upper solution was introduced as 200 μL aliquots to size-exclusion-chromatography. Aloin content was less than 10 ppm by HPLC analysis[14], water content: 3% ± 0.5%, colony formulating unit: less than 300/g, Na: 430 mg/100 g, Ca: 2100 mg/100 g. AHM contained the neutral polysaccharides with MW of about 1000 kDa, and 90% carbohydrate and 7% protein. Glycoprotein and verectin composed of carbohydrate and protein in a ratio of 10.7% and 82.0%, respectively, with MW of 29 kDa[15], was obtained in a ratio of 20% by immunochemical assay in AHM. Chemical shifts of AHM were determined in D_2O with a JOEL JNM α-400 and 100 MHz for proton and carbon, respectively. The infrared spectra were determined with a FTIR-8600PC, Shimadzu, Japan.

Patients

The subjects in this study were selected from the Internal Medicine Department, Tanta University Hospitals. They included 15 healthy volunteers as the control group and 40 patients (32 men and 8 women, ranged 25-56 years). Among the 40 patients, 15 had HCV, 24 had HBV and 1 had bilharziasis. Patients were included in the study if they were positive for serum hepatitis B surface antigen or C antibodies and had persistently elevated serum aminotransferase concentrations 1.5 times higher than the upper limit of the reference range for at least 6 mo. All the patients were diagnosed according to the International Autoimmune Hepatitis Group Report protocol[16].

For assessment of liver fibrosis scores, all patients underwent liver biopsy as part of the normal diagnostic procedure and were sub-classified according to the score for the histological activity index (HAI). Patients with a history of gastrointestinal bleeding and chronic liver disease (Wilson's disease, hemochromatosis, α 1-antitrypsin deficiency, or hepatocellular carcinoma), active intravenous drug abuse, and liver transplantation were excluded.

All the patients were subjected to full history taking, thorough clinical examination, biopsy and histological examinations, and laboratory investigations (Table 1).

Informed consent was obtained from all the participants. The protocol of the study was approved by the Ethical Committee of the University.

Table 1 Characteristics of the study populations (mean ± SD)			
Parameter	Control group (*n* = 15)	Conventional group[1] (*n* = 20)	AHM group[2] (*n* = 20)
Age (yr)	40.2 ± 10.4	41.6 ± 15.4	40.5 ± 13.9
Sex (M/F)	15/0	15/5	17/3
HBV	-	12	12
HCV	-	8	7
Bilharziasis	-	0	1
Fibrosis Stage			
F1	0	3	3
F2	0	9	10
F3	0	8	7
F4	0	0	0
Total bilirubin (mg/dL)	0.55 ± 0.20	1.19 ± 0.20	1.18 ± 0.19
ALT (IU/L)	19.4 ± 8.2	87.4 ± 8.4	85.9 ± 9.1
AST (IU/L)	25.0 ± 4.3	50.8 ± 6.7	51.0 ± 4.2
ALP (IU/L)	55.0 ± 14.3	225.0 ± 85.6	223.0 ± 74.5
Albumin (g/dL)	4.4 ± 0.1	4.2 ± 0.2	4.3 ± 0.1
INR	0.88 ± 0.22	1.47 ± 0.3	1.51 ± 0.2

[1]Patients treated with the conventional treatment with placebo (starch) for 12 consecutive weeks; [2]Patients treated with the conventional treatment with 0.15 g/d AHM for 12 consecutive weeks. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; INR: International normalization ratio; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AHM: Aloe vera high molecular weight fractions.

Treatment was initiated if they met the inclusion criteria. Treatment of each patient was according to a standard protocol. Hepatitis C patients were treated with pegylated interferon (180 μg/wk) + ribavirin (800-1200 mg/d). Hepatitis B patients were treated with adefovir (10 mg/d) or lamivudin (100 mg/d).

The patients were randomly subdivided into two equal groups: the conventional group treated with the conventional treatment with placebo (starch) for 12 consecutive weeks, and the AHM group treated with the conventional treatment with 0.15 g/d AHM (0.05 g three times daily) for 12 consecutive weeks. The dosage was calculated according to Williams *et al*[10]. The AHM preparation was provided in sachets, which contained powder to be dissolved in 50 mL fresh water. Liver and blood samples were collected at the start and at the end of the study period for assessment.

Histological assessment
Liver biopsy fragments were fixed in 10% neutralized formaldehyde, embedded in paraffin, and then stained with hematoxylin and eosin. Liver biopsy samples were examined in a double-blinded fashion using a METAVIR scoring system. Blood samples were withdrawn; serum was separated and utilized for biochemical analysis of aspartate aminotransferase (AST), alanine aminotransferase (ALT) alkaline phosphatase (ALP), hyaluronic acid (HA), transforming growth factor-β (TGF-β) and matrix metalloproteinase-2 (MMP-2). Liver sample was dried, cut into portions and kept frozen at -80 ℃ until used for analysis. Liver tissues were weighed and homogenized in solution containing ice-cold isotonic saline[17]. The homogenates were centrifuged at 4500 rpm for 15 min at 4 ℃ and the supernatants were taken for determination of reduced glutathione (GSH), and malondialdehyde (MDA). The protein content of the tissue aliquots was determined by the Lowry method[18].

Biochemical assays
Measurement of liver enzyme activities: The serum enzyme activities of ALT and AST were measured colorimetrically according to the method of Reitman and Frankel[19], using Boehringer Mannheim Kit. The optical density was read at 546 nm using UV-160A Shimadzu Spectrophotometer. Serum total alkaline phosphatase activity was estimated by commercially available kits (BioMerieux, France) according to the method of Kind and King[20].

Measurement of fibrosis markers: Serum hyaluronic acid was measured using a kit provided by Corgenix Inc. (Colorado, United States, under license of Chugai Diagnostic Science Co.). It was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Serum TGF-β1 and MMP-2 levels were evaluated using a commercially available human TGF-β1 and MMP-2 ELISA kit according to the manufacturer's instructions (TGF-β1: R and D System, Abingdon, United Kingdom; MMP-: Amersham, Bucks, United Kingdom).

Measurement of liver oxidative markers: Hepatic GSH concentration was determined by the method of Richardson and Murphy[21], using Ellman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid; DTNB) and the absorbance was measured at 412 nm. The results were calculated as μmol GSH/g tissue. Lipid peroxidation was assessed by measuring MDA using thiobarbituric acid according to the method of Yoshioka *et al*[22]. Thiobarbituric acid reactive substances was measured in μmol/g of tissue according to the absorbance at 532 nm.

Immunohistochemical analysis
For immunohistochemical analysis, sections were incubated with anti-α-smooth muscle actin (α-SMA) (1/1000; Dako North America, Inc., Carpenteria, CA) for 30 min. Staining was visualized using the horseradish peroxidase-conjugated Dako staining system (Dako In-Vision; Dako North America, Inc.).

Statistical analysis
Data were statistically analyzed by 2-way analysis of variance (ANOVA) (repeated measure type) to compare the results before (baseline) and after treatment within the same group, and unpaired Student's *t* test was used to compare the means of different groups. Significant differences between the groups were statistically analyzed using one-way ANOVA using the computer program SPSS for Windows version 10 (Chicago, IL, United States). All results were expressed as mean ± SD. The level of significance was set at $P < 0.05$.

Figure 1 Hematoxylin and eosin staining of liver tissues (HE, × 200). A: Control group showed normal lobular architecture and cell structure; B: Patients before treatment showed fibro-proliferated bile ductules, thick fibrous septa and dense inflammatory cellular infiltration; C: The conventional group showed moderate fibrosis with inflammatory infiltration and slight ballooning of liver cells; D: Aloe vera high molecular weight fractions group showed minimal infiltration and minimal fibrosis.

RESULTS

Macroscopic presentation and histological evaluation

The histological findings of liver tissues are presented in Figure 1. There were thick fibrous septa and dense inflammatory cellular infiltration in the patients before treatment. However, the control group showed normal lobular architecture and cell structure. The conventional group showed moderate fibrosis with inflammatory infiltration and slight ballooning of liver cells after treatment. AHM treatment could inhibit the inflammation, and showed minimal infiltration and minimal fibrosis compared to the conventional group. The histopathological evaluation of the two groups before and after treatment is shown in Table 2.

Determination of liver enzyme activities

The serum ALT, AST and ALP activities were significantly higher in the patients at the beginning of the study as compared with the control group. These increases were attenuated after treatment in both the conventional group and the AHM group, and the decrease in the AHM group was more significant than in the conventional group ($P <$ 0.05, Figure 2). There was no significant difference in the liver enzyme activities between HBV-induced fibrosis and HCV after treatment ($P <$ 0.05, Table 3).

Determination of liver oxidative markers

Similar to the aminotransferase activities, the MDA levels were significantly higher in the patients before treatment,

Table 2 Histopathological evaluation of the patients before and after treatment n (%)

	Conventional group			AHM group		
	Before	**After**	χ^2/**P**	**Before**	**After**	χ^2/**P**
Grade						
0	0 (0)	3 (15)	4.390/	0 (0)	3 (15)	9.390/
1	3 (15)	5 (25)	0.223	3 (15)	9 (45)	0.024[a]
2	9 (45)	6 (30)		10 (50)	4 (20)	
3	8 (40)	6 (30)		7 (35)	4 (20)	
Stage						
0	0 (0)	0 (0)	0.000/	0 (0)	2 (10)	6.170/
1	3 (15)	3 (15)	1.000	3 (15)	7 (35)	0.103
2	9 (45)	9 (45)		10 (50)	4 (20)	
3	8 (40)	8 (40)		7 (35)	7 (35)	

[a]$P < 0.05$ *vs* conventional group. AHM: Aloe vera high molecular weight fractions.

and were attenuated after treatment in both groups ($P <$ 0.05, Figure 3). In contrast, the hepatic glutathione content in the patients was decreased to about 49% of the control. Twelve weeks of treatment could increase the concentration of reduced glutathione. The increase was more significant in the AHM group ($P <$ 0.05, Figure 3). There was no significant difference in the liver oxidative markers between HBV-induced fibrosis and HCV after treatment ($P <$ 0.05, Table 3).

Determination of fibrosis markers

As shown in Figure 4, a significant increase in the serum

Table 3 Biochemical parameters of hepatitis B virus-induced fibrosis and hepatitis C patients after treatment

Parameter	Conventional group		AHM group	
	HBV (*n* = 12)	HCV (*n* = 8)	HBV (*n* = 12)	HCV (*n* = 7)
ALT (IU/L)	50.1	47.2	50.1	37.3
AST (IU/L)	40.4	38.3	30.3	28.1
ALP (IU/L)	173.3	177.8	156.3	160.4
MDA (μmol/g)	596.1	607.4	571.0	565.0
GSH (μg/g)	22.9	20.4	23.7	27.6
TGF-β (pg/mL)	38.2	41.1	34.6	38.4
HA (ng/mL)	63.3	59.7	58.1	52.1
MMP-2 (ng/mL)	259.1	277.8	238.8	256.7

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; MDA: Malondialdhyde; GSH: Reduced glutathione; TGF-β: Transforming growth factor-β; HA: Hyaluronic acid; MMP-2: Matrix metalloproteinase-2; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

level of TGF-β1, HA and MMP-2 was observed in the patients before treatment. Both the conventional and the AHM groups showed significant decrease in the levels after treatment ($P < 0.05$). There was no significant difference of fibrosis markers between HBV-induced fibrosis and HCV after treatment ($P < 0.05$, Table 3).

The expression of α-SMA was modified in patients before and after treatment as compared with the normal control. Before treatment, α-SMA positive cells were detected in portal space, sinusoid, lobule and areas where fibrotic septum appeared. After treatment, activation of HSC appeared to be strikingly decreased. After 12 wk of conventional treatment, there were only thin and incomplete parenchymal α-SMA positive septum joining thickened centrilobular veins. α-SMA positive cells were mainly found in portal space and areas around fibrotic septum. In AHM group, few α-SMA positive cells were present in sinusoid and lobule (Figure 5).

DISCUSSION

The present study evaluates the anti-inflammatory, anti-oxidative and anti-fibrotic effect of aloe vera in hepatic fine periportal fibrosis. AHM treatment for the hepatic fibrosis patients markedly attenuated the release of ALT, AST and ALP as compared with the control group and the conventional group. Histological findings of liver samples strongly supported the release of aminotransferases by damaged hepatocytes and the protective effect of AHM.

GSH constitutes the first line of defense against free radicals and is a critical determinant of tissue susceptibility to oxidative damage. This is evident in this study by the significant increase in hepatic content of MDA and depletion of GSH in the patients before treatment. AHM exhibited hepato-protective effects by impairing oxidative stress through decreased production of free radical derivatives, as evidenced by the decreased MDA level. Furthermore, it attenuated hepatic glutathione depletion.

Figure 2 Serum activity of alanine aminotransferase, alkaline phosphatase and aspartate aminotransferase of hepatic fibrosis patients and controls. A: Serum activity of alanine aminotransferase (IU/L) of hepatic fibrosis patients and controls; B: Serum activity of aspartate aminotransferase (IU/L) of hepatic fibrosis patients and controls; C: Serum activity of alkaline phosphatase (IU/L) of hepatic fibrosis patients and controls. [a]$P < 0.05$, [c]$P < 0.05$, [e]$P < 0.05$ *vs* before treatment and conventional group; Data are presented as mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; AHM: Aloe vera high molecular weight fractions.

This increase in the hepatic glutathione level could result from either its effect on the *de novo* synthesis of glutathione and its regeneration, or both. These results suggest that the antioxidant properties may be one mechanism by which AHM protects against liver damage.

Previous findings are in agreement with the finding by Anilakumar *et al*[23] who showed that aloe vera gel extract is able to reduce azoxymethane (AOM) induced-oxidative stress and toxicity in rat liver. Rajasekaran *et al*[24] also revealed that aloe vera leaf extract has a modulatory effect on oxidative stress in rats treated with streptozotocin by decreasing the thiobarbituric acid reactive substances, and improving reduced glutathione in the pancreas of STZ-

Figure 3 Hepatic malondialdhyde and glutathione of hepatic fibrosis patients and controls. A: Hepatic malondialdhyde (μmol/g) of hepatic fibrosis patients and controls; B: Hepatic reduced glutathione (μg/g) of hepatic fibrosis patients and controls. [a]P < 0.05, [c]P < 0.05 *vs* before treatment and conventional group. Data are presented as mean ± SD. MDA: Malondialdehyde; GSH: Glutathione; AHM: Aloe vera high molecular weight fractions.

induced diabetic rats. However, Yang *et al*[25] showed that oral aloe supplementation caused increase in liver enzymes and subsequent acute liver injury.

TGF-β, a multifunctional growth factor, is the most potent fibrogenic cytokine[26]. It is involved in regulation of liver growth and induction of hepatocyte apoptosis. TGF-β can promote the development of liver fibrosis by inducing the synthesis of ECM proteins and down-regulating the expression of matrix[27]. The effect of oral administration of aloe vera gel on the simulation of TGF-β was studied by Atiba *et al*[28]. The present study showed that TGF-β1 increased in the serum of fibrotic patients and decreased after treatment. These results suggest that TGF-β1 is closely correlated with hepatic fibrosis and that the improvement of hepatic fibrosis is related to the decreasing expression of TGF-β1. The serum level of TGF-β1 was low in the AHM group compared with the conventional group and this decrease was not significantly different as compared with the control group, suggesting that AHM can inhibit the expression of TGF-β1. These results coincide with that of Kim *et al*[7] who showed that ACTIValoe®N-931 complex decreased the TGF-β1 level and the hepatic hydroxyproline content in CCl-4-induced hepatotoxicity rats.

HA is mostly synthesized by the hepatic stellate cells and degraded by the sinusoidal endothelial cells[29]. Some investigators[30,31] have shown that there is good correlation between HA and the degree fibrosis. In the present

Figure 4 Serum hyaluronic acid, transforming growth factor-β and matrix metalloproteinase-2 of hepatic fibrosis patients and controls. A: Serum hyaluronic acid (ng/mL) of hepatic fibrosis patients and controls; B: Serum transforming growth factor-β (pg/mL) of hepatic fibrosis patients and controls; C: Serum matrix metalloproteinase-2 (ng/mL) of hepatic fibrosis patients and controls. [a]P < 0.05, [c]P < 0.05, [e]P < 0.05 *vs* before treatment and conventional group. Data are presented as mean ± SD. HA: Hyaluronic acid; TGF-β: Transforming growth factor-β; MMP-2: Matrixmetalloproteinase-2; AHM: Aloe vera high molecular weight fractions.

study, 12 wk co-treatment of AHM and the conventional treatment decreased the serum HA by 25% as compared before treatment. It showed a trend toward greater improvement in the AHM group, however due to the short duration of treatment in our study, this was statistically insignificant as compared with control group.

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent enzymes that degrade extracellular matrix components and act as a marker of HSCs activation[32]. It was recently shown that it promoted HSCs apoptosis by cleavaging N-cadherin as an essential HSCs survival factor[33]. During fibrogenesis, the expression of MMP-2 increased and decreased significantly after treat-

Figure 5 Immunohistochemical staining of α-smooth muscle actin. A: Section of liver from controls showing negative staining (PAP × 125); B: Section of liver from liver fibrosis patients before treatment showing dense fibrotic reaction with strong positivity for α-smooth muscle actin (α-SMA) ↑↑ and dense mononuclear cellular infiltration ↑ (PAP × 225); C: Section of liver from conventional group after treatment showing dense mononuclear inflammatory infiltration ↑ and moderate positivity for α-SMA in fibrotic lesion↑↑ (PAP × 125); D: Section of liver from AHM group after treatment showing moderate mononuclear cellular infiltration ↑ surrounded by minimal fibrotic reaction stained mildly with α-SMA ↑↑ (PAP × 125).

ment, suggesting that the treatment favored a collagenolytic activity.

The present study showed no significant difference in the serum liver fibrosis markers between HBV and HCV patients which is in agreement with Elmetwally *et al*[34], who revealed that serum HA concentrations did not differ significantly among chronic hepatitis subtypes (HBV *vs* HCV), while its level correlates with the degree of fibrosis. This suggested that progression of liver fibrosis (and inflammation) was accompanied by impairment in the liver endothelial cell function and reduced degradation of this hetero-polysaccharide, eventually resulting in elevation of serum HA concentrations.

α-SMA is a reliable marker of hepatic stellate cell activation which precedes fibrous tissue deposition, and it can be used for identification of the earliest stage of hepatic fibrosis and for monitoring the efficacy of the therapy[35]. In the present study, the expression of α-SMA was detected by immunohistochemistry; it was activated in the fibrotic patients, and the lowest level was observed in the AHM group. These data were consistent with Dechene *et al*[36] who revealed significant increase in serum tissue inhibitors of metalloproteinases and MMP as well as histological evidence of collagen formation and α-SMA expression in acute liver failure patients. They demonstrated an ongoing profibrotic process together with an increased HSC activity. It is possible that a collagen matrix is synthesized and deposited as a structural framework to preserve the liver architecture. Acute liver fibrosis may serve as a part of beneficial wound healing process by transiently conserve the organ's structure until defective tissue areas are replaced by functional hepatocytes.

In conclusion, AHM has antifibrotic effects which could be attributed to its ability to attenuate oxidative stress, and enhance the collagenolytic activity. This study provides evidences that AHM could be used as adjunct treatment to prevent or treat hepatocellular damage in hepatic fine periportal fibrosis.

COMMENTS

Background

Acute and chronic liver diseases constitute a global concern, but medical

treatment up to now has limited efficacy. The therapeutically effective herbal medicine from documented medicinal plants may reduce the risk of drug toxicity. Aloe vera gel has been demonstrated to have liver protective effect in rats, and many toxicity studies have been conducted to determine the LD50 of aloe vera. This study investigated the antifibrotic effects of aloe vera for patients with acute liver fibrosis.

Research frontiers

Various *in vitro* studies have been conducted in an attempt to restore the integrity of damaged hepatocytes and reveal the hepatoprotective role of aloe vera in hepatic fibrosis. This study was undertaken to evaluate the antifibrotic effect of aloe vera in patients with hepatic fine periportal fibrosis and its mechanism of action.

Innovations and breakthroughs

The antifibrotic effect of aloe vera high molecular weight fractions (AHM) is attributed to its ability to attenuate oxidative stress, and enhance the collagenolytic activity. In the light of the potential of aloe vera plant extract, the discoveries of novel low-cost drug of natural non-toxic origin are promising for developing countries.

Applications

This study provides evidences that AHM could be used as adjunct treatment to prevent or treat hepatocellular damage in hepatic fine periportal fibrosis.

Peer review

The study is interesting and goes along with previous studies that showed hepatoprotective effect of aloe vera extracts in experimental models.

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ORIGINAL ARTICLE

Transactivation of the TIEG1 confers growth inhibition of transforming growth factor-β-susceptible hepatocellular carcinoma cells

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Abstract

AIM: To investigate the role of transforming growth factor (TGF)-β-inducible early gene 1 (TIEG1) in TGF-β-induced growth inhibition in hepatocellular carcinoma (HCC) cells.

METHODS: Human hepatocyte and HCC cell lines with varied susceptibilities to TGF-β1 were tested by methylthiazoletetrazolium (MTT) assay. The expression changes of *Smad2*, *Smad3*, *Smad4*, *Smad7*, *TIEG1* and *TIEG2* gene following treatment with TGF-β1 in a TGF-β-sensitive hepatocyte cell line (MIHA), a TGF-β-sensitive hepatoma cell line (Hep3B) and two TGF-β-insensitive hepatoma cell lines (HepG2 and Bel7404) were examined. SiRNA targeting TIEG1 was transfected into Hep3B cells and the sensitivity of cells to TGF-β1 was examined. Overexpression of TIEG1 was induced by lentiviral-mediated transduction in TGF-β1-resistant hepatoma cell lines (Bel7404 and HepG2). MTT assay and 4',6-Diamidino-2-phenylindole staining were used to identify cell viability and apoptosis, respectively. The expression level of stathmin was measured by reverse transcriptase polymerase chain reaction and Western-blotting analysis, and stathmin promoter activity by TIEG1 was monitored by a luciferase reporter gene system.

RESULTS: TIEG1 was significantly upregulated by TGF-β1 in the TGF-β1-sensitive HCC cell line, Hep3B, but not in the resistant cell lines. The suppression of TIEG1 by siRNAs decreased the sensitivity of Hep3B cells to TGF-β1, whereas the overexpression of TIEG1 mediated growth inhibition and apoptosis in TGF-β1-resistant HCC cell lines, which resembled those of TGF-β1-sensitive HCC cells treated with TGF-β1. Our data further suggested that stathmin was a direct target of TIEG1, as stathmin was significantly downregulated by TIEG1 overexpression, and stathmin promoter activity was inhibited by TIEG1 in a dose-dependent manner.

CONCLUSION: Our data suggest that transactivation of TIEG1 conferred growth inhibition of TGF-β-susceptible human HCC cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer death worldwide, and there are few effective therapeutic options available for those suffering from advanced disease[1]. HCC poses a major challenge because of its clinical heterogeneity and lack of good diagnostic markers and treatment strategies[2]. Transforming growth factor-β (TGF-β) is a multifunctional cytokine which regulates cell proliferation, migration and differentiation[3]. TGF-β has been shown to inhibit cell proliferation and to induce apoptosis to control excessive growth of hepatocytes and maintain liver size, and is considered a liver tumor suppressor[4]. However, many HCC cells are thought to have lost their sensitivity to TGF-β, and thus escape the antiproliferative effect of TGF-β[4,5]. The loss of TGF-β activities resulting in hyperproliferative disorders and cancer in the liver and derangement of TGF-β signaling are associated with an increased incidence of HCC[6]. TGF-β has shown dual effects on tumors, in that it can either be pro- or anti-tumorigenetic, depending on the stage of tumorigenesis and the responsiveness of the tumor cells[7]. Various studies have shown that TGF-β signaling may suppress human hepatocarcinogenesis, possibly *via* cyclin D1 deregulation[8], and that TGF-β could serve as a potential senescence inducer in HCC cells and thereby inhibit tumor growth *in vivo*[9]. However, in advanced cancers, TGF-β has been found to be a tumor enhancer because it can promote tumor progression by facilitating tumor invasion, neoangiogenesis, and immunosuppression[10].

The TGF-β-inducible early gene 1 (*TIEG1*) can be activated at the initial stage of the TGF-β pathway. Previous studies have shown that TIEG1 has an important role in regulating cell growth[11,12]. This gene is classified as a member of the Krüppel-like family of transcription factors (KLF10), all of which bind to GC-rich Sp1-like binding sites to regulate gene transcription[12]. In addi-tion, TIEG1 is also regarded as a potent transcriptional repressor. The overexpression of TIEG1 has been found to induce apoptosis in pancreatic cancer cells, and this indicates that it has a pivotal role in mediating TGF-β-induced apoptosis[13,14]. In addition, TIEG1 was found to induce apoptosis *via* a mechanism which involves the formation of reactive oxygen species[15]. The transcription level of TIEG1 was found to be predominantly expressed in various tissues, and the associated levels were regulated by cytokines and growth factors[12]. It is well known that different HCC cell lines respond differently to TGF-β treatment, and that some HCC cell lines are sensitive to TGF-β, whereas others are resistant. However, the molecular mechanism underlying the differential responses has never been elucidated. In the present study, we investigated the role of TIEG1 in TGF-β-induced growth inhibition in HCC cells. We deduced that transactivation of the TIEG1 conferred growth inhibition of TGF-β-susceptible human HCC cells.

MATERIALS AND METHODS

Cell culture

Immortalized human hepatocyte (MIHA), HCC cell lines (HepG2, Hep3B, Bel7404, Huh-7, and PLC), and human HEK293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, United States). All cultures were maintained in a humidified 37 ℃ incubator with 5% CO_2.

TGF-β1 treatments

Prior to treatment with TGF-β1, the cells were seeded, allowed to attach for 24 h and then starved in a serum-free medium for another 24 h. The cells were then treated with 5 ng/mL TGF-β1 (R&D System, Minneapolis, MN, United States) for the indicated time periods. Cell survival and changes in nuclei morphology were respectively monitored using methylthiazoletetrazolium (MTT) assays and 4',6-Diamidino-2-phenylindole (DAPI) staining[13] after 96 h of treatment.

Reverse transcriptase-polymerase chain reaction

For gene response studies, the total RNA was extracted using TRIZOL (Invitrogen), and then cDNA synthesis was performed using the Superscript First-Strand Synthesis Kit (Promega, United States)[13]. The mRNA levels of genes were determined by quantitative real-time polymerase chain reaction (PCR) or semi-quantitative reverse transcriptase (RT)-PCR[13]. The following forward and reverse primers were used respectively: TIEG1: 5'-GT-CACATCTGTAGCCACCCA-3' and 5'-CCTCCTTTCA-CAACCTTTCC-3'; TIEG2: 5'-TCTGACTCTGGGGAT-GTCAC-3' and 5'-CGGCAATCTGGAGTCTGGA-3'; Smad2: 5'-GCCACGGTAGAAATGACAAG-3' and 5'-CAGACTGAGCCAGAAGAGCA-3'; Smad3: 5'-GAACGGGCAGGAGGAGAAAT-3' and 5'-ACAG-GCGGCAGTAGATGACA-3'; Smad4: 5'-CCATTTC-

CAATCATCCTGCT-3' and 5'-ACCTTTGCCTATGTG-CAACC-3'; Smad7: 5'-CTTAGCCGACTCTGCGAACT-3' and 5'-CCCAGGCTCCAGAAGAAGTT-3'; Stathmin (STMN): 5'-TTTTCAATCCCAATTCTGTC-3' and 5'-GAAAGTAACAGCTGACCTGG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control): 5'-CCAGCCGAGCCACATCGCTC-3' and 5'-AT-GAGCCCCAGCCTTCTCCAT-3'. Quantitative real-time PCR was performed using SYBRs GREEN PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) and an ABI 7500 Real-time PCR system. The relative amount of mRNA expression was normalized based on the expression of human GAPDH. Each of the normalized gene expression values was calibrated to the normalized gene expression of cells with TGF-β1 treatment at time zero. The experiments were repeated thrice to build a geometric mean. Alternatively, RT-PCR was employed for semi-quantitative analysis of gene expression levels. GAPDH acted as the internal control[13].

siRNA transfection

For siRNA transfection, Hep3B cells were transfected with 50 pmol control siRNA or TIEG1 siRNA (Santa Cruz) using oligofectamime (Invitrogen). A second identical transfection was carried out 24 h later. Seventy-two hours after the first transfection, the total RNA was extracted and real-time RT-PCR was performed to evaluate the downregulatory effects. Moreover, 5×10^3 cells were seeded onto 96-well plates and transfected with siRNA twice and then treated with 5 ng/mL TGF-β1 for 72 h. MTT assay was then performed to determine the changes in cell growth.

Lentiviral transduction

Lentiviral vectors expressing TIEG1 were constructed, as previously described[13]. The VSV-G pseudotyped lentiviruses were produced by cotransfecting 293T cells with the transfer vector and three packaging vectors. The cells were transduced with lentivirus, as described[13].

Western blotting

The SDS-PAGE and Western blotting analysis were performed, as previously described[13]. The primary antibodies used were polyclonal antibodies against TIEG1 (sc-23159; Santa Cruz) and Actin (80-50; Abcam).

Luciferase reporter assays

The promoter of STMN was constructed into the pGL3-basic luciferase reporter vector (Promega, Madison, WI, United States) using primer: F-Kpn I : 5'-CCGG-TACCTCTAAGGCACGGTCAGACCA-3', R-Bgl Ⅱ: 5'-CGCAGATCTCCTGACCACACTCTGAGC-3'. For the luciferase assay, 293T cells were co-transfected with pGL3-promoter-STMN vector, a renilla plasmid, along with different amount of TIEG1-expressed construct or pEGFP-N1 vector. Cell extracts were lysed 48 h post-transfection and assayed for luciferase activities using the Dual-Luciferase Reporter Assay System (Promega).

Figure 1 Susceptibilities of various human hepatocyte (MIHA) and hepatocellular carcinoma cells (Hep3B, PLC, Huh7, HepG2, and Bel7404) to transforming growth factor-β**.** A: Cells were treated with 5 ng/mL transforming growth factor (TGF)-β1 for 96 h and cell survival was then determined by methylthiazoletetrazolium assays; B: Nuclear morphology of apoptotic TGF-β-treated Hep3B cells demonstrated by 4',6-Diamidino-2-phenylindole staining and examined by fluorescence microscopy. Scale bar: 50 μm, 400 ×.

Statistical analysis

Data are expressed as the mean ± SD error of the mean. Statistical differences between groups were compared using the Student's *t* test. *P* values less than 0.05 were considered significant.

RESULTS

Susceptibilities of human hepatocyte and HCC cell lines to TGF-β1

The cell proliferation inhibitory effect of TGF-β1 on various cell lines was evaluated by MTT assay (Figure 1A). TGF-β1 exerted the highest inhibitory effect on Hep3B (> 80%), a known TGF-β-sensitive hepatoma cell line, exhib-

Figure 2 Differential responses of TIEGs and Smads in various TGF-β1-treated cells. Cells were treated with 5 ng/mL TGF-β1 for up to 4 h. At indicated time intervals, the mRNA levels of TIEG1, TIEG2, Smad2, Smad3, Smad4, and Smad7 were determined by quantitative real-time RT-PCRs. Data were presented as mRNA levels relative to that of time 0 from three independent experiments.

ited a moderate inhibition (approximately 60%) in MIHA cells, a TGF-β-sensitive hepatocyte cell line, and an inhibition rate of approximately 60% was observed in HCC PLC cells. In addition, TGF-β1 exerted only marginal inhibitory effects on Huh7 cells (approximately 30%). Lastly, HepG2 and Bel7404 cells exhibited resistance to growth inhibition by TGF-β1 and completely lost their sensitivity to TGF-β. Using DAPI staining and subsequent fluorescence it was revealed by microscopic examination that the growth inhibitory effect of TGF-β1 on Hep3B cells was *via* the induction of apoptosis (Figure 1B).

Differential responses of TIEGs and Smads in various TGF-β1-treated cells

Firstly, we employed semi-quantitative PCR analysis to determine the basal mRNA level of eight different genes,

namely, the TGF-β1 receptor 1, the TGF-β1 receptor 2, Smad2, Smad3, Smad4, Smad7, TIEG1 and TIEG2, which are involved in the TGF-β signaling pathway in all the cell lines studied. We found no correlation between the expression of TGF-β-related genes and their sensitivity to TGF-β. All interested genes were present in all cell lines at slightly varied levels (data not shown). Because there was no correlation between the static expression of the TGF-β-related genes and the sensitivity of different HCC cells to TGF-β, we next examined the expression changes of these genes after they had been treated with TGF-β1 in a TGF-β-sensitive hepatocyte cell line (MIHA), a TGF-β-sensitive hepatoma cell line (Hep3B) and two TGF-β-insensitive hepatoma cell lines (HepG2 and Bel7404). Figure 2 shows the relative changes in expression of various genes in response to TGF-β1 over time. The TIEG1 was

Figure 3 Downregulation of transforming growth factor-β-inducible early gene 1 by RNA interference (siRNA) decreased the transforming growth factor-β1 susceptibility of Hep3B cells. A: Decreases in the transforming growth factor (TGF)-β-inducible early gene 1 (TIEG1) mRNA level in siRNAs targeting TIEG1 (siTIEG1)-transfected Hep3B cells with or without TGF-β1 treatment. Cells were transfected with siTIEG1 prior to the TGF-β1 treatments described above. After 4 h of treatment, mRNA levels of TIEG1 were determined by quantitative reverse transcriptase-polymerase chain reaction; B: Increases in the survivability of the siTIEG1-transfected and the TGF-β1-treated Hep3B cells. The decreased growth inhibitory response of Hep3B cells to TGF-β1 treatment was determined by methylthiazoletetrazolium assays after 72 h of TGF-β1 treatment. [a]$P < 0.05$.

sharply upregulated as early as 30 min after TGF-β1 treatment in MIHA and Hep3B cells, which were sensitive to TGF-β. One hour after treatment, TIEG1 was upregulated seven-fold in MIHA and more than ten-fold in Hep3B cells, respectively. However, there was a slight increase in TGF-β-insensitive cells (HepG2 and Bel7404). TIEG1 mRNA levels were more potently induced by TGF-β1 in TGF-β1-sensitive cell lines (Hep3B and MIHA) than in TGF-β1-insensitive cell lines (HepG2 and Bel7404).

We also observed that the *Smad3* gene was upregulated in TGF-β-sensitive cells after TGF-β1 treatment for 4 h, and that there was no significant change in insensitive hepatoma cells. However, the upregulation of TIEG1 expression appeared earlier than upregulation of the *Smad3* gene in TGF-β-sensitive cells. In the case of another gene, namely Smad7, the expression was sharply upregulated after treatment in TGF-β-sensitive cells and weakly upregulated in insensitive hepatoma cells. There was no significant change in *TIEG2*, *Smad2* and *Smad4* gene expression after TGF-β1 treatment. Smad7 is thought to be a TGF-β-inducible antagonist of TGF-β signaling[16], and there are autoregulatory negative-feedback signals in the signal transduction of the TGF-β superfamily[17]. These data imply that the *TIEG1* gene might play a critical role in TGF-β-mediated growth inhibition of HCC cells.

siRNA targeting TIEG1 decreased TGF-β susceptibility of Hep3B cells
To study the role of TIEG1 in TGF-β induced growth inhibition in HCC cells, we used siRNA to target TIEG1 in the TGF-β-sensitive hepatoma cell line Hep3B. The siRNA targeting significantly decreased the mRNA expression of TIEG1 with or without TGF-β1 treatment (Figure 3A), and consequently increased the survival rate of the cells after treatment with TGF-β1 for 72 h (Figure 3B).

Overexpression of TIEG1 by lentiviral-mediated transduction inhibited cell growth and induced apoptosis in TGF-β1-resistant hepatoma cells
As seen in Figure 4, the overexpression of TIEG1 was

successfully induced by lentiviral-mediated transduction in the TGF-β1-resistant hepatoma cell lines (Bel7404 and HepG2) both at transcription (Figure 4A) and translational levels (Figure 4B). MTT assay revealed a very significant inhibitory effect on growth of the two cell lines after the induction of TIEG1 by the lentivirus (Figure 4C). DAPI staining demonstrated a significantly higher amount of apoptotic cells in the two HCC cell lines after the overexpression of TIEG1 (Figure 4D).

Transcriptional regulation of TIEG1 on STMN by binding on STMN promoter
In the present study, the correlation between TIEG1 and STMN was also evaluated. Overexpression of TIEG1 was found to decrease STMN expression at both the transcription and translational levels (Figure 5A and B). STMN promoter activity was reduced in a dose-dependent manner with the induction of *TIEG1* gene (Figure 5C).

DISCUSSION
The present study determined the molecular mechanism underlying the differential susceptibilities of HCC cells to TGF-β treatment. In this study, one TGF-β-sensitive hepatocyte cell line (MIHA) and five TGF-β-various-sensitive hepatoma cell lines were investigated. In general, treatment with TGF-β1 significantly inhibited the growth of the two TGF-β-sensitive cell lines (Hep3B and MIHA). However, HepG2 and Bel7404 cells did not respond to TGF-β1. Taken together, treatment with TGF-β1 caused varied levels of inhibition in different cell lines. Some HCC cell lines were sensitive to TGF-β, whereas others were resistant. Recently, studies on the relationship between HCC and the TGF-β signaling pathway have been extensive. One recent study has reported a negative relationship between interleukin-6, a major stem cell signaling pathway, and the TGF-β signaling pathway in human HCC[18]. TGF-β signaling and Smad adaptor embryonic liver fodrin could suppress HCC *via* cyclin D1 deregulation[8]. Another study showed

Figure 4 The overexpression of transforming growth factor-β-inducible early gene 1 by lentiviral-mediated transfection-induced growth inhibition and apoptosis in transforming growth factor-β1-resistant hepatoma Bel7404 and HepG2 cells. A and B: Increases in mRNA and protein levels of transforming growth factor (TGF)-β-inducible early gene 1 (TIEG1) in Bel7404 and HepG2 cells transfected by lenti-TIEG1, respectively, revealed by reverse transcriptase-polymerase chain reaction and Western blotting analysis; C: The overexpression of TIEG1 by lentiviral-mediated transfection-inhibited cell growth in Bel7404 and HepG2 cells. Cell survival was determined by methylthiazoletetrazolium assays; D: Induction of apoptosis in Bel7404 and HepG2 cells after lenti-TIEG1 transfection for 72 h as shown by 4',6-Diamidino-2-phenylindole staining. Scale bar: 50 μm, 400 ×.

Figure 5 Transcriptional regulation of STMN (stathmin) by transforming growth factor-β-inducible early gene 1. A and B: Decreases in mRNA and protein levels of STMN in Bel7404 and HepG2 cells transfected by lenti-transforming growth factor-β-inducible early gene 1 (TIEG1), revealed by reverse transcriptase-polymerase chain reaction and Western blotting analyzes; C: Dose-dependent suppression of STMN promoter activity by TIEG1 monitored by a luciferase reporter gene system. Control: pEGFP-N1.

that human HCC cells could be protected by interleukin-4 from TGF-β-induced apoptosis, which indicated another therapeutic option by the targeting of interleukin-4[19]. Lost sensitivity to TGF-β has been postulated to be an early event in HCC development[18,20].

In the present study, the role of TIEG1 in TGF-β-induced growth inhibition was analyzed in established TGF-β-sensitive and -insensitive cell systems. Our studies revealed that TIEG1 mRNA was dramatically upregulated by TGF-β1 in TGF-β1-sensitive cell lines but not in resistant cell lines. However, expression of the endogenous TIEG1 protein was not detected in all cell lines (namely, Hep3B, MIHA, Bel7404, and HepG2) before and after the TGF-β1 treatment. One of the reasons for this could be that the amounts of endogenous TIEG1 protein in the cells were too low to be detected. We found that the induction of TIEG1 was transient and occurred before the phenomenon of cell growth inhibition. The time course for the induction of TIEG1 expression was similar to that found in human osteoblast cells[21] and

pancreatic epithelial cells[22] following TGF-β1 treatment. Although TIEG1 induction was transient following TGF-β1 treatment, it might participate in the TGF-β1 signaling processes or amplify the TGF-β1 signaling events that inhibited cell growth.

The suppression of TIEG1 by siRNAs decreased the sensitivity of Hep3B cells to TGF-β1, whereas the overexpression of TIEG1 mediated growth inhibition and apoptosis in TGF-β1-resistant HCC cell lines (HepG2 and Bel7404), which resembled those of TGF-β1-sensitive HCC cells treated with TGF-β1. This indicated the pivotal role of TIEG1 in TGF-β1-induced growth inhibition in HCC. In a previous study, the overexpression of TIEG1 was shown to inhibit cell proliferation and growth in TGF-β-sensitive Hep3B cells[15] and in pancreatic carcinoma cell lines[13,22]. Also, TIEG1 plays a role in TGF-β-induced inhibition of cell proliferation and apoptosis in human osteoblast cells[12]. Nevertheless, our data indicated that TIEG1 overexpression alone was capable of inducing sufficient inhibition or apoptosis in HCC tumor cells, despite the cells' sensitivity to TGF-β. We also found that TIEG1 was identified as a transcriptional repressor of STMN, as the mRNA expression and the promoter activity of STMN were significantly reduced in the presence of overexpressed TIEG1. Bioinformatics analysis revealed several Sp1-binding sites in the promoter region of STMN. TIEG1 regulates STMN transcription by binding to the STMN promoter. These findings indicate the pivotal role of STMN in promoting tumor cell survival which has also been reported elsewhere[23-25]. Various strategies have been suggested to target the expression of STMN for treating tumors, including prostate, cervical[26] and breast cancer[27]. In pancreatic carcinoma cells, we have revealed that overexpression of TIEG1 could induce cell growth inhibition and promote gemcitabine chemosensitivity through downregulation of STMN[13]. In this study, the data again suggest a pivotal role for STMN (i.e., downregulation) in diminishing HCC proliferation, or in facilitating tumor cell apoptosis.

Taken together, these results demonstrate that TIEG1 is involved in TGF-β1-mediated growth inhibition. Transactivation of TIEG1 conferred growth inhibition of TGF-β-susceptible human HCC cells. However, it should be noted that this study was based on *in vitro* investigations and *in vivo* models should be explored.

COMMENTS

Background
Transforming growth factor-β (TGF-β) has been shown to inhibit cell proliferation and to induce apoptosis to control excessive growth of hepatocytes and maintain liver size, and is considered a liver tumor suppressor. However, many hepatocellular carcinoma (HCC) cells are thought to have lost their sensitivity to TGF-β, and thus escape the antiproliferative effect of TGF-β. Lost sensitivity to TGF-β has been postulated to be an early event in HCC development. The reasons why some HCC cells are sensitive yet others are resistant to TGF-β mediated growth inhibition are still poorly understood.

Research frontiers
The TGF-β-inducible early gene 1 (*TIEG1*) can be activated at the initial stage of the TGF-β pathway. Recent reports have highlighted the importance of

TIEG1 in the TGF-β signaling pathway and in regulating cell growth. In the present study, the authors demonstrated the role of the *TIEG1* gene in TGF-β-induced growth inhibition in HCC cells.

Innovations and breakthroughs

This study indicated the TIEG1 was significantly upregulated by TGF-β1 in the TGF-β1-sensitive HCC cell line, Hep3B, but not in the resistant cell lines. The suppression of TIEG1 by siRNAs decreased the sensitivity of Hep3B cells to TGF-β1, whereas the overexpression of TIEG1 mediated growth inhibition and apoptosis in TGF-β1-resistant HCC cell lines, which resembled those of TGF-β1-sensitive HCC cells treated with TGF-β1. The studies suggest that transactivation of TIEG1 conferred growth inhibition of TGF-β-susceptible human HCC cells.

Applications

By understanding the molecular mechanism underlying the differential susceptibility of HCC cells to TGF-β, this study may provide new molecular targets for therapeutic intervention in HCC.

Peer review

This paper deals with the transactivation of the TIEG1 in growth inhibition of TGF-β-susceptible HCC cells. The authors aimed to investigate the role of TIEG1 in TGF-β-induced growth inhibition in HCC. They found that transactivation of the TIEG1 conferred growth inhibition of the TGF-β-susceptible human HCC cells. The results are interesting and the design of this study is appropriate.

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ORIGINAL ARTICLE

Aberrant methylation of *SPARC* in human hepatocellular carcinoma and its clinical implication

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Abstract

AIM: To investigate the methylation status of secreted protein acidic and rich in cysteine (*SPARC*) in human hepatocellular carcinoma (HCC) and evaluate its clinical implication.

METHODS: The methylation status of *SPARC* was analyzed in one HCC cell line (SMMC-7721) and 60 pairs of HCC and corresponding nontumorous tissues by methylation-specific polymerase chain reaction and bisulfite sequencing. The expression of *SPARC* mRNA and protein were examined by reverse transcription polymerase chain reaction and immunohistochemistry, respectively. The correlations between the methylation status and the gene expression, the clinicopathological parameters, as well as the prognosis after surgery were analyzed.

RESULTS: In the SMMC-7721 cell line, the loss of *SPARC* expression was correlated with the aberrant methylation and could be reactivated by the demethylating agent 5-aza-2'-deoxycytidine. Methylation frequency of *SPARC* in HCC was significantly higher than that in the corresponding nontumorous tissues (45/60 *vs* 7/60, $P < 0.001$), and it was correlated with the pathological classification ($P = 0.019$). The downregulation of the *SPARC* mRNA expression in HCC was correlated with the *SPARC* methylation ($P = 0.040$). The patients with methylated *SPARC* had a poorer overall survival than those without methylated *SPARC* (28.0 mo *vs* 41.0 mo, $P = 0.043$).

CONCLUSION: Aberrant methylation is an important mechanism for *SPARC* inactivation in HCC and *SPARC* methylation may be a promising biomarker for the diagnosis and prognosis of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most

common malignancies and the third leading cause of cancer death in the world[1,2]. To date, surgical resection is still considered the most important treatment for patients with resectable HCC[3]. Unfortunately, most patients are at inoperable stages when the tumor is diagnosed[4]. In addition, the high incidence of tumor recurrence after curative resection also leads to poor clinical outcomes[5,6]. Therefore, the development of biomarkers for early diagnosis and accurate prognosis of HCC is valuable for improving patients' survival.

Although the detailed molecular mechanisms of hepatocarcinogenesis remain largely unclear, the accumulating evidences have shown that aberrant methylation of promoter CpG islands causes inactivation of tumor suppressor genes, which is involved in the occurrence and development of HCC[7-10]. Detections of such an aberrant DNA methylation of tumor suppressor genes could be used as a diagnostic or a prognostic marker for HCC.

Secreted protein acidic and rich in cysteine (*SPARC*) is a matricellular glycoprotein involved in some biological processes, including tissue remodeling, angiogenesis, extracellular matrix production and so on[11-13]. It has been reported that *SPARC* has tumor suppressing properties to various cancers, such as ovarian cancer and pancreatic cancer[14-16]. Moreover, *SPARC* is epigenetically silenced through promoter hypermethylation in these cancers, and the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR) can rescue *SPARC* expression[17-20]. The *SPARC* promoter methylation is an important factor in the carcinogenesis of these cancers and may be a promising epigenetic marker for them. However, up to date, there have been few reports about the methylation status in HCC.

In this study, in order to explore the status of *SPARC* methylation in HCC, we examined the methylation and expression of *SPARC* in HCC cell line and tissues. We correlated the methylation status with clinicopathologic features and evaluated whether the methylation of *SPARC* can serve as a potentially diagnostic or prognostic biomarker for HCC.

MATERIALS AND METHODS

Cell line and patient samples

The SMMC-7721 cell line used in this study was obtained from the Shanghai Institute of Cell Biology (Shanghai, China). HCCs and their corresponding nontumorous tissues were obtained from 60 patients who were diagnosed and treated at the Department of Hepatobiliary Surgery, Tianjin Third Central Hospital in China from October 2003 to June 2008. This study protocol was approved by the Clinical Research Ethics Committee of our institution and the informed consent was obtained from each of these patients. After surgical resection, samples were immediately stored in the liquid nitrogen for later analysis. For the gene expression analysis, the hematoxylin-eosin-stained samples from each tumor block were examined microscopically to confirm

the presence of more than 80% tumor cells. The nontumorous samples from each patient were also microscopically confirmed.

Cell culture and 5-Aza-CdR treatment

SMMC-7721 cells were grown in DMEM supplemented with 100 g/L fetal bovine serum and incubated in 37 ℃ and 50 mL/L CO$_2$. For the 5-Aza-CdR (Sigma, St Louis, MO, United States) treatment, cells were split to 5×10^5 per 75-cm^2 culture bottle and incubated overnight in the growth media. The normal growth media was replaced with the growth media supplemented with 5-Aza-CdR (10 μmol as a final concentration) for 6 d with the media change on day 4. Cells cultured with vehicle alone served as 5-Aza-CdR negative control. After the culture, cells were harvested for the extraction of genomic DNA and total RNA. In order to detect the *SPARC* protein in different groups by immunocytochemical staining, SMMC-7721 cells were also seeded onto 6-well plates containing coverslips to induce cells to spread and adhere to the glass.

DNA extraction and bisulfite treatment

The genomic DNA was extracted from the cell line and tissue samples by digesting with sodium dodecyl sulfate/proteinase K in Tris ethylenediamine tetraacetic acid (TE) buffer followed by a standard phenol/chloroform extraction. The extracted DNA was subjected to the bisulfite treatment as previously described[21-23]. Briefly, 1-2 μg genomic DNA was denatured with 0.3 mol/L NaOH at 37 ℃ for 20 min, and incubated in 3.0 mol/L sodium bisulfite and 10 mmol/L hydroquinone at 55 ℃ for 16 h. The DNA was desalted with a QIAquick gel extraction kit (Qiagen, Valencia, CA, United States) and dissolved in 50 μL of 10 mmol/L TE buffer (pH 8.0). Then, 5.5 μL of 3.0 mol/L NaOH was added and incubated at 37 ℃ for 20 min to desulfonate it. The modified DNA was neutralized with 30 μL of 10 mol/L ammonium acetate, precipitated using 2 volumes of ethanol, and resuspended in 40 μL of 1.0 mmol/L TE buffer (pH 7.6).

Methylation specific polymerase chain reaction and sequencing

Methylation specific polymerase chain reaction (MSP) was performed to examine the methylation status at CpG island of *SPARC* promoter in both SMMC-7721 cells and tissue samples. The primers used in this study for polymerase chain reaction (PCR) are shown in Table 1. A PCR mixture contained 1 × PCR buffer (10 mmol/L Tris, 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$ and 10 mmol/L β-mercaptoethanol), deoxynucleotide triphosphates (each at 0.2 mmol/L), primers (10 pmol each), bisulfite-modified DNA templates (2 μL) and 1 U of Taq polymerase, and the final volume was 25 μL. The PCR conditions were as follows: 94 ℃ for 2 min; then 40 cycles of 94 ℃ for 30 s, at optimum annealing temperature for 30 s and 72 ℃ for 30 s; and final extension for 5 min at 72 ℃. The normal leukocyte DNA methylated *in vitro* with SssI

Table 1 Primer sequences for polymerase chain reaction

Gene	Primer sequences (forward/reverse 5'-3')	Accession No.	Location to transcription start	Product size (bp)	Annealing temperature (℃)
SPARC methylation	GAGAGCGCGTTTTGTTTGTC	NM_003118.2	+52 to +71	112	54
	AACGACGTAAACGAAAATATCG		+142 to +163		
SPARC unmethylation	TTTTTTAGATTGTTTGGAGAGTG	NM_003118.2	+36 to +58	132	59
	AACTAACAACATAAACAAAAATATC		+143 to +167		
SPARC BS	GATAGAGATAGTTTTGGTTATGGGA	NM_003118.2	-119 to -95	401	55
	CCACCTTCTAAAAAACA ACAAAC		+260 to +282		
SPARC mRNA	CGCATGCGGGACTGGCTCAA	NM_003118.2	+601 to +620	148	60
	GCTCCACGGGG TGGTC TCCT		+729 to +748		
GAPDH mRNA	GGGCATCCTGGGCTACACTGA	NM_002046.3	+915 to +935	143	58
	CAAATTCGTTGTCATACCAGGAAATG		+1032 to +1057		

SPARC: Secreted protein acidic and rich in cysteine; BS: Bisulfite sequencing; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase.

methyltransferase (New England Biolabs Inc., Beverly, MA, United States) was used as the positive control of methylation, and the normal leukocyte DNA was used as the negative control. The distilled water without template DNA was used as a blank control for all tests. Five microliters of PCR products underwent electrophoresis on 25 g/L agarose gel, and was visualized under ultraviolet illumination with the ethidium bromide staining. To verify the accuracy of MSP, the PCR products of both methylation and unmethylation were randomly chosen and cloned into the pMD-18-T vector (TaKaRa, Dalian, China) followed by a sequencing analysis.

To investigate the status of CpG sites in the region of *SPARC* promoter of SMMC-7721 cells, bisulfite sequencing analysis was performed for the bisulfite-treated DNA. The PCR products were cloned into a pMD-18-T vector and 8 individual clones of each group were sequenced.

RNA preparation and reverse transcription-PCR

RNA was extracted from the cell line and tissues using the Trizol (Tiangen, Beijing, China) according to the manufacturer's instructions. The total mRNA was digested with the DNase I (Ambion, Austin, TX, United States) to remove the genomic DNA contamination and then subjected to reverse transcription using the reverse transcription system (Promega, Madison, WI, United States). *SPARC* expression of SMMC-7721 cells and tissues were tested by reverse transcription (RT)-PCR and quantitative RT-PCR, respectively. Real-time quantitative RT-PCR was done on the ABI Prism 7000 sequence detection system in combination with the SYBR green real-time PCR master mix (Toyobo, Shanghai, China). The PCR amplification was carried out for 2 min at 94 ℃ for the initial denaturation, followed by 35 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. Melting curve analyses following amplification were performed to assure the product specificity. The relative expression of *SPARC* mRNA was normalized to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) in the same cDNA using the comparative CT method. For the quantification of gene expression, the target gene (*SPARC*) value normalized to the expression of *GAPDH* was designated as ΔCT [ΔCT = CT (*SPARC*) - CT (*GAPDH*)]. The ΔCT for the nontumorous samples was then subtracted from the ΔCT for the tumorous samples to generate ΔΔCT [ΔΔCT = ΔCT (tumor) - ΔCT (nontumorous sample)]. The ΔΔCT measurement was used to calculate the relative expression ($2^{-\Delta\Delta CT}$).

Immunohistochemistry

The protein expression of *SPARC* was examined in 23 primary HCCs and the corresponding nontumorous tissues by immunohistochemistry. Sections (5 μm) from the tumor and nontumorous tissues were cut onto coated slides and deparaffinized by the routine techniques. The antigen retrieval was performed in 10 mmol sodium citrate buffer (pH 6.0), and heated at 95 ℃ for 10 min. After endogenous peroxidase activity was blocked with 30 g/L H_2O_2 for 5 min, the sections were incubated with an anti-*SPARC* monoclonal antibody at a 1:100 dilution (Santa Cruz Biotecfology, United States) overnight. Labeling was detected with the PV-9000 Kit (Zhongshan, Beijing, China), following the protocol afforded by the manufacturer, and all sections were counterstained with hematoxylin. Cytoplasm staining of more than 90% parenchyma cells (tumor cells or liver cells) was regarded as positive for *SPARC*.

Similarly, *SPARC* protein was also tested in SMMC-7721 cells growing on the coverslips by immunocytochemistry.

Analysis for clinicopathological data and statistics

The gene methylation status in HCC was evaluated in the correlation with the clinicopathological parameters of patients, including age, gender, tumor size, virus infection, liver function, tumor number, vascular infiltration, pathology class and the level of alpha-fetal protein (AFP). The Pearson χ^2 test or the Fisher's exact test was used to analyze associations between methylation frequencies and categorical variables. Disease free or overall survival was calculated from the date of the operation until tumor recurrence or death or the date of the last follow-up (censored). Survival was analysed by the Kaplan-Meier method, and differences in their distribution were evaluated by the log-rank test. A multivariate Cox's proportional-hazard model was developed to evaluate the covariates' joint effects. All *P* values were two-sided, and *P* value less

Figure 1 Secreted protein acidic and rich in cysteine methylation and expression in SMMC-7721 cell line. *SPARC*: Secreted protein acidic and rich in cysteine; Aza: 5-aza-2'-deoxycytidine; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase; M: Methylation; U: Unmethylation; L: 50 bp ladder; Scale bar: 50 μm.

Table 2 Methylation frequencies of secreted protein acidic and rich in cysteine in 60 cases			
	SPARC methylation status		
Tissue	Methylated (%)	Unmethylated (%)	*P* value
Tumorous	45 (75.00)	15 (25.00)	< 0.001
Nontumorous	7 (11.67)	53 (88.33)	

SPARC: Secreted protein acidic and rich in cysteine.

than 0.05 was defined as being statistically significant. Analyses were performed with SPSS V 13.0 software for Windows (SPSS, Chicago, United States).

RESULTS

Methylation status and expression of SPARC in SMMC-7721 cells

We used MSP to measure both methylated and unmethylated segments in the *SPARC* promoter region. The results demonstrated that only the methylated segment was detected in SMMC-7721 cells of the control group. However, both methylated and unmethylated segments were found in the cells after treated with 5-Aza-CdR. These results indicated that *SPARC* was homologously methylated in SMMC-7721 cells and 5-Aza-CdR could convert the methylation status of *SPARC*. RT-PCR revealed that the *SPARC* mRNA expression was absent in the cells without the 5-Aza-CdR treatment, however, the cells treated with the 5-Aza-CdR restored the *SPARC* mRNA expression. Consistently, the immunocytochemical analysis of the cultured cells displayed that the *SPARC* protein expression was restored in the cells previously lacking

of the *SPARC* expression. The concordance between the loss of gene expression and the aberrant methylation suggested that the DNA methylation played a causal role in the loss of the *SPARC* expression in SMMC-7721 cells. The representative results are shown in Figure 1.

The bisulfite sequencing displayed that the control cells were methylated at almost all the 18 CpG sites in the 8 clones. On the contrary, most of CpG sites were unmethylated in the cells treated with 5-Aza-CdR. Figure 2 shows the methylation pattern of the *SPARC* promoter in SMMC-7721 cells.

Frequent SPARC hypermethylation in human HCC

We used MSP to evaluate the *SPARC* methylation status of the CpG island in 60 pairs of tissues. Methylation alleles in 75.00% (45/60) of HCC samples were detected, however, only 11.67% (7/60) methylated alleles could be found in the corresponding nontumorous tissues. The methylation frequence of *SPARC* in HCC was significantly higher than that in noncancerous liver tissues (Table 2). If methylation was used as an indicator for distincting HCC from nontumorous tissues, the sensitivity, specificity and accuracy were 86.54%, 77.94% and 81.67%, respectively. To validate the accuracy of MSP, we randomly chose the PCR products of methylation or unmethylation for sequencing. The results were according to the PCR aim segments. The representative results of PCR and sequencing are demonstrated in Figure 3.

Correlation between SPARC methylation and mRNA expression

The expression of *SPARC* mRNA was examined in 60 pairs of HCC and nontumorous tissues by quantitative

Figure 2 Bisulfite sequencing of secreted protein acidic and rich in cysteine in SMMC-7721 cell line. *SPARC*: Secreted protein acidic and rich in cysteine; TSS: Transcription start site; BS: Bisulfite sequencing; M: Methylation; U: Unmethylation; 5-Aza-CdR: 5-aza-2'-deoxycytidine; 1-18: CpG sites; - 119 to - 95, + 260 to + 282, + 52 to + 71, + 142 to + 163, + 36 to + 58, + 143 to + 167: Polymerase chain reaction primers position; Black dots: Methylation; Blank rings: Unmethylation.

Figure 3 Representative results of methylation specific polymerase chain reaction analysis and sequencing in tissues. L: 50 bp ladder; PC: Positive control; NC: Negative control; BC: Blank control; M: Methylation; U: Unmethylation; T: Hepatocellular carcinoma tissue; NT: Nontumorous tissue.

RT-PCR. Most of primary HCC tissues (65.00%, 39/60) showed a lower expression level when compared with their corresponding nontumorous livers (Figure 4A). Moreover, the median of relative expression was statistically different between the methylated and unmethylated *SPARC* samples of HCC ($P = 0.040$) (Figure 4B). The methylated samples had a lower median of expression.

Methylation and protein expression

The protein expression of *SPARC* was examined in 23 pairs of HCC and nontumorous tissues by immunostaining. The positive frequency of tumor cells in HCC was relatively lower than that of liver cells in nontumorous tissues, but there was no statistical significance between two groups (Table 3). We divided all 46 samples into

Figure 4 Expression of secreted protein acidic and rich in cysteine mRNA in hepatocellular carcinoma. Horizontal lines represent the median, and range indicates a 25%-75% quartile. *SPARC*: Secreted protein acidic and rich in cysteine.

Table 3 Protein expression frequencies in 23 pairs of samples

| Tissue | *n* | Protein expression | | *P* value |
		Positive (%)	Negative (%)	
Tumorous	23	12 (52.2)	11 (47.8)	0.552
Nontumorous	23	14 (60.9)	9 (39.1)	

Table 4 Association of secreted protein acidic and rich in cysteine methylation with protein expression

| *SPARC* | *n* | Protein expression | | *P* value |
		Positive (%)	Negative (%)	
Methylated	14	6 (42.9)	8 (57.1)	0.216
Unmethylated	32	20 (62.5)	12 (37.5)	

SPARC: Secreted protein acidic and rich in cysteine.

methylation and unmethylation groups (14 *vs* 32). There was no statistical correlation between the methylation and the protein expression (Table 4). In some HCC samples, stromal cells around tumor cells showed a positive signal even if the parenchyma cells had no expression of *SPARC*. The representative immunohistochemical staining is shown in Figure 5.

Relationship between methylation and clinical data

We analyzed the association of *SPARC* methylation with clinicopathological parameters in patients with HCC.

Table 5 Correlation between methylation status and clinico-pathological data

Parameters	*n*	Methylated	Unmethylated	*P* value
Age (yr)				0.766
> 53	30	23	7	
≤ 53	30	22	8	
Gender				0.835
Male	51	38	13	
Female	9	7	2	
Tumor size (cm)				1.000
≤ 5	20	15	5	
> 5	40	30	10	
Virus infection				0.661
HBV or HCV	52	40	12	
Negative	8	5	3	
Liver function				1.000
Child-Pugh A	46	35	11	
Child-Pugh B	14	10	4	
AFP (μg/L)				0.125
≤ 400	35	29	6	
> 400	23	15	8	
Tumor number				0.174
Single	35	24	11	
Multiple	25	21	4	
Vascular invasion				0.122
Positive	22	19	3	
Negative	38	26	12	
Edmondson classification				0.019
I / II	21	12	9	
III / IV	39	33	6	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetal protein.

There was significant association between the methylation status and the pathological class. The *SPARC* methylation was more frequently observed in cases with a high pathologic grade (33 of 39, 84.6%) than in those with a low grade (12 of 21, 57.1%) (Table 5). However, there was no statistically significant correlation between the methylation status and other clinicopathologic factors.

Prognostic value of SPARC methylation in HCC

We also divided all cases into two groups according to the methylation status of *SPARC* to determine whether this factor had prognostic value. The disease free survival between the two groups had no statistical difference. Patients whose primary tumors exhibited *SPARC* methylation had a lower overall survival rate after resection (28.0 mo *vs* 41.0 mo, *P* = 0.043, Table 6 and Figure 6). Five clinicopathological factors and methylation status of *SPARC* found to be prognostic on the univariate analysis were entered into a multivariate model to identify independent predictors of overall survival. The Cox's multivariate proportional-hazard model indicated that the factors significantly affecting overall survival were tumor size, AFP level and *SPARC* methylation (Table 7).

DISCUSSION

In this current study, we determined the methylation status of *SPARC* gene promoter in SMMC-7721 cell line

Figure 5 Immunohistochemical analysis of secreted protein acidic and rich in cysteine expression. A: A tumor with positive staining; B: A tumor with negative result, but stromal tissues with positive signal (arrows); C: Nontumorous tissues with positive staining; D: Nontumorous tissues with negative staining; Scale bar: 50 μm.

Figure 6 Disease free (A) and overall (B) survival analysis of patients with different secreted protein acidic and rich in cysteine methylation status.

Table 6 Survival analysis of patients with different methylation status

Gene	M/U	*n*	Disease free survival				Overall survival			
			Estimate (mo)	Scope (mo)	Log-Rank	*P* value	Estimate (mo)	Scope (mo)	Log-Rank	*P* value
SPARC	M	37	15.0	9.6-20.4	2.094	0.148	28.0	17.8-38.2	4.096	0.043
	U	14	24.0	12.6-35.5			41.0	36.5-45.5		

SPARC: Secreted protein acidic and rich in cysteine; M: Methylation; U: Unmethylation.

and HCC tissues. The data suggested that in SMMC-7721 cell line, hypermethylation of the promoter was an important mechanism for *SPARC* downregulation, which was most likely involved in the development and progression of HCC. Moreover, the methylation frequency of *SPARC* was significantly higher in the HCC tissues than in the corresponding nontumorous tissues. The hypermethylation of *SPARC* was associated with pathological class and patients without *SPARC* methylation had higher rates of overall survival after resection. Our results showed that methylation of *SPARC* could be further evaluated as a tumor marker for the diagnosis and

Table 7 Cox regression model of overall survival

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Methylation						
Positive	2.672	0.999-7.147	0.044	3.207	1.290-7.975	0.012
Negative	1			1		
Tumor size (cm)						
> 5	5.293	1.560-17.959	0.008	8.045	2.125-30.456	0.002
≤ 5	1			1		
AFP (μg/L)						
> 400	3.306	1.421-7.694	0.006	7.105	1.798-28.080	0.005
≤ 400	1			1		
Age (yr)						
> 53	0.663	0.279-1.576	0.353			
≤ 53	1					
Gender						
Male	1.104	0.373-3.266	0.859			
Female	1					
Tumor number						
Multiple	3.330	1.440-7.704	0.005			
Single	1					
Vascular invasion						
Positive	2.776	1.186-6.502	0.019			
Negative	1					
Edmondson classification						
I / II	0.379	0.147-0.982	0.046			
III /IV	1					

AFP: Alpha-fetal protein; RR: Relative risk.

prognosis of HCC.

In some tumor cell lines, aberrant methylation of *SPARC* has been tested. Functional studies have shown that methylation of *SPARC* could induce gene silence and possess tumor suppressing effects[24-26]. Transcription factors were incapable of binding to the methylated DNA of their recognition sequences, therefore, the gene transcription was blocked[24,25]. However, the demethylating agent could convert the methylation status and restore the gene expression. *SPARC* involved in the occurrence and development of certain cancers[27-31]. In concordance with these studies, we observed that the loss of *SPARC* expression correlated with the aberrant methylation and this loss of expression could be rescued by the demethylating agent 5-Aza-CdR. These data suggested that hypermethylation of the promoter is also an important mechanism for *SPARC* inactivation in SMMC-7721 cell line. The results of our DNA bisulfite sequencing of the *SPARC* promoter also displayed that 5-Aza-CdR could convert the methylation status and affect the expression of *SPARC*.

We observed that *SPARC* methylation occurred more frequently in HCC tissues than in nontumorous tissues. We tested the same segments of putative CpG island near the transcription start site in HCC samples, and compared with the previous groups[15,32]. The results showed that *SPARC* methylation was also a relatively higher frequent incident in HCC and the sequencing results validated that there were high-density methylated CpG sites in the amplified region. The distinct methylation status of *SPARC* gene in the benign and malignant tissues was the prereq-

uisite to determine it as an effective molecular biomarker. *SPARC* could discriminate HCC from the nontumorous tissues with a high sensitivity and a specificity, suggesting that *SPARC* methylation may be a promising epigenetic biomarker for the assistant diagnosis of HCC.

In this study, we observed that 65.0% of the HCC samples showed a relatively lower expression level of *SPARC* mRNA compared with the nontumorous tissues. On the contrary, previous groups have reported that *SPARC* was overexpressed in HCC tissues as compared with the nontumorous tissues, nevertheless, *SPARC* mRNA and protein were mainly detected in the tumor capsule, and fibrous bands within HCC[26,33]. *SPARC* was strongly expressed by the stromal myofibroblasts of HCC[26]. In our study, except for different patient population, we used exclusively tumors with more than 80% of epithelial tumor cells to test the *SPARC* mRNA expression, which could minimise the potential contamination of stromal cells in HCC. Some studies in other cancers have revealed aberrant hypermethylation of the *SPARC* promoter to be responsible for low levels of *SPARC* expression[15,16]. In concordance with these studies, we found that the *SPARC* expression of samples with methylation was significantly lower than that without methylation. Although there were other possible mechanisms for the downregulation of the *SPARC* expression, the concordance between the mRNA expression and the DNA methylation indicated that the gene was downregulated, at least partially, through the DNA methylation in HCC. We found no significant correlation between the *SPARC* protein expression and the DNA methylation. The regulation of the translation process or the degradation of protein might also influence the *SPARC* protein abundance in HCC tissues. On the other hand, the *SPARC* protein might be variably expressed by the heterogeneous hypermethylation in one allele of tumor cells. But, interestingly, we also found the *SPARC* expression in the stromal cells in HCC even though the tumor cells had a negative signal, which was accordant with the report[33].

We demonstrated that the pathological class was the only clinicopathological variable associated with the *SPARC* methylation and patients with the *SPARC* methylation tended to have a poorer overall survival after resection in this study. It may be explained by the function of this gene, which was involved in the tumor progression. *SPARC* could inhibit the progress of tumor by restraining the angiogenesis and affecting the extracellular matrix production[34-36]. Our results suggested a potential clinical use of *SPARC* methylation as a prognostic marker in patients with HCC. Because *SPARC* methylation was a kind of DNA marker, it will be possible to detect the status of *SPARC* methylation in peripheral blood in the future, which might be more convenient and less traumatic than using the pathological tissues. However, since the number of patients in this study is relatively small, these findings need to be verified in a study with more patients and a longer follow-up period.

In conclusion, the results in this study indicated that

SPARC promoter hypermethylation in HCC was most likely related to a disease state, which may provide potential diagnostic or predictive markers of this disease.

COMMENTS

Background
Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. The development of biomarkers for early diagnosis and accurate prognosis of HCC is important for improving patients' survival. Aberrant DNA methylation of tumor suppressor genes could be used as a new marker for HCC in the future.

Research frontiers
It has been reported that secreted protein acidic and rich in cysteine (*SPARC*) has tumor suppressing properties to some cancers. Moreover, the *SPARC* promoter methylation is an important factor in the carcinogenesis of these cancers and may be a promising epigenetic marker for them. However, up to date, there have been few reports about the methylation status in HCC. In this study, the authors detected the status of *SPARC* methylation in HCC and estimated its clinical implication.

Innovations and breakthroughs
This is the first study to report that *SPARC* hypermethylation is a high frequent event in HCC. The downregulation of the *SPARC* mRNA expression in HCC is correlated with the *SPARC* methylation. The patients with methylated *SPARC* had a poorer overall survival than those without methylated *SPARC*.

Applications
The results in this study indicated that *SPARC* hypermethylation in HCC is most likely related to a disease state, which might be helpful for finding potential diagnostic or predictive markers of this disease.

Peer review
This is a good descriptive study in which authors investigate the methylation status of *SPARC* in HCC and evaluate its clinical implication. The results are interesting and suggest aberrant methylation is an important mechanism for *SPARC* inactivation in HCC and *SPARC* methylation may be a promising biomarker for the diagnosis and prognosis of HCC.

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ORIGINAL ARTICLE

Affinity peptide developed by phage display selection for targeting gastric cancer

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Abstract

AIM: To develop an affinity peptide that binds to gastric cancer used for the detection of early gastric cancer.

METHODS: A peptide screen was performed by biopanning the PhD-12 phage display library, clearing non-specific binders against tumor-adjacent normal appearing gastric mucosa and obtaining selective binding against freshly harvested gastric cancer tissues. Tumor-targeted binding of selected peptides was confirmed by bound phage counts, enzyme-linked immunosorbent assay, competitive inhibition, fluorescence microscopy and semi-quantitative analysis on immunohistochemistry using different types of cancer tissues.

RESULTS: Approximately 92.8% of the non-specific phage clones were subtracted from the original phage library after two rounds of biopanning against normal-appearing gastric mucosa. After the third round of positive screening, the peptide sequence AADNAKTKSFPV (AAD) appeared in 25% (12/48) of the analyzed phages. For the control peptide, these values were 6.8 ± 2.3, 5.1 ± 1.7, 3.5 ± 2.1, 4.6 ± 1.9 and 1.1 ± 0.5, respectively. The values for AAD peptide were statistically significant ($P < 0.01$) for gastric cancer as compared with other histological classifications and control peptide.

CONCLUSION: A novel peptide is discovered to have a specific binding activity to gastric cancer, and can be used to distinguish neoplastic from normal gastric mucosa, demonstrating the potential for early cancer detection on endoscopy.

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INTRODUCTION

New methods for the early detection of gastric cancer (GC) are urgently needed. GC is the second most common cause of cancer-related mortality worldwide[1,2]. Early detection is of paramount importance to improve the 5-year survival rate of the patients. Periodic endoscopic surveillance is the only currently available means to diagnose early gastric cancer in high-risk populations who have pre-cancerous lesions such as atrophic gastritis and intestinal metaplasia. However, the current surveillance program and mode of endoscopic diagnosis are labor-intensive and economically unfeasible. White light endoscopy has limited effectiveness for early GC screening. Neoplastic lesions can be less than a millimeter in size which is difficult to localize within regions of precancerous mucosa that usually are several square centimeters. Thus, a rigorous method is needed for selecting and validating molecular probes that bind specifically and highlight neoplastic lesions.

Molecular imaging is a technique that identifies and characterizes tumors and other lesions based on their protein expression pattern, rather than by their macroscopic morphology[3]. The molecular expression pattern of cells and tissues can be visualized with the help of disease-specific molecular probes such as antibodies, antibody fragments, peptides, radioactive probes and nanoparticles[4-6]. Such molecular probes enable the diagnosis of disease *in situ* and in real time. In a previous study, a heptapeptide was isolated from a phage library and conjugated with fluorescein for labeling of colonic dysplasia[7]. Although the molecular target of this sequence has not yet been identified, preferential binding of this targeting moiety to neoplastic cells *in vivo* with a high sensitivity and specificity was observed. In recent clinical studies, molecular imaging has been developed for guiding biopsy of high-grade dysplasia in Barrett's esophagus using fluorescent-labeled peptides. An affinity peptide selected using phage display techniques was administered over a region of intestinal metaplasia in resected specimens of the distal esophagus. The wide-area stereoscopic images of increased fluorescence intensity could predict and localize high-grade dysplasia[8].

In this study, we screened a peptide that has highly specific binding activity to human GC tissues. When labeled with fluorescein isothiocyanate (FITC), the peptide has the potential for *in vivo* use to produce increased fluorescence intensity at the site of neoplastic mucosa. This method can be used as a more specific strategy for early detection of GC.

MATERIALS AND METHODS

Cell culture

The human gastric cancer cell line BGC823 and Epstein-Barr virus-transformed human gastric epithelial cell line GES-1 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 ℃ in an atmosphere with 5% CO_2.

Human tissue specimens

Peptide screen was conducted in the patients ($n = 3$) with histologically validated intestinal-type gastric adenocarcinoma (Lauren's classification). Paraffin-embedded human tissues from 36 cases of gastric cancer (21 intestinal and 15 diffuse) and 15 cases of adjacent normal appearing gastric mucosa, 12 cases of breast cancer, and 15 cases of colorectal cancer were used for validating the screened peptide. The study was approved by the Bioethics Committee of the First Affiliated Hospital of Xian Jiaotong University Medical College, and written informed consent was obtained from all the patients. For the peptide screen, fresh specimens of cancer and adjacent normal appearing gastric mucosa (5 cm away from the macroscopic margin of the tumor) were collected during subtotal gastrectomy. Half of the tissue was cut into 0.5 cm × 0.5 cm × 0.3 cm pieces immediately and washed with magnesium-free Dulbecco's phosphate-buffered saline (PBS) for 2 min at 4 ℃ to be used for biopanning or immunofluorescence procedures[9]. The other half of the tissue was embedded in optimal cutting temperature freezing compound (Sakura Finetek United States, Torrance, CA) immediately. The tissue was cut into 6-μm sections, mounted onto Poly-D-Lysine-coated slides, and stored at -80 ℃ for the peptide binding assay. All the histopathological specimens were evaluated by two gastrointestinal pathologists who were blinded to each other according to the common procedural criteria for such studies and to the imaging results[10].

Peptide screening

Peptides were selected using the PhD-12™ phage display peptide library (New England BioLabs, Beverly, MA)[11-13]. This library has 1×10^{13} pfu/mL phages, with a diversity of 1.28×10^9 unique peptide sequences and about 70 copies of each sequence. For screening, non-specific binding phage was cleared from the library by panning against normal appearing gastric mucosa adjacent to the tumor. Tissue blocks were placed into 12-well cell culture plates and blocked by adding one mL of 1% bovine serum albumin (BSA) diluted in PBS for 30 min at 4 ℃. Phage (1×10^{11} pfu) in one mL of blocking buffer was incubated with tissue at room temperature (RT) for 30 min with gentle agitation. The supernatant containing unbound phages was collected and added to another well for the second round of clearance. The resulting supernatant was incubated with the gastric cancer specimens for positive selection. After 30 min of biopanning at RT, the tissue specimens were transferred to 1.5 mL tubes and washed 10 times with PBST (PBS/0.1% Tween-20, v/v). The bound phages on the tissue surface were eluted with one mL of 0.2 mol glycine, pH 2.2, 0.1% BSA for 8 min and immediately neutralized with 150 μL of 1 mol Tris, pH 9.5. The eluted phage was amplified and tittered according to the manufacturer's instructions. The resulting phage (10^{11} pfu) was used to perform another round of positive selection, as described above. In the last 2 rounds, elution was first performed for 2 min to remove the weakly bound phages, and new elution buffer was

then added to obtain the stronger bound phage.

Phage clones (n = 48) obtained from the last round of biopanning were randomly selected and sequenced. Peptide sequences that appeared more than twice were selected as candidates for further analysis. These peptide sequences were analyzed by searching the UniProtKB/Swiss-Prot database for homology using the basic local assignment search tool (BLAST, National Center for Biotechnology Information, Bethesda, MD) with the option for short, nearly exact matches to identify potential human protein targets.

Cell enzyme-linked immunosorbent assay

The protocol used for performing the cell enzyme-linked immunosorbent assay (C-ELISA) has been described previously[14]. BGC823 and GES-1 cells were allowed to reach an 80%-90% confluency in 96-well plates. The wells were blocked for 30 min at 37 ℃ with 200 μL BSA. Next, 2×10^7 pfu of candidate phages were incubated separately with each cell type in triplicate at RT for 30 min. The insertless wild-type phage (M13KE, New England Biolabs, Beverly, MA) was used as a control. Bound phages were detected using a horseradish peroxidase-conjugated polyclonal anti-M13 phage antibody (Pharmacia, United States). Tetramethylbenzidine working substrate solution (50 μL/well; Sigma, St Louis, MO) was added and incubated for 20 min at RT. The reaction was stopped by adding 4 mol H_2SO_4. Between each incubation step, the plates were washed three times with 300 μL TBST (0.5% Tween-20). Absorbance was measured at 490 nm using a microplate reader (Bio-Rad model 550, Hercules, CA). Untreated cells were used as controls. The absorbance (A) values between different groups were compared.

Phage binding affinity on human tissues

Specific binding of the candidate phages to gastric cancer was validated by incubating 2×10^{11} pfu of each phage (candidates and M13KE) with fresh gastric cancer or adjacent normal appearing gastric mucosa in wells in triplicate. The steps of incubation, two-step elution, and titration of phages were performed as described above. All of the eluted phages were tittered to determine the mean phage plaque numbers. The ratio of binding of each phage group to gastric cancer relative to that of M13KE was calculated. The level of binding of each phage clone to gastric cancer and normal appearing gastric mucosa was analyzed using the Student's t test.

Peptide synthesis

The candidate peptides were synthesized (Shanghai Biochem, Shanghai, China) using standard solid-phase fluorenylmethyloxycarbonyl chloride chemistry and purified to a minimum purity of 98% using high-performance liquid chromatography (HPLC). Analysis was performed by reverse phase HPLC and mass spectrometry[15]. FITC or biotin was conjugated to the C-terminus of the peptide *via* a flexible linker with the 5 amino acid sequence GGGSK (12-mer peptide-GGGSK-FITC or 12-mer peptide-GGGSK-biotin), the sequence of which is the same as that for the linker on the coat protein pⅢ of the M13 phage. For the control, the candidate peptide was scrambled to form a peptide sequence containing the same amino acids.

Competitive inhibition assay

Preferential binding of the candidate peptide to gastric cancer was further validated by a competitive binding assay. The candidate peptides at concentrations of 0.5, 5, 50, 500 and 5000 μmol were incubated with fresh gastric cancer or adjacent normal appearing gastric mucosa in wells in triplicate. Each phage (2×10^{11} pfu; candidate or M13KE) was then added. Incubation, elution, and tittering of the binding phages were performed as described above. The ratio of binding of each phage clone to gastric cancer and normal appearing gastric mucosa was analyzed.

Peptide binding on fresh human tissues

Peptide-based immunofluorescence analysis was performed to validate binding of the candidate peptide to human gastric cancer[16,17]. Frozen sections of human gastric cancer and adjacent normal appearing gastric mucosa tissues were blocked with PBS containing 3% BSA for 30 min at RT. Slides were then incubated with 100 μmol of the candidate peptide (peptide-FITC) for 30 min at 37 ℃, rinsed 3 times with PBST and fixed in acetone at 4 ℃ for 90 s, counterstained with propidium iodide, and mounted using PBST. Fluorescent images of the sections were recorded at 400× magnification. A FITC-labeled scrambled peptide was used as a negative control.

Peptide binding affinity on paraffin-embedded human tissues

The streptavidin-peroxidase-biotin immunohistochemical method was performed to detect candidate peptide binding on paraffin-embedded human tissues[18] from 36 cases of gastric cancer (21 intestinal and 15 diffuse) and 15 cases of adjacent normal appearing gastric mucosa, 12 cases of breast cancer, and 15 cases of colorectal cancer. In brief, paraffin-embedded specimens were cut into 4-μm sections and kept at 60 ℃ for 60 min. The sections were deparaffinized with xylene and rehydrated. Sections were submerged into ethylenediaminetetracetic acid antigenic retrieval buffer, microwaved for antigenic retrieval, and then cooled at RT for 20 min. The sections were pretreated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with normal serum to block non-specific binding. Then the sections were incubated with 100 μmol biotin-conjugated peptide for one hour at 37 ℃. The unbound peptide was rinsed off with PBS. The tissue sections were incubated with the streptavidin-horseradish peroxidase complex (Zhongshan Biotechnology, Beijing, China), and stained with diaminobenzidene (DAB). Finally, the sections were counterstained with hematoxylin. A biotin-labeled scrambled peptide was used as a negative control.

Semi-quantitative image analysis was performed as reported previously[19]. In brief, 3 images with typical

Figure 1 Preferential phage-binding to BGE823 and GES-1 cells. Phage capture enzyme-linked immunosorbent assay revealed a greater optical density at binding sites of AADNAKTKSFPV (AAD) phage to BGE823 cells compared with that of wild type phage ([b]*P* < 0.01) or no phage. No significant difference was found in binding of AAD phage to the control cells. WT: Wild type.

Figure 2 Phage binding affinity. AADNAKTKSFPV (AAD) phage showed an about 615 times higher binding efficiency in gastric cancer (GC) tissues than wild type (WT)-phage, and the binding of AAD phage was about 591 times greater in GC tissues than in gastric mucosa.

features were selected from each slide. The quantitative labeling index was calculated as the ratio of brown membranous area stained by DAB to round blue areas stained by hematoxylin, for the assessment of tumor cell density in the selected image. The extractions of the brown *vs* blue signal were carried out based on an RGB color parameter. The blue areas larger than 0.005 mm^2 were eliminated because of the nuclear staining in cells such as fibroblasts and lymphocytes but not in carcinoma cells. Images were analyzed using NIH Image J software.

Statistical analysis

Differences in the mean *A* value, number of eluted phages, and image intensity for all tissue classifications were compared using a one-way analysis of variance (ANOVA) or two-sided Student's *t* test with unequal variance. Statistical significance was assessed at the level of *P* = 0.01. All results were presented as mean ± SD unless otherwise noted.

RESULTS

Enrichment of phage with specific binding to tumor tissues

Approximately 92.8% of the non-specific phage clones were subtracted from the original phage library after two rounds of biopanning against normal appearing gastric mucosa. After the third round of positive screening, 50 phage clones that specifically bound to human gastric cancer were randomly selected from the enriched phage library. Phage clones were amplified and sequenced. The peptide sequence AADNAKTKSFPV (AAD) appeared in 25% (12/48) of the analyzed phages. Except for 2 phage clones which expressed the same peptide sequence IVWPTSPRALDA, the other 36 clones expressed unique amino acid sequences. These peptide sequences were analyzed by searching the UniProtKB/Swiss-Prot database using BLAST. Peptide AAD has identities = 10/14 (71%) with methyltransferase, which belongs to UbiE/COQ5 family.

Selective phage binding verified by C-enzyme-linked immunosorbent assay

The C-ELISA demonstrated selective binding of the AAD phage to BGC823 cells. As shown in Figure 1, the *A* value for the AAD phage binding to BGC823 cells was 1.15 ± 0.09 compared to 0.61 ± 0.07 and 0.65 ± 0.05 for the wild type (WT)-phage (*P* < 0.01) and no phage (*P* < 0.01), respectively. The *A* for AAD phage binding to the GES-1 cells was 0.123 ± 0.035 compared to 0.189 ± 0.045 and 0.271 ± 0.035 for the WT-phage (*P* > 0.05) and for no phage (*P* > 0.05), respectively. These results suggest that the AAD phage binds specifically to the BGC823 (cancer) cells and not to the GES-1 (control) cells. WT-phage and no phage did not bind significantly to any of the cells.

Phage binding affinity to gastric cancer tissues

The AAD phage showed about 615 times greater binding to gastric cancer than did the WT-phage, with a total phage number of 4.8 × 10^6 *vs* 7.9 × 10^3, as shown in Figure 2 (*P* < 0.01). Similarly, the binding of AAD phage was 591 times greater to gastric cancer than normal appearing gastric mucosa with a total phage number of 7.1 × 10^6 *vs* 1.2 × 10^4, respectively (*P* < 0.01, Figure 2). These results suggest that AAD phage binds specifically to gastric cancer (target) and not to the adjacent normal appearing gastric mucosa (control).

Competitive binding assay

As shown in Figure 3, we observed that the addition of 0.5, 5, 50, 500 and 5000 μmol of the compound consisting of the AAD peptide with the GGGSK linker (AAD-GGGSK) resulted in a significant reduction in the number of bound phages, corresponding to values of 2900 × 10^4, 1680 × 10^4, 1320 × 10^4, 80 × 10^4 and 0 (*P* < 0.01), respectively. Moreover, we did not see any significant change in the number of bound phages with the addition of 0.5, 5, 50, 500 and 5000 μmol of the control peptide (PAKFKAANSDVT), which resulted in a total of (2300 ± 41) × 10^4 bound phage (*P* < 0.01) at 5000 μmol. These

Figure 3 Competition binding assay. Binding of AADNAKTKSFPV (AAD) phage to gastric cancer tissues is reduced by competition with increasing concentrations of AAD peptide ($P < 0.01$) in a dose-dependent manner. The addition of the control peptide at concentrations of 0.5, 5, 50, 500 and 5000 μmol revealed no competitive inhibition.

results suggest that the AAD peptide competes with the AAD phage for binding to gastric cancer, and that binding is determined by the specific sequence of the expressed peptide, rather than by the phage coat proteins.

Peptide-based immunofluorescence assay

The peptide-based immunofluorescence assay was performed to confirm the selective binding of the AAD phage to fresh gastric cancer tissues. As shown in Figure 4, the fluorescence images displayed that the AAD peptide binds to both the tumor cell membrane and cytoplasm (C), but not to adjacent normal appearing gastric mucosa (B). Fluorescence was seen on the membrane and in the perinuclear cytoplasm of gastric cancer cells. The FITC-labeled scrambled control peptide, PAKFKAAN SDVT, did not bind to tumor tissues.

Binding analysis of biotin-AAD by immunohistochemistry

Tissue slides from multiple types of other human cancers were prepared to evaluate specific binding of biotin-labeled AAD peptide. From the results shown in Figure 5, biotin-AAD demonstrates specific binding to intestinal (Figure 5A) and diffuse (Figure 5B) gastric cancer. In contrast, no staining was observed in normal appearing gastric mucosa (Figure 5C), or breast cancer (Figure 5D) and colon cancer (Figure 5E). Weak binding of the AAD peptide to gastric mucosa dysplasia (Figure 5F) and intestinal metaplasia (Figure 5G) was also observed. The negative results were obtained when gastric cancer tissues were stained with biotin-conjugated scramble peptide (Figure 5H) and PBS (Figure 5I). In the positive slides, the area stained dark brown was located at the membrane and perinuclear cytoplasm, which is the same as FITC-conjugated AAD binding on fresh GC tissues, indicating the positive binding region of peptide AAD to GC cells.

Semi-quantitative image analysis was then performed. For the AAD peptide, the values in 37 specimens of gastric cancer (21 intestinal and 15 diffuse), 15 specimens of normal appearing adjacent gastric mucosa, 12

Figure 4 Immunofluorescence analysis of fluorescein isothiocyanate-conjugated AADNAKTKSFPV binding to human gastric cancer tissues. Frozen sections for biopanning were incubated with fluorescein isothiocyanate-conjugated AADNAKTKSFPV (AAD); scrambled peptide PAKFKAANSDVT was used as the control. Immunofluorescence stain with FITC-conjugated AAD showed selective signals (green) located in tumor membrane, cytoplasm (C), but no binding to normal gastric mucosae (B). As a control, the scramble peptide displayed no signals in tumor tissues (A).

specimens of breast cancer, and 15 specimens of colon rectal cancer were 150.0 ± 11.0, 135.5 ± 13.2, 43.5 ± 3.4, 52.3 ± 6.4 and 39.6 ± 5.0, respectively (Figure 6). For the control peptide, these values were 6.8 ± 2.3, 5.1 ± 1.7, 3.5 ± 2.1, 4.6 ± 1.9, and 1.1 ± 0.5, respectively. A one-way ANOVA showed an F-value of 1149.2 ($P < 0.01$), and the pair-wise t test yielded a t value of 15.3 ($P < 0.01$), demonstrating that the result for the AAD peptide is statistically significant for gastric cancer as compared with other histological classifications and control peptides.

DISCUSSION

Other investigators have used phage display technology

Figure 5 Binding analysis of biotin-AADNAKTKSFPV by immunohistochemistry. The results demonstrate that biotin showed a specific binding affinity to gastric cancer (GC) (A: Intestinal; B: Diffuse). In contrast, no positive staining was observed in gastric mucosae (C). In addition, peptide AADNAKTKSFPV (AAD) did not bind to breast cancer (D) or colon cancer (E), suggesting that the AAD peptide is specific to GC. A small amount of binding of the peptide AAD to the gastric mucosa dysplasia (F) and intestinal metaplasia (G) was also observed. The negative results were also obtained when GC tissues were stained with the biotin-conjugated scramble peptide (H) or phosphate-buffered saline (I).

Figure 6 Semi-quantitative image analysis. AADNAKTKSFPV (AAD) peptide is statistically significant for gastric cancer as compared with other histological classifications and control peptide. GC: Gastric cancer.

to select peptides that target specific organs, tumors, and proteins without prior knowledge of the target's molecular structure[20-22]. These libraries often contain more than 10 billion unique sequences, which enable peptide

selection with highly specific binding properties. Peptides specific for endothelial markers in dysplasia have been identified in mice[23-26]. Biopanning using freshly harvested human tissues has successfully isolated peptides that specifically bind to polarized luminal surfaces of dysplastic colonocytes[27,28]. In this study, we selected the 12-mer peptide AAD using the PhD-12 library. This peptide exhibited specific binding to human gastric cancer cells in culture and tissues.

The biopanning protocol used in this study was different from that used by most other investigators. The original phage library was first panned against freshly harvested normal-appearing mucosa adjacent to cancer to clear non-specific phages. After the clearing of normal mucosa binding phage from the original phage library, the likelihood of obtaining gastric cancer-specific peptides in the following tumor-targeted screen increased. We removed 92.8% of the phage clones from the original library after two rounds of subtractive biopanning. To avoid biasing the library, we did not amplify the remaining phage pool between each round. The pep-

tide sequence AAD appeared in more than 20% (12/50) of the analyzed phages after the third round of positive screening. This peptide was found to have no more than a 50% amino acid residue homology to the reported protein sequence. Phage expressing this peptide demonstrated preferential binding to cultured gastric cancer cells and fresh gastric cancer mucosa, and was validated by ELISA and bound phage counts. The binding was inhibited by the addition of competing peptide AAD, thus supporting cell surface binding. Moreover, when conjugated with FITC or biotin, the peptide AAD can be used as an *in vitro* peptide probe to distinguish tumor-adjacent mucosa from gastric cancer.

There is a great clinical need to improve the cancer screening and surveillance methods for diseases such as Barrett's esophagus, gastric intestinal metaplasia, flat and depressed sporadic colonic adenomas, and bladder carcinoma *in situ*. In nuclear medicine, imaging with radioactively labeled probes is routinely used. In contrast, fluorescent-labeled probes in gastrointestinal endoscopy are still being developed. Tumor-specific molecular probes have been used to improve the lesion contrast during gastrointestinal endoscopy to guide tissue biopsies[29]. Most digestive tract neoplasia arises from the epithelial layer, which is compatible with topical administration of the probe. Thus, molecular imaging has a particular advantage in the diagnosis or treatment of disorders of the gastrointestinal and other hollow organs, compared with lesions from solid tumors. Antibodies against epitopes that are over-expressed in gastrointestinal cancers, such as vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR), have been fluorescently labeled and used for *in vivo* imaging[30,31]. These antibodies have highly selective binding affinities to their target structures, with an optimized signal-to-background ratio. With the disclosure of the biologic relevance of their targets, therapeutic antibodies were developed such as cetuximab and panitumumab against EGFR, and bevacizumab against VEGF.

Peptides have several advantages over antibodies as disease-specific probes for molecular imaging. Peptides consist of only a few amino acids, and have much smaller structures with lower molecular weight. Therefore, peptides have better tissue penetration, shorter plasma half-life, and less associated immunogenicity[32-34]. In this study, peptide AAD showed a weak binding affinity to gastric dysplasia, but a significantly higher binding affinity to gastric cancer. A possible reason is that the targets are expressed at a lower level in pre-cancerous lesions as compared with cancer cells. As a molecular probe, peptide AAD may be used for grading dysplastic tissue and diagnosis of cancerous mucosa in early stage gastric cancer. The difference in binding affinity was not as significant between peptide AAD and the control peptide as reported in some published studies[35,36]. This may indicate a lower sensitivity as a tumor-specific probe and influence its future use. However, because of their pharmacokinetic advantages for *in vivo* imaging, tumor-targeting peptides do not necessarily have the highest binding

affinity. Multiple excitation and detection wavelengths, in conjunction with multiple labels, may further enhance the applicability of this strategy. More than one tumor-specific peptide, each with a different target and fluorescent-label, could be mixed to increase the sensitivity, which may be used as a promising strategy for *in vivo* detection. Even with the limitations of current approaches, molecular imaging has the potential to greatly affect future imaging in gastroenterology. Future efforts should focus on the validation of peptides binding to malignantly-transformed mucosa *in vivo*.

Great progress has been made in molecular imaging in recent years, and technological and scientific advancement in endoscope compatible instruments have provided new imaging tools to improve the detection of early neoplastic lesions. Fluorescence endoscopes and confocal microendoscopes have been developed with a high sensitivity[37-39]. Once integrated with novel screening and surveillance methods, molecular endoscopy will prove effective real-time localization of dysplasia or neoplastic mucosa. Molecular probes that bind to suspected mucosal lesions may guide the doctor to perform a targeted biopsy. *In vivo* molecular imaging of live tissues may be less sensitive to bias from sampling error and tissue processing artifact than conventional histopathology, thus increasing the efficiency of endoscopic screening and surveillance. The peptide AAD identified in this study has the potential to guide tissue biopsy and improve the detection of pre-cancerous lesions in gastric mucosa.

COMMENTS

Background
Periodic endoscopy in high risk populations is most helpful in improving the early detection of gastric cancer (GC). However, the current endoscopic surveillance program for GC is labor-intensive and ineffective. Molecular probes are being developed to increase image contrast from early cancer during endoscopy to guide biopsy in some pioneered reports.

Research frontiers
Molecular imaging is a technique that identifies and characterizes tumors and other lesions based on their protein expression pattern, rather than by their macroscopic morphology. The molecular expression pattern of cells and tissues can be visualized with the help of disease-specific molecular probes such as antibodies, antibody fragments, peptides, activatable probes and nanoparticles. Such molecular probes enable the diagnosis of disease *in situ* and in real time.

Innovations and breakthroughs
In this study the authors discovered a novel peptide that has specific binding activity to GC and can be used to distinguish neoplastic from normal gastric mucosa.

Applications
The peptide AADNAKTKSFPV identified in this study has the potential to guide tissue biopsy and improve the detection of pre-cancerous lesions in gastric mucosa.

Peer review
The topic of the study is interesting and the authors tried to tackle very relevant and clinical important issues, the early detection of GC. The authors identified a peptide that seems to bind to GC tissue like an antibody.

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BRIEF ARTICLE

Experience after 100 patients treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy

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Abstract

AIM: To investigate perioperative patient morbidity/mortality and outcome after cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC).

METHODS: Of 150 patients 100 were treated with cytoreductive surgery and HIPEC and retrospectively analyzed. Clinical and postoperative follow-up data were evaluated. Body mass index (BMI), age and peritoneal carcinomatosis index (PCI) were chosen as selection criteria with regard to tumor-free survival and perioperative morbidity for this multimodal therapy.

RESULTS: CRS with HIPEC was successfully performed in 100 out of 150 patients. Fifty patients were excluded because of intraoperative contraindication. Median PCI was 17 (1-39). In 89% a radical resection (CC0/CC1) was achieved. One patient died postoperatively due to multiorgan failure. Neither PCI, age nor BMI was a risk factor for postoperative complications/outcome according to the DINDO classification. In 9% Re-CRS with HIPEC was performed during the follow-up period.

CONCLUSION: Patient selection remains the most important issue. Neither PCI, age nor BMI alone should be an exclusion criterion for this multimodal therapy.

INTRODUCTION

Peritoneal carcinomatosis (PC) is generally considered to be a terminal disease and for a long time was viewed as incurable. Based on the rationale of a disease limited to the abdominal compartment[1-8], the pioneering work of Sugarbaker made it possible for certain tumor entities with PC to have the option to be cured by radical cytoreduction and hyperthermic intraperitoneal chemotherapy (HIPEC). Patient prognosis is determined by the

feasibility of complete cytoreduction (CC) and therefore a compulsory patient selection remains the Achilles heel[9]. Since the surgical procedure itself is challenging, postoperative morbidity and mortality must be considered in the preoperative evaluation process in addition to the extent of tumor spread. A high tumor load, causing a high peritoneal carcinomatosis index (PCI), is associated with poor prognosis with regard to disease-free and overall survival[10]. Age is commonly widely accepted as a selection criteria *per se* for major tumor resections. Most groups restrict cytoreductive surgery and HIPEC to patients aged under 65 years. Similarly, a high body mass index (BMI) often hampers major surgery and obese patients have more complications in general. A valid surgical complication score was developed by Dindo *et al*[11]. He defines 5 grades of complications, whereas grade 1 is any deviation from normal postoperative course; grade 2 requiring pharmacological treatment; grade 3 any radiological, endoscopic or surgical intervention; grade 4 a life threatening complication and grade 5 death.

We here report our experience with 100 consecutive cytoreductive surgeries (CRS) and HIPEC and lessons learned with respect to the perioperative period.

MATERIALS AND METHODS

During the last five years 150 consecutive patients underwent surgery with the intent to perform complete cytoreduction (CRS) and HIPEC. All patients underwent preoperative anesthesiological and cardiologic evaluation. Extraabdominal metastases were excluded and intraperitoneal tumor load was detected by computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET)-CT scan. After surgical exploration 50 (33%) patients were found to not be suitable for CRS and HIPEC due to either extensive intraoperative tumor load, retroperitoneal tumor infiltration or deep infiltration of the mesenteric axis.

In 100 consecutive patients with peritoneal carcinomatosis of various origins (Table 1) cytoreductive surgery was performed with intraoperative hyperthermic chemotherapy. For colorectal or appendiceal cancer intraabdominal locally heated (42 Celcius) mitomycin C 25-35 mg/m^2 was routinely administered for 90 min. For gastric or ovarian cancer cisplatin 50 mg/m^2 was used. For gastric cancer a combination chemotherapy consisting of mitomycin and cisplatin was administered in some cases. Data were analyzed retrospectively.

Surgical procedure

After explorative laparotomy and complete adhesiolysis PCI score was determined, in particular with respect to the ligamentum teres, right upper suphrenic quadrant, space between the vena cava and liver segment 1, retrosplenic sulcus and bursa omentalis, which are most likely to be tumor-infiltrated. Then, after exclusion of contraindications, cytoreductive surgery was performed according to the technique described by Sugarbaker[1-8].

Table 1 Tumor type and primary tumor nodes status	
Tumor type	n
Colon	21
Rectal	5
Appendiceal	10
Ovarian	33
Pseudomyxoma	13
Stomach	11
Mesothelioma	1
Other	6
Tumor	n (%)
1	5 (5)
2	9 (9)
3	44 (44)
4	42 (42)
Nodes	n (%)
0	33 (33)
1	39 (39)
2	24 (24)
3	4 (4)

After maximal cytoreduction and reconstruction of intestinal continuity, if required, HIPEC was administered to the open abdomen for 90 min at 42 degrees Celsius. A rubber drain was routinely placed in the pelvis and an additional drain inserted in the left upper abdominal quadrant for splenectomy. Finally, the abdomen was closed with interrupted sutures.

Statistical analysis

Data are presented as median (min-max) or n (%), unless otherwise stated. Qualitative differences were compared using the χ^2 test, quantitative differences using the Mann-Whitney U test. Survival analysis was performed with the Kaplan-Meier method. For overall survival (OS) and disease-free survival, time to event was calculated as time from cytoreductive surgery until death or time to last contact, if the patient was alive. A P value less than 0.05 was considered significant. R was used for all statistical analysis[12].

RESULTS

Clinical characteristics, tumor types and intraoperative data are listed in Tables 1-3. Most tumors treated were of ovarian ($n = 33$) or colorectal origin ($n = 26$). Median age was 54 (17-76) years. Median BMI was 24 cm/kg^2. CRS with HIPEC was performed in 100 consecutive patients. The resection types are listed in Table 4. In 26 of the 50 patients without HIPEC an explorative laparotomy was performed. The other 24 patients underwent palliative bowel resection or debulking due to tumor obstruction. 66% of these patients died during follow-up with a 50% probability of survival within 224 d. Median operating time was 593 (178-1076) min. Median PCI was 17 (1-39). In 89% a radical resection (CC0/CC1) was achieved. Mitomycin C was used in 60% and Cisplatin in 32% of patients. In 5% a combination of Mitomycin C and Cisplatin was administered. A ureteral splint was perioperatively

Table 2 Clinical characteristics	
Patients	***n* = 100**
Age (yr)	54 (17-76)
American Society of Anesthesiologists [*n* (%)]	
1	7 (7)
2	52 (52)
3	41 (41)
BMI (cm/kg²)	24 (17-41)
Time to PC from primary diagnosis (d)	365 (103-1009)

Data are presented as mean (min-max) or *n* (%). PC: Peritoneal carcinomatosis; BMI: Body mass index.

Table 3 Intraoperative data	
PCI score	17 (1-39)
Operating time (min)	593 (178-1076)
CC Status	*n* (%)
0	56 (56)
1	33 (33)
2	11 (11)

Intraoperative peritoneal carcinomatosis index (PCI) and complete cytoreduction (CC) status, operating time.

Table 4 Type of hyperthermic intraperitoneal chemotherapy and resection	
HIPEC type	**%**
Mitomycin C	60
Cisplatin	32
Mitomycin C and Cisplatin	5
Other	3
Parietal peritonectomy	90
Gastrectomy	18
Ileo-coecal resection	18
Colonic resection	19
Anterior rectal resection	35
Right hemicolectomy	28
Sigmoideal resection	11
Small bowel resection	28
Omental resection	33
Cholecystectomy	22
Hysterectomy	22
Ovarectomy/adnexectomy	14
Splenectomy	35
Atypical liver resection	9
Pancreatic resection	5
Removal of part of the diaphragm	15
Tumor resection in the abdominal wall	1
Ureteral resection	1

Type of operation during complete cytoreduction and hyperthermic intraperitoneal chemotherapy.

Table 5 Complications and mortality	
Cumulative complications, *n*	94
30-d mortality, *n* (%)	1
90-d mortality, *n* (%)	0

Cumulative complications and mortality; data are presented as median (min, max).

Table 6 Types of complication	
Types of complication	***n* (%)**
Cardiac	1 (1)
Pneumonia	5 (5)
Sepsis	3 (3)
Thrombembolic	9 (9)
Postoperative bleeding	2 (2)
Ureter injury	3 (3)
Wound infection	21 (21)
Leukopenia	29 (29)
Anastomotic leakage	8 of 139 (5.8)
Compartment syndrome	1 (1)
Transient paresthesia in the legs	1 (1)
Pancreatic fistula	0
Reoperation due to complication	21 (21)
DINDO complication classification	%
0	52
1	23
2	10
3	3
4	11
5	1

Complications and complications according to DINDO classification.

on perioperative complications according to the DINDO classification (Table 7). Median time of follow-up was 538 (17-1932) d.

Recurrence-free survival is shown in Figure 1B. Overall survival is shown in Figure 1A. In 9% Re-CRS with HIPEC was performed during the follow-up period.

DISCUSSION

CRS with HIPEC is now a procedure with the potential to cure selected patients suffering from PC[13-17]. PC can be considered a disease limited to the abdominal compartment, and based on this rationale maximal cytoreduction may be justified for various histological entities such as pseudomyxoma, ovarian cancer and colorectal cancer, *etc*., thus improving overall and recurrence-free survival[13-21].

Patient selection is certainly, as already mentioned, the "achilles heel" when including patients in this multimodal therapy. Radiological imaging estimates intraoperative tumor load, but reliable tumor identification in the critical regions such as the small bowel or ligamentum hepatoduodenale is still poor. Especially small lesions of about 1 cm or less are difficult to detect, even by PET-CT scan[22].

In this article we describe our first experiences with CRS and HIPEC. Since this procedure entails a certain morbidity, also due to long operating time, intraoperative

implanted in 17%. Intra- and postoperative complications are listed in Tables 5 and 6. The anastomotic leakage rate was 5.8%. HIPEC was not completed for 90 min in one patient due to cardiac arrhythmia. One patient died due to multi-organ failure. Leucopenia was observed in 29% of patients. Median hospital stay was 18 (3-105) d.

Neither PCI, BMI nor age had a significant influence

Table 7 Neither peritoneal carcinomatosis index, body mass index nor age had a significant influence on perioperative complications according to the DINDO classification *n* (%)

	PCI < 20	PCI ≥ 20	*P* value	Correlation coefficient
DINDO 0	34 (55)	18 (48)	0.128	0.07
DINDO 1-2	22 (35)	10 (26)		
DINDO 3-5	6 (10)	10 (26)		
	Age < 65	Age ≥ 65		
DINDO 0	45 (55)	9 (50)	0.917	-0.0028
DINDO 1-2	25 (30)	6 (33)		
DINDO 3-5	12 (15)	3 (17)		
	BMI < 25	BMI ≥ 25		
DINDO 0	35 (60)	17 (41)	0.075	0.19
DINDO 1-2	17 (30)	14 (33)		
DINDO 3-5	6 (10)	11 (26)		

PCI: Peritoneal carcinomatosis index; BMI: Body mass index.

Figure 1 Overall survival (A) and recurrence-free survival (B).

chemotherapy and multivisceral resection, we attempted to detect risk factors for patient selection with a view to perioperative morbidity. The literature currently available gives no conclusive data on age or BMI of patients for the purpose of patient selection for this multimodal therapy.

Since it is generally known that numeric age does not correlate with biological age, it is not acceptable to generally exclude older patients who are in good condition. Moreover, patients with a high BMI are often viewed negatively, because they are more challenging to operate and have a greater risk for perioperative complications.

Additionally, one of the main prognostic factors is PCI and when it exceeds 20 in colorectal cancers no survival benefit is achieved. However, for other entities it is still unclear and in pseudomyxoma the completeness of cytoreduction is the only prognostic factor, not tumor load.

From our results we concluded that tumor load, age and BMI had no significant impact on the perioperative complication rate according to the DINDO classification. Therefore, if desired, a biologically young patient should be included in this therapy if CC0/CC1 resection appears possible. We therefore hypothesize that the probability to achieve a CC0/CC1 resection should be the determining criterion for selection, and not PCI. Patients with a BMI over 25 had complication rates similar to those of patients with a BMI under 25. At any rate, we recommend that caution be exercised with superobese patients, because they were not represented in this study.

In obese patients with a low PCI, a laparoscopic approach with HIPEC might be an option and should be discussed[23].

For patients with a high PCI this also seems valuable. The results of this study show that from the standpoint of postoperative morbidity more patients could be included in this therapy. Resectability should remain the main criteria for performing CRS and HIPEC.

In the beginning we generously applied a ureteral splint during peritonectomy for better orientation in patients with pelvic recurrence. Because of extensive pre- and postoperative pain and the questionable necessity of the splint during the operation we abandoned ureteral splinting completely in patients without hydronephrosis. In one case we had to perform a ureteral resection and end-to-end anastomosis because of tumor infiltration.

Our anastomotic leakage rate of 5.8% is acceptable and comparable with that of the current literature[10]. However, we tended to avoid anastomoses or stomas in favor of meticulous cleaning of the small bowel and large bowel of tumor seedings whenever possible, especially in the most recent patients. Only four patients received a loop ileostomy after anterior rectal resection, two received a terminal ileostomy after colectomy and four patients a terminal colostomy. In our opinion resection of the colon should not be performed according to oncologic criteria with removal of a maximum of lymph nodes, except when there is a synchronous PC of colorectal cancer. More importantly, all macroscopically visible tumor seedings must be removed and the organs should be preserved whenever possible.

Most recurrences occurred in the right upper quadrant or in the retroperitoneum (data not shown). This might have been induced by the large wound surfaces and the increasing risk for tumor adherence[24]. This observation has been known for a long time[25-27]. In this regard, CRS should be performed only in the tumor-affected peritoneum and never in healthy tissue. Nonetheless, it is sometimes easier to begin with the parietal peritonectomy in the healthy region, for example by removing the peritoneum of the whole pelvis and not only the affected region

in the Douglas space.

In the summary of the decision to include or not include a patient in this multimodal therapy is based on a variety of factors and should be done only at centers offering interdisciplinary evaluation by internal medicine specialists, surgical oncologists, anesthetists and radiologists. Lastly, the decision should be taken individually for each patient and high PCI, BMI or age should not be an exclusion criterion *per se* with regard to perioperative morbidity.

COMMENTS

Background
Perioperative patient morbidity/mortality after cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) is a major concern in the selection process for this multimodal therapy.

Research frontiers
Of 150 patients 100 were treated with cytoreductive surgery and HIPEC and retrospectively analyzed. Clinical and postoperative follow-up data were evaluated. Body mass index (BMI), age and peritoneal carcinomatosis index (PCI) were chosen as selection criteria with regard to perioperative morbidity.

Innovations and breakthroughs
Neither PCI, age nor BMI was a risk factor for postoperative complications/outcome according to the Dindo classification. The decision to include or not to include a patient in this multimodal therapy regimen is based on a variety of factors and should be made only at centers offering interdisciplinary evaluation by internal medicine specialists, surgical oncologists, anesthesiologists and radiologists. Finally, the decision should be made individually for each patient and high PCI, BMI or age should not be an a priori exclusion criterion with regard to perioperative morbidity.

Applications
The study results suggest that a high PCI, BMI or advanced age itself are not a contraindication for this multimodal therapy concerning perioperative morbidity.

Terminology
HIPEC is administered with 42 degrees Celcius for 90 min; PCI describes the intraperitoenal tumor load and was developed by Professor Sugarbaker.

Peer review
This is a good descriptive study in which authors analyze perioperative patient morbidity/mortality and outcome after CRS and HIPEC. The results are interesting and suggest that patient selection remains the most important issue. Neither PCI, age nor BMI alone should be an exclusion criterion for this multimodal therapy.

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Efficacy and safety profile of LCR35 complete freeze-dried culture in irritable bowel syndrome: A randomized, double-blind study

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Abstract

AIM: To assess the effects and safety of *Lactobacillus casei rhamnosus* LCR35 complete freeze-dried culture (LCR35) in patients suffering from irritable bowel syndrome (IBS).

METHODS: A randomized, double-blind pilot study was performed in 50 patients complaining of IBS symptoms complying with Rome III criteria. Patients were allocated to receive either LCR35 (n = 25) at a minimum daily dose of 6×10^8 colony forming units or placebo (n = 25) for 4 wk. At inclusion, after treatment and 2 wk later, patients completed the IBS severity scale. Change from baseline in the IBS severity score at the end of treatment was the primary efficacy criterion. Changes were compared between groups in the whole population and in IBS subtypes (IBS with predominance of constipation, IBS with predominance of diarrhoea, mixed IBS, unsubtyped IBS). The presence of *lactobacillus casei rhamnosus* in stools was investigated at inclusion and at the end of treatment. The gastrointestinal quality of life questionnaire and the hospital anxiety and depression (HAD) scale were also completed.

RESULTS: Both groups were balanced for baseline characteristics. In 85% of patients, stool analyses showed that *lactobacillus casei rhamnosus* able to survive in the digestive tract. In the whole population, improvements in the IBS severity score did not differ significantly between treatments with a 25% decrease after 4-wk treatment, and a 15% decrease from baseline 2 wk later in both groups. In IBS subgroups, statistical analysis could not be performed due to small sample size, but a clinical response in favour of LCR35 was observed in IBS patients with predominance of diarrhoea: no change in the symptom severity score was seen with the placebo after 4 wk treatment, whereas a clinically relevant decrease occurred with LCR35 (-37% *vs* -3%). Furthermore, in spite of an increase in symptom intensity, the IBS severity score was maintained below the baseline value 2 wk later with LCR35 (-19% from baseline), whilst a slight 5% increase from baseline was observed with placebo. In the IBS subgroup with predominance of diarrhoea only, a clinically relevant decrease in abdominal pain severity score (-36%)

was observed with LCR35, whereas no change occurred with placebo. In mixed IBS patients, the 20% and 30% decreases in the IBS severity score observed after treatment with LCR35 and placebo, respectively, were maintained 2 wk later in both groups. A clinical response slightly in favour of placebo was observed at the end of the treatment period in IBS patients with predominance of constipation (-41% *vs* -20%) and unsubtyped IBS patients (-47% *vs* -17%), with the same value maintained 2 wk later. In both groups, no clinically relevant changes were observed either for the gastrointestinal quality of life index or HAD score. Thus, these results suggest that sub-grouping of IBS patients may be important for optimizing treatment responses by the physician.

CONCLUSION: This pilot study suggests that LCR35 could have some efficacy in IBS patients complaining of diarrhoea. These preliminary results need to be confirmed in larger studies.

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional bowel disorder, with an estimated worldwide prevalence of 10%-20% among adults and adolescents[1]. IBS is the most common diagnosis made by gastroenterologists. IBS contributes considerably to disability, absence from work or school and increased health-care costs[2]. As no curative treatment is available, therapy for IBS is palliative and supportive, targeting specific symptoms, but is notoriously unsatisfactory[3,4].

Studies have observed altered intestinal microflora in IBS patients and an increase in symptoms after enteric infections, suggesting that restoration of the intestinal microflora may be a useful therapeutic goal[5-8].

Lactobacilli are a component of the commensal microbiota of both the small and large intestinal tract of humans and animals. They are frequently used as probiotics and have a long history of safe consumption in food[9]. Probiotics, live microbiologic organisms found in foods and supplements, are supported by enough evidence to recommend their use in the treatment of IBS. This therapeutic class is gaining popularity for the treatment of multiple gastrointestinal disorders and a recent meta-analysis suggests that probiotics offer promise for the treatment of IBS[10]. Probiotics reportedly bind to small and large bowel epithelium and produce substances with antibiotic properties that may inhibit attachment and invasion by pathogenic organisms[11,12]. Probiotics may also modulate gastrointestinal luminal immunity by changing the cytokine and cellular milieu from a pro-inflammatory to anti-inflammatory state[13]. This immunomodulatory effect also attenuates the visceral hypersensitivity characteristic of IBS[7,14]. It has been speculated that each individual bacterial strain or a combination of strains may affect select subclasses of symptoms[15]. Whatever the underlying mechanism, in order to produce their health effects, the probiotic microorganisms must be able to survive within the gastrointestinal tract.

LCR35 complete freeze-dried culture has been successfully exploited commercially as a pharmaceutical product for its antidiarrhoeal properties for more than 50 years. *In vitro* investigations showed that this strain has probiotic activities such as the ability to adhere to intestinal cells and antibacterial activity against a large variety of pathogens[16]. Colonization by this probiotic in the gastrointestinal tracts of mice and humans has been studied and the findings suggest that LCR35 is able to survive *in vivo*[17]. In a study on mouse dendritic cells, *Lactobacillus casei* appears to be a probiotic which, in small concentrations, induces the production of large quantities of anti-inflammatory interleukins[18].

Thus, the therapeutic potential of probiotic bacteria - especially lactobacilli- reported in literature, as well as the research performed on LCR35, suggest a beneficial effect of this strain on the symptomatology of IBS patients.

The objective of this pilot study was to assess the efficacy and tolerability of the completely freeze-dried culture of *Lactobacillus casei rhamnosus,* LCR35, by measuring its effects on the symptomatology of IBS and evaluating its impact on the gastrointestinal quality of life and anxiety/depression level in patients suffering from IBS satisfying the Rome Ⅲ diagnostic criteria.

MATERIALS AND METHODS

This was a prospective, multicentre, randomized, double-blind, placebo-controlled pilot trial on two parallel groups. Patients were recruited from the outpatient clinics of the Department of Gastroenterology of 3 university hospitals (in Clermont-Ferrand, Nice and Rouen) and one private medical centre in Clermont-Ferrand. The study was conducted in accordance with Good Clinical Practice (CPMP/ICH/135/95), the French regulations, and the Declaration of Helsinki and subsequent World Medical Assemblies. The trial was approved by the regional Ethics Committee (CPP Sud Est VI) on March 7th, 2008 and was registered by the French Health Authorities with the identifier number 2008-A00010-55.

Patient enrolment

Eligible patients were those fulfilling the Rome Ⅲ criteria for IBS[19], whatever the subtype of IBS: IBS with predominance of constipation, IBS with predominance of diarrhoea, mixed IBS and unsubtyped IBS. At screening, the Hamilton scale[20] was used to exclude depressive patients. Inclusion criteria were: both genders, age between 18 and 70 years, availability of morphological, radiological and/or endoscopic data verifying the integrity of the digestive tract during the last 5 years, moderate symptom intensity (IBS severity score between 150 and 300 -see below-), efficient contraceptive method for women of child-bearing age. The non-inclusion criteria were: denied written informed consent, immunodeficiency or any serious illness or any progressive disease.

The following treatments were prohibited throughout the trial: other probiotics, antibiotics, anti-inflammatories, and any drugs aiming to treat IBS (antispasmodics, clays, *etc*.). Paracetamol was authorized to relieve pain at a daily dose ≤ 3 g/d; bisacodyl (no more than one tablet per day) and loperamide (≤ 6 capsules per day) could be used for no more than 2 consecutive days for constipation and diarrhoea, respectively. Psychotropic drugs (antidepressants or anxiolytics) were authorized if patients had been previously treated for several weeks without any modification of the dosage within the month preceding their enrolment into the study.

Procedures and treatment

After a screening visit (V1) performed 10 to 14 d before inclusion, patients had to attend 3 visits over a 6-wk period: V2 on day 0 involved randomization and treatment initiation; V3 was scheduled at the end of the 4-wk treatment period (between day 28-day 32) and V4 was planned 2 wk after the end of treatment (between day 42-day 46).

At screening, after obtaining informed consent, the Rome Ⅲ criteria were checked. Patients were instructed not to change their eating habits as to dietary fibre intake except for fermented milk and any food supplement likely to contain probiotics which were forbidden throughout the entire study period.

At visit 2, each potentially eligible patient was evaluated by a full review of clinical history and physical examination and their transit was assessed using the Bristol stool form scale[21]. Each subject completed the IBS severity scale[22], the gastrointestinal quality of life index (GIQLI) questionnaire[23] and the hospital anxiety and depression (HAD) scale[24-26]. Subjects eligible for the treatment phase were identified by a serial number and were randomly assigned to receive either LCR35 complete freeze-dried culture or the placebo, in a 1:1 ratio. Each treatment was provided in gelatine capsules and 3 capsules had to be taken once daily in a fasting state for 4 wk. One capsule of LCR35 contained 250 mg of product (total freeze-dried culture of *Lactobacillus casei* variety *rhamnosus* with a concentration of at least 2×10^8 CFU). Placebo capsules were identical in all aspects to the verum, thus allowing effective blinding. All capsules had to be taken in the morning while fasting, with a glass of non-alcoholic drink at ambient temperature in order to avoid a decrease in the number of LCR35.

At visit 3, after 4 wk treatment, a clinical examination was performed and patients completed the IBS severity scale, the GIQLI questionnaire, the HAD scale and the Bristol stool form scale. They did the same at visit 4, 2 wk after the end of the treatment.

Adverse events and medication compliance were monitored throughout the study period.

Compliance was also evaluated by the presence or absence of *Lactobacillus* in the faeces which were collected at inclusion and at the end of the 4-wk treatment period. All samples were aliquoted into 2 faecal culture cups and frozen at -80 ℃. After extraction of total bacterial DNA (kit QIAamp Mini Kit for stool QIAGEN), the presence of *Lactobacillus casei* variety *rhamnosus* was specifically determined by qualitative polymerase chain reaction (PCR - primer pairs hyb-21[27]) - cycles of amplification [(94 ℃, 5 mn - 94 ℃, 30 s; 56 ℃, 30 s; 72 ℃, 1 mn/kb) × 33, 72 ℃, 7 mn].

Questionnaires

The IBS severity scoring system is a self-administered questionnaire initially developed and validated by Francis *et al*[22] of which the French version has been previously validated[28]. It is composed of: (1) two items concerning the presence of abdominal pain and bloating (response yes or no); (2) four visual analogue scales measuring intensity of pain, bloating, relief following defecation and impact of symptoms on general QoL; and (3) an item on the number of days of suffering during the preceding 10 d. It provides a quantitative score ranging from 0 to 500 enabling grouping patients by symptom severity from mild to severe forms [(0-150) = mild, (150-300) = moderate, > 300 = severe]. Furthermore, previous studies have shown a positive correlation between this severity score and QoL of IBS patients[28,29].

In this study, the IBS severity score was used as the primary efficacy variable. Patients with an IBS severity score reduced by 50% after 4 wk of treatment were considered "responders".

The GIQLI[23] is a validated tool to measure quality of life related to gastrointestinal diseases. The GIQLI questionnaire includes 36 items asking about symptoms, physical status, emotions, social dysfunction, and effects of medical treatment. Higher scores, better GI-specific health-related quality of life.

The HAD scale[24-26] was designed to assess the contribution of mood disorder, especially anxiety and depression, in order to understand the experience of suffering in the setting of medical practice. The lower the HAD score, the lower the depression and anxiety level.

Ethical issues

All patients provided informed consent. Participation in the study was voluntary, and patients were allowed to withdraw at any point without giving an explanation.

Statistical analysis

For this pilot study, due to the lack of significant data in the literature, we arbitrarily considered that 60 subjects

Figure 1 Disposition of patients.

Table 1 Demographic and disease-related baseline characteristics (mean ± SD)

	Placebo (n = 25)	LCR35 (n = 25)
Sex, n (%)		
Male	5 (20.0)	10 (40.0)
Female	20 (80.0)	15 (60.0)
Age (yr)	48.0 ± 10.8	46.1 ± 11.3
Height (cm)	163.2 ± 7.6	168.2 ± 7.6
Weight (kg)	65.5 ± 13.1	66.4 ± 14.9
BMI (kg/m²)	24.5 ± 4.0	23.4 ± 4.9
IBS severity score	247.1 ± 43.8	261.5 ± 39.4
Abdominal pain score	36.7 ± 20.6	44.6 ± 13.2
GIQLI	62.9 ± 8.6	63.9 ± 7.8
HAD score	16.5 ± 6.4	16.3 ± 6.5
IBS subgroups		
IBS with predominance of constipation, n (%)	7 (28.0)	4 (16.0)
IBS severity score	270.4 ± 28.4	281.5 ± 9.9
IBS with predominance of diarrhoea	8 (32.0)	7 (28.0)
IBS severity score	259.6 ± 53.7	286.1 ± 11.2
Abdominal pain score	36.6 ± 27.2	51.4 ± 12.4
Mixed IBS, n (%)	6 (24.0)	11 (44.0)
IBS severity score	222.3 ± 36.7	245.0 ± 42.0
Unsubtyped IBS, n (%)	4 (16.0)	3 (12.0)
IBS severity score	218.5 ± 27.1	238.0 ± 63.6

NB: No statistical difference was found between the groups; BMI: Body mass index; IBS: Irritable bowel syndrome; GIQLI: Gastrointestinal quality of life index; HAD: Hospital anxiety and depression.

would be enrolled.

Statistical analysis was performed using version 9.1.3 Windows of SAS® software. Inclusion was considered as baseline.

The primary efficacy endpoint was the change in the IBS severity score at the end of the 4-wk treatment period. Other efficacy variables were considered as secondary: changes in the IBS severity composite score at the end of the study, changes in the IBS severity score referring to IBS subtypes, distribution of patients according to symptom severity classes, number of responders, changes in the abdominal pain severity score (sub-item of the IBS

severity score; pain is one of the key features of many of the functional gastrointestinal disorders), changes in the GIQLI and HAD score.

Efficacy results were similar on the "full analysis set" (FAS) and the "per-protocol set", therefore, only results based on the FAS are reported.

Absolute and relative changes from baseline in the IBS severity score, the GIQLI and the HAD score were compared between both treatment groups using the two-sided Student's *t*-test with a 5% significance level. The same test was used to assess changes in the IBS severity score in the IBS sub-groups (IBS with predominance of constipation, IBS with predominance of diarrhoea, mixed IBS and unsubtyped IBS). The distribution of patients according to IBS severity score classes was described in both groups at each visit. In the whole population and in the four IBS sub-groups, the percentages of "responders" were compared between treatment groups using the χ^2 test or the Fisher's exact test. Data from the Bristol stool form scale could not be analysed because of an important number of missing data.

RESULTS

The flow of subjects through the protocol is described in Figure 1.

Fifty-two patients were screened for the study. All of them fulfilled the inclusion criteria and were randomized equally into two groups. Among the 52 included patients, 5 discontinued and 47 completed the study. Prior to un-blinding of the data, 2 patients without primary criterion evaluation at V3 were excluded from the FAS, and 8 subjects were deemed non-evaluable because of major deviations (among them 3 were premature dropouts), thus providing a FAS of 50 patients (25 in each group) and a PP population of 44 (21 in the LCR35 group and 23 in the placebo group).

Baseline characteristics of IBS patients

Table 1 summarizes demographic data and disease-related

Table 2 Absolute and relative changes from baseline in the irritable bowel syndrome severity score referring to irritable bowel syndrome type (mean ± SD)

	Placebo (*n* = 25)		LCR35 (*n* = 25)	
	Absolute changes	Relative changes (%)	Absolute changes	Relative changes (%)
IBS with predominance of constipation	*n* = 7		*n* = 4	
Post-treatment (V3-V2)	-109.4 ± 93.1	-41.0 ± 32.7	-56.8 ± 43.9	-20.5 ± 16.5
End of study (V4-V2)	-61.0 ± 96.0	-23.5 ± 35.0	-27.5 ± 31.6	-10.1 ± 11.3
IBS with predominance of diarrhoea	*n* = 8		*n* = 7	
Post-treatment (V3-V2)	-1.9 ± 82.8	-3.1 ± 35.6	-105.0 ± 128.4	-36.6 ± 44.7
End of study (V4-V2)	23.9 ± 119.7	4.9 ± 46.8	-54.9 ± 151.7	-19.1 ± 53.5
Mixed IBS	*n* = 6		*n* = 11	
Post-treatment (V3-V2)	-70.0 ± 91.4	-31.2 ± 38.8	-50.3 ± 99.4	-21.8 ± 39.7
End of study (V4-V2)	-68.3 ± 110.6	-30.7 ± 50.1	-53.3 ± 97.4	-20.5 ± 39.9
Unsubtyped IBS	*n* = 4		*n* = 3	
Post-treatment (V3-V2)	-101.8 ± 96.2	-46.7 ± 46.7	-22.0 ± 99.9	-17.4 ± 45.9
End of study (V4-V2)	-63.8 ± 97.7	-31.3 ± 50.8	21.3 ± 140.8	4.3 ± 51.0

IBS: Irritable bowel syndrome.

Figure 2 Relative changes in irritable bowel syndrome severity score between V2 and V3, V2 and V4 in irritable bowel syndrome patients with predominance of diarrhoea. V2: Baseline; V3: At the end of the 4-wk treatment period, a more marked decrease in irritable bowel syndrome (IBS) severity score occurred with the test drug (-36.6% *vs* -3.1% with placebo); V4: Two weeks later, the IBS severity score was maintained below baseline with the test drug (-19.1%), whilst it slightly increased over baseline (+4.9%) with placebo.

baseline characteristics of the IBS patients. Except for a higher percentage of mixed IBS patients in the LCR35 group (44.0% *vs* 24.0%), no clinically relevant difference was observed between the groups. As required by the protocol, patients suffered from IBS symptoms of moderate intensity within the interval (150-300), with a mean value close to the upper values of the class in both groups.

Compliance

The presence of *Lactobacillus casei rhamnosus* in stools was investigated in 27 patients (14 LCR35 and 13 Placebo). In 85% of patients treated with LCR35, *Lactobacillus* was found by qualitative PCR. In one patient in the placebo group, no data was available to explain the presence of *Lactobacillus casei* in the faeces collected before and after treatment. Such a result may reflect the presence of LCR35 at a commensal level in some people. Furthermore, in this study, patients suffering from IBS may have been previously treated with probiotics.

Response to treatment

In both groups, no clinically relevant changes *vs* baseline

were observed during the study either for the GIQLI score or for the HAD score.

At the end of the treatment period, a similar improvement in the abdominal pain severity score was observed with the test drug (-13.1 ± 20.5) and the placebo (-11.9 ± 27.5). In patients with predominance of diarrhoea, no change in the abdominal pain severity score was observed with the placebo at the end of the 4-wk treatment period (-0.1 ± 26.5), whereas a clinically relevant decrease occurred with the test drug (-18.4 ± 26.3, i.e., 36%).

In the whole population, the improvements in the IBS severity score observed with LCR35 and placebo were not significantly different. Indeed, after a 25% decrease at the end of the treatment period (-63.2 ± 100.6 and -64.3 ± 95.9, respectively; *P* = 0.9692), a 15% decrease from baseline was observed 2 wk later in both treatment groups (-40.6 ± 110.1 and -36.0 ± 109.5, respectively; *P* = 0.8829).

Absolute and relative changes in the four IBS subgroups are presented in Table 2.

In IBS patients with predominance of diarrhoea, the clinical response was in favour of the active drug. Indeed, no change in the symptom severity score was observed with the placebo at the end of the 4-wk treatment period, whereas a more marked decrease occurred with the test drug (-36.6% *vs* -3.1%). Furthermore, in spite of an increase in the symptom intensity, the IBS severity score was maintained below the baseline value 2 wk later with the test drug (-19.1% from baseline), whilst a slight 4.9% increase from baseline was observed with the placebo.

Even if no statistical analysis could be performed due to the small sample size of this subgroup, the graphic representation of these results (Figure 2) clearly shows the differences in the clinical responses induced by the test drug and the placebo in IBS patients with predominance of diarrhoea.

In mixed IBS patients, the response observed at the end of the treatment (a 20% and 30% decrease in the IBS severity score with LCR35 and placebo, respectively) was maintained at the end of the study for both treat-

Table 3 Distribution of patients according to the irritable bowel syndrome severity score classes *n* (%)

	Placebo (*n* = 25)	LCR35 (*n* = 25)
Baseline IBS severity score (V2)		
150-300 (moderate symptoms)	25 (100.0)	25 (100.0)
Post-treatment IBS severity score (V3)		
0-150 (mild symptoms)	12 (48.0)	8 (32.0)
150-300 (moderate symptoms)	8 (32.0)	13 (52.0)
> 300 (severe symptoms)	5 (20.0)	4 (16.0)
IBS severity score at the end of study (V4)		
0-150 (mild symptoms)	9 (36.0)	6 (24.0)
150-300 (moderate symptoms)	8 (32.0)	13 (52.0)
> 300 (severe symptoms)	8 (32.0)	6 (24.0)

IBS: Irritable bowel syndrome.

ment groups, with no relevant clinical difference between treatments.

A clinical response slightly in favour of placebo was observed at the end of the treatment period in IBS patients with predominance of constipation (-41% *vs* -20%) and unsubtyped IBS patients (-47% *vs* -17%). The same value was maintained 2 wk later.

After 4 wk of treatment, the patient distribution according to the IBS severity score classes was slightly in favour of the placebo (48% *vs* 32% of improved patients, Table 3), and this observation correlates with the results observed on the main criterion.

The results obtained on the responder rates were in accordance with the results reported above. Indeed, the percentage of patients with a 50% reduction in the IBS severity score was higher in the placebo group (40% *vs* 28%), except for IBS patients with predominance of diarrhoea who showed a responder rate higher with LCR35 compared to placebo (43% *vs* 12%).

Adverse events
There were no adverse effects attributable to treatment with either LCR35 or placebo.

DISCUSSION
The results of this placebo-controlled pilot study showed that IBS symptoms assessed by the IBS severity score did not improve with LCR35 complete freeze-dried culture when considering the whole population, and no clinically relevant changes *vs* baseline were observed either for the GIQLI score or the HAD score. Yet, when considering IBS subtyped patients, it can be seen on the graphic representation of the data that a deterioration in the baseline symptom score was never observed with the test drug, and the line graphs show that the evolution pattern of the IBS severity score differed between the IBS subtypes. Indeed, a clinical response in favour of LCR35 complete freeze-dried culture was observed in IBS patients with predominance of diarrhoea.

The efficacy of therapeutics for IBS is undoubtedly impacted by the heterogeneous pathogenesis of IBS, and

up to now there is no recognised reference treatment for this pathology. Results observed in the present study are not surprising because the fact that subgroups of patients with IBS are likely to respond differently to a treatment is often discussed in the literature. Thus, sub-grouping of IBS patients may be important both for optimizing treatment responses by the practicing clinician as well as improving the outcome from clinical trials of novel therapeutic modalities. Thus, some authors also recommend that limiting trials to defined subgroups of patients should be considered to enhance homogeneity of the study population[30,31]. More recently, when validating the Rome III criteria, Longstreth *et al*[19] emphasize that "due to heterogeneity of IBS and to the fact that bowel pattern subtypes are highly instable, it may be desirable, in both research and practice, to base drug use on a stronger bowel pattern predominance".

Many papers have discussed the difficulties of the methodology to be used in IBS clinical research, and recommendations have been drawn to minimize bias in trials of functional GI disorders. Nevertheless, there is no consensus on IBS clinical trial methodology; in particular, there is no standardized outcome assessments[10,32]. Major problems with clinical trial design are the multiple presentations of the disease and the placebo response which is extremely variable and high, up to 70%. Therefore, it is recommended that all IBS trials be placebo controlled and it is essential that clinical trials are conducted on consistently identified patients with clearly defined outcome measures. These outcome measures should not only deal with symptom relief but also improvement in quality of life[30]. As the symptomatology of IBS is highly unstable, the so-called placebo responses may equally well be the temporary spontaneous improvements that are part of the condition[33]. Furthermore, there is evidence that psychiatric disorders have an adverse influence on the outcome of irritable bowel syndrome. Thus, accurate measurement of psychological symptoms as predictors of outcome is an important aspect of trial design for IBS therapy, and selection criteria need to take both physical and psychological domains into account[34]. The results of the present study observed in the placebo group confirm the importance of the psychological impact in IBS patients.

The design of the present study complied with the recommendations in the literature. It was double blinded and placebo controlled and used internationally approved diagnostic criteria for a clinical trial in IBS ("Rome III criteria"[19,35]), in order to allow a homogeneous population to be selected. For the assessment of efficacy, a clear well defined outcome measure was chosen as the primary efficacy parameter. Indeed, the IBS severity scale is a tool which was described in the literature as the only IBS symptom severity scale "shown to be responsive to treatment effects"[36]. Thus, the study complied with the recommendations of the Rome Committee[37]. The duration of treatment was based on the evaluation of medicinal products recommendations with a main efficacy criterion assessed after a 4-wk treatment period[38]. As recom-

mended in a recent meta-analysis highlighting important considerations for the design of probiotic controlled trials[32], every effort was made by the investigators to minimize loss-to-follow-up (none occurred in our study) and to adhere to "Intent-to-Treat" principles analyzing all subjects with the group to which they were originally assigned (our main analysis was done on the FAS set).

In our study population, the female predominance for IBS (70%), the mean age of 47.1 years, and the symptom severity as assessed by the IBS severity score were similar to data published in the literature and support the pertinence of our results. The IBS severity score at inclusion was close to the one reported in a French observational study on 1407 patients in gastroenterological practice (254.3 ± 41.9 with a range of 161-299 *vs* 268.5 ± 85.2 with a range of 10-487) but all of our patients had moderate symptom severity, whilst the observational study included 45% of patients with severe symptom intensity[39]. The distribution of patients according to IBS subtypes (IBS with predominance of constipation: 22%; IBS with predominance of diarrhoea: 30%; mixed IBS: 34%; unsubtyped IBS: 14%) was also similar to that of an observational study carried out in 1092 patients recruited by 159 GPs and 75 gastroenterologists (IBS with predominance of constipation: 22%; IBS with predominance of diarrhoea: 26%; mixed IBS: 29%; unsubtyped IBS: 22%)[40]. The mean value of the GIQLI score at inclusion showed clearly the negative impact of IBS on the QoL of our patients. The baseline value (63.4 ± 8.1) was lower than the value reported in patients in the study carried out to validate the French version of this questionnaire: the mean score was 126 for healthy individuals and 96 for patients[23].

Factors which might explain the absence of statistically significant results in the present trial are as follows: This study was a pilot study performed on a rather small sample size. The results in favour of the test drug might be confirmed with a statistically significant difference *vs* placebo in a future trial on a larger number of patients and, as discussed above, on a defined subgroup of patients (IBS patients with predominance of diarrhoea and mixed IBS subtypes).

Regarding the tool used to assess QoL, it must be pointed out that the GIQLI questionnaire is a generalist questionnaire for gastroenterological practice. As the QoL is known to be particularly altered in patients complaining of diarrhoea, it may be argued that this evaluation tool was not adapted to assess accurately the impact of diarrhoea on daily QoL.

In our study performed by gastroenterologists, patients suffered from marked IBS symptoms with a marked negative impact on QoL as shown by baseline values of IBS severity score (mean value close to the upper values of the moderate intensity class) and GIQLI (30% lower than in patients involved in the study which validated the French version of this questionnaire). The question of the likely impact of recruitment site has been often addressed in the literature[19].

In the French observational study carried out in 2000, the descriptive analysis of management practices demonstrated that patients who referred to gastroenterologists have a rather severe chronic form of IBS. Moreover, a search for a relationship between the qualitative score and the number of consultations nevertheless demonstrated that most patients first consult a general practitioner despite the fact that at that time there was access to specialists in the French healthcare system[39].

Two recent meta-analyses of randomized controlled trials on probiotics for the treatment of IBS showed heterogeneity across studies as to the outcome measures used to assess the severity of IBS symptoms, making it challenging to compare results across studies. Both meta-analyses selected the proportion of subjects with improvement in global IBS symptoms as the primary outcome to demonstrate that probiotics may improve IBS symptoms[10,32]. Thus, it is not possible to compare the results obtained with LCR35 complete freeze-dried culture in this study to results published for other probiotics.

The tolerability of LCR35 complete freeze-dried culture prescribed at the minimum daily dose of 6×10^8 CFU for 4 wk was excellent, and no adverse event was reported throughout the trial in the active group. This dose, used in several published studies, is the dose usually prescribed in daily practice for IBS patients[41-44]. The good tolerability displayed in this study is in accordance with the McFarland's review of probiotics controlled trials which did not find any evidence of significant adverse effects due to these treatments[32]. Given their superior safety profile compared to drug therapies usually prescribed in IBS, and the efficacy results observed with some probiotics against all of the primary IBS symptoms[13], as well as the impact of many probiotics on "gas-related" symptoms[45], probiotics may ultimately prove more acceptable for long-term therapy than medications with adverse effects.

As functional bowel disorders are diseases without morbi-mortality, treatments prescribed should not be more deleterious than the disorder itself[46,47]. Therapies should focus on specific gastrointestinal dysfunctions (e.g., constipation, diarrhoea, pain), and medications only should be used when non-prescription remedies do not work or when symptoms are severe.

This study showed that in 85% of patients treated with LCR35, *Lactobacillus* was found in their stools with a concentration of at least 10^4 living bacteria per gram, indicating that survival in the digestive tract is possible.

As in any pilot study, this study did not aim to definitely demonstrate the efficacy of LCR35 complete freeze-dried culture in IBS patients. It was designed to test the trend in the magnitude of variation in clinical response measures, to evaluate the effect size in an attempt to predict an appropriate sample size and improve upon the study design prior to performance of a full-scale research project. Thus, it is not surprising that small sample size, a strong placebo effect and the lack of uniformity of patients led to results that did not reach statistical sig-

nificance in the global population. Nevertheless, in IBS patients complaining of diarrhoea, the trend to lower global symptom score and abdominal pain sub-score (pain being the most bothersome symptom in IBS patients) after treatment observed with the test drug but not with the placebo, is an interesting observation suggesting that LCR35 complete freeze-dried culture might be useful in this subgroup of IBS patients. This observation made in sub-typed patients is in accordance with the fact that it is recognized that no drug is effective in treating all IBS symptoms because a variety of processes appear to be at work in this disorder and IBS sufferers are not a homogeneous population. As a precise characterization of patients is likely to lead to better therapeutic results, our results are encouraging and need to be confirmed in larger studies. Safety is a main concern in patients with gastrointestinal disorders, and deleterious adverse events are not acceptable in a relatively mild, non-fatal condition. The excellent safety profile of LCR35 complete freeze-dried culture shown in this study makes this probiotic strain, demonstrated to survive in the digestive tract, a reasonable choice for IBS.

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COMMENTS

Background

Irritable bowel syndrome (IBS) is the most common diagnosis made by gastro-enterologists. Despite its prevalence and its impact on quality of life and health expenditures, conventional medical treatment is notoriously unsatisfactory, and many patients try alternative or complementary therapies. Among them, probi-otics are generating great interest.

Research frontiers

Studies have observed altered intestinal microflora in IBS patients and an in-crease in symptoms after enteric infections, suggesting that restoration of the intestinal microflora may be a useful therapeutic goal. One strategy to restore normal flora is the use of probiotics. Probiotics are beneficial bacteria or yeasts that are ingested to improve health. Probiotics are also known to modulate the immune response and reduce cytokine production. This pilot study investigated the efficacy and safety of *Lactobacillus casei rhamnosus* LCR35, a probiotic used for its antidiarrhoeal properties for more than 50 years and shown *in vitro* to adhere to intestinal cells and to display antibacterial activity against a large variety of pathogens. Major problems in clinical research on IBS are the multiple presentations of this disease, the high placebo response and the absence of any consensus on the main outcome measure.

Innovations and breakthroughs

This study showed a clinically significant improvement in global symptomatol-ogy, and especially in abdominal pain (the most bothersome symptom in this pathology), in one subgroup of patients called "IBS patients with predominance of diarrhoea". Stool analysis demonstrated that *Lactobacillus casei rhamnosus* LCR35 was able to survive in the digestive tract. The improvement in symptom severity observed in sub-typed patients is in accordance with the fact that it is recognized that no drug is effective in treating all IBS symptoms because IBS sufferers are not a homogeneous population. The small sample size, a strong

placebo effect and the lack of uniformity of patients may contribute to the ab-sence of significant results in the global population. The clinical results and the excellent safety profile of LCR35 shown in this study make this probiotic strain a reasonable choice for IBS.

Applications

The findings in this pilot study indicate that subgrouping of patients with IBS may be important both for optimizing treatment responses by the practicing clinician as well as improving the outcome from future clinical trials on larger numbers of patients.

Peer review

In this pilot study, the authors evaluate the efficacy and safety profile of a newer probiotic in IBS patients. Treatment of IBS is still largely unsatisfactory, and thus newer treatments would add to the armamentarium of IBS therapy. The ques-tion posed by the authors is novel and well defined. However, the title should probably be changed to better reflect the nature of the study (e.g., "Efficacy and safety profile of LCR35 complete freeze-dried culture in irritable bowel syndrome: A randomized, double-blind study"). The methods are appropriate and well described. The data are sound and well controlled. The discussion and conclusions are well balanced and adequately supported by the data. On the other hand, the sample size is small, though the authors have stated this clearly as a limitation of their study.

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BRIEF ARTICLE

Age, smoking and overweight contribute to the development of intestinal metaplasia of the cardia

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Abstract

AIM: To assess the role of *Helicobacter pylori* (*H. pylori*), gastroesophageal reflux disease (GERD), age, smoking and body weight on the development of intestinal metaplasia of the gastric cardia (IMC).

METHODS: Two hundred and seventeen patients scheduled for esophagogastroduodenoscopy were enrolled in this study. Endoscopic biopsies from the esophagus, gastroesophageal junction and stomach were evaluated for inflammation, the presence of *H. pylori* and intestinal metaplasia. The correlation of these factors with the presence of IMC was assessed using logistic regression.

RESULTS: IMC was observed in 42% of the patients. Patient age, smoking habit and body mass index (BMI) were found as potential contributors to IMC. The risk of developing IMC can be predicted in theory by combining these factors according to the following formula: Risk of IMC = a + s - 2B where a = 2,...6 decade of age, s = 0 for non-smokers or ex-smokers, 1 for < 10 cigarettes/d, 2 for > 10 cigarettes/d and B = 0 for BMI < 25 kg/m^2 (BMI < 27 kg/m^2 in females), 1 for BMI > 25 kg/m^2 (BMI > 27 kg/m^2 in females). Among potential factors associated with IMC, *H. pylori* had borderline significance (*P* = 0.07), while GERD showed no significance.

CONCLUSION: Age, smoking and BMI are potential factors associated with IMC, while *H. pylori* and GERD show no significant association. IMC can be predicted in theory by logistic regression analysis.

INTRODUCTION

The development of gastric cancer involves an interplay of bacterial, host, and environmental facts, including dietary factors, lifestyle factors and *Helicobacter pylori* (*H. pylori*)[1-3].

The worldwide incidence of gastric cancer has declined over the recent decades[4]. Part of this decline is due to the recognition of risk factors such as *H. pylori* infection and other environmental risk factors[3,5]. Despite the overall decline in gastric cancer, there has been a significant increase in the incidence of cancer of the gastric cardia[6]. The shift from distal to proximal stomach may be due to the decrease in the distal cancers.

However, it has also been proposed that adenocarcinomas at the cardia represent a different entity of antral gastric adenocarcinomas[7]. Indeed, environmental factors or chemical carcinogens may be more strongly associated with cardia carcinomas compared with more distal gastric carcinoma[8]. On the other hand, the proximal gastric carcinomas differ from distal gastric carcinomas as they are not associated with a severe form of gastritis characterized by atrophy and/or intestinal metaplasia[9,10].

Carcinomas of the gastric cardia appear to be similar to those associated with Barrett's esophagus, with which they share some demographic features[9]. Several studies indicated that obesity satisfies several criteria for a causal association with gastroesophageal reflux disease (GERD) and some of its complications, including erosive esophagitis, and esophageal adenocarcinoma[11-13]. Therefore, obesity could represent an important factor in the development of cardia carcinoma.

The development of cardia carcinoma seems to be preceded by intestinal metaplasia, which is secondary to chronic inflammation[1,14-16]. However, the etiology of intestinal metaplasia of the gastric cardia (IMC) remains controversial. To our knowledge, no study has evaluated the effects of age, smoking and body weight on IMC. Thus, our aim was to set up a cross-sectional study to examine these the role of these factors, and also *H. pylori* and GERD. To evaluate the incidence of IMC and the respective role of these factors on the development of IMC at a particular point in time, we enrolled a population of outpatients with upper gastrointestinal (GI) endoscopy scheduled for various reasons.

MATERIALS AND METHODS

Study design and population
The study complied with the Declaration of Helsinki regarding investigation in humans and was approved by our institutional Ethics committee (Commission Centrale d'Ethique) (Controlled-trials.com, Number ISRCTN15324190, www.con trolled-trials.com). This was an investigator-initiated study with no involvement of industry.

Inclusion criteria
All outpatients scheduled for an elective upper GI endoscopy were eligible for inclusion in the study. Exclusion criteria were: age < 18 years, pregnancy, patients unable to give their own consent, a previous history of upper GI surgery, severe bleeding diathesis (platelet count < 50 000/mm^3, prothrombin rate < 50%), psychiatric dis-

eases, allergy to lidocaine. All patients signed an informed consent form.

For this study, 250 consecutive patients scheduled for upper GI endoscopy for a variety of conditions were recruited over a 2.5-year period. Among them, 217 accepted the study protocol (acceptance rate of 86%). Endoscopy was performed in the left lateral position after local anesthesia using a 10% xylocaine spray with the patient under conscious sedation using midazolam as reported previously[17]. A standard endoscopy was performed, including retroflexion in the stomach (Table 1).

Endoscopic biopsies
Each patient had 2 biopsies performed in the esophagus 2 cm above the Z-line, 4 biopsies at the esophagogastric junction, 2 biopsies in the cardia located within 10 mm below the Z-line, 2 biopsies in the fundus (greater and lesser curvature), 2 biopsies in the antrum (greater and lesser curvature), and one biopsy in the angulus. In the case of Barrett's esophagus, 4 biopsies were performed every cm in the Barrett's segment. All biopsy specimens were fixed in 0.5% formaldehyde solution and stained with haematoxylin eosin, Giemsa, and Gomori-aldehyde-fuschin. Two experienced GI pathologists (BH, MB), who were blinded to the clinical diagnosis, analyzed the biopsies. The diagnosis of IMC was reserved for patients with intestinal metaplasia detected in biopsy specimens sampled from the macroscopically normal-appearing gastroesophageal junction.

Study variables and data collection
As previously defined by Vakil *et al*[18], clinically significant GERD was diagnosed when reflux of the gastric contents caused troublesome symptoms and/or complications. It was considered negative if there were no such symptoms (Score 0), positive if the patient presented symptoms once a month (Score 1) or more than once a month (Score 2).

The presence of a hiatus hernia was defined as widening of the muscular hiatal tunnel and circumferential laxity of the phrenoesophageal membrane[19], allowing a portion of the stomach to slide into the thorax.

Smoking habit (never smoking: score 0; past smoker: score 1; current smoker < 1 pack per day: score 2; current smoker > 1 pack per day: score 3), body mass index (BMI) and age were recorded.

Endoscopic biopsies from the esophagus, the Z-line, the cardia, the fundus and the antrum were histologically evaluated for the following criteria: (1) Acute and chronic inflammation using a visual analogue scale from 0 to 3 as proposed by Dixon *et al*[20] in an updated Sydney system, 0 being none and 3 being marked; (2) Absence or presence of incomplete (types Ⅰ, Ⅱ) or complete (type Ⅲ) intestinal metaplasia as defined by Filipe *et al*[21]; and (3) Absence or presence of *H. pylori*.

Barrett's esophagus was defined as "a change in the distal esophageal epithelium of any length that can be recognized as metaplastic mucosa at endoscopy and confirmed to have intestinal metaplasia by biopsy of the tubu-

Table 1 Characteristics of the cohort study group *n* (%)

	Total	Men	Women	*P* value	OR (95% CI)
No. of patients (%)	217	97 (45)	120 (55)		
Age (yr)	45.3 ± 15.3	49.9 ± 16.5	43.3 ± 13.5		
Reason for endoscopy[1]					
Epigastric pain	62 (29)	31	31	NS	
Barrett's esophagus	26 (12)	20	6	0.01	4.94 (1.9-12.8)
Reflux grade 1	17 (8)	6	11	NS	
Reflux grade 2	141 (65)	69	72	NS	
Dyspepsia	9 (4)	3	6	NS	
Dysphagia	2 (1)	2	0	NS	
Gastric bypass	48 (22)	7	41	0.01	0.15 (0.06-0.35)
Anemia	5 (2)	2	3	NS	
Celiac disease suspicion	7 (3)	4	3	NS	
Ulcer follow-up	8 (4)	6	2	NS	
Helicobacter antibiogram	4 (2)	1	3	NS	
Personal history of reflux	141 (65)	69	72	NS	
Tobacco use					
Non smoker	119 (55)	46	73	NS	
Past smoker	11 (5)	5	6	NS	
Current smoker all	87 (40)	46	41	NS	
Current smoker 0.5 p/d	48 (22)	27	21	NS	
Current smoker > 1.0 p/d	39 (18)	19	20	NS	
BMI	29.8 ± 10.6	32.4 ± 11.5	27.7 ± 7.7		
PPI users	96 (44)	52	44	NS	
NSAID users	37 (17)	20	17	NS	

P value indicates results of the comparison of male and female patients. [1]Patients may have more than one pathology. OR: Odds ratio; BMI: Body mass index; PPI: Proton pump inhibitor; NSAID: Non steroidal antiinflammatory drug; NS: Not significant.

lar esophagus"[22]. Short segment Barrett's esophagus was considered if metaplasia extended < 3 cm into the tubular esophagus or long segment Barrett's esophagus if metaplasia extended > 3 cm into the tubular esophagus. Patients taking nonsteroidal anti-inflammatory drugs and/or proton pump inhibitors (PPI) during the last month before inclusion and continuously for more than 6 mo were defined as regular non steroidal antiinflammatory drug or PPI users.

Statistical analysis

The analysis of group variables was studied primarily using cross-tabulation analysis (Fischer's exact test). The Kruskal-Wallis test was used to test equality of the population. Multivariate analysis of predictors of IMC was performed by linear logistic regression. To accomplish this goal, a model was created that included all predictor variables. Multiple logistic regression analysis was used to develop an equation to predict a logit transformation of the probability of IMC based on risk factors that included in the equation: age (years), BMI (kg/m^2), smoking habit, sex, reflux disease and hiatal hernia. Age and BMI, were modeled as continuous variables, and sex was modeled as a categorical variable (0 = male and 1 = female). The final mathematical equation provided an estimate of a subject's likelihood of having IMC. *P* values < 0.05 were interpreted as statistically significant. *P* values > 0.05 were taken as non significant (NS). SPSS advanced models 10.0 was used for statistical analysis.

RESULTS

A total of 217 patients were enrolled in the current study.

Table 1 presents the characteristics of the group according to the sex distribution. There were no significant differences among the two groups except a higher frequency of Barrett's esophagus in men and a higher rate of gastric bypass in women. BMI exceeded 25 kg/m^2 in 54 males (56%) and 27 kg/m^2 in 61 females (51%). Because our surgical team has a long lasting program of bariatric surgery[23], a substantial number of patients evaluated for gastric bypass (22%) was included but the mean BMI in the overall cohort was < 30 kg/m^2. Epigastric pain and gastroesophageal reflux were the major causes for endoscopy. Of note, almost half of the patients was taking a PPI and 45% of the patients were past or current tobacco users.

Endoscopy was normal in 36% of the patients whereas erosive esophagitis and hiatus hernia were the main endoscopic findings in our population (Table 2). Barrett's esophagus was histologically confirmed in 13% of the patients. *H. pylori* was present in the gastric biopsies of 70 patients (32%).

We analyzed the potential relationship between the presence of *H. pylori* and various factors including GERD, Barrett's esophagus, hiatus hernia, tobacco use, sex and BMI (Table 3). There were statistically more patients infected by *H. pylori* with a normal esophagogastric junction (45%) than with long (16%) or short segment Barrett's esophagus (11%) (*P* < 0.05). Patients with a hiatus hernia were less frequently infected by *H. pylori* than patients without (*P* < 0.05). Current tobacco users were more frequently infected by *H. pylori* than non or past smokers (*P* < 0.05).

Table 2 Results of upper gastrointestinal endoscopy *n* (%)				
	Total	**Men**	**Women**	***P* value**
No. of patients	217	97	120	
Normal	79 (36)	43	36	NS
Esophagitis[1]	26 (12)	14	12	NS
Hiatus hernia[1]	60 (28)	36	24	NS
Short segment Barrett's	9 (4)	6	3	NS
Long segment Barrett's	19 (9)	11	8	NS
Esophageal tumor	4 (2)	3	1	NS
Gastric ulcer	4 (2)	3	1	NS
Gastric tumor	1 (0.5)	1	0	NS
Gastritis	9 (4)	5	4	NS
Duodenal ulcer	3 (1)	2	1	NS
Duodenal atrophy	2 (1)	1	1	NS
Other	1 (0.5)	1	0	NS
H. pylori				
Positive	70 (32)	33	37	NS
Negative	147 (68)	64	83	NS

P value indicates results of the comparison of male and female patients. [1]Patients may have more than one pathology. NS: Not significant; *H. pylori*: *Helicobacter pylori*.

The correlation between the presence of reflux symptoms, and Barrett's esophagus, hiatus hernia, tobacco use, sex, age and BMI was shown in Table 4. All patients with short segment Barrett's esophagus and 90% of patients with long segment Barrett's esophagus had significant reflux ($P < 0.01$). The presence of reflux was not statistically significantly associated with the presence of hiatus hernia or increased BMI.

IMC was observed in 92 patients (42%), 48 were men and 44 were female, and the mean age was 27.3 years. IMC was not statistically associated (although there was a tendency) with the presence of *H. pylori* ($P > 0.05$), whereas *H. pylori* was strongly associated with the presence of inflammation of the cardia (carditis) since 82% of patients infected with *H. pylori* had carditis compared with 30% in the group without *H. pylori* infection ($P < 0.001$). Furthermore, there was a strong relationship between IMC and metaplasia found in other gastric areas, including the antrum and the corpus of the stomach. This relationship was not present in patients with Barrett's esophagus. BMI was found to be significantly lower and age significantly greater in patients with IMC than in patients with normal cardia mucosa. *H. pylori* played a pivotal role in the development of metaplasia of the antrum and the fundus, with an odds ratio reaching 7.4 and 11.2 respectively compared with patients not infected with *H. pylori* (Table 5).

GERD was not significantly associated with acute and chronic inflammation of the gastroesophageal junction, and it was not associated with the presence of IMC (borderline significance 0.06) (Table 5). Compared with patients with either short or long segment Barrett's esophagus, patients with IMC had significantly fewer GERD symptoms.

In univariate analysis, age, BMI and tobacco use were statistically significantly associated with IMC (Table 5). Using logistic regression analysis, the presence of IMC could be predicted by the patient's age, smoking habit

Figure 1 The curve indicates the actual risk (with standard deviation) of cardia metaplasia as a function of the personal clinical score.

and the absence of overweight according to the following formula: Risk score of IMC = a + s - 2B where a = 2,… 6 decade of age, s = 0 for non-smokers or ex-smokers, 1 for < 10 cigarettes per day, 2 for > 10 cigarettes per day, and B = 0 for BMI < 25 kg/m^2 (BMI < 27 kg/m^2), 1 for BMI > 25 kg/m^2 (BMI > 27 kg/m^2) for males (females), respectively. In the presence of these factors, *H. pylori* had only a borderline significance ($P = 0.07$) and would contribute only 1 point on the above scale which ranges from 0 to 8. Therefore, for a 50-year-old patient smoking 15 cigarettes per day and with a BMI of 26 kg/m^2, the theoretical risk of having IMC is 5. The data of 82 patients with IMC were analyzed to obtain the actual frequency in percent of IMC as a function of the calculated risk score for a given patient using the above equation (Figure 1). Finally, the presence of *H. pylori* was associated with severe acute and chronic inflammation in the antrum ($P < 0.01$), corpus ($P < 0.01$) and in the Z-line area ($P < 0.01$).

DISCUSSION

During the last few decades there has been a marked increase in the incidence of adenocarcinoma of the esophagogastric junction in Western countries in contrast to the reduction in incidence of distal gastric cancer. Most observers believe that this is the consequence of an increased rate of adenocarcinoma of the distal esophagus and a decreased incidence of distal gastric cancer related to *H. pylori* eradication. If the above-mentioned epidemiologic relationships are correct, this could indicate that the so-called cardia adenocarcinomas are not related to *H. pylori* infection but to other factors, and eventually may not be considered to be "gastric" cancers.

Because of the debated roles of different clinical factors in the emergence of malignant neoplasia of the gastric cardia, and because many reports have stated that IMC precedes the development of cardiac cancer, our study was aimed at assessing the respective roles of *H. pylori*, GERD, age, smoking habit and body weight on the development of IMC in a large group of patients. To this end our cross-sectional study enrolled outpatients scheduled for upper GI endoscopy for various reasons and specifi-

Table 3 Relationship between the presence of *Helicobacter pylori* and reflux, Barrett's esophagus, hiatus hernia, tobacco, sex and body mass index *n* (%)

	Total	*H. pylori* negative	*H. pylori* positive	*P* value	OR (95% CI)
H. pylori	217	147	70		
Reflux = 0	59 (27)	36 (61)	23 (39)	NS	
Reflux = 1	17 (8)	11 (65)	6 (35)	NS	
Reflux = 2	141 (65)	100 (71)	41 (29)	NS	
Short segment Barrett's	9 (4)	8 (89)	1 (11)	NS	
Long segment Barrett's	19 (9)	16 (84)	3 (16)	NS	
Normal gastroesophageal junction	189 (87)	122 (65)	67 (45)	0.01	4.58 (1.33-15.73)
Hiatus hernia					
Absent	157 (72)	100 (64)	57 (36)	0.04	2.06 (1.03-4.13)
Present	60 (28)	47 (78)	13 (22)	0.03	0.49 (0.24-0.98)
Tobacco					
Non smoker	119 (55)	85 (71)	34 (29)	NS	
Past smoker	11 (5)	9 (82)	2 (18)	NS	
Current smoker (all)	87 (40)	53 (61)	36 (39)	0.03	1.88 (1.08-3.35)
Current smoker 0.5 p/d	48 (22)	26 (54)	22 (46)	0.03	5.2 (1.1-4.12)
Current smoker > 1.0 p/d	39 (18)	27 (69)	12 (31)	NS	
Age					
Mean age	45.3	46.57	42.78	0.06	
Sex					
Male	97 (45)	64 (66)	33 (34)	NS	
Female	120 (55)	83 (69)	37 (31)	NS	
Body Mass Index					
Mean BMI	29.8	30.6	28.1	NS	

P value indicates results of the comparison of *H. pylori* positive patients and *H. pylori* negative patients. OR: Odds ratio; BMI: Body mass index; NS: Not significant; *H. pylori*: *Helicobacter pylori*.

Table 4 Relationship between reflux and Barrett's esophagus, hiatus hernia, tobacco use, sex, age and body mass index *n* (%)

	Total	Reflux			*P* value
		Grade 0	Grade 1	Grade 2	
No. of patients	217	59	17	141	
Short segment Barrett's	9 (4)	1 (11)	0	8 (89)	0.010
Long segment Barrett's	19 (9)	1 (5)	1 (5)	17 (90)	0.010
Normal gastroesophageal junction	189 (87)	59 (31)	16 (9)	114 (60)	0.002
Hiatus hernia					
Absent	157 (72)	49 (31)	13 (8)	94 (61)	0.060
Present	60 (28)	10 (17)	4 (7)	46 (76)	NS
Tobacco					
Non smoker	119 (55)	32 (27)	13 (11)	74 (62)	NS
Past smoker	11 (5)	1 (10)	1 (10)	9 (80)	NS
Current smoker all	87 (40)	26 (29)	11 (13)	51 (48)	NS
Current smoker 0.5 p/d	48 (22)	14 (29)	7 (15)	27 (56)	NS
Current smoker > 1.0 p/d	39 (18)	12 (30)	3 (8)	24 (62)	NS
Sex					
Male	97 (45)	22 (23)	6 (6)	69 (71)	NS
Female	120 (55)	37 (31)	11 (9)	72 (60)	NS
Age					
Mean age	45.3	42	45.2	46.8	0.080
Body Mass Index					
Mean BMI	29.8	27.9	33.1	30.2	0.080

BMI: Body mass index; NS: Not significant.

cally searched for the presence of IMC. The study then examined all the above mentioned variables to evaluate their respective role on the development of IMC at a particular point in time. For this purpose, each patient had endoscopic gastric and esophageal biopsies. We paid specific attention to the cardia area because the location and extent of the gastric cardia are controversial. Today,

the vast majority of the data available on the cardia and cardia cancer are not comparable because of variations in the diagnostic criteria. Thus, to avoid any problem in "cardia" definition, we followed rigorous anatomist and endoscopist recommendations that defined the cardia as being the part of the stomach that lies around the orifice of the tubular esophagus, and which corresponds to the

Table 5 Factors associated with intestinal metaplasia of the cardia *n* (%)

	Total	Intestinal metaplasia		*P* value	OR (95% CI)
		Absent	Present		
No. of patients (%)	217	125 (58)	92 (42)		
Indications for endoscopy					
GERD[1]	141 (65)	106 (75)	35 (25)	NS	
Epigastric pain[1]	62 (29)	41 (66)	21 (34)	NS	
Gastric bypass	48 (22)	30 (63)	18 (37)	NS	
Results of endoscopy					
Normal	79 (36)	41 (52)	38 (48)	NS	
GERD	26 (12)	10 (38)	16 (62)	0.060	
Hiatus hernia	60 (28)	26 (43)	34 (57)	NS	
Barrett's esophagus	28 (13)	13 (46)	15 (54)	NS	
Other	29 (13)	17 (59)	12 (41)	NS	
H. pylori					
Positive	70 (32)	34 (49)	36 (51)		
Negative	147 (68)	91 (62)	56 (28)	0.060	
Reflux = 0	59 (27)	33 (56)	26 (44)		
Reflux = 1	17 (8)	15 (88)	2 (12)		
Reflux = 2[2]	141 (65)	77 (55)	64 (45)	0.060	
BMI	29.8 ± 10.6	31.61	27.33		
Age	45.3 ± 15.3	43.29	50.68		
Sex					
Male	97 (45)	55 (57)	42 (43)	NS	
Female	120 (55)	70 (58)	50 (42)	NS	
Tobacco use					
Non smoker	119	89	30		
Past smoker	11	8	3	0.500	0.6 (0.13-1.9)
Current smoker all	87	28	59	0.001	6.19 (3.4-11.2)
Current smoker 0.5 p/d	48	14	32	0.001	4.23 (2.1-8.5)
Current smoker > 1.0 p/d	39	12	27	0.010	4.33 (2.1-9.1)
Antrum metaplasia	19	5	14	0.010	4.31 (1.49-12.4)
Fundus metaplasia	9	1	8	0.001	11.8 (1.45-96.1)

P value indicates results of the comparison of patients with or without intestinal metaplasia of the cardia. [1]patients may have more than one reason for endoscopy; [2]data are missing. GERD: Gastroesophageal reflux disease; OR: Odds ratio; BMI: Body mass index; NS: Not significant; *H. pylori*: *Helicobacter pylori*.

point at which the tubular esophagus joins the saccular stomach[24-26].

In our study, IMC was histologically found in 92 patients (42%). Whereas *H. pylori* was present in 70 patients (32%), the distribution of *H. pylori* was similar in patients with or without IMC, suggesting that no significant relationship between *H. pylori* and IMC exists. We found that *H. pylori* was strongly associated with the presence of carditis without IMC, as previously reported. Indeed, Sotoudeh *et al*[3] found a significant relationship between carditis and *H. pylori* infection in an Iranian cohort of patients in which the infection rate was as high as 85% whereas our infection rate was close to 32%. In contrast to our study, Goldblum *et al*[10] found that not only was *H. pylori* associated with carditis but also with IMC. In our series there was a strong correlation between the presence of *H. pylori* and acute or chronic inflammation in biopsies taken at different sites of the stomach. This finding is consistent with several other eastern observations showing that carditis is more associated with *H. pylori* infection than with GERD. In our study, GERD was present in 141 patients (65%) and was not found to be significantly associated with IMC, whereas GERD was strongly associated with acute and chronic inflammation of the gastroesophageal junction, a feature already

reported by others[27,28]. Voutilainen *et al*[14] reported that there are two dissimilar types of chronic inflammation of the gastric cardiac mucosa that seem to occur: one existing in conjunction with chronic *H. pylori* infection and the other with normal stomach and erosive GERD. Patients with IMC also had significantly higher rates of metaplasia of other gastric areas. Therefore finding antral or corporeal metaplasia should alert the gastroenterologist to redo a biopsy of the cardia in a subsequent endoscopy. The absence of any correlation between the presence of IMC and Barrett's esophagus might indicate that IMC is a distinct entity from Barrett's esophagus as proposed by Golblum[16]. Similar inflammation and mucosal alteration in the antrum and the corpus should be regarded as a potential sign of predisposition to IMC. We found that the risk of having IMC was associated with increasing age since 70.5% of patients with IMC were > 40 years old, a feature also reported by McNamara *et al*[29] in a cohort of 36 patients with IMC. Among other factors associated with IMC, tobacco use was found to be strongly associated with the development of IMC and 68% of patients with IMC were smokers, data never reported before, to our knowledge. Indeed, Koizumi *et al*[5] reported an increased risk of IMC of 1.84 (1.39-2.43 95% CI) only for antral cancer in current smokers compared with subjects

who had never smoked. BMI was surprisingly found to be a protective factor: the greater the BMI the less it contributed to the risk of IMC. This specific point should be balanced by the fact that the majority of our population was under the threshold of obesity. This finding could seem controversial against the current literature that suggests a link between obesity, GERD and carcinoma[30].

Although a real prospective study design is needed to assess a prediction, our statistician has tried to find the best fitting model to describe the relationship between the presence of IMC and various predisposing factors. The final mathematical equation provided an evaluation (and not the risk) of a subject's likelihood of having IMC. In the current study, the presence of IMC could be predicted by age, smoking habit and low or normal BMI. The evaluation showed no association with GERD. *H. pylori* had only a borderline significance ($P = 0.07$) if any. Nevertheless, this point must be emphasized because recent studies have found two types of cardia cancer, one linked to *H. pylori*-associated atrophic gastritis, and the other associated with nonatrophic gastritis, resembling esophageal adenocarcinoma[7].

Although our study was not prospective, it gives results at a particular point in time, and points out the greater need for endoscopic surveillance of gastric cardia mucosal changes in individuals aged 40 and over. Regarding the epidemiology of adenocarcinoma of the esophagus and gastroesophageal junction, the patterns of each disease are sufficiently alike to implicate shared risk factors or even to represent a single neoplastic entity. Although studies at the molecular level have produced conflicting results, many have demonstrated the similarity between adenocarcinomas of the cardia and esophagus. Most important is the evidence that the overall survival is similar in patients with gastroesophageal junction and esophageal adenocarcinoma.

In summary, this study indicates that chronically inflamed gastric mucosa in the cardia area can be replaced by intestinal metaplasia, a finding which has been shown to precede the development of cardia cancer. Whereas esophageal adenocarcinomas are strongly associated with GERD and obesity and are inversely associated with *H. pylori*, we suggest that IMC is not convincingly associated with GERD, is inversely correlated with BMI and has a dubious association with the presence of *H. pylori,* but is associated with increased age and a smoking habit. A prospective study aimed at evaluating the true incidence and natural history of IMC is therefore clearly needed.

COMMENTS

Background
Development of carcinoma of the cardia seems to be preceded by intestinal metaplasia. Gastroesophageal reflux and *Helicobacter pylori* (*H. pylori*) infection are together believed to cause intestinal metaplasia of the cardia (IMC).

Research frontiers
Despite the overall decline in gastric cancer, there has been a significant increase in the incidence of cancer of the gastric cardia. Because the roles of different clinical factors in the emergence of cardia malignancy are still debated,

there is a need to carry out further studies aiming at defining the risk factors for developing IMC.

Innovations and breakthroughs
This cross-sectional study indicated that IMC is associated with increased age and smoking, but is not strongly associated with *H. pylori infection* and gastroesophageal reflux disease.

Applications
The study indicates there is a greater need for endoscopic surveillance of gastric cardia mucosal changes in individuals aged 40 and over.

Terminology
Intestinal metaplasia of the cardia represents a mucosal change of the gastric cardia that is secondary to chronic inflammation and that can be considered as a potential precursor of cancer.

Peer review
The paper is a well-written manuscript with an important topic in clinical epidemiology. The authors tried to study the risk factors for intestinal metaplasia of the cardia. In addition they compared these factors with the epidemiological criteria of patients with Barrett's esophagus. The results show that Barrett mucosa and IMC differed according their etiological factors.

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BRIEF ARTICLE

Rifaximin, but not growth factor 1, reduces brain edema in cirrhotic rats

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Abstract

AIM: To compare rifaximin and insulin-like growth factor (IGF)-1 treatment of hyperammonemia and brain edema in cirrhotic rats with portal occlusion.

METHODS: Rats with CCl_4-induced cirrhosis with ascites plus portal vein occlusion and controls were randomized into six groups: Cirrhosis; Cirrhosis + IGF-1; Cirrhosis + rifaximin; Controls; Controls + IGF-1; and Controls + rifaximin. An oral glutamine-challenge test was performed, and plasma and cerebral ammonia, glucose, bilirubin, transaminases, endotoxemia, brain water content and ileocecal cultures were measured and liver histology was assessed.

RESULTS: Rifaximin treatment significantly reduced bacterial overgrowth and endotoxemia compared with cirrhosis groups, and improved some liver function parameters (bilirubin, alanine aminotransferase and aspartate aminotransferase). These effects were associated with a significant reduction in cerebral water content. Blood and cerebral ammonia levels, and area-under-the-curve values for oral glutamine-challenge tests were similar in rifaximin-treated cirrhotic rats and control group animals. By contrast, IGF-1 administration failed to improve most alterations observed in cirrhosis.

CONCLUSION: By reducing gut bacterial overgrowth, only rifaximin was capable of normalizing plasma and brain ammonia and thereby abolishing low-grade brain edema, alterations associated with hepatic encephalopathy.

Òdena G, Miquel M, Serafin A, Galan A, Morillas R, Planas R, Bartolí R. Rifaximin, but not growth factor 1, reduces brain edema in cirrhotic rats. *World J Gastroenterol* 2012; 18(17): 2084-2091 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i17/2084.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i17.2084

INTRODUCTION

Hepatic encephalopathy (HE) is a complication of advanced hepatic insufficiency characterized by a wide range of neurological and neuropsychiatric symptoms, ranging from subclinical manifestations to hepatic coma[1]. When cirrhotic patients develop HE, their survival prognosis considerably worsens[2], and liver transplantation has to be considered[3].

It is well known that high plasma ammonia levels play a central role in the multifactorial network of mechanisms leading to HE[4-6]. In fact, ammonia reaches the liver *via* the portal vein from the intestine as a result of bacterial degradation of nitrogenous compounds and as a consequence of the metabolism of glutamine by the enzyme glutaminase[7]. In addition, urea cycle activity in cirrhotic patients is decreased due to the reduction of liver cell mass[8]. Both the presence of portosystemic shunts and the loss of parenchymal cells in the liver of cirrhotic patients lead to an increase in plasma ammonia levels and are key factors in the development of HE in these patients[9]. A traditional therapeutic approach to HE is to decrease plasma ammonia levels by decreasing ammoniagenic substrates, as well as inhibiting ammonia generation, reducing its intestinal absorption, and facilitating its elimination[10].

Non-absorbable antibiotics and/or non-absorbable disaccharides have been used as a standard treatment of HE in human cirrhosis[10-14]. Recent studies have demonstrated that rifaximin reduces the risk of hospitalization involving HE without producing side effects[11,15]. Rifaximin is a non-absorbable rifamycin derivative with activity against aerobic and anaerobic microorganisms, which are an important source of ammonia[7,11,16]. Furthermore, rifaximin is not absorbed by the gut, thereby allowing the antibiotic to reach high concentrations in the intestinal tract and to remain in the feces in its active form[10,17].

Insulin-like growth factor (IGF)-1 is a powerful anabolic hormone that exerts anabolic and trophic effects in many tissues, acting in an endocrine, paracrine and autocrine manner[18]. Levels of IGF-1 are markedly decreased in liver cirrhosis. Several studies have shown that the administration of low doses of IGF-1 (i.e., 4 μg/100 g body weight per day) reduces liver fibrosis, improves liver function, increases intestinal absorption of nutrients and corrects osteopenia and hypogonadism in experimental liver cirrhosis[19-22]. Previous work from our laboratory has demonstrated that IGF-1 therapy enhances intestinal barrier function, and reduces endotoxemia and bacterial translocation in cirrhotic rats[23]. Most of these alterations are considered to be precipitating factors leading to HE, therefore, the administration of IGF-1 could be a novel therapeutic approach for this condition.

The aim of this study was to compare the efficacy of rifaximin and IGF-1 in the treatment of HE using a combined model of intrahepatic hypertension (CCl4-induced cirrhosis plus ascites) and extrahepatic hypertension generated through portal vein occlusion - a proven new animal model of hyperammonemia and brain edema related to decompensated advanced liver cirrhosis, recently described in our laboratory[24], which exhibits most of the alterations present in type C HE.

MATERIALS AND METHODS

Male Sprague-Dawley OFA rats weighing about 100 g were included in the study. All animals were caged individually at a constant room temperature of 21 ℃, exposed to a 12/12-h light/dark cycle and provided free access to a standard rodent chow (A04; Harlan Ibérica S.A, Barcelona, Spain). Rats received 1.5 mmol/L phenobarbital, an inducer of cytochrome P450 enzymatic activity, in their drinking water. The study was conducted according to guidelines established by the Guide for the Care and Use of Laboratory Animals and was approved by the Ethical and Research Committee of our research institute.

Experimental design

Six groups of rats were studied. (1) Cirrhosis (group 1; $n = 9$): rats with CCl4-induced liver cirrhosis with ascites plus portal vein occlusion treated with placebo (saline); (2) Control (group 2; $n = 10$): sham-operated control rats treated with placebo; (3) Cirrhosis + IGF-1 (group 3; $n = 9$): rats with CCl4-induced liver cirrhosis with ascites plus portal vein occlusion treated with IGF-1 (2 μg/100 g s.c. twice daily for 14 d); (4) Control + IGF-1 (group 4; $n = 9$): sham-operated control rats treated with IGF-1 (2 μg/100 g s.c. twice daily for 14 d); (5) Cirrhosis + R (group 5; $n = 9$): rats with CCl4-induced liver cirrhosis with ascites plus portal vein occlusion treated with rifaximin (50 mg/kg daily by gavage for 14 d); and (6) Control + R (group 6; $n = 9$): sham-operated control rats treated with rifaximin (50 mg/kg daily for 14 d).

Animal procedures

Ascitic cirrhotic rats with portal occlusion were assessed as previously described[24]. Briefly, when animals reached a body weight of 200 g, cirrhosis was induced by intragastric administration of CCl4 through an orogastric stainless steel tube (Poper and Sons, New Hyde Park, NY, United States). The initial dose was 20 μL, and subsequent doses were adjusted based on changes in body weight[25]. Six weeks after starting cirrhosis induction, animals underwent partial portal vein occlusion (> 0.9 mm portal diameter) achieved by ligating around a 20 G needle, followed by complete portal vein occlusion 48 h later[26]. Surgical procedures were performed under strict aseptic conditions; animals were anesthetized for surgery using ketamine, diazepam and atropine, and were subsequently administered 30 μg (s.c.) buprenorphine (Buprex; Schering-Plough, Madrid, Spain) for 3 d. Cirrhosis induction was continued (CCl4 administration) until ascites developed. When ascites was diagnosed (by abdominal paracentesis), animals were randomized to receive the corresponding treatment (placebo, rifaximin or IGF-1) for 14 d. Control rats were subjected to sham operation and were also randomized in parallel. Twelve hours after

finishing treatment, rats underwent an oral glutamine-challenge test, and immediately afterward were sacrificed by bilateral thoracotomy. Peripheral and portal blood, cecum fecal content, and solid tissue (brain and liver) were obtained.

Oral glutamine-challenge test

A load of 100 mg/kg of L-glutamine (SHS S.A., Barcelona, Spain) was administered through an orogastric stainless steel tube. Venous blood samples (150 μL) from the femoral vein were drawn preload (baseline) and every 30 min for 4 h for ammonia determination. Body temperature was monitored and maintained between 36 ℃ and 38 ℃ using an infrared lamp. Samples were centrifuged *in situ* for 10 min at 2000 × *g*, and plasma was stored at -80 ℃ until analysis. The area under the curve (AUC) of the ammonemia response was also calculated using Graph Pad Prism for Windows version 5.01 (La Jolla CA, United States).

Biochemical characterization

Blood samples were obtained during sacrifice. Biochemical determinations [aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and glucose] were made using an autoanalyzer (Dimension Clinical Chemistry System, Dade Behring-Siemens, Madrid, Spain).

Endotoxin levels

Endotoxemia was quantified in all groups of rats using a Limulus amebocyte lysate kinetic test (Endosafe Charles River, L'Abresle Cedex, France*)*. Briefly, plasma samples were diluted 1:5 with endotoxin-free water and then heated to 70 ℃ for 5 min. Afterwards, samples were further diluted (final dilution, 1:50) and assessed.

Cecal bacterial content

During the course of laparotomy and after harvesting all other samples, the cecal region was identified and 1 mL of content was obtained by cecal puncture. Cecal bacterial content was measured by culturing serially diluted samples (1/8000 and 1/160 000) on non-selective blood agar plates. All samples were cultured in triplicate. After an incubation period of 48 h, the number of colony-forming units (CFU) was counted. The composition of isolated flora was determined using standard bacteriological identification techniques. The results were expressed as CFU/mL of cecal content. Cecal bacterial overgrowth was defined as a stool bacterial count greater than the mean of healthy control rats plus two standard deviations[27].

Determination of plasma and brain ammonia

Ammonia was measured in plasma and cerebral cortex. Briefly, blood (150 μL) was drawn from the femoral vein and centrifuged in heparinized tubes. The resulting plasma samples were stored at -80 ℃ until analysis. Brain samples were weighed, homogenized, and deproteinized by adding five volumes of cold perchloric acid (6%)

and then centrifugation at 12 000 × *g* for 20 min. After neutralization with KHCO₃ (25% w/v), samples were stored at -80 ℃ until analysis, which was performed using a commercial enzymatic Ammonia Assay Kit (Sigma-Aldrich, Madrid, Spain).

Low-grade brain edema

Low-grade brain edema was measured as brain water content. Briefly, a frontal left hemisphere brain sample from each rat was excised, weighed, and heated to 90 ℃ for 48 h in a drying oven to evaporate all water content. Then, dried samples were weighed again. The difference between initial and final weight was considered as the water content[28].

Hepatic histology

Liver samples for histological examination were collected in 4% formaldehyde, subsequently embedded in paraffin wax, sliced into 5-μm sections, and stained with hematoxylin and eosin. Liver samples were evaluated using the Scheuer scoring system[29].

Statistical analysis

Unless otherwise indicated, results are expressed as mean ± SE or proportions, as appropriate. Comparisons of means among groups were performed using one-way analysis of variance or corresponding non-parametric (Kruskal-Wallis) tests; *post hoc* comparisons to identify pairs of groups significantly different at the 0.05 level were made using the Duncan test or the Mann-Whitney *U* test, respectively. Differences in proportions among groups were compared using the χ^2 test. Statistical analysis were performed with SPSS for Windows version 13.0 (Chicago, IL, United States).

RESULTS

General features

No differences in any of the parameters studied, except for fecal bacterial count (as expected), were observed among the three sham-operated control groups. In contrast, all parameters were significantly altered in cirrhosis plus portal vein occlusion groups compared to control groups.

Body weight and ascites development

Body weight at sacrifice was similar in cirrhotic groups and was significantly lower than in controls (overall *P* = 0.017). No differences in the time elapsed between the first CCl₄ dose and ascites development were observed among the groups (range: 8-15 wk). None of the ascitic rats showed any signs of infection or sepsis.

Biochemical characterization

Liver function and liver damage parameters are summarized in Table 1. Liver cirrhosis plus portal vein occlusion resulted in a significant increase in serum AST, ALT and bilirubin, and a decrease in serum glucose con-

	Endotoxin (EU)	Glucose (mmol/L)	Bilirubin (μmol/L)	ALT (UI/L)	AST (UI/L)
Table 1 Biochemical features of groups studied					
Cirrhosis	0.582 ± 0.069[a]	9.41 ± 2.17[a]	22.0 ± 6.71[a,c]	181.2 ± 16.1[a]	333.2 ± 84.2[a]
Controls	0.374 ± 0.037	27.63 ± 2.39	2.9 ± 0.15	68.9 ± 9.9	228.3 ± 24.6
Cirrhosis + IGF-1	0.432 ± 0.033	9.43 ± 0.70[a]	10.2 ± 2.96[a]	210.3 ± 58.6[a]	482.3 ± 121.8[a]
Controls + IGF-1	0.363 ± 0.032	25.17 ± 2.42	3.2 ± 0.10	94.4 ± 22.1	179.2 ± 30.1
Cirrhosis + R	0.410 ± 0.041	17.75 ± 3.06[e]	5.0 ± 1.23	121.4 ± 18.4	262.1 ± 49.1
Controls + R	0.368 ± 0.027	29.39 ± 2.08	3.1 ± 0.20	97.7 ± 23.4	236.2 ± 43.2

[a]$P < 0.05$ *vs* all control groups; [c]$P < 0.05$ *vs* cirrhosis + R; [e]$P < 0.05$ *vs* control + R. IGF: Insulin-like growth factor; R: Rifaximin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Figure 1 Blood ammonia levels. Comparison of the concentrations of basal plasma ammonia in cirrhotic groups (closed bars) and control groups (open bars). Cirrhosis plus portal vein occlusion resulted in a significant increase in basal ammonia levels. Rifaximin-treated cirrhotic rats showed plasma ammonia levels similar to those observed in controls; by contrast, insulin-like growth factor (IGF)-1 treatment was unable to normalize these values. [a]$P < 0.05$ *vs* each control group. Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

Figure 2 Brain ammonia levels. Comparison of the concentrations of brain ammonia in cirrhotic groups (closed bars) and control groups (open bars). Liver cirrhosis plus portal vein occlusion resulted in an increase in brain ammonia levels compared to controls, whereas in rifaximin-treated cirrhotic rats, these levels remained similar to those in controls. insulin-like growth factor (IGF)-1 did not significantly decrease these values compared to the respective controls. [a]$P < 0.05$ *vs* each control group. Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

centrations. However, in rifaximin-treated cirrhotic rats, these alterations tended to be less marked. In fact, no differences in bilirubin or transaminases were observed in this group compared to controls. By contrast, all of these biochemical parameters remained significantly altered in the IGF-1-treated group compared to control groups, indicating that IGF-1 treatment was unable to improve liver function.

Endotoxin levels
Portal blood endotoxin levels were significantly increased only in placebo-treated cirrhotic rats (0.582 ± 0.069 *vs* 0.374 ± 0.037; $P = 0.044$). By contrast, both IGF-1 and rifaximin treatments normalized endotoxemia levels, producing similar values relative to their respective controls (Table 1).

Blood and brain ammonia levels and oral glutamine-challenge test
Liver cirrhosis plus portal vein occlusion resulted in hyperammonemia. Blood ammonia levels were increased in placebo-treated cirrhotic rats compared to placebo-treated controls (284 ± 29 μmol/L *vs* 144 ± 47 μmol/L; $P = 0.007$). Rifaximin treatment improved ammonemia in cirrhotic rats, reducing ammonia to levels similar to those observed in rifaximin-treated controls (205 ± 47 μmol/L *vs* 128 ± 37 μmol/L; $P = 0.122$). By contrast,

IFG-1 treatment failed to reduce plasma ammonia levels, which remained significantly increased compared to those observed in controls (323 ± 58 μmol/L *vs* 142 ± 35 μmol/L; $P = 0.004$; Figure 1).

Similarly to ammonemia, brain ammonia levels were significantly higher in placebo-treated cirrhotic rats than in placebo-treated controls (0.38 ± 0.03 mmol/kg *vs* 0.22 ± 0.01 mmol/kg; $P = 0.006$). Rifaximin treatment normalized brain ammonia levels in cirrhosis, yielding values similar to those observed in rifaximin-treated control rats (0.27 ± 0.03 mmol/kg *vs* 0.24 ± 0.03 mmol/kg; $P = 0.429$). Again, IGF-1 treatment was ineffective; brain ammonia levels in IGF-1-treated cirrhotic rats were significantly higher than those in controls (0.35 ± 0.04 mmol/kg *vs* 0.25 ± 0.02 mmol/kg; $P = 0.039$; Figure 2).

Further analysis of these results (Figure 3) showed that AUCs after oral glutamine-challenge tests were significantly increased in placebo and IGF-1 treatments in cirrhotic rats compared with each control group (3770 ± 501 *vs* 2419 ± 501; $P = 0.043$ and 5216 ± 1144 *vs* 2120 ± 428; $P = 0.021$). By contrast, the AUC in rifaximin-treated cirrhotic rats was similar to that observed in controls (3310 ± 870 *vs* 2525 ± 302; $P = 0.240$).

Cecal bacterial content
Cecal bacterial content was significantly increased in

Figure 3 Ammonia response to oral glutamine-challenge tests. The area under the curve (AUC) was significantly increased by both placebo and insulin-like growth factor (IGF)-1-treatment in cirrhotic rats. By contrast, the AUC in rifaximin-treated cirrhotic rats was similar to that observed in controls. Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

Figure 4 Cecal bacterial populations. Comparison of cecal bacterial content in cirrhotic groups (●) and control groups (○). Bacterial overgrowth was defined as a colony-forming units (CFU) count greater than the mean ± 2SD of the control group (dashed line). CCl₄-induced cirrhosis plus portal vein occlusion resulted in cecal bacterial overgrowth in placebo and insulin-like growth factor (IGF)-1-treated cirrhotic rats ([a]$P < 0.05$ *vs* all others). Rifaximin treatment reduced bacterial content in cirrhotic rats to a level similar to that observed in the rifaximin-treated control group; bacterial counts in these latter groups were the lowest among all groups ([c]$P < 0.05$). Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

both the placebo-treated cirrhosis group and cirrhotic rats treated with IGF-1 compared with their respective controls ($18.6 \pm 3.6 \times 10^6$ CFU/mL *vs* $8.4 \pm 1.3 \times 10^6$ CFU/mL; $P = 0.042$ and $20.4 \pm 4.6 \times 10^6$ CFU/mL *vs* $8.9 \pm 1.4 \times 10^6$ CFU/mL; $P = 0.047$). Only rifaximin treatment reduced bacterial content in cirrhotic rats; the values in rifaximin-treated cirrhotic rats were similar to those observed in the rifaximin-treated control group ($4.4 \pm 1.4 \times 10^6$ CFU/mL *vs* $4.4 \pm 1.3 \times 10^6$ CFU/mL; $P = 0.931$), and were significantly lower than those in the placebo-treated cirrhosis group ($P = 0.003$) and cirrhosis + IGF-1 group ($P = 0.002$). Moreover, as shown in Figure 4, rifaximin eliminated bacterial overgrowth (threshold value, 17.64×10^6 CFU/mL) in cirrhotic rats, whereas almost 50% of rats in the placebo-treated cirrhosis group (4/9 rats) and cirrhosis + IGF-1 group (5/9 rats) showed bacterial overgrowth (overall $P < 0.05$ *vs* each control and cirrhosis + rifaximin groups).

Brain water content
Liver cirrhosis plus portal vein occlusion resulted in a significant increase in brain water content compared to placebo-treated controls ($79.17\% \pm 0.22\%$ *vs* $78.26\% \pm 0.04\%$;

Figure 5 Low-grade brain edema. Low-grade brain edema was determined by measuring the percentage brain water content in cirrhotic groups (closed bars) and control groups (open bars). Placebo-treated cirrhotic rats showed the presence of low-grade brain edema. Brain water content in rifaximin-treated cirrhotic rats was similar to that in control groups, and was significantly lower to that observed in placebo-treated cirrhotic rats. Insulin-like growth factor (IGF)-1 treatment did not diminish brain edema; brain water content in the cirrhosis + IGF-1 group was similar to that observed in the cirrhosis + placebo group. [a]$P < 0.05$ *vs* all control groups; [c]$P < 0.05$ *vs* cirrhosis + R. Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

$P = 0.002$). Rifaximin treatment was accompanied by a significant reduction in low-grade brain edema, as demonstrated by the fact that brain water content in this group of cirrhotic rats was similar to that measured in rifaximin-treated control rats ($78.61\% \pm 0.31\%$ *vs* $78.24\% \pm 0.19\%$; $P = 0.233$) and significantly lower than that observed in placebo-treated cirrhotic rats ($P = 0.046$). IGF-1 treatment did not diminish brain edema; brain water content in the cirrhosis + IGF-1 group was similar to that observed in the placebo-treated cirrhosis group ($P = 0.566$) and was significantly higher than that observed in the control + IGF-1 group ($78.94\% \pm 0.08\%$ *vs* $78.34\% \pm 0.19\%$; $P = 0.009$; Figure 5).

Histology
All ascitic cirrhotic rats with portal vein occlusion developed liver cirrhosis with regeneration nodules, necrosis, and steatosis regardless of treatment received. All CCl₄-treated animals scored F4 with the Scheuer system. As expected, all control groups showed normal hepatic histology (Figure 6).

DISCUSSION
In this study, we demonstrated the effectiveness of rifaximin in normalizing both ammonemia and brain ammonia levels, and consequently averting the appearance of low-grade brain edema in ascitic cirrhotic rats with portal vein occlusion; an experimental model of hyperammonemia related to decompensated advanced cirrhosis. These alterations play a central role in the multifactorial mechanisms leading to HE as a result of chronic liver disease.

Although administration of low doses of IGF-1 has been proposed as a promising therapy for cirrhotic patients on the basis of preclinical data showing that this hormone displays hepatoprotective and antifibrogenic

Figure 6 Photomicrographs of liver sections. All cirrhotic rats developed micronodular cirrhosis with regeneration nodules, necrosis and steatosis regardless of treatment (A: Cirrhosis; B: Cirrhosis + IGF-1; C: Cirrhosis + rifaximin). All control rats showed normal cellular architecture (D: Control; E: Control + IGF-1; F: Control + rifaximin).

activities[7,9,21], we observed virtually no positive effect of IGF-1 on most of the alterations that lead to HE in this experimental model.

Given that the major precipitating factor leading to HE in cirrhosis is the presence of large amounts of ammonia, not only in the bloodstream but especially in the brain, and further considering that the main source of this ammonia is production by enteric bacteria, the two key factors that warrant particular attention are: (1) Deranged function of the liver, which introduces ammonia into the urea cycle and is the main ammonia-detoxifying organ; and (2) The presence of bacterial overgrowth related to disturbed intestinal transit.

In this context, rifaximin treatment was accompanied by a slight, but significant, improvement of some parameters of liver function, such as glucose, bilirubin and ALT. This improvement cannot be mainly attributed to a decrease in endotoxin levels, which is known to promote activation and release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α[30,31], because these parameters were also diminished in the IGF-1 treated group (group 3) without producing any positive effect on liver function.

Notwithstanding these observations, a recent study

has observed a direct effect of norfloxacin, another "non-absorbable" antibiotic widely used for selective intestinal bacterial decontamination, in cirrhotic patients. Norfloxacin actively accumulates in polymorphonuclear cells, leading to a decrease in plasma TNF-α and interferon-γ levels, and a reduction in oxidative stress[32]. Although these mechanisms were not explored in the present study, we cannot rule out the possibility that a similar action of rifaximin could explain our results. Further studies are needed to examine this possibility.

However, we did not observe the hepatoprotective effects of IGF-1 administration in experimental cirrhosis that have been reported by others[20]. In our study, hepatic function (glucose, bilirubin, AST, ALT) in IGF-1-treated cirrhotic rats was similar to that observed in untreated cirrhotic rats. We attribute these differences to the fact that, in our study, all animals presented with well-established cirrhosis plus ascitic decompensation.

As mentioned previously, cirrhotic patients present several alterations in gut motility that could lead to an increase in gut bacterial content[33]. A close cause and effect relationship between bacterial overgrowth and plasma ammonia levels has been reported, reflecting the fact that enteric bacterial fermentation is the main source

of ammonia. In our study, cirrhotic groups treated with placebo or IGF-1 (groups 1 and 2) showed a significant increase in cecal bacterial content compared with control groups. By contrast, rifaximin treatment dramatically reduced cecal bacterial content, not only in cirrhotic rats but also in control rats (groups 3 and 6), as reported in other studies[34,35]. As a consequence of this reduction in bacterial content, plasma ammonia levels in rifaximin-treated cirrhotic rats (group 3) remained similar to those observed in controls (groups 4-6). Similarly, brain ammonia levels were normalized in rifaximin-treated rats. In keeping with this, low-grade brain edema was absent in this group of rats. Again, consistent with its inability to modify cecal bacterial content, IGF-1 failed to improve any of these parameters.

In conclusion, our data indicate that, by reducing gut bacterial overgrowth and improving liver function, rifaximin may be useful in the treatment of most alterations associated with HE in experimental cirrhosis, whereas the administration of low doses of IGF-1 is not indicated in this condition.

COMMENTS

Background
Hepatic encephalopathy (HE) in cirrhosis appears as a consequence of hepatic failure and/or the presence of portosystemic shunting that leads to the passage of nitrogenate compounds such as ammonia from the gut to the systemic circulation, which in turn, lead to an increase of brain water content. Non-absorbable antibiotics and/or non-absorbable disaccharides have been used as a standard treatment of HE in human cirrhosis.

Research frontiers
Rifaximin has been reported to be useful for its activity against aerobic and anaerobic microorganisms, which are an important source of ammonia. However, no data regarding the effect of rifaximin on low-grade brain edema in experimental HE in cirrhotic rats has been reported. Also, insulin-like growth factor (IGF)-1 could be useful for the treatment of that pathology for its antifibrogenic and anabolic effects.

Innovations and breakthroughs
For the first time, authors have demonstrated in an experimental model of hyperammonemia related to decompensated cirrhosis that the effectiveness of rifaximin for the treatment of HE is mainly due to its efficacy in reducing low-grade brain edema. On the contrary, low doses of IGF-1 have no effects in preventing liver damage or hyperammonemia.

Applications
By understanding unequivocally that rifaximin is a useful therapy in the treatment of hyperammonemia and most of the alterations associated with HE in cirrhosis. The data suggest that the use of non-absorbable antibiotics could be a good therapeutic strategy.

Terminology
Rifaximin is a non-absorbable rifamycin derivative, with activity against enteric aerobic and anaerobic microorganisms. IGF-1 is a powerful anabolic hormone with diverse endocrine, paracrine and autocrine effects. In cirrhosis, the reduction of functional hepatocellular mass causes a marked fall in IGF-1 serum levels.

Peer review
This is an experimental study comparing the efficacy of rifaximin and IGF-1 in the treatment of some of the alterations observed in HE such as hyperammonemia and low-grade brain edema. The results showed that only rifaximin, by abolishing bacterial overgrowth, is capable of reducing hyperammonemia and low-grade brain edema in cirrhotic rats. By contrast, low doses of IGF-1 are not indicated for this pathology.

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BRIEF ARTICLE

New reduced volume preparation regimen in colon capsule endoscopy

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Abstract

AIM: To evaluate the effectiveness of our proposed bowel preparation method for colon capsule endoscopy.

METHODS: A pilot, multicenter, randomized controlled trial compared our proposed "reduced volume method" (group A) with the "conventional volume method" (group B) preparation regimens. Group A did not drink polyethylene glycol electrolyte lavage solution (PEG-ELS) the day before the capsule procedure, while group B drank 2 L. During the procedure day, groups A and B drank 2 L and 1 L of PEG-ELS, respectively, and swallowed the colon capsule (PillCam COLON® capsule). Two hours later the first booster of 100 g magnesium citrate mixed with 900 mL water was administered to both groups, and the second booster was administered six hours post capsule ingestion as long as the capsule had not been excreted by that time. Capsule videos were reviewed for grading of cleansing level.

RESULTS: Sixty-four subjects were enrolled, with results from 60 analyzed. Groups A and B included 31 and 29 subjects, respectively. Twenty-nine (94%) subjects in group A and 25 (86%) subjects in group B had adequate bowel preparation (ns). Twenty-two (71%) of the 31 subjects in group A excreted the capsule within its battery life compared to 16 (55%) of the 29 subjects in group B (ns). Of the remaining 22 subjects whose capsules were not excreted within the battery life, all of the capsules reached the left side colon before they stopped functioning. A single adverse event was reported in one subject who had mild symptoms of nausea and vomiting one hour after starting to drink PEG-ELS, due to ingesting the PEG-ELS faster than recommended.

CONCLUSION: Our proposed reduced volume bowel preparation method for colon capsule without PEG-ELS during the days before the procedure was as effective

as the conventional volume method.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer mortality in developed countries[1,2]. In recent years, colon capsule endoscopy has received widespread attention as an emerging minimally invasive endoscopic technique that is likely to impact on colorectal examination[3-10]. It is gradually being accepted as a useful diagnostic technique, particularly in Europe. Van Gossum *et al*[6] evaluated the first generation PillCam colon capsule and reported that the sensitivity for detecting patients with advanced polyps was 73% regardless of colon cleansing level, increasing to 88% in the subgroup of patients having adequate bowel preparation. These results have clearly shown that colon cleanliness plays an important role in providing optimal colon visualization when using colon capsule endoscopy.

However, the most commonly used preparation method may require as much as 6 L of fluid intake over two days. Reducing the volume of fluid intake is an important consideration in increasing patient acceptance of colon capsule endoscopy. Regarding traditional colonoscopy, there have been efforts to reduce patient stress by limiting fluid intake for bowel preparation to the day of examination which has resulted in better cleansing quality compared with the conventional volume method[11,12]. With respect to colon capsule endoscopy, there is no presently published report on bowel preparation during the day of examination only.

We intended to simplify the bowel preparation method by eliminating polyethylene glycol electrolyte lavage solution (PEG-ELS) on the day before the examination and to increase acceptance for colon capsule endoscopy. The aim of this study was to evaluate the effectiveness of the proposed reduced bowel preparation method for colon capsule endoscopy in terms of colon cleanliness and colon capsule excretion rates within the capsule's

battery life.

MATERIALS AND METHODS

Study group

The study was a pilot, multicenter (six medical facilities), prospective, randomized controlled trial comparing our proposed "reduced volume method" with the "conventional volume method" of bowel preparation used with colon capsule endoscopy. The subjects were recruited between October 2009 and March 2010, and included men and women between 18 and 79 years of age who were either asymptomatic healthy volunteers or symptomatic patients. The study protocol was approved by the institutional review boards at each of the six participating medical facilities. This study was registered in the *UMIN Clinical Trials Registry* (registration ID number: UMIN000002562). Written informed consent was obtained from all subjects prior to enrollment in the study.

Subjects were stratified according to their specific medical facility, gender, age ($\geq$ 40 years or < 40 years), and whether they were asymptomatic or symptomatic. Subjects were randomly assigned to one of the two study groups with different PEG-ELS (Muben®; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) administration protocols: Group A (reduced volume method) received 2 L PEG-ELS during the procedure day, before capsule ingestion; Group B (conventional volume method) received 2 L PEG-ELS on the night before the procedure day and an additional 1 L during procedure day, before capsule ingestion.

Exclusion criteria included presence of dysphagia, constipation, congestive heart failure, renal insufficiency, diabetes, digestive tract diverticulum, a history of radiotherapy, accompanying cancerous peritonitis, Crohn's disease or ulcerative colitis, familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer; individuals taking non-steroidal anti-inflammatory drugs, morphine hydrochloride or tranquilizers or having a history of allergic reaction to any of the medications planned for use in this study, a cardiac pacemaker or other implanted electromedical devices; as well as anyone currently pregnant, having had abdominal surgery, suspected of symptoms or having a history of intestinal obstruction or stenosis; and any other cases in which a doctor considered it inappropriate.

According to a four-point scale grading system assessing colon cleanliness[5], based on an assumption that the average cleansing score of groups A and B are 3.5 and 3.0 with a standard deviation of 0.70 within each group, and α error of 0.05 and β error of 0.20, the required sample size is 27 subjects per group, with a total of 54 subjects.

Bowel preparation

The bowel preparation procedure is shown in Table 1. On the day before examination, all subjects had three meals consisting of a low fiber diet using ENIMACLIN® (Glico,

Table 1 Bowel preparation procedures for colon capsule endoscopy

	Group A	Group B
Day 1		
Three regular meals	Low fiber diet	Low fiber diet
Evening (7-9 pm)	-	2 L PEG-ELS
Bedtime	24 mg sennoside	24 mg sennoside
Day 0[1]		
1st Step	100 mL water including 1 g pronase and 2.5 g sodium bicarbonate	100 mL water including 1 g pronase and 2.5 g sodium bicarbonate
2nd Step	15 mg mosapride	15 mg mosapride
3rd Step	2 L PEG-ELS with 400 mg dimethicone	1 L PEG-ELS with 400 mg dimethicone
4th Step[2]	Additional 300 mL PEG-ELS	Additional 300 mL PEG-ELS
	Maximum, administered twice: Maximum dosage 600 mL	Maximum, administered twice: Maximum dosage 600 mL
0 h	Colon capsule ingestion	Colon capsule ingestion
2 h (Booster I [3])	50 g magnesium citrate/900 mL water	50 g magnesium citrate/900 mL water
6 h (Booster II)	50 g magnesium citrate/900 mL water	50 g magnesium citrate/900 mL water
7 h	5 mg mosapride	5 mg mosapride

PEG-ELS: Polyethylene glycol electrolyte lavage solution. [1]If capsule excreted early, remaining regimen discontinued immediately; [2]If bowel preparation judged as complete by checking the color of evacuation, 4th step skipped and subjects permitted to ingest colon capsule. In cases of inadequate preparation, additional PEG-ELS permitted in both groups; [3]In the case of capsule still present in stomach, subject received 5 mg of mosapride as prokinetic agent (maximum dosage, 15 mg).

Osaka, Japan) and 24 mg oral sennoside prior to bedtime. Group A did not receive any PEG-ELS the night before examination, while group B received 2 L PEG-ELS at 7 p.m.

On the day of examination, all subjects drank 100 mL water which contained 1 g pronase and 2.5 g sodium bicarbonate followed by 15 mg mosapride. Then, group A subjects drank 2 L PEG-ELS with 400 mg dimethicone over 2 h, while group B subjects drank 1 L PEG-ELS with 400 mg dimethicone over 1 h. Experienced medical staff assessed the quality of the bowel preparation by checking the clarity of subjects' evacuation before the subjects were given the colon capsule. A reference which we use to evaluate the quality of bowel preparation as a standard procedure of total colonoscopic examination in our facilities is outlined in Figure 1. Using this reference, we define that the capsule is ready to be ingested when a grade 5 quality is achieved.

In cases of unsatisfactory preparation, additional amounts of PEG-ELS (maximum total dosage, 0.6 L) were administered prior to capsule ingestion (Table 1).

When bowel preparation was judged to be complete, each subject ingested the first generation colon capsule (PillCam colon capsule, Given Imaging Inc., Yoqneam, Israel). Two hours later, the capsule location was checked with a real-time viewing monitor (RAPID® Access Real Time Tablet PC; Given Imaging). If the capsule had

passed through the stomach, the subject received the first booster consisting of 50 g magnesium citrate (Magcorol P®, Horii Pharmacological Co. Ltd, Osaka, Japan) in 900 mL water in which 200 mg dimethicone was dissolved. If the capsule was in the stomach, the subjects received 5 mg mosapride as a prokinetic agent every 15 min until the capsule passed through the stomach or up to a maximum mosapride dosage of 15 mg. If the capsule was not excreted by 6 h after ingestion, subjects received a second booster similar to the first booster.

If the capsule was not excreted by 7 h after ingestion, subjects ingested 5 mg mosapride and were then permitted to eat dinner. Defecation should have been completed within eight hours so a suppository of 10 mg bisacodyl was administered to those subjects who had not excreted the colon capsule within that timeframe.

Colon capsule examination
The study employed a first generation PillCam COLON capsule, and the examinations were conducted without colon intubation and insufflations or sedation. The capsule enabled recording of images for 3 min after activation, then became inactive for 1 h 45 min (sleep mode) to save battery energy. After the capsule reactivated ("woke up"), its normal operational time was approximately 6-8 h depending on the capsule's actual battery life.

Evaluation of colon cleanliness and capsule excretion
Overall colon cleanliness was determined in accordance with a four-point grading scale consisting of excellent (no more than small bits of adherent feces), good (small amount of feces or dark fluid not interfering with the examination), fair (enough feces or dark fluid present to prevent a reliable examination) and poor (large amount of fecal residue precluding a complete examination) based on previously published reports[3,4,6]. Excellent or good grades were categorized as adequate cleansing, and fair or poor as inadequate. We also scored colon cleanliness using a four-point scale grading system from 1 to 4 (excellent, good, fair and poor)[5].

Excretion of the capsule was defined as occurring when the capsule was either expelled from the subject's body or had reached and visualized the hemorrhoidal plexus within the capsule's battery life. Location within the colon was determined using both colorectal images and the rapid localization system.

Before commencing this study, the 20 principal investigators received two half-days of training on managing the colon capsule endoscopy examination with particular emphasis on estimation of colon cleanliness levels and procedure completion. Three selected clinicians (Kakugawa Y, Saito S and Watanabe K) who were blinded to the study groups graded the cleanliness levels. An additional independent physician (Saito Y) supervised the blinding process.

Adverse events
All subjects were interviewed for any associated adverse

Figure 1 Reference used to evaluate the quality of bowel preparation prior to capsule ingestion. Experienced medical staff assessed the quality of the bowel preparation by checking the clarity of subjects' evacuation before the subjects were given the colon capsule. We define that the capsule is ready to be ingested when a grade 5 quality is achieved.

Table 2 Subject characteristics and bowel preparation

		Group A (*n* = 31)	Group B (*n* = 29)
Median age	yr (range)	39 (28-70)	39 (29-78)
Gender	Male/female	19/12	20/9
Reason for referral	Symptomatic/asymptomatic	4/27	4/25
Standard PEG-ELS preparation before capsule ingestion	Without/with additional PEG-ELS prescription	30/1	26/3
Total volume amount of intake for 2 d	Median, liter (range)	3.8 (2.9-4.1)	4.8 (3.9-5.1)

PEG-ELS: Polyethylene glycol electrolyte lavage solution.

symptoms at the outpatient clinic following the colon capsule endoscopy. Adverse events were recorded as mild, moderate or severe by the physicians who performed the colon capsule procedures. A condition not requiring treatment was defined as mild, a condition needing any kind of treatment was regarded as moderate, and a condition that required any emergency treatment was considered severe.

Statistical analysis

Univariate analysis using Fisher's exact test was performed to compare differences in colon cleansing level and capsule excretion rate between the two groups. Values of $P < 0.05$ were considered significant.

RESULTS

Subjects

Sixty-four subjects enrolled in this study. Thirty-three sub-

jects were randomly assigned to group A and 31 to group B. The colon capsules failed to "wake up" in 2 subjects of group A and 2 subjects in group B. As a result, 31 subjects in group A and 29 subjects in group B were included in our analysis (Table 2). All subjects consumed the specified initial amount of PEG-ELS. In four subjects, the quality of the bowel preparation by checking the clarity of subjects' evacuation was not adequate prior to capsule ingestion, so an additional 0.3 L of PEG-ELS was prescribed prior to capsule ingestion for one subject in group A and 3 subjects in group B. Median fluid solution intake including boosts was 3.8 L (range, 2.9-4.1 L) in group A, while it was 4.8 L (range, 3.9-5.1 L) in group B.

Location when capsule "woke up"

The colon capsule was located in the stomach of six subjects, the small bowel of 44 subjects, and the colon of 10 subjects when it "woke up" at 1 h 45 min post-ingestion. Of the latter 10 subjects, capsules were located in either the cecum (*n* = 9) or the ascending colon (*n* = 1).

Colon cleanliness

Colon cleanliness is shown in Table 3. Colon cleanliness was evaluated as adequate in 29 subjects (94%) in group A, compared to 25 subjects (86%) in group B. The average scores of groups A and B were 3.60 ± 0.61 and 3.50 ± 0.72, respectively (ns). The level of colon cleanliness was evaluated to be excellent in all 4 of those subjects who were prescribed additional PEG-ELS just prior to colon capsule endoscopy.

Capsule excretion

In 25% of subjects (15/60) the capsule was excreted from the body within 6 h post-ingestion, in 53% (32/60)

Table 3 Colon cleansing level

	Group A (*n* = 31)	Group B (*n* = 29)
Colon cleansing level		
Adequate	29 (94%)	25 (86%)
Excellent	13	14
Good	16	11
Inadequate	2 (6%)	4 (14%)
Fair	2	4
Poor	0	0

Table 4 Capsule excretion within battery life (%)

		Group A (*n* = 31)	Group B (*n* = 29)	Total
Capsule excretion within battery life		22/31 (71)	16/29 (55)	38/60 (63)
According to colon cleansing level	Adequate	21/29 (72)	15/25 (60)	36/54 (67)
	Inadequate	1/2 (50)	1/4 (25)	2/6 (33)
According to location when capsule woke up	Stomach	2/3 (67)	0/3 (0)	2/6 (33)
	Small bowel	16/24 (66)	11/20 (55)	27/44 (61)
	Colon	4/4 (100)	5/6 (83)	9/10 (90)

within 8 h, in 58% (35/60) within 10 h, and in 63% (38/60) within the capsule's battery life. Twenty-two (71%) of the 31 subjects in group A excreted the capsule compared to 16 (55%) of the 29 subjects in group B (ns) (Table 4). Of the remaining 9 subjects in group A and 13 subjects in group B whose capsules were not excreted, all of the capsules were located in the left side colon when they stopped functioning.

Adverse events

Only one subject, a 70-year-old female in group A, experienced mild symptoms of nausea and vomiting 1 h after starting to drink PEG-ELS, due to ingesting the PEG-ELS faster than recommended. She was advised to slow down the rate of ingestion for the remaining PEG-ELS and was able to continue with the colon capsule procedure. After capsule examination, the colon cleansing level was evaluated as being excellent.

DISCUSSION

This is the first study aimed at reducing the total amount of bowel preparation intake for colon capsule endoscopy procedures. While colon cleanliness is essential for optimal visualization during colon capsule endoscopy, fluid intake during bowel preparation can be as high as a total of 6 L over two days[4,6,7,9,10], an amount considered excessive for some patients. Reducing the volume of fluid intake is an important consideration in increasing patient acceptance of colon capsule endoscopy. We propose reducing the total amount of bowel preparation fluid by eliminating PEG-ELS intake on the day before the examination. Therefore, we conducted a multicenter, prospective, randomized controlled trial, comparing our proposed "reduced volume method" with the "conventional volume method" of bowel preparation for colon capsule endoscopy.

In this study, the level of cleansing was defined as adequate in 94% of group A subjects (reduced volume method), despite the elimination of drinking any PEG-ELS on the day before the examination. This result was higher than that of previous studies (52%-88%)[3-7,9]. We successfully reduced median fluid intake to 3.8 L (range, 2.9-4.1 L) using the reduced volume method compared to total fluid intake ranging from 4.5 to 6 L using the conventional volume method in previously reported studies[3-10]. These results indicate that the intake

of PEG-ELS on the day of the examination promotes colon cleansing, whereas intake on the day before the examination may have very limited, if any, impact on the cleansing. A possible reason for this is that after ingesting and evacuating 2 L PEG-ELS on the day before the examination, biliary and intestinal secretion occurs (processes necessary for stool production) and the ingestion of only 1 L PEG-ELS on the day of the examination is then insufficient to clean the whole colon. Regarding total colonoscopy, in most Japanese facilities patients take 2 L PEG-ELS in the endoscopy waiting room on the day of the examination and the examination then starts when the fecal matter becomes liquid and transparent. In this way, we usually obtain an adequate preparation for performing total colonoscopy. The Japanese preparation method for total colonoscopy has evidence of achieving an adequate cleansing level[11,12]. Therefore, we believe that our proposed reduced volume method for colon capsule endoscopy is also adequate.

There has been no previously published report on the use of experienced medical staff in this respect. In this study, we established a method in which experienced medical staff assessed the quality of the bowel preparation by checking the clarity of subjects' evacuation before the subjects were given the colon capsule (Figure 1). In cases of unsatisfactory preparation, an additional PEG-ELS was prescribed, with additional PEG-ELS required for one subject in group A and three subjects in group B. Following this additional PEG-ELS, colon cleanliness was judged as adequate in all four subjects. In most Japanese institutes, experienced medical staff assess the quality of the bowel preparation by checking the clarity of subjects' evacuation before starting total colonoscopy. This may be one of the key points why we achieved a high cleansing level. Experienced medical endoscopy staff could also be utilized to help achieve a high level of colon cleanliness when performing colon capsule endoscopy.

It is also important to note that small changes in technique can make significant differences in achieving a high quality of colon cleansing. The difference between this study and previous reports[3-7,9,10] is that we used pronase and dimethicone as adjuncts, and isotonic magnesium citrate as a booster. It has previously been re-

Figure 2 Example of the contribution of dimethicone to improved mucosal visualization by the colon capsule. A: A lesion (arrow) is clearly observed on the transverse colon by colon capsule endoscopy. Dimethicone worked to join numerous microbubbles to form groups of large bubbles, resulting in better mucosal visualization; B: Colonoscopy image of the lesion post-capsule procedure. The lesion was diagnosed as a laterally spreading tumor. Endoscopic submucosal dissection was performed, and intramucosal cancer consisting of well differentiated adenocarcinoma was identified on the resected specimen.

ported that pronase was effective as a mucolytic agent[13] and dimethicone was useful in dissolving intraluminal air bubbles[14,15]. Accordingly, we used pronase dissolved into 100 mL of water as the first step on the day of examination, and dimethicone was added to the solution of PEG-ELS and magnesium citrate. We believe that dimethicone worked to join numerous microbubbles to form groups of large bubbles, resulting in better mucosal visualization, and that pronase and dimethicone should be used routinely as adjuncts to bowel preparation (Figure 2).

It is possible to conclude that our method is superior in terms of safety. Sodium phosphate has served as a booster in a number of studies[3-10], and PEG-ELS in one published report[16], but the use of magnesium citrate as a booster has not been reported. In this study, we used isotonic magnesium citrate instead of sodium phosphate as a booster, principally because it is very easy to drink since it has a similar taste to sports drinks. Japan is the only country in the world as far as we know in which isotonic magnesium citrate is available as a laxative. Secondly, isotonic magnesium citrate might reduce the level of electrolytic imbalance, while sodium phosphate has been reported as causing major problems including acute phosphate nephropathy[17,18]. Therefore, both patient ac-

ceptance and safety are increased using isotonic magnesium citrate instead of sodium phosphate as a booster.

Van Gossum *et al*[6] reported 6.7% adverse events related to the bowel preparation; however, in our study, a mild adverse event related to the bowel preparation was observed in only one case (3%). Moreover, this single case was then able to continue with the procedure, and the subject's colon cleanliness level was subsequently rated as excellent. These results may indicate the higher degree of acceptance and improved safety of the reduced volume method.

Our results do not deny the cleansing ability of the conventional volume method (group B) which achieved an adequate cleansing level of 86%. There is no statistically significant difference between the two groups. However, in terms of a better quality of life and improved acceptability for patients, the reduced volume method (group A) is a preferred option for colon cleansing associated with colon capsule endoscopy.

Our proposed reduced volume method had a lower rate of capsule excretion before the battery life ended. The excretion rate was 71%, which was lower in comparison with the 64%-94% reported in previous studies[3-7,9,10]. Those reports and the present study differed in the use of bisacodyl suppository. Such use was mandatory in the earlier studies, but it was left to each individual subject in this study, with a bisacodyl suppository requested in only one case. Considering the fact that the capsule was located in the left descending colon in all cases where the capsule was not excreted within the capsule's battery life, the use of bisacodyl suppository could have increased the excretion rate to be much higher. There may be a possibility that some subjects feel unwilling to use the suppository because of embarrassment; therefore, it may be an effective option to administer a third oral booster in order to achieve a higher excretion rate.

The size of this study was small because it was a pilot study and the median age of 39 years for all participating subjects was relatively young. Further studies are necessary to clarify the efficiency of the reduced volume method, especially in terms of increasing the excretion rate of the colon capsule. PillCam COLON2 capsule, the second generation of colon capsule, has no sleep mode and has a small bowel detection function to indicate the presence of the capsule in the small bowel[8,19]. This should allow more appropriate timing for administering the first booster, which can be expected to shorten the procedure time and improve the capsule excretion rate.

In this study, we clarified that our newly proposed regimen with a reduced volume of bowel preparation when conducting colon capsule endoscopy was as effective as the commonly used higher volume method. An important advantage of the reduced volume method is that subjects do not need to drink any PEG-ELS and can eat three low fiber meals on the day before the examination. Our proposed reduced volume bowel preparation method could be useful in encouraging subjects who have not undergone colorectal screening to under-

take colon capsule endoscopy in the future.

COMMENTS

Background
Adequate colon cleanliness is essential for optimal visualization during colon capsule endoscopy, but the widely used preparation method may require as much as 6 L of fluid intake over two days.

Research frontiers
The authors propose a reduced volume method without polyethylene glycol electrolyte lavage solution intake in the days before the capsule procedure.

Innovations and breakthroughs
In this study, the authors demonstrate that a new preparation method for colon capsule endoscopy was as effective as the conventional method.

Applications
In terms of a better quality of life and improved acceptability for patients, the reduced volume method is a useful option for colon cleansing when undertaking colon capsule endoscopy.

Peer review
A well conducted study on a very important issue, even if as stated by the authors "the size of this study was small because it was a pilot study".

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BRIEF ARTICLE

Differences between diffuse and focal autoimmune pancreatitis

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Abstract

AIM: To investigate differences in clinical features between diffuse- and focal-type autoimmune pancreatitis (AIP).

METHODS: Based on radiological findings by computed tomography and/or magnetic resonance imaging, we divided 67 AIP patients into diffuse type (D type) and focal type (F type). We further divided F type into head type (H type) and body and/or tail type (B/T type) according to the location of enlargement. Finally, we classified the 67 AIP patients into three groups: D type, H type and B/T type. We compared the three types of AIP in terms of clinical, laboratory, radiological, functional and histological findings and clinical course.

RESULTS: There were 34 patients with D-type, 19 with H-type and 14 with B/T-type AIP. Although obstructive jaundice was frequently detected in D-type patients (88%) and H-type patients (68%), no B/T-type patients showed jaundice as an initial symptom ($P < 0.001$). There were no differences in frequency of abdominal pain, but acute pancreatitis was associated more frequently in B/T-type patients (36%) than in D-type patients (3%) ($P = 0.017$). Serum immunoglobulin G (IgG)4 levels were significantly higher in D-type patients (median 309 mg/dL) than in B/T-type patients (133.5 mg/dL) ($P = 0.042$). Serum amylase levels in B/T-type patients (median: 114 IU/L) were significantly greater than in H-type patients (72 IU/L) ($P = 0.049$). Lymphoplasmacytic sclerosing pancreatitis (LPSP) was histologically confirmed in 6 D-type, 7 H-type and 4 B/T-type patients; idiopathic duct-centric pancreatitis was observed in no patients. Marked fibrosis and abundant infiltration of CD20-positive B lymphocytes with few IgG4-positive plasma cells were detected in 2 B/T-type patients. Steroid therapy was effective in all 50 patients (31 D type, 13 H type and 6 B/T type). Although AIP relapsed during tapering or after stopping steroids in 3 D-type and 3 H-type patients, no patients relapsed in B/T type. During follow-up, radiological features of 6 B/T-type patients were not changed and 1 B/T-type patient improved naturally.

CONCLUSION: Clinical features of H-type AIP were similar to those of D-type, but B/T-type differed from D and H types. B/T-type may involve diseases other than LPSP.

INTRODUCTION

Autoimmune pancreatitis (AIP) is a type of pancreatitis that is thought to have an autoimmune etiology. AIP responds dramatically to steroid therapy, therefore, differentiating AIP from pancreatic cancer is important to avoid unnecessary pancreatic resection[1-3]. According to the Asian Diagnostic Criteria for AIP[4], AIP is diagnosed based on the following items: diffuse or focal enlargement of the pancreas and narrowing of the main pancreatic duct; increased serum immunoglobulin G (IgG) or IgG4 levels or the presence of autoantibodies in the serum; histological findings of lymphoplasmacytic infiltration and fibrosis in the pancreas [lymphoplasmacytic sclerosing pancreatitis (LPSP)[5]]; and responsiveness to steroids. AIP occurs frequently in elderly men. The primary initial symptom is obstructive jaundice, and diabetes mellitus occurs in half of patients. AIP is rarely associated with acute pancreatitis or ulcerative colitis. AIP is associated with several sclerosing extrapancreatic lesions such as sclerosing cholangitis, sclerosing sialadenitis, and retroperitoneal fibrosis, and is currently recognized as a pancreatic lesion of IgG4-related systemic disease. AIP responds well to steroid therapy, but it sometimes relapses[1,2].

Recently, another histological pattern of AIP, characterized by ductal epithelial granulocytic infiltration, has been recognized[6,7]. This pattern is referred to as idiopathic duct-centric pancreatitis (IDCP)[6]. Clinical features of IDCP patients are different from those of LPSP patients. The terms, type 1 and type 2 AIP, are proposed to describe the clinical features associated with LPSP and IDCP, respectively[8-11].

Radiologically, AIP is classified into diffuse type (D type) and focal type (F type)[1-3,12-14]. Although diffuse enlargement of the pancreas is rather specific to AIP, F-type AIP should be strictly differentiated from pancreatic cancer. However, only a few studies[13,14] have investigated the differences between D- and F-type AIP, and it is unknown whether F-type AIP is an initial stage of D-type AIP, or a different type of AIP. This study aimed to clarify differences in clinical features between D- and F-type AIP.

MATERIALS AND METHODS

Study patients

From 1988 to 2010, 67 AIP patients (47 men and 20 women; median age: 60.3 years; range: 27-83 years) were diagnosed according to the Asian Diagnostic Criteria for AIP[4] in Tokyo Metropolitan Komagome Hospital. Based on radiological findings by computed tomography

and/or magnetic resonance imaging, we divided the AIP patients into D type and F type. We further divided F type into head type (H type) and body and/or tail type (B/T type) according to the location of enlargement. Finally, we classified the 67 AIP patients into three groups: D type, H type, and B/T type. D-type AIP patients showed diffuse enlargement of the pancreas (Figure 1A-C), H-type patients showed focal enlargement of only the pancreatic head (Figure 2A-C), and B/T-type patients showed enlargement of only the pancreatic body and/or tail (Figure 3A and B). We compared clinical, laboratory, radiological, functional, and histological findings and clinical course among the three types of AIP.

Clinical, radiological and laboratory analysis

Clinical assessments were as follows: age at time of diagnosis; sex; drinking and smoking habits; presence and/or history of allergic diseases; initial symptoms such as obstructive jaundice and abdominal pain; and associated diseases such as diabetes mellitus, acute pancreatitis and ulcerative colitis. Drinking habit was defined as drinking > 80 g/d alcohol for > 7 years. Smoking habit was defined as smoking > 20 pack-years. Diabetes mellitus was diagnosed if fasting serum glucose levels and/or hemoglobin A1c levels were higher than normal levels (126 mg/dL and 6.1%, respectively)[15]. Acute pancreatitis was diagnosed when both severe abdominal pain and elevation of serum amylase level (> 3 times normal; normal: 115 IU/L) were seen.

Stenosis of the lower bile duct was evaluated on endoscopic retrograde cholangiopancreatography and/or magnetic resonance cholangiopancreatography. Three extrapancreatic lesions that are frequently associated with AIP (sclerosing cholangitis of the hilar or intrahepatic bile duct, swelling of salivary glands, and retroperitoneal fibrosis) were evaluated radiologically.

Laboratory findings were assessed including serum IgG ($n = 67$), IgG4 ($n = 65$) and immunoglobulin E (IgE) ($n = 43$) levels, peripheral eosinophil count ($n = 56$), serum amylase levels ($n = 66$), and autoantibodies including antinuclear antigen ($n = 60$) and rheumatoid factor ($n = 58$). Serum IgG4 levels were measured by nephelometry using IgG subclass (BS-NIA) kits. A cutoff value of 135 mg/dL, which is widely accepted, was used.

Histological and immunohistological studies

The pancreas was assessed by surgical resection ($n = 7$), surgical or ultrasound-guided biopsy ($n = 6$), and endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA, $n = 10$) and examined histologically and immunohistochemically using anti-CD3, anti-CD20, and anti-IgG4 antibodies.

Clinical course

Ten patients were initially treated with surgical procedures on suspicion of pancreatic cancer (pancreatoduodenectomy, $n = 6$; distal pancreatectomy, $n = 1$; choledochoduodenostomy with pancreatic biopsy, $n = 3$). Fifty patients,

Figure 1 Computed tomography scan showing diffuse type autoimmune pancreatitis. Swelling of the head (A), body (B) and tail (C) of the pancreas was seen.

Figure 2 Computed tomography scan showing head type autoimmune pancreatitis. There was swelling of the only pancreatic head (A), but the pancreatic body (B) and tail (C) were a normal size.

Figure 3 Computed tomography scan showing body and/or tail type autoimmune pancreatitis. Pancreatic body and tail was swollen (A), but the pancreatic head was within a normal size (B).

including one surgically treated, were treated with oral steroids. Prednisolone at an initial dose of 30-40 mg/d was given for 2-3 wk. It was then tapered by 5 mg every 1-3 wk to 5 mg/d. Maintenance therapy (2.5-5 mg/d, 1-3 years) was used in all patients. Recurrence of AIP was defined as the reappearance of symptoms with the development or reappearance of pancreatic and/or extrapancreatic abnormalities on imaging studies. Eight patients were followed up conservatively without steroid therapy.

Statistical analysis
Differences between groups were analyzed using Fisher's exact probability test, and Mann-Whitney's *U* test. *P* values were corrected by Bonferroni's method, and *P* < 0.05 was considered statistically significant.

RESULTS
Clinical, radiological and laboratory differences
Sixty-seven AIP patients were classified into D-type (*n* = 34), H-type (*n* = 19) and B/T-type (*n* = 14) AIP. There were no significant differences in age, sex, drinking,

smoking habits and history of allergic diseases among the groups. Although obstructive jaundice was frequently detected in D-type (88%) and H-type (68%) patients, no B/T-type patients showed jaundice as an initial symptom (*P* < 0.001). Although there were no differences in frequency of abdominal pain, acute pancreatitis was seen more frequently in B/T-type (36%) than in D-type (3%) patients (*P* = 0.017). Although stenosis of the lower bile duct was seen frequently in D-type (94%) and H-type (95%) patients, no B/T-type patients showed stenosis of the lower bile duct (*P* < 0.001). There were no differences between groups in associated extrapancreatic lesions (Table 1).

Serum IgG4 levels were significantly higher in D-type (median: 309 mg/dL) than in B/T-type (133.5 mg/dL) patients (*P* = 0.042). Serum IgG4 levels were more frequently elevated in D-type (88%) than in B/T-type (50%) patients (*P* = 0.030). There were no significant differences in serum IgE levels and peripheral eosinophil count among the groups. Serum amylase levels in B/T-type patients (median: 114 IU/L) were significantly greater than in H-type patients (72 IU/L) (*P* = 0.049). There were no

Table 1 Clinical and radiological findings of three types of autoimmune pancreatitis

	D type, *n* = 34	H type, *n* = 19	B/T type, *n* = 14	*P* value, D *vs* H	*P* value, D *vs* B/T	*P* value, H *vs* B/T
Median age (yr) (quartile range)	67.5 (59-71.5)	64.0 (56-71)	61.5 (50.3-72.5)	> 0.99	> 0.99	> 0.99
Male/female	24/10	15/4	8/6	> 0.99	> 0.99	> 0.99
Drinking habit +/- (%)	4/27 (15)	4/15 (21)	2/12 (14)	> 0.99	> 0.99	> 0.99
Smoking habit +/- (%)	16/27 (59)	12/16 (75)	5/13 (39)	> 0.99	> 0.99	0.198
Allergies +/- (%)	8/25 (32)	6/18 (33)	7/13 (54)	> 0.99	0.885	0.879
Jaundice +/- (%)	30/4 (88)	13/6 (68)	0/14 (0)	0.420	< 0.001	< 0.001
Abdominal pain +/- (%)	6/28 (18)	7/13 (37)	5/9 (36)	0.546	0.771	> 0.999
Diabetes +/- (%)	10/24 (29)	11/8 (58)	4/10 (29)	0.231	> 0.999	> 0.999
Acute pancreatitis +/- (%)	1/33 (3)	1/18 (5)	5/9 (36)	> 0.999	0.017	0.183
Ulcerative colitis +/- (%)	1/33 (3)	0/19 (0)	1/13 (7)	> 0.999	> 0.999	> 0.999
Stenosis of the lower vile duct +/- (%)	32/34 (94)	18/19 (95)	0/14 (0)	> 0.999	< 0.001	< 0.001
Extrapancreatic lesions +/- (%)	13/21 (38)	6/13 (32)	5/9 (36)	> 0.999	> 0.999	> 0.999

D: Diffuse; H: Head; B/T: Body and/or tail.

Table 2 Laboratory findings of three types of autoimmune pancreatitis

	D type, *n* = 34	H type, *n* = 19	B/T type, *n* = 14	*P* value, D *vs* H	*P* value, D *vs* B/T	*P* value, H *vs* B/T
Median IgG, mg/dL (quartile range)	2163.5 (1637.5-2682.8)	1920 (1398.5-2300)	1619 (1369.5-2364.5)	0.642	> 0.999	> 0.999
Median IgG4, mg/dL (quartile range)	309 (181-1015)	351 (228-780)	133.5 (60.8-326.5)	> 0.999	0.042	0.057
IgG4 > 135, mg/dL (%)	28/32 (88)	16/19 (84)	7/14 (50)	> 0.999	0.030	0.168
Median IgE, IU/L (quartile range)	406.9 (265.5-877)	249.0 (74.75-589.75)	264.0 (134.1-856)	0.447	> 0.999	> 0.999
IgE > 580, (IU/L) (%)	8/20 (40.0)	3/12 (25.0)	4/11 (36.4)	> 0.999	> 0.999	> 0.999
Median eosinophil, /µL (quartile range)	180 (72-440)	238.5 (152.5-447.75)	473 (114.5-621.5)	> 0.999	0.354	> 0.999
Eosinophil > 600 /µL (%)	3/27 (11)	2/16 (13)	3/13 (23)	> 0.999	> 0.999	> 0.999
Median amylase (IU/L) (quartile range)	86 (49.5-119.5)	72 (43-100)	114 (73.3-589.3)	> 0.999	> 0.999	0.049
Amylase > 115 (IU/L) (%)	8/33 (24)	4/19 (21)	7/14 (50)	> 0.999	0.300	0.408
Positive antinuclear antigen (%)	14/29 (48)	4/18 (22)	6/13 (46)	0.366	> 0.999	0.738
Positive rheumatoid factor (%)	10/28 (36)	2/17 (12)	1/13 (7)	0.288	0.378	> 0.999

D: Diffuse; H: Head; B/T: Body and/or tail; IgG: Immunoglobulin G; IgE: Immunoglobulin E.

Table 3 Histological findings of the pancreas of three types of autoimmune pancreatitis

	D type	H type	B/T type
Resection	LPSP (*n* = 3)	LPSP (*n* = 3)	LPSP (*n* = 1)
Biopsy	LPSP (*n* =2)	LPSP (*n* = 3)	Fibrosis with abundant infiltration of B lymphocytes (*n* = 1)
EUS-FNA	LPSP (*n* = 1)	LPSP (*n* = 1)	LPSP (*n* = 3)
	Inadequate material (*n* = 1)	Inadequate material (*n* = 2)	Fibrosis with abundant infiltration of B lymphocytes (*n* = 1) Inadequate material (*n* = 1)

D: Diffuse; H: Head; B/T: Body and/or tail; LPSP: Lymphoplasmacytic sclerosing pancreatitis; EUS-FNA: Endoscopic ultrasonography-guided fine-needle aspiration.

differences among the groups in terms of the ratio of antinuclear antigen and rheumatoid factor (Table 2).

Histological and immunohistochemical studies

LPSP was histologically confirmed with abundant infiltration of CD3-positive T lymphocytes and IgG4-positive plasma cells in 6 D-type, 7 H-type and 4 B/T-type patients. In the pancreas of 2 B/T-type patients (1 surgical biopsy specimen and 1 EUS-FNA specimen), marked fibrosis and abundant infiltration of CD20-positive B lymphocytes rather than T lymphocytes were detected, but few IgG4-positive plasma cells or neutrophils were detected. IDCP was not observed in any patients. EUS-FNA of 4 AIP patients could not confirm the histological diagnosis due to insufficient specimens (Table 3).

Clinical course

Steroid therapy was effective in all 50 patients (31 D type, 13 H type and 6 B/T type). AIP relapsed during tapering or after stopping steroids in 3 D-type and 3 H-type patients. In 8 conservatively followed-up patients (1 H type and 7 B/T type), radiological features were not changed in seven patients, but enlargement of the pancreatic tail improved naturally in 1 B/T-type patient. During the course prior to our hospitalization, focal enlargement of the pancreatic head developed to diffuse enlargement in three cases, and focal enlargement of the pancreatic tail

DISCUSSION

Radiologically, AIP is classified into diffuse and focal forms. Diffuse enlargement of the pancreas, called sausage-like enlargement, is a typical feature of AIP. However, F-type AIP, sometimes forming a mass, is frequently difficult to differentiate from pancreatic cancer[1-3,12-14]. Possible differences in clinical presentations between the D and F types of AIP are unclear.

In the present study, we classified 67 AIP patients into D type (*n* = 34), H type (*n* = 19) and B/T type (*n* = 14). D and H types showed similar clinical features. However, the B/T-type was different from D and H types in several aspects. Obstructive jaundice and stenosis of the lower bile duct were frequently detected in D- and H-type patients, but no B/T-type patients showed these features. According to Ghazale *et al*[16], stenosis of the lower bile duct was present in 70% of 53 AIP patients. Hirano *et al*[17] have stated that both pancreatic edema due to inflammation of the pancreatic head and biliary wall thickening influence stenosis of the lower bile duct in AIP, based on EUS findings and the fact that 93% of pancreatic head lesion-positive AIP patients had stenosis of the lower bile duct, compared with only 17% of lesion-negative patients.

In this study, the serum IgG4 level was significantly lower in B/T-type than in D-type patients, and elevation of serum IgG4 levels was less in B/T-type than in D-type patients. Acute pancreatitis was seen more frequently in B/T-type than in D-type patients, and serum amylase levels were significantly higher in B/T-type than in H-type patients. As to the diagnosis and frequency of acute pancreatitis, the high percentage in B/T type patients seems to depend mostly on the higher amylase levels. Acute pancreatitis or some acute inflammatory attack in chronic pancreatitis cases may not be associated with high amylase levels because of the atrophic acinar tissue. There was no difference in the frequency of abdominal pain, therefore, some cases in D-type and H-type might have been overlooked.

No B/T-type patients relapsed after steroid therapy, compared with 10% of D-type and 23% of H-type patients. It has become clear that there are two histological subtypes (LPSP and IDCP) in AIP[6-11]. Most AIP patients in Asia have LPSP, and half of AIP patients in Europe have IDCP[8,10]. The clinical features of these two subtypes differ substantially. It is generally reported that IDCP patients are younger at diagnosis, and IDCP patients are less likely to show elevated serum IgG4 levels. IDCP is more likely associated with acute pancreatitis and inflammatory bowel disease. According to a comparative study of LPSP and IDCP patients by Sah *et al*[9], IDCP patients tended to have more focal features than LPSP (84% *vs* 60%), and no relapse of IDCP was seen in any patient, whereas LPSP relapsed in 47% of patients. To diagnose IDCP, histological examination of

an adequate pancreatic specimen is needed, and the need for histological examination to diagnose IDCP at present makes clinical diagnosis difficult.

In histological examination of the present cases, LPSP was confirmed in 17, but IDCP was not detected. Clinical files of D-type and H-type AIP were compatible with those of LPSP patients. However, considering low serum IgG4 levels and frequent association with acute pancreatitis, there is a possibility that some IDCP cases may be involved in B/T-type cases. For example, a 32-year-old man with B/T-type AIP showed normal serum IgG4 levels, association with ulcerative colitis, and good responsiveness to steroid therapy, but histological diagnosis by EUS-FNA could not be confirmed due to an insufficient biopsy specimen. Interestingly, histology of two B/T-type patients included abundant infiltration of B cells with little infiltration of IgG4-positive cells or neutrophils, which might be another type of AIP other than LPSP and IDCP. B/T-type AIP may involve a disease other than LPSP.

Three H-type and one B/T-type patient progressed to D type during the natural course. It has also been reported that H-type AIP progresses to D-type[18,19], and B/T-type AIP to D-type[20]. There have also been reports of relapse in the remnant pancreas after resection, such as relapse in the remnant pancreatic head 1 year after distal pancreatectomy[21], and relapse in the remnant pancreatic body and tail 4 mo after pancreatoduodenectomy[22]. Steroid therapy was effective for those lesions that had progressed or relapsed. These findings indicate that the focal inflammation may advance to subsequent diffuse changes throughout the pancreas or develop repeatedly at different sites and at different times in some AIP patients. Diffuse change in the pancreas may be the final appearance of AIP, and whether this inflammatory process affects the gland diffusely or focally may merely reflect the stage of the disease. In seven conservatively followed up B/T-type patients, six showed no change and one improved naturally. Kubota *et al*[23] have reported that all eight AIP patients who improved spontaneously showed focal pancreatic enlargement. B/T-type AIP may involve a disease other than D-type and H-type AIP.

In conclusion, although clinical features of H-type AIP are similar to those of D-type AIP, B/T-type AIP differed in several aspects from D and H types. B/T-type AIP may involve a disease other than LPSP.

COMMENTS

Background
Autoimmune pancreatitis (AIP) is a particular type of pancreatitis that is thought to have an autoimmune etiology. Recently, AIP has been radiologically classified into diffuse type (D type) and focal type (F type). However, the differences between D- and F-type AIP are not well known.

Research frontiers
Differences in clinical, radiological, laboratory and histological findings between D- and F-type AIP were investigated.

Innovations and breakthroughs
AIP patients were divided into D type and F type. Furthermore, F type was

divided into head type (H type) and body and/or tail type (B/T type). No B/T-type patients showed jaundice as an initial symptom, but acute pancreatitis was associated more frequently in B/T type than in D type. Serum amylase levels were significantly higher in B/T-type than in H-type patients. Serum immunoglobulin G (IgG)4 levels were significantly higher in D-type than in B/T-type patients. No B/T-type patients relapsed after steroid therapy, compared with 10% of D-type and 23% of H-type patients. Two B/T-type patients included abundant infiltration of B cells with little infiltration of IgG4-positive cells or neutrophils.

Applications
B/T-type AIP differed in several aspects from D and H types, and some B/T-type AIP may involve a disease other than lymphoplasmacytic sclerosing pancreatitis.

Peer review
The study itself seems reasonable and the results are interesting and of etiological importance.

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BRIEF ARTICLE

Study of *Helicobacter pylori* genotype status in saliva, dental plaques, stool and gastric biopsy samples

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Abstract

AIM: To compare genotype of *Helicobacter pylori* (*H. pylori*) isolated from saliva, dental plaques, gastric biopsy, and stool of each patient in order to evaluate the mode of transmission of *H. pylori* infection.

METHODS: This cross-sectional descriptive study was performed on 300 antral gastric biopsy, saliva, dental plaque and stool samples which were obtained from patients undergoing upper gastrointestinal tract endoscopy referred to endoscopy centre of Hajar hospital of Shahrekord, Iran from March 2010 to February 2011. Initially, *H. pylori* strains were identified by rapid urease test (RUT) and polymerase chain reaction (PCR) were applied to determine the presence of *H. pylori* (*ureC*) and for genotyping of voculating cytotoxin gene A (*vacA*) and cytotoxin associated gene A (*cagA*) genes in each specimen. Finally the data were analyzed by using statistical formulas such as Chi-square and Fisher's exact tests to find any significant relationship between these genes and patient's diseases. *P* < 0.05 was considered statistically significant.

RESULTS: Of 300 gastric biopsy samples, 77.66% were confirmed to be *H. pylori* positive by PCR assay while this bacterium were detected in 10.72% of saliva, 71.67% of stool samples. We were not able to find it in dental plaque specimens. The prevalence of *H. pylori* was 90.47% among patients with peptic ulcer disease (PUD), 80% among patients with gastric cancer, and 74.13% among patients with none ulcer dyspepsia (NUD) by PCR assay. The evaluation of *vacA* and *cagA* genes showed 6 differences between gastric biopsy and saliva specimens and 11 differences between gastric and stool specimens. 94.42% of *H. pylori* positive specimens were *cagA* positive and all samples had amplified band both for *vacA s* and *m* regions. There was significant relationship between *vacA s1a/m1a* and PUD diseases (*P* = 0.04), *s2/m2* genotype and NUD diseases (*P* = 0.05). No statically significant relationship was found between *cagA* status with clinical outcomes and *vacA* genotypes (*P* = 0.65). The evaluation of *vacA* and *cagA* genes showed 6 differences between gastric biopsy and saliva specimens and 11 differences between gastric and stool specimens.

CONCLUSION: Regard to high similarity in genotype of *H. pylori* isolates from saliva, stomach and stool, this study support the idea which fecal- oral is the main route of *H. pylori* transmission and oral cavity may serve as a reservoir for *H. pylori*, however, remarkable genotype diversity among stomach, saliva and stool samples showed that more than one *H. pylori* genotype may exist in a same patient.

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Key words: *Helicobacter pylori*; Gastric biopsy; Saliva; Dental plaque; Stool

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is the organism responsible for diseases such as atrophic gastritis, chronic gastritis, duodenal ulcers, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer[1]. *H. pylori* is distributed worldwide and is found in developing countries in particular. For instance, more than 90% of Iranian individuals are infected with *H. pylori*[2]. Although there is much information about *H. pylori* infection, several aspects of the pathogenesis and epidemiology of this organism remains unclear[3]. The transmission route of *H. pylori* infection has been the topic of several studies. Most infections are probably acquired in childhood, mainly *via* oral-oral or fecal-oral routes[4], however, the exact mode(s) of transmission is still unknown.

H. pylori has been found in saliva, dental plaques and feces, which shows that oral and fecal cavities are probably involved in *H. pylori* transmission[5]. The role of *H. pylori* in the oral cavity remains controversial since the detection rate of the bacterium in the mouth is very diverse, ranging between 0% and 100%[6]. Different typing methods have been proposed for the study of correlations between *H. pylori* isolates from different anatomical sites for epidemiological purposes[7]. Genotyping using some well-known virulence marker genes, such as the cytotoxin associated gene A (*cagA*) and voculating cytotoxin gene A (*vacA*), are considered as one of the best approaches[8]. The *cagA* gene is located at the end of the *cag* pathogenicity island (PAI) and has been proposed as a marker for the *cag* PAI, and the presence of certain *cagA* alleles (e.g., *cagA1a* in East Asian strains) have been associated with severe clinical outcomes[9]. The *vacA* gene is present in virtually all strains of *H. pylori* but it is polymorphic, comprising variable signal regions (type *s1* or *s2*) and mid-regions (type *m1* or *m2*). Type *s1/m1 vacA* causes more epithelial cell damage than type *s1/m2*, whereas type *s2/m2* and the rare *s2/m1* are non-toxic due to the presence of a short 12-residue hydrophilic extension on the *s2* form[10,11]. The s-region is classified into *s1* and *s2* types and the m-region into *m*1 and *m*2 types. The *s*1 type is further subtyped into *s1a*, *s1b* and *s1c* subtypes, and the *m1* into *m1a* and *m1b* subtypes. The mosaic combination of sand m-region allelic types determines the particular cytotoxin and, con-

sequently, the pathogenicity of the bacterium[12,13]. Recently, several studies have examined the presence of *H. pylori* in saliva, dental plaque, gastric biopsies and stool, but few studies have evaluated the relationship between genotypes *of H. pylori* isolated from these specimens in a single patient. Therefore, we aimed to compare *H. pylori cagA* and the *vacA* allelic status among strains isolated from saliva, dental plaque, gastric biopsies and stool samples in the same patient with dyspepsia manifestations in order to evaluate the mode of transmission of *H. pylori* infection.

MATERIALS AND METHODS

Patients and samples

Samples were obtained over a year (March 2010 to February 2011) from patients with gastroduodenal diseases that were referred to the endoscopy center of Hagar Hospital of Shahrekord, Iran.

Prior to sampling, the questionnaire, including medical history and demographic data, were recorded for each patient. All studied patients signed an informed consent form before endoscopy and declared their willingness to allow the application of their anonymous data for research purposes. Gastric biopsies, saliva, dental plaques and stool samples were collected from each patient. Saliva and dental plaque sampling was done in the morning before undergoing endoscopy. All patients were asked to wash their mouth with normal saline prior to saliva and dental plaque sampling. Saliva samples, in a volume of 2-3 mL, were collected using sterile toothpicks and filter paper. Dental floss was used to remove the dental plaque from the interdental spaces and both samples were transported in sterile flasks containing digestion buffer [100 mmol NaCl, 10 mmol Tris-HCl (pH 8.0), 250 mmol ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and 1% sodium lauryl sarcosine] on the day of sampling and were stored at -70 ℃ until DNA extraction[6]. For each patient, two biopsy specimens from the antrum were taken using a disinfected endoscope. One was used for screening of *H. pylori* positive specimens by a rapid urease test (RUT). The second piece from RUT-positive patients was placed in 1 mL of sterile phosphate buffer saline solution. Stool was collected in a container with a screw cap and was transported immediately to the biotechnology research center of Islamic Azad University, Shahrekord Branch for molecular analysis.

Rapid urease test

One biopsy piece from each patient was inoculated immediately after collection into 1.5 mL to 2 mL of urea broth (Merck, Germany). It was incubated at 37 ℃ in the incubator for 1.5 h. The change in color of the broth from yellow to pink was taken as a positive test.

Genomic DNA extraction and polymerase chain reaction

DNA was extracted from biopsies and stool specimens using a Genomic DNA Purification kit (Fermentas, Ger-

Table 1 Primers used for polymerase chain reaction analysis of voculating cytotoxin gene A and cytotoxin associated gene A

Region	Primer	Sequence (5'-3')	Size and location of PCR product
s1a	vacA s1a-F	CTC TCG CTT TAG TAG GAG C	213 bp
	VA1-R	CTG CTT GAA TGC GCC AAA C	(843-1055)
s1b	SS3-F	AGC GCC ATA CCG CAA GAG	187 bp
	VA1-R	CTG CTT GAA TGC GCC AAA C	(869-1055)
s1c	vacA s1c-F	CTC TCG CTT TAG TGG GGY T	213 bp
	VA1-R	CTG CTT GAA TGC GCC AAA C	(843-1055)
s2	SS2-F	GCT AAC ACG CCA AAT GAT CC	199 bp
	VA1-R	CTG CTT GAA TGC GCC AAA C	(433-631)
m1a	VA3-F	GGT CAA AAT GCG GTC ATG G	290 bp
	VA3-R	CCA TTG GTA CCT GTA GAA AC	(2741-3030)
m1b	VAm-F3	GGC CCC AAT GCA GTC ATG GA	291 bp
	VAm-R3	GCT GTT AGT GCC TAA AGA AGC AT	(2741-3031)
m2	VA4-F	GGA GCC CCA GGA AAC ATT G	352 bp
	VA4-R	CAT AAC TAG CGC CTT GCA	(976-1327)
cagA	cagA-U	GGA ATA CCA AAA ACG CAA AAA CCA	300 bp
	cagA-L	CCC CAC AAT ACA CCA GCA AAA CT	
ureC (glmM)	GlmM1-R	GCTTACTTTCTAACACTAAC-GCGC	296 bp
	GlmM1-F	GGATAAGCTTTTAGGGGTGT-TAGGGG	

PCR: Polymerase chain reaction; *cagA*: Cytotoxin associated gene A; *vacA*: Voculating cytotoxin gene A.

many) according to the manufacturer's instructions. To prepare DNA from saliva and dental plaque, one volume of the digestion buffer and 100 g/mL proteinase K were added to the saliva samples and incubated at 55 ℃ for 3 h. DNA was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with 3 mol sodium acetate and 0.7 mL volume of isopropanol. Rinsed and dried DNA pellets were dissolved in Tris-EDTA (TE) buffer (Tris 10 mmol, EDTA 1 mmol and pH 8.0)[8]. The concentration and quality of DNA preparations were determined spectrophotometrically by measuring absorbance at 260 nm and 280 nm by agarose gel electrophoresis. The DNA preparations were stored at -20 ℃. The presence of *ureC* and *cagA* and the genotypes of *vacA* alleles (*s1a, s1b, s1c, m1a, m1b* and *m2*) were determined by polymerase chain reaction (PCR). The primer sequences are shown in Table 1[8,11,14].

DNA samples from *H. pylori* (D0008, Genekam, Germany) were used as positive controls for *ureC, cagA* and *vacA* genes, and sterile distilled water was used as a negative control. All PCR mixtures were prepared in a volume of 25 μL containing 1X PCR buffer, 0.4 μmol of each primer, 0.3 U Taq DNA polymerase and 2 μL DNA sample[5]. The mixture was placed in a thermocycler (Eppendrof Mastercycler 5330; Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany), and PCR products were visualized by electrophoresis in a 1.5% agarose gel,

strained with ethidium bromide, and examined under ultraviolet illumination.

Statistical analysis
The data were analyzed using SPSS software (Version 17.SPSS Inc, United States) and *P* values were calculated using Chi-square and *F* test to find any significant relationship. *P* < 0.05 was considered statistically significant.

RESULTS
The study population consisted of 300 patients; 143 men and 157 women with mean age 46 ± 17 years. The patients were classified at the time of endoscopy and histopathology as having peptic ulcer disease (PUD: *n* = 63), gastric cancer (GC: *n* = 5) and none ulcer dyspepsia (NUD: *n* = 232) regardless of *H. pylori* status. Based on RUTs, 271 (90.33%) patients were positive for *H. pylori* while 233 (77.66%) patients had positive PCR results by using specific primers (*ureC*) looking for *H. pylori* DNA in their gastric specimens. *H. pylori* was detected in 25 (10.72%) of saliva and 167 (71.67%) of stool samples but we were not able to detect this bacterium in the dental plaques of studied patients.

According to gastric specimen results, the prevalence of *H. pylori* was 90.47% (57 of 63) among patients with PUD, 80% (4 of 5) among patients with GC, and 74.13% (172 of 232) among patients with NUD by PCR assay. Generally, of 233 *H. pylori* positive isolates from gastric biopsy specimens, 220 samples (94.42%) were *cagA* positive and all samples had amplified bands both for *vacA* s and m regions. Overall, 114 (48.92%) samples had *vacA s1a*, 32 (13.73%) had *vacA s1b*, 52 (22.31%) had *vacA s1c* and 35 (15.00%) had *vacA s2* alleles, whereas the frequency of *m1a, m1b* and *m2* alleles were 76 (32.61%), 13 (5.57%) and 144 (61.80%), respectively. There was a significant relationship between *vacA s1a/m1a* and PUD diseases (*P* = 0.04) and the *s2/m2* genotype and NUD diseases (*P* = 0.05). No statically significant relationship was found between *cagA* status with clinical outcomes and *vacA* genotypes (*P* = 0.65). There was a statistically significant correlation between *H. pylori s2/m2* genotypes and the development of NUD (*P* = 0.05) and among *s1a/m1a* and PUD outcomes (*P* = 0.04).

Of 25 saliva samples positive for *H. pylori*, all were *cagA* positive while 18 (72.00%) samples had *s1a/m2*, 5 (20.00%) samples had *s1a/m1a*, 2 (8.00%) samples had *s2/m2* genotypes and all of the samples were *cagA* positive (Table 2). There was no association between genotypes of *H. pylori* from saliva with clinical outcomes (*P* > 0.05).

In stool samples, of 167 positive strains, the *cagA* gene was positive in 162 (97.00%) specimens. One hundred twenty (71.85%) had *s1a/m2*, 22 (13.17%) had *s2/m2*, 14 (8.38%) had *s1a/m1a*, 3 (1.79%) had *s1c/m2*, 3 (1.79%) had *s1c/m1a*, 2 (1.19%) had *s1b/m2*, 2 (1.19%) had *s1b/m1a* and *s1a/m1b* genotypes (Table 2). There was a significant relationship between NUD manifestation and the *s2/m2* genotype of *H. pylori* from stool samples (*P* = 0.04).

Table 2 The frequency of cytotoxin associated gene A and voculating cytotoxin gene A genotypes in gastric biopsy, saliva and stool samples

| | cagA *n* (%) | vacA *n* (%) | | | | | | | | | | | |
		s1a/m1a	s1a/m1b	s1a/m2	s1b/m1a	s1b/m1b	s1b/m2	s1c/m1a	s1c/m1b	s1c/m2	s2/m1a	s2/m1b	s2/m2
Gastric biopsy	220 (94.42)	36 (15.45)	9 (3.86)	60 (25.75)	7 (3)	5 (2.14)	13 (5.57)	17 (7.29)	5 (2.14)	39 (16.73)	12 (5.15)	0	30 (12.87)
Saliva	25 (100)	5 (20)	-	18 (72)	-	-	-	-	-	-	-	-	2 (8)
Stool	162 (97)	14 (8)	2 (1.19)	120 (71.85)	2 (1.19)	-	2 (1.19)	3 (1.79)	-	3 (1.79)	-	-	22 (13.17)

cagA: Cytotoxin associated gene A; *vacA*: Voculating cytotoxin gene A.

Table 3 The list of patients with incompatible *Helicobacter pylori* voculating cytotoxin gene A genotypes

Patient number	Gastric biopsy strain	Saliva strain	Stool strain
1	s1a/m1a	s1a/m2	s2/m2
2	s1a/m1a	s1a/m2	-
3	s2/m1a	s1a/m2	-
4	s1c/m2	s1a/m2	s1c/m2
5	s2/m2	s1a/m2	-
6	s2/m2	s1a/m2	s2/m2
7	s1a/m1a	s1a/m1a	s2/m2
8	s1a/m2	s1a/m2	s2/m2
9	s1a/m2	-	s1a/m1a
10	s1a/m1b	-	s1b/m2
11	s2/m2	s2/m2	s1a/m2
12	s2/m2	-	s1a/m2
13	s2/m2	-	s1a/m2
14	s1a/m2	s1a/m2	s1a/m1a
15	s2/m2	-	s1c/m2
16	s2/m1a	-	s2/m2

PCR tests for dental samples looking for *H. pylori* gene clues were negative. The *H. pylori* detection rate was statistically associated with the type of sample ($P = 0.01$). All patients with positive *H. pylori* in their saliva had a positive PCR reaction for gastric biopsy samples simultaneously.

Upon analysis of the results, in some cases we found different genotypes of *H. pylori* from the saliva, gastric biopsies and stool of the same patient. As presented in Table 3, in 6 (24.00%) patients, isolated *H. pylori* strains from gastric biopsies and the saliva of every patient showed a different genotype. In 11 (6.58%) patients, the genotypes of stool strains differed from genotypes of gastric isolates, and in one (4.00%) patient there were three different genotypes in his gastric biopsy, saliva and stool specimen (Table 3). However, variation of *H. pylori* genotypes in different studied sites were statistically non-significant ($P > 0.05$).

DISCUSSION

Infection by *H. pylori* remains one of the most important scientific phenomena in the biomedical literature worldwide and represents the most prevalent chronic bacterial disease because it affects more than half of the world's population, with a distribution related to the degree of economic development in each country[3]. The prevalence of *H. pylori* differs significantly both between and within countries, with high rates of infection being associated with low socioeconomic status and high densities of living[15]. For instance, in Japan, South America, Turkey and Pakistan, the prevalence is more than 80%, while in Scandinavia and England, the prevalence is between 20% and 40%[11]. The prevalence of this bacterium in Iran is 60%-90%, indicating Iran is a high risk region for *H. pylori* infection. The prevalence of this bacterium was 77.66% in our study and it was therefore compatible with other reports in Iran[2,11]. In our study, the rate of *H. pylori* in different sites of the gastric tract (0% dental plaques, 10.72% saliva, 77.66% gastric biopsy and 71.67% stool) varied, which is inconsistent with other studies[16,17]. There are several hypotheses which can explain the low rate of *H. pylori* in oral cavity compare to gastric biopsy and stool samples. First may be due to the fact that eradication therapy usually removes the gastric infection while it does not necessarily affect oral and intestinal colonization[16]. The second reason for such decreasing level of the rate of bacterium can be related to the presence of oral normal flora, which is able to affect the *H. pylori* growth by producing bacteriocin-like inhibitory proteins against *H. pylori* strains[1]. The third reason is based on the hypothesis that the *H. pylori* may persists in yeast while is in mouth. The *Candida spp.* could be the reservoir for *H. pylori* and play an important role in the bacterial re-inoculation in gastric tissue or transmission to a new host[18], so may yeast protects *H. pylori* from the stressful conditions in the mouth and carries it to the gastrointestinal tract of human[19]. According to Gatti *et al*[20] from Brazil and Bindayna *et al*[21] from India in 2006, there was a significant relationship between *cagA* gene and the inflammation of gastric tissue. The prevalence of *cagA*+ gene in their samples was 79% and 59% respectively. However, Kangsadalampai *et al*[22] from Thailand in 2005 and Cirak *et al*[23] from Turkey in 2003, and Gutiérrez *et al*[24] from Cuba in 2005 failed to confirm such relationship between *cagA* status and gastric disorders. The prevalence of *cagA* gene was 31% in Thailand, 71% in Turkey and 88.5% in Cuba. In our survey, the prevalence of *cagA* gene was 94.42% in gastric biopsy samples and due to high prevalence of *cagA* in our studied isolates, we did not find any significant relationship between this gene and gastric disorders. The prevalence of *cagA* gene in our study was in accordance with our previous report[9] and similar to East Asian countries where the most of isolates are positive for *cagA* gene. Also this finding was different with major of previous

Table 4 Summary of studies which analysed *Helicobacter pylori* status in different oral cavity, stool and gastric sample

Author name	Country	Target population	Number of sample	Type of specimens	Method	Positive rate %
Cześnikiewicz-Guzik et al[16]	Poland	Gastrointensinal patients	100	Gastic biopsy, saliva and gingival plaques	ELISA	51 biopsy 54 saliva and 48.3 gingival pockets
Medina et al[3]	Argentina	Gastroduodenal patients	98	Saliva, dental plaque and gastric biopsy	PCR	88.4 biopsy and 18.98 oral samples
Iamaroon et al[7]	Thailand	Recurrent auphtus ulcer patients and healthy volunteers	22 patients/15 normal people	Mocusa	Nested PCR	4.5 auphtus patients and 4.5 normal patients
Tanahashi et al[37]	Northern California	Gastric patients	16 infected 10 uninfected	Stool, saliva and vomits	PCR and culture	18.8 saliva, 21.8 stool and 37.5 vomits
Silva et al[6]	Brazil	Gastric patients	30	Gastric biopsy, saliva and dental plaque	Single step and nested PCR	80 gastric biopsy, 30 saliva and 20 dental plaque
Fernández-Tilapa et al[5]	Mexico	Adults without dyspepsia	200	Gastric biopsy, saliva and dental plaque	Nested and semi nested PCR, ELISA	62 biopsy and 17 oral samples
Wang et al[8]	Tennessee	Gastric patients	31	Gastric biopsy and saliva	PCR and DNA sequencing	100 gastric biopsy and 71 saliva
Current study	Iran	Gastroduodenal patients	300	Gastric biopsy, saliva, dental plaque and stool	PCR	77.66 biopsy, 10.72 saliva, 0 dental plaque and 71.67 stool

ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction.

reports from Iran, which the *cagA* positive rate was 44% to 91% and similar to European isolates[11,25,26]. This phenomenon may be because of changes in Iranian isolates status or targeting different part of *cagA* gene for amplification. According to López-Vidal et al[27] from Mexico in 2008 *vacA s1b/m1*, Linpisarn et al[28] from Thailand in 2007, *vacA s1a/m1* and *vacA s1c/m1*, Ahmad et al[29] from Pakistan in 2009, *vacA s1b/m2* and *vacA s1a/m1a*, Rudi et al[30] from Germany in 1998, Miciuleviciene et al[31] from Lithuania in 2008 and Saribasak et al[32] from Turkey in 2004, Hussein et al[33] from Iraq in 2008 and Momenah et al[34] from Saudi Arabia in 2006, *vacA s1a/m2* were the prominent strains in their country. We have found *vacA s1a/m2* as a predominant genotype in gastric specimens of Iranian patients with gastroduodenal diseases which was similar to Germany, Lithuania, Turkey, Iraq and Saudi Arabia but far different with Mexico, Thailand and Pakistan. There was statically significant correlation between *vacA s2/m2* genotype and NUD ($P = 0.05$) and *vacA s1a/m1a* genotype with PUD ($P = 0.04$). This finding is in accordance with the major of studies which believe *s1/m1* isolate are more virulent than *s2/m2*[27,29]. Similar to the previous reports from Iran, from statistical point of view no relationship was found between gastric cancer and *vacA* status ($P = 0.1$)[2,11]. Gastric epithelial cells seem to be the main niche of the *H. pylori*, however there are limited studies considering *H. pylori* status in oral cavity. Some of studies have detected *H. pylori* from different sites of the oral cavity[7,9] and some other groups failed to detect *H. pylori* from saliva, subgingival plaques and gingival pockets[35,36].

Medina et al[3] from Argentina in 2010 found *H. pylori* in 18.4% of saliva and Fernández-Tilapa et al[5] from Mexico in 17% of dental plaque during 2011. Cześnikiewicz-Guzik et al[16] from Poland in 2004 find this bacterium in 54% of saliva and 48.3% of gingival packets while Iamaroon et al[7] from Thailand in 2003

did not find *H. pylori* in oral aphtous ulceration patients (Table 4). In this study we found *H. pylori* in 10.72% of saliva and none of dental plaques. That's may be because of the high level of hygiene in our studied population[1]. Some authors have suggested that *H. pylori* may belong to the normal oral flora of the human oral cavity, maintaining a commensal relation with the host, but sometimes present in very low numbers which is difficult for identification. Others have suggested that *H. pylori* are not consistently present in dental plaque and saliva so when present, may be the result of occasional gastroesophageal reflux[1]. Some researchers suggest that *H. pylori* in oral cavity may serve as a source of gastric reinfection by this bacterium[7]. According to Tanahashi et al[37] from Austria, 93.7% of stool samples were *H. pylori* positive and Parsonnet et al[17] found this bacterium in 88% of the specimens. Both of them applied PCR assay for detection of *H. pylori*. In our study, 71.67% of stool samples of infected patients were *H. pylori* positive which is somehow accordance with other studies[17,38]. The lower prevalence of *H. pylori* in feces rather than stomach may is due to the effect of the intestinal tract normal flora. Our results showed high homology (58%) in *vacA* genotype in saliva and gastric samples from the same patients. This result was consistent with the findings of study by Wang et al[8] which showed 64% homology between saliva and gastric samples from the same patients. These findings support the hypothesis that saliva is a possible source of *H. pylori* infection. The major difference between gastric biopsy, stool and saliva is that saliva represents the entire oral cavity, but punch biopsy and stool sample serve only as a fraction of the total gastric mucosal surface. Interestingly the *H. pylori* isolated from gastric samples showed high diversity compare to those isolated from saliva and stool which may indicate that gastric biological nature support survive of all different genotype of *H. pylori*. Saliva is more likely to contain the

entire DNA from every strain colonizing the oral cavity but at concentrations that may be close to or below the detection level of our PCR assay. In current study we found several genotypic diversities between *H. pylori* strains isolated from saliva, stool and stomach of the same patient. Our data indicated that isolates from different sites of a single individual tend to be more a like than strains isolated from the same site of different individuals ($P = 0.001$). This is in agreement with our previous report which there was 61% homology between *H. pylori vacA* genotypes in saliva and gastric biopsy of same individuals[9]. The heterogeneity of *H. pylori* may be due to genotypic variation among strains and/or variations in *H. pylori* populations within an individual host, as proposed by Blaser[13]. Genotypic variation of *H. pylori* has been documented in point mutations and variation in the gene order[31,32]. Although high rate of similarity was seen among *H. pylori* isolates from different anatomical sites, but 16% of patients were infected with 3 different strains. This finding supports the idea that humans can be simultaneously infected with two or more *H. pylori* genotypes[39]. Variation might be because of co-existence of these bacteria together or occurring mutations[1].

In conclusion, there is high similarity between *H. pylori* strains isolated from saliva, stool and gastric specimens so it indicates that the possibly role of saliva and stool as *H. pylori* infection sources. However, the diversity of *H. pylori* genotypes between stomach, stool and saliva in the same patient suggest that more than one *H. pylori* strains may exist in the saliva and stomach of the same patient due to co-infection or genetic variation.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is widespread throughout the world, and it is estimated that more than half of people are infected with this bacterium, but the exact route of transmission has not yet been fully clarified and remains poorly understood.

Research frontiers

Overall there are limited studies considering *H. pylori* status in oral cavity or feces. Some limited studies suggest that dental plaques, oral cavity and feces have important role in infection transmission and may serve as a reservoir for *H. pylori*, however some other studies did not find such correlation.

Innovations and breakthroughs

To date there has been a very limited study considering genotyping of *H. pylori* in oral cavity and feces. In this study, the authors employed genotyping in more detail using well-known virulence marker genes such as cytotoxin associated gene A (*cagA*) and voculating cytotoxin gene A (*vacA*). Furthermore, more anatomical sites of each patient including dental plaques, saliva, gastric and stool were analyzed for *H. pylori* genotyping by authors. Current study confirmed the significant role of saliva and feces but not dental plaques as a possible mean of

H. pylori transmission and reservoir.

Applications

By finding correlation between *H. pylori* genotypes isolated from saliva and stool with gastric biopsy, the authors concluded that control of *H. pylori* in saliva and stool is crucial for managing of *H. pylori* infection in gastric tissue.

Terminology

Genotype: The genotype is the genes makeup and characteristic of an organism, a cell or an individual which reflect genetic profile of the cell. Genotyping is the process of determining and classification of organisms or cell based on differences in the genetic makeup (genotype) using biological techniques. Compare to observable characteristics (phenotype) of organisms, genotyping can provide a more accurate view of the biological and genetical status and be expected to be more useful for evaluating, for example, the source of infection, the mode of infection transmission and genetic variation.

Peer review

In the current cross-sectional study on high number of patients, the authors analyzed *H. pylori* genotype status in digestive system from mouth to rectum by targeting 8 regions of two important virulence marker genes, *cagA* and *vacA* alleles. The result indicate that although saliva and stool seems to be major source of *H. pylori* which infects gastric, however remarkable number of patients carry different genotypes in their gastrointestinal tract.

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BRIEF ARTICLE

Association of NOD1 and NOD2 genes polymorphisms with *Helicobacter pylori* related gastric cancer in a Chinese population

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Abstract

AIM: To investigate the association between the tag single nucleotide polymorphisms (TagSNPs) of NOD1 and NOD2 and the risk of developing gastric cancer.

METHODS: We conducted a hospital-based case-control study including 296 incident gastric cancer patients and 160 gastritis controls. Eight TagSNPs in the NOD1 and NOD2 genes were selected from the Hapmap database using the haploview software and genotyped by the Sequenom MassArray system. The serum levels of anti-*Helicobacter pylori* (*H. pylori*) IgG were measured by enzyme-linked immunosorbent assay to indicate *H. pylori* infection. The odds ratios (OR) and 95% confidence intervals (CI) were calculated by unconditional logistic regression, including sex and age as confounding factors.

RESULTS: The NOD1 rs2907749 GG genotype showed a decreased risk for gastric cancer (OR 0.50, 95% CI: 0.26-0.95, P = 0.04) while the rs7789045 TT genotype showed an increased risk (OR 2.14, 95% CI: 1.20-3.82, P = 0.01). An elevated susceptibility to gastric cancer was observed in the subjects with *H. pylori* infection and the NaOD1 rs7789045 TT genotype (OR 2.05, 95% CI: 1.07-3.94, P = 0.03) or the NOD2 rs7205423 GC genotype (OR 2.52, 95% CI: 1.05-6.04, P = 0.04). Haplotype analysis suggested that the distribution of AGT (rs2907749, rs2075820 and rs7789045) in NOD1 between the cases and control groups was significantly different (P corrected: 0.04), and the diplotype AGT/AGT was associated with an elevated gastric cancer risk (OR 1.98, 95% CI: 1.04-3.79, P = 0.04). The association of the NOD1 rs7789045 TT genotype and the diplotype AGT/AGT was significant with *H. pylori*-related diffuse-type gastric cancer (OR 3.00, 95% CI: 1.38-6.53, P = 0.01; OR 4.02, 95% CI: 1.61-10.05, P < 0.01, respectively).

CONCLUSION: Genetic polymorphisms in NOD1 and NOD2 may interact with *H. pylori* infection and may play important roles in promoting the development of gastric cancer in the Chinese population.

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INTRODUCTION

The average prevalence of *Helicobacter pylori* (*H. pylori*) infection in people worldwide is approximately 50%. An epidemiology meta analysis has indicated that the *H. pylori* prevalence ranges from 35% to 81% in different districts within China, and the average infection rate is 58%[1]. *H. pylori* was estimated to be responsible for approximately 65% of all stomach cancers worldwide[2]. It has been reported that gastric cancer-associated mortality rates accounted for nearly one-quarter of the total malignant tumor-related mortalities in China[3]. Together with *H. pylori* infection, host genetic susceptibility, diet, a high salt intake and smoking have all been proposed to be risk factors for gastric cancer.

Clinical and epidemiologic studies have suggested a strong association between chronic infection, inflammation, and cancer[4-6]. Gastric cancer develops very rarely in the normal gastric mucosa. Most of the *H. pylori*-infected individuals showed gastritis, but very few people develop gastric cancer. The genetic variations between the gastritis and gastric cancer patients may play important roles in the *H. pylori*-related clinical outcomes[7]. The host immune response has a strong role in determining the outcome of *H. pylori* infection, and the polymorphisms in genes that control this immune response have been shown to affect the risk of gastric cancer[8-11]. *H. pylori* trigger inflammation through activation of the receptors that recognize pathogen-associated molecular patterns (PAMPs), and these PAMPs are recognized through a set of germline-encoded pattern recognition receptors (PRRs). The activation of PRRs leads to rapid production of a range of pro-inflammatory cytokines with a profound impact on both the innate and adaptive immune responses.

Among the cytosolic PRRs is the nucleotide-binding oligomerization domain (NOD)-like receptor family. Two members of this family, known as NOD1 and NOD2, have been recently identified[12]. NOD1 and NOD2 are characterized by a central NOD, an N-terminal effector-binding domain (CARD) and a C-terminal ligand recognition domain that is comprised of leucine-rich repeats (LRR)[13]. NOD1 senses a muropeptide found mostly in Gram-negative bacterial peptidoglycans, whereas NOD2 senses bacterial molecules produced during peptidoglycan synthesis or degradation[14]. NOD1 and NOD2 are be-

coming known as key regulators of chronic inflammatory conditions[15]. The NODs ultimately activate transcription factors such as nuclear factor (NF)-κB, STAT1 and so on, which play important roles in inflammation-linked tumor development. It is important to understand how the NOD family proteins work together in coordinating the host response to a given pathogen. Direct evidence for NOD family-mediated host defense derived mostly from an *in vivo* study in which NOD1-deficient mice were reported to be more susceptible to infection by *H. pylori* strains with functional type IV secretion systems[16]. Additionally, NOD2 was reported to regulate antimicrobial peptide synthesis as part of the host defense strategies against *L. monocytogenes* infection *in vivo*[17].

There are several reports that demonstrated that the polymorphisms of the NOD1 and NOD2 genes in different populations were related to variant clinical outcomes of *H. pylori* infection. Although two studies have shown that the NOD1 E266K (rs2075820) mutation increased the risk of peptic ulceration, antral atrophy and intestinal metaplasia[18,19], there is little research related to the association between NOD1 polymorphisms and gastric cancer. NOD2 polymorphisms have been proven to significantly correlate with the incidence of gastric cancer in European populations[20-22], whereas all of the SNPs studied proved to be monomorphic sites in the Chinese population. To verify that the polymorphisms of the *H. pylori*-recognized NOD1 and NOD2 contribute to gastric cancer carcinogenesis through gene-gene and gene-environment interactions, we performed a hospital-based case-control study with 296 incident gastric cancer patients (hospital case subjects) and 160 gastritis patients (hospital control subjects).

MATERIALS AND METHODS

Study population

The hospital-based case-control study consisted of 466 hospitalized patients recruited sequentially in the China People's Liberation Army General Hospital from January 2009 to June 2010. The 296 case subjects were histopathologically verified gastric cancer patients (GC group) and the 160 control subjects were gastritis patients (GA group) who had undergone gastroscopy. All subjects were unrelated Han Chinese. The exclusion criteria for the hospital control subjects included previous cancer and previous chemotherapy or radiotherapy. Upon recruitment, informed consent was obtained from each subject or their relatives, and this study was approved by the Institutional Review Board of the Institute of Biotechnology.

Genotyping and tag single nucleotide polymorphisms selection

Eight TagSNPs for the NOD1 and NOD2 genes were chosen from the designable set of common SNPs [minor allele frequency (MAF) ≥ 0.05] genotyped in the Han Chinese (CHB) population samples of the HapMap Project (Data Release 24/Phase II, NCBI B36 assembly, dbSNP b126). The TagSNPs selection was done using

Table 1 Primer details for genotyping of single nucleotide polymorphisms from NOD1 and NOD2 genes

SNP ID	PCR 1st primer	PCR 2nd primer	Amplication length (bp)	Extension sequence primer
rs17159048	ACGTTGGATGTCAAGAGGAGGGT ATTAGGC	ACGTTGGATGCTGTGTGCTTGGG CAGTAAC	93	TTTGGCAGTAACAGTGACAAG
rs2907749	ACGTTGGATGGCTGTGAAGAACA GCAAATC	ACGTTGGATGCACACAGCAGGTT GTACCAC	99	GTAGGTTGTACCACATACATCC
rs2075820	ACGTTGGATGAAGCGCAGCAGG AAGGCAAA	ACGTTGGATGACCTGCTCTTCAA GCACTAC	90	CACTCCTGCTACCCAGAGCGGGAC CCC
rs7789045	ACGTTGGATGAGCAGACACAGA CAGGGTTC	ACGTTGGATGTTGAGATTGCTGA CTGGTGG	95	GTGGTCTCTTCCAGC
rs2067085	ACGTTGGATGATCAGGTTGCCGA TCTTCAC	ACGTTGGATGCCTTTCCTGAGAA CTCTGTG	95	GTGCCTCACCCTCTG
rs1861759	ACGTTGGATGTGACATTTCTCTTG GCTTCC	ACGTTGGATGTGATGTGAAGGAA TTCCAGG	100	AAGACACGACACCTTTGGC
rs3135500	ACGTTGGATGGGCCATGTTGTCT ATAAGAG	ACGTTGGATGGATGTGTGAAAAC TGGTTAA	99	ATTTGTGAAAACTGGTTAATATTTA TAG
rs7205423	ACGTTGGATGCTTGGCTCCAGCC CATTTTG	ACGTTGGATGTATGGCTGCTGCA GGAAATG	99	CACAAATTATCCCCTTATAGTC

SNP: Single nucleotide polymorphism; PCR: Polymerase chain reaction.

the software Haploview version 4.0 with pairwise tagging mode. For the NOD1 gene, four TagSNPs were selected (rs17159048, rs2907749, rs2075820 and rs7789045), which captured 36 out of 45 (80%) of the SNPs covering the whole gene. For the NOD2 gene, four TagSNPs were selected (rs2067085, rs1861759, rs3135500 and rs72054230), which captured 17 out of 21 (80%) of the SNPs covering the whole gene, and the 3'-flanking 2 kb regions; TagSNPs were selected with pairwise $r^2 \geq 0.80$.

The genotypes of all the SNPs were determined by the MassArray system (Sequenom iPLEXassay, San Diego, United States). The polymerase chain reaction (PCR) primers (MassExtend; Sequenom) used in this study were listed in Table 1. Briefly, approximately 15 ng of genomic DNAs isolated from the peripheral blood lymphocytes of the study subjects were used to genotype each sample. Locus-specific PCR and detection primers were designed using the MassARRAY Assay Design 3.0 software (Sequenom, San Diego, United States). The sample DNA was amplified by a multiplex PCR reaction, and the PCR products were then used for locus-specific single-base extension reaction. The resulting products were desalted and transferred to a 384-element SpectroCHIP array. The alleles were discriminated by mass spectrometry. (Sequenom, San Diego, United States). Genotyping was performed without knowledge of the case or control status. Twenty random samples were tested in duplicate by different persons, and the reproducibility was 100%.

Helicobacter pylori detection
The *H. pylori* infection status was evaluated by the detection of serum-specific IgG antibodies against *H. pylori* in duplicate with enzyme-linked immunosorbent assay procedures. The sonicated *H. pylori* strain SS1 antigen was used to coat 96-well microplates at a concentration of 2 μg/mL. The sera of the samples were diluted 1:100 when measured. Twenty serum samples, which were verified by *H. pylori* histology culture, rapid urease test and

Carbon-14-Urea Breath Test, were considered the candidate positive controls, whereas twenty serum samples were considered candidate negative controls by the same three tests. Six serum samples were ultimately confirmed as the negative control criteria, the mean absorbance of which was identical to that of the 20 candidate negative controls. Finally, the samples with a mean absorbance 2.1-fold or greater than the mean absorbance of the six negative reference samples were considered to be positive reactions. The sensitivity of the *H. pylori* detection system was 100% (20 of 20) in the control groups.

Haplotype construction and statistical analysis
The Pearson's χ^2 test was used to examine the differences between the case and the control groups in sex, *H. pylori* infection and age groups. The genotype frequencies in the cases and controls were compared and both the OR and 95% CI: of each genotype were estimated by applying unconditional logistic regression adjusting for age, sex and *H. pylori* infection when it was appropriate. The homozygotes of the most frequent allele in controls were used as the reference group. The Hardy-Weinberg equilibrium was performed using PLINK version1.07[23]. The haplotypes were inferred using Haploview 4.0[24]. The pairwise linkage disequilibrium (LD) among the SNPs was assessed using Haploview 4.0. The case-control comparisons of the haplotype distributions were carried out by applying the inbuilt permutation test based on 10 000 permutations. SPSS, version 15.0 (Chicago, IL, United States) was used for all the statistical analyses.

RESULTS
Characteristics of the study population
A total of 296 incident patients with gastric cancer and 160 incident patients with gastritis were enrolled in this case-control study. Table 2 shows that the distributions of sex between the two groups were not significantly dif-

Table 2 Baseline clinical characteristics of cases and controls *n* (%)

	GC group (*n* = 296)	GA group (*n* = 160)	*P*[1]
Sex			0.455
Male	222 (75.0)	125 (78.1)	
Female	74 (25.0)	35 (21.9)	
Helicobacter pylori infection			0.946
Positive	221 (74.7)	119 (74.4)	
Negative	75 (25.3)	41 (25.6)	
Age (yr)			0.193
≤ 55	131 (43.9)	81 (50.6)	
> 55	165 (56.1)	79 (49.4)	
Histological type			
Intestinal	129 (43.6)		
Diffuse	125 (42.2)		
Unknown	42 (14.2)		

[1]Two-sided χ^2 test. GC: Gastric cancer group; GA: Gastritis group.

ferent. The age groups distribution between the gastric cancer patients and gastritis controls was also similar. The percentage of patients having *H. pylori* infection was almost the same in both the cases and controls. Among the gastric cancer cases, 129 (44%) were intestinal-type cancer, 125 (42%) were diffuse-type cancer, and 42 (14%) were unknown histology-type cases.

Genetic association of the polymorphisms in NOD1 and NOD2 with gastric cancer

The distribution of each of the eight SNPs genotyped in the gastric cancer and gastritis group fitted the Hardy-Weinberg equilibrium law except for NOD1 rs7789045. For rs7789045, this Hardy-Weinberg equilibrium option is available for the gastritis subjects (χ^2 = 0.735, *P* = 0.391) but not for the gastric cancer subjects (χ^2 = 5.221, *P* = 0.022). The major allele homozygotes in all the SNPs were used as the reference genotypes. There were no significant differences between the gastric cancer case and gastritis control in the genotype frequency of the 4 polymorphisms of NOD2 gene. For NOD1 gene, the rs2907749 GG homozygote genotype and the recessive model (genotype GG *vs* GA + AA) showed a reduced risk for gastric cancer (adjusted OR, 0.50, 95% CI: 0.26-0.95, *P* = 0.04 and adjusted OR, 0.52, 95% CI: 0.28-0.96, *P* = 0.04, respectively), whereas the rs7789045 TT homozygote genotype and both the dominant model (genotype TT + TA *vs* AA) and the recessive model (genotype TT *vs* TA + AA) showed an elevated risk for gastric cancer (adjusted OR, 2.14, 95% CI: 1.20-3.82, *P* = 0.01; adjusted OR, 1.50, 95% CI: 1.01-2.22, *P* = 0.04 and adjusted OR, 1.87, 95% CI: 1.09-3.20, *P* = 0.02, respectively) (Table 3).

We next examined the joint effects of NOD1, NOD2 polymorphisms and *H. pylori* infection. Because of the limited number in *H. pylori* seronegative subjects (with 75 and 41 subjects in gastric cancer and gastritis groups, respectively), only the *H. pylori* seropositive subjects were considered for analysis. Logistic regression analysis

showed that the NOD1 rs7789045 TT homozygote and the recessive model (genotype TT *vs* TA+AA) carriers had an elevated risk for gastric cancer, with adjusted OR, 2.05, 95% CI: 1.07-3.94, *P* = 0.03 and adjusted OR, 2.06, 95% CI: 1.13-3.76, *P* = 0.02, respectively. For the NOD2 gene, the rs3135500 AG heterozygote genotype had an increased risk for gastric cancer in *H. pylori*-positive subjects (adjusted OR, 2.65, 95% CI: 1.02-6.89, *P* = 0.05). Both the GC heterozygote and the dominant model (genotype CC+GC *vs* GG) of rs7205423 showed an elevated risk for gastric cancer (adjusted OR, 2.52, 95% CI: 1.05-6.04, *P* = 0.04 and adjusted OR, 2.38, 95% CI: 1.03-5.48, *P* = 0.04, respectively). We examined the association of the gene variations in NOD1 and NOD2 with intestinal-type and diffuse-type gastric cancer as well. The results showed that the NOD1 rs2907749 AA homozygote and the dominant model (genotype AA+GA *vs* GG) carriers (adjusted OR, 2.66, 95% CI: 1.10-6.44, *P* = 0.03 and adjusted OR, 2.47, 95% CI: 1.05-5.81, *P* = 0.04, respectively) together with the rs7789045 TT homozygote and the recessive model (genotype TT *vs* TA+AA) carriers (adjusted OR 2.97, 95% CI: 1.49-5.95, *P* < 0.01 and adjusted OR 2.53, 95% CI: 1.35-4.74, *P* < 0.01, respectively) had a significantly elevated risk for developing diffuse-type gastric cancer. When *H. pylori* infection and the gastric cancer type were considered simultaneously, both the recessive model of rs2075820 (genotype GG *vs* GA+AA) and the rs7789045 TT homozygote or the recessive model (genotype TT *vs* TA+AA) in the NOD1 gene showed a significantly elevated risk for developing diffuse-type gastric cancer in *H. pylori*-positive subjects (adjusted OR 1.89, 95% CI: 1.07-3.32, *P* = 0.03; adjusted OR 3.00, 95% CI: 1.38-6.53, *P* < 0.01; and adjusted OR 2.91, 95% CI: 1.45-5.87, *P* < 0.01, respectively) (Table 4).

Haplotype and diplotype analysis of NOD1 tag single nucleotide polymorphisms selection

In a linkage disequilibrium analysis for all of the polymorphisms, we found suggestive evidence for the linkage of rs2907749, rs2075820 and rs7789045 polymorphisms (for rs2907749 and rs2075820, D':0.935, LOD:19.93, r2:0.168; for rs2075820 and rs7789045, D':1.0, LOD:49.28, r2:0.307; for rs2907749 and rs7789045, D':0.949, LOD:38.97, r2:0.251) in the NOD1 gene. The five common haplotypes (AGT, AAA, GGA, GAA and AGA) in the gastritis control group accounted for 99% of all haplotypes (Table 5). The most common haplotype was AGT, occurring in 42% and 33% of the case and control groups, respectively, and the distribution of AGT was significantly different between cases and controls (*P* = 0.01).

For the NOD1 gene, the diplotypes with frequencies > 5% include AGT/AAA, AAA/GGA, AGT/GGA, AGT/AGT, GGA/GGA and AAA/AAA, which accounted for 94% of all the diplotypes in the controls. Using the most common diplotype AGT/AAA as a reference group, our data showed that diplotype AGT/AGT was significantly associated with elevated gastric cancer risk, with OR, 1.98, 95% CI: 1.04-3.79, *P* = 0.04 (Table 6).

Table 3 Adjusted odds ratios for gastric cancer associated with NOD1 and NOD2 polymorphisms

Gene	rsID	Chr	1[1]	2[1]	GC group	GA group	AOR[2] (95% CI)			
							Heterozygote	Homozygote	Dominant	Recessive
					11/12/22	11/12/22				
NOD1	rs17159048	7	G	T	1/62/233	3/38/118[3]	0.82 (0.52-1.31)	/	0.78 (0.49-1.22)	/
	rs2907749	7	G	A	23/117/156	22/62/76	0.92 (0.61-1.39)	0.50 (0.26-0.95)[a]	0.81 (0.55-1.19)	0.52 (0.28-0.96)[a]
	rs2075820	7	A	G	32/118/145[3]	12/78/68[3]	0.70 (0.47-1.06)	1.27 (0.62-2.64)	0.78 (0.53-1.16)	1.51 (0.75-3.04)
	rs7789045	7	T	A	65/126/105	21/67/72	1.30 (0.85-1.99)	2.14 (1.20-3.82)[a]	1.50 (1.01-2.22)[a]	1.87 (1.09-3.20)[a]
NOD2	rs2067085	16	G	C	0/46/250	0/24/136	1.04 (0.61-1.78)	/	/	/
	rs1861759	16	C	A	8/69/219	3/43/114	0.79 (0.50-1.23)	1.29 (0.33-4.97)	0.82 (0.53-1.27)	/
	rs3135500	16	A	G	16/111/169	12/51/97	1.22 (0.80-1.85)	0.73 (0.33-1.61)	1.13 (0.76-1.67)	0.68 (0.31-1.47)
	rs7205423	16	G	C	20/135/141	14/61/85	1.31 (0.87-1.96)	0.82 (0.39-1.72)	1.22 (0.82-1.79)	0.72 (0.35-1.48)

[1]"1"designates the minor allele,"2"designates the major allele; [2]ORs were adjusted for the covariates (age, sex and Helicobacter pylori infection); [3]One or two subjects failed to be genotyped. GC: Gastric cancer group; GA: Gastritis group. [a]P < 0.05, GC vs GA.

Table 4 Association of the risk single nucleotide polymorphisms in NOD1 and NOD2 with two major gastric cancer types and Helicobacter pylori infection status

Type	Genotype			Heterozygote		Homozygote		Dominant model		Recessive model	
	AA[1]	Aa	aa	AOR[2] (95% CI)	P	AOR (95% CI)	P	AOR (95% CI)	P	AOR (95% CI)	P
NOD1 rs2907749 risk allele:A											
Diffuse 125 cases	68	49	8	2.24 (0.91-5.53)	0.08	2.66 (1.10-6.44)	0.03[a]	2.47 (1.05-5.81)	0.04[a]	1.39 (0.86-2.24)	0.18
Intestinal 129 cases	66	52	11	1.61 (0.71-3.67)	0.26	1.58 (0.71-3.55)	0.27	1.59 (0.73-3.46)	0.24	1.09 (0.68-1.75)	0.72
160 controls	91	61	8	/	/	/	/	/	/	/	/
NOD1 rs2075820 risk allele:G											
Diffuse 125 cases	60	55	13	0.45 (0.19-1.07)	0.07	0.82 (0.36-1.90)	0.65	0.63 (0.28-1.41)	0.26	1.55 (0.96-2.51)	0.08
Intestinal 128 cases	68	41	16	0.54 (0.22-1.31)	0.17	0.77 (0.32-1.85)	0.56	0.65 (0.28-1.51)	0.32	1.29 (0.79-2.08)	0.31
158 controls	68	78	12	/	/	/	/	/	/	/	/
HP+ 220 cases	119	78	23	0.39 (0.16-0.99)	0.05	0.674 (0.27-1.68)	0.40	0.53 (0.22-1.28)	0.16	1.47 (0.93-2.31)	0.10
HP+ diffuse 93 cases	56	27	10	0.38 (0.13-1.13)	0.08	0.85 (0.29-2.46)	0.76	0.60 (0.21-1.70)	0.34	1.89 (1.07-3.32)	0.03[a]
HP+ 117 controls	52	58	7	/	/	/	/	/	/	/	/
NOD1 rs7789045 risk allele:T											
Diffuse 125 cases	32	51	42	1.36 (0.79-2.33)	0.26	2.97 (1.49-5.95)	0.00[a,b]	1.719 (1.045-2.828)	0.03[a]	2.53 (1.35-4.74)	0.00[a,b]
Intestinal 129 cases	22	60	47	1.39 (0.83-2.32)	0.21	1.55 (0.76-3.16)	0.23	1.42 (0.88-2.31)	0.15	1.31 (0.67-2.53)	0.43
160 controls	20	85	55	/	/	/	/	/	/	/	/
HP+ 221 cases	56	86	79	0.99 (0.60-1.62)	0.96	2.05 (1.07-3.94)	0.03[a]	1.24 (0.79-1.97)	0.35	2.06 (1.13-3.76)	0.02[a]
HP+ diffuse 93 cases	28	34	31	1.06 (0.56-2.01)	0.87	3.00 (1.38-6.53)	0.01[a,b]	1.50 (0.84-2.69)	0.17	2.91 (1.45-5.87)	0.00[a,b]
HP+ 119 controls	17	53	49	/	/	/	/	/	/	/	/
NOD2 rs3135500 risk allele:G											
HP+ 221 cases	132	79	10	2.65 (1.02-6.89)	0.05[a]	2.20 (0.88-5.51)	0.091	2.35 (0.96-5.79)	0.06	0.98 (0.62-1.55)	0.92
HP+ 119 controls	73	35	11	/	/	/	/	/	/	/	/
NOD2 rs7205423 risk allele:C											
HP+ 221 cases	112	97	12	2.52 (1.05-6.04)	0.04[a]	2.26 (0.96-5.35)	0.06	2.38 (1.03-5.48)	0.04[a]	1.04 (0.66-1.63)	0.88
HP+ 119 controls	61	45	13	/	/	/	/	/	/	/	/

[1]Genotypes are shown as AA for risk allele homozygotes, Aa for heterozygotes and aa for the nonrisk allele homozygotes; [2]ORs were adjusted for the covariates (age, sex and/or Helicobacter pylori infection). [a]P < 0.05, [b]P < 0.01, cases vs controls.

The distribution of the NOD1 diplotypes between gastric cancer and gastritis subjects infected with *H. pylori* was significantly different from when the *H. pylori* infection status was not considered. The results showed that the diplotypes AGT/GGA, AGT/AGT and AAA/AAA were significantly associated with elevated gastric cancer risk when compared with the diplotype AGT/AAA, with adjusted OR, 2.14, 95% CI: 1.01-4.51, P = 0.05, adjusted OR, 3.07, 95% CI: 1.47-6.41, P < 0.01, adjusted OR, 2.96, 95% CI: 1.10-7.92, P = 0.03, respectively. The risks of intestinal-type and diffuse-type gastric cancer associated with diplotypes in the NOD1 and NOD2 genes were also estimated. The results showed that AGT/AGT was significantly associated with elevated diffuse-type gastric cancer risk compared with the diplotype AGT/AAA, with adjusted OR, 2.56, 95% CI: 1.17-5.58, P = 0.02. The risk of diffuse-type gastric cancer related to the NOD1 diplotype AGT/AGT was further examined with stratification by *H. pylori* infection. As expected, the OR value of diffuse-type gastric cancer with *H. pylori* infection for subjects carrying the AGT/AGT diplotype was 4.02, 95% CI: 1.61-10.05, P < 0.01, which was higher than that of the *H. pylori* infection group or diffuse-type group alone (Table 7). The NOD2 polymorphism was associated with neither the intestinal-type nor the diffuse-type gastric cancer in this study.

Table 5 Frequencies of haplotype of NOD1

Haplotype[1]	Frequency		P	P corrected[2]
	GC (n = 296)	GA (n = 160)		
AGT	0.42	0.33	0.01[a]	0.04
AAA	0.29	0.32	0.37	0.84
GGA	0.25	0.32	0.02[a]	0.08
GAA	0.02	< 0.01	0.05	0.23
AGA	0.01	0.01	0.55	0.96

[1]The order of the haplotype is rs2907749, rs2075820 and rs7789045; [2]Corrected by 10 000 times permutation test. GC: Gastric cancer group; GA: Gastritis group. [a]P < 0.05, GC vs GA.

Table 6 Associations of NOD1 diplotypes and gastric cancer risk n (%)

Diplotype	GC (n = 296)	GA (n = 160)	AOR[1] (95% CI)	P
AGT/AAA	58 (20)	38 (24)	1	
AAA/GGA	47 (16)	35 (22)	0.87 (0.48-1.59)	0.65
AGT/GGA	54 (18)	25 (16)	1.45 (0.77-2.74)	0.25
AGT/AGT	63 (21)	21 (13)	1.98 (1.04-3.80)	0.04[a]
GGA/GGA	22 (7)	20 (13)	0.72 (0.34-1.50)	0.38
AAA/AAA	30 (10)	12 (8)	1.69 (0.76-3.72)	0.20
Others	22 (7)	9 (6)	1.58 (0.65-3.81)	0.31

[1]ORs were adjusted for the covariates (age, sex and Helicobacter pylori infection). GC: Gastric cancer group; GA: Gastritis group. [a]P < 0.05, GC vs GA.

DISCUSSION

In the present study, we investigated the association between NOD1 and NOD2 gene polymorphisms and the risk of gastric cancer in a Chinese population. To clarify the impact of genetic variation in NOD1 and NOD2 on the difference of H. pylori-related clinical outcomes, gastritis patients and gastric cancer patients were selected as cases and controls. We found that subjects who carried the NOD1 rs7789045 TT genotype had an increased risk for gastric cancer. Furthermore, the risk was even more distinct when stratified by H. pylori infection and in the diffuse-type gastric cancer group. Moreover, individuals with certain haplotypes and diplotypes derived from three TagSNPs of the NOD1 gene had a significantly elevated risk of gastric cancer, suggesting that the combined effects of several SNPs may be detected by haplotype-based analyses. To our best knowledge, this is the first study investigating the impact of the NOD1 and NOD2 polymorphisms on susceptibility to gastric cancer in a Chinese population.

NOD1 consists of a C-terminal LRR (Leucine-rich region), a central NOD, and an N-terminal CARD (caspase-activating domain) domain[14]. NOD1 has emerged as a crucial factor for maintaining a basal level of immune activation. Clarke et al[25] showed the important role for peptidoglycan in priming systemic innate immunity and for NOD1 as a homeostatic regulator. The majority of patients with H. pylori-associated gastritis have higher NOD1 expression in gastric epithelial cells as compared

with controls or H. pylori-non-associated gastritis[21], which suggests the involvement of NOD1 signaling in the development of human gastric inflammation. Recently, it has been demonstrated that H. pylori virulence factors and the NOD1 receptor ubiquitin-activating enzyme E1 accumulated in human superficial-foveolar epithelium and its metaplastic or dysplastic foci in a discrete cytoplasmic structure named the particle-rich cytoplasmic structure (PaCS). PaCS modulates immune-inflammatory and proliferative responses of the gastric epithelium of potential pathologic relevance[26]. Therefore, the function alteration of NOD1 due to the gene polymorphisms may contribute to the development of H. pylori-related gastric cancer.

It has been suggested that the AA homozygote of the E266K (rs2075820) NOD1 gene polymorphism increases the risk of peptic ulceration in H. pylori-positive patients in the Hungarian population[18]. Another report indicated that E266K A allele carriers have an increased risk of occurrence of intestinal metaplasia and atrophy and eradication failure in the Turkish population[19]. The rs2075820 SNP was chosen in the coding sequence of the NOD1 gene in exon 3 as it was earlier reported to encode a changed protein (E266K) in the nucleotide-binding domain altering a glutamic acid residue, suggesting a potential functional effect of the mutation[27]. Our result indicated that the AG heterozygote of rs2075820 was protective against the risk of gastric cancer (P = 0.026) while the AA homozygote showed moderate risk of gastric cancer (P = 0.397) in the H. pylori-positive subjects. There are no exact data that demonstrate how the NOD1 polymorphism alters the function of NOD1, but our results suggest that the change of negatively-charged glutamine to positively-charged lysine may cause a drastic change in the structure or regulation of the NOD1 protein that alters the reactivity to H. pylori or the nature of downstream inflammatory pathways.

Two studies focusing on the association of several NOD1 polymorphisms with colorectal and endometrial cancer, which include SNP rs2907749, did not find any relationship between individual NOD1 genotypes and the susceptibility to these cancers[28,29]. However, an association of the NOD1 polymorphisms with atopic eczema in the German population has been reported in a study that examined the effects of 11 SNPs, which covering the complete NOD1 gene, on atopy phenotypes[30]. One NOD1 haplotype and three polymorphisms (rs2907748, rs2907749, and rs2075822) were significantly associated with atopic eczema in a population-based cohort, case-control population, and/or family-based association analysis. The results indicated that genetic variants within the NOD1 gene were important determinants of atopy susceptibility. Especially, it showed that the A allele at rs2907749 is significantly associated with elevated IgE levels. Similarly, our study found that the A allele at rs2907749 elevated the risk of gastric cancer; moreover, the risk association was strengthened in diffuse-type gastric cancer patients. Rs2907749 is located in intron 9 of the NOD1 gene where two putative transcription factor-

Table 7 Associations of NOD1 diplotypes and gastric cancer with two major types and *Helicobacter pylori* infection status

	Diplotype						
	AGT/AAA	AAA/GGA	AGT/GGA	AGT/AGT	GGA/GGA	AAA/AAA	Others
HP+ GC (*n* = 221)	34 (15)	35 (16)	41 (19)	54 (24)	18 (8)	22 (10)	17 (8)
HP+ GA (*n* = 119)	32 (27)	22 (18)	18 (15)	17 (14)	16 (13)	7 (6)	7 (6)
AOR[1] (95% CI)	1	1.49 (0.72-3.08)	2.14 (1.01-4.51)[a]	3.07 (1.47-6.41)[a]	1.09 (0.47-2.53)	2.96 (1.10-7.92)[a]	2.44 (0.89-6.71)
P		0.28	0.05[a]	0.00[2,a,b]	0.83	0.03[a]	0.08
Diffuse GC (*n* = 125)	21 (17)	17 (14)	26 (21)	30 (24)	8 (6)	16 (13)	7 (6)
GA (*n* = 160)	38 (24)	35 (22)	25 (16)	21 (13)	20 (13)	12 (8)	9 (6)
AOR (95% CI)	1	0.84 (0.38-1.88)	1.66 (0.76-3.61)	2.56 (1.17-5.58)[a]	0.66 (0.24-1.77)	2.01 (0.78-5.16)	1.33 (0.43-4.14)
P		0.68	0.20	0.02[a]	0.41	0.15	0.62
Intestinal GC (*n* = 129)	29 (23)	22 (17)	25 (19)	22 (17)	11 (9)	12 (9)	8 (6)
GA (*n* = 160)	38 (24)	35 (22)	25 (16)	21 (13)	20 (13)	12 (8)	9 (6)
AOR (95% CI)	1	0.83 (0.40-1.73)	1.64 (0.76-3.54)	1.43 (0.65-3.14)	0.84 (0.34-2.06)	1.62 (0.619-4.26)	1.13 (0.38-3.34)
P		0.62	0.20	0.38	0.69	0.32	0.83
HP+ diffuse GC (*n* = 93)	12 (13)	13 (14)	19 (20)	26 (28)	7 (8)	10 (11)	6 (7)
HP+ GA (*n* = 119)	32 (27)	22 (19)	18 (15)	17 (14)	16 (13)	7 (6)	7 (6)
AOR (95% CI)	1	1.42 (0.53-3.76)	2.36 (0.91-6.08)	4.02 (1.61-10.05)[a]	1.09 (0.35-3.38)	3.12 (0.94-10.40)	2.30 (0.63-8.44)
P		0.48	0.08	0.00[2,a,b]	0.88	0.06	0.21

[1]ORs were adjusted for the covariates (age, sex and/or *Helicobacter pylori* infection); [2]Remained significant after Bonferroni adjustment for multiple comparisons. GC: Gastric cancer group; GA: Gastritis group. [a]*P* < 0.05, [b]*P* < 0.01, GC *vs* GA.

binding sites for Pax1 are found. The alteration of the allele changes the computer-predicted Pax1-binding probability next to exon 9, which may influence the T-regulatory cell development[30].

The NOD1 rs7789045 TT genotype was at a significantly elevated risk for gastric cancer in this study. Few studies have addressed the relationship of the polymorphism in rs7789045 with clinical diseases before, whereas our result indicated that this polymorphism was worthy to be further studied because it may play important roles in the *H. pylori*-related gastric carcinogenesis. Rs7789045 is located in intron 5 of the NOD1 gene, which is in the splicing region. The possible significance of T/A alteration was predicted by NetGene2 and SpliceView computer programs[31-33]. Different splicing may lead to the alteration of NOD1 caspase activity in the CARD domain; therefore, this may imply a difference in the signal pathway regulation downstream.

Because haplotype analyses may be of higher informative value to draw associations between the phenotypes and genetic variation than SNPs[34], we also assessed the effects of haplotypes and diplotypes in our studies. Analyses revealed significant association between the NOD1 haplotype AGT (rs2907749, rs2075820, and rs7789045) and gastric cancer, and the difference remained significant after a 10 000-times permutation test. Our results also showed that the AGT/AGT diplotype was associated with an increased risk of diffuse-type gastric cancer, and the risk was more evident in *H. pylori*-positive subjects. Some studies have observed the inverse associations of *H. pylori* and atopic diseases such as asthma and atopic eczema[35,36]. Epidemiological observations are consistent with the hypothesis that *H. pylori*, which has been colonizing the human stomach for ≥ 58 000 years and is usually acquired within the first few years of life, may play distinct roles in the maturation of the immune system[37]. Weidinger *et al*[30] demonstrated that the haplotype A-G-T-A-C-C-G-T-A-

C-G, defined by the eleven polymorphic alleles of NOD1 including rs2907749 A allele and rs2075818 C allele, rs2235099 C allele, rs2075821 G allele, the last three of which are in a linkage with rs2075820 G allele in the AGT haplotype, is significantly protective against the development of atopic eczema, whereas haplotype AGT is associated with an increased risk of *H. pylori*-related gastric cancer in this study. The different relationships of the similar haplotype of NOD1 between two diseases may imply the distinct roles of NOD1 in the pathogenesis of atopy and gastric cancer.

Some studies have investigated the relationships among the three major mutations, R702W (rs2066844), G908R (rs2066845) and 3020insC (rs5743293), in the coding region of the NOD2 gene with colorectal cancer[38], gastric cancer[20] and gastrointestinal diseases[22] in the European population. The results showed that NOD2 polymorphisms increase the susceptibility to gastrointestinal cancer. These three polymorphisms were shown to be monomorphic sites in the Chinese population due to the ethnic difference (Hapmap and Genome Variation Server). In this study, the association between the other four NOD1 SNPs and gastric cancer was investigated in the Chinese population. Although no significant differences on genotype distribution were found between gastric cancer and gastritis patients, our results indicated that the AG heterozygote genotype of rs3135500 and CC genotype of rs7205423 were associated with an increased risk for gastric cancer in *H. pylori*-positive subjects. It is notable that rs3135500 is located in the 3'UTR of the NOD2 gene while rs7205423 is located in the intergenic region between the NOD2 gene and the CYLD gene. The latter is a de-ubiquitinating enzyme that inhibits the activation of the NF-κB, which has key roles in inflammation, immune responses, carcinogenesis, and protection against apoptosis[39]. And the G allele of rs7205423 may be at a splice site, which was predicted by NetGene2

and SpliceView computer programs[31-33]. Another article of Weidinger *et al*[40] showed that the presence of the A allele at rs3135500 was significantly associated with an increased risk of developing asthma. On the contrary, our results showed that A allele at rs3135500 was associated with a slightly reduced risk of developing gastric cancer. The results of the two association studies of the NOD2 polymorphisms were in accordance with those of the NOD1 polymorphisms we mentioned above. These results emphasized that polymorphisms of NOD1 and NOD2 may contribute differently to the development of atopic diseases and gastric cancer.

Our study has some limitations. The number of participants in this study was relatively small, and thus, future replication studies with large cohorts are needed. Further expression analysis and transcription factor-binding studies are needed to clarify the functional role of NOD1 and NOD2 polymorphisms. Finally, *H. pylori* is genetically a highly diverse bacteria, and the virulence of *H. pylori* is related to different subtypes that contribute differently to clinical outcomes. However, anti-CagA antibodies were not available in our study.

In conclusion, to our knowledge, this study is the first one to indicate that the NOD1 rs7789045 polymorphism increases the genetic susceptibility of gastric cancer in a Chinese population, and it is observed to be enhanced in *H. pylori*-positive and diffuse-type gastric cancer subjects. The other two polymorphisms, rs2907749 and rs2075820, showed an association with gastric cancer as well. In addition, *H. pylori*-positive subjects carrying the NOD2 rs7205423 C allele have an increased risk of gastric cancer. These findings suggest that the polymorphisms of the NOD1 and NOD2 genes may play a role between *H. pylori* infection and development of gastric cancer. The underlying mechanism needs further investigation.

COMMENTS

Background

The role of *Helicobacter pylori* (*H. pylori*) in the development of gastric cancer has been confirmed. It is known that *H. pylori* is an important factor in both the induction of gastritis and the histological progression to gastric cancer. The NOD (nucleotide-binding oligomerization domain) proteins NOD1 and NOD2 play distinct roles in innate immunity as sensors of *H. pylori* components derived from bacterial peptidoglycan. The *H. pylori* infection may interact with the polymorphisms of NOD1 and NOD2, which influence the development of gastric cancer. In this hospital-based case-control study, the author analyzed the associations between the polymorphisms of NOD1 and NOD2 and the risk for *H. pylori*-related gastric cancer in a Chinese population.

Research frontiers

It has been confirmed that the *H. pylori* peptidoglycan delivered by the type IV secretion system can be sensed *via* NOD1. The polymorphisms of NOD2 was associated with gastric lymphoma. The current study is the first to access the impact of the TagSNPs of NOD1 and NOD2 and disease susceptibility to gastric cancer in a Chinese population.

Innovations and breakthroughs

This study indicated that genetic polymorphisms of NOD1 and NOD2 may interaction with *H. pylori* infection and may play distinct roles in developing gastric cancer in the Chinese population.

Applications

This is an original report of the association between NOD1 and NOD2 polymorphisms and Chinese patients with gastric cancer. It is believed these findings

will be valuable in clarifying the relationship between genetic variation within innate immune molecules and *H. pylori* infection-related gastric cancer.

Peer review

The study examined NOD1/NOD2 polymorphisms in association with *H. pylori* infection in the patients of gastric cancer (296) *vs* gastritis (160). The results indicate that *H. pylori*-induced gastric cancer is associated with the genetic background of the patients. The data are useful. The study is in focus but can be expanded to include more factors such as smoking status, body mass index, age, *etc*. The written English needs some improvement.

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BRIEF ARTICLE

Role of serum carcinoembryonic antigen in the detection of colorectal cancer before and after surgical resection

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Abstract

AIM: To determine whether serum levels of carcinoembryonic antigen (CEA) correlate with the presence of primary colorectal cancer (CRC), and/or recurrent CRC following radical resection.

METHODS: A total of 413 patients with CRC underwent radical surgery between January 1998 and December 2002 in our department and were enrolled in this study. The median follow-up period was 69 mo (range, 3-118 mo), and CRC recurrence was experienced by 90/413 (21.8%) patients. Serum levels of CEA were assayed preoperatively, and using a cutoff value of 5 ng/mL, patients were divided into two groups, those with normal serum CEA levels (e.g., $\leq$ 5 ng/mL) and those with elevated CEA levels (> 5 ng/mL).

RESULTS: The overall sensitivity of CEA for the detection of primary CRC was 37.0%. The sensitivity of CEA according to stage, was 21.4%, 38.9%, and 41.7% for stages Ⅰ-Ⅲ, respectively. Moreover, for stage Ⅱ and stage Ⅲ cases, the 5-year disease-free survival rates were reduced for patients with elevated preoperative serum CEA levels (P < 0.05). The overall sensitivity of CEA for detecting recurrent CRC was 54.4%, and sensitivity rates of 36.6%, 66.7%, and 75.0% were associated with cases of local recurrence, single metastasis, and multiple metastases, respectively. In patients with normal serum levels of CEA preoperatively, the sensitivity of CEA for detecting recurrence was reduced compared with patients having a history of elevated CEA prior to radical resection (32.6% *vs* 77.3%, respectively, P < 0.05).

CONCLUSION: CRC patients with normal serum CEA levels prior to resection maintained these levels during CRC recurrence, especially in cases of local recurrence *vs* cases of metastasis.

INTRODUCTION

Globally, colorectal cancer (CRC) is the third most common cancer diagnosed, and is associated with high rates of incidence and mortality for both men and women[1]. Furthermore, despite progress that has been made in the treatment of advanced cases of CRC, the clinical outcome of this disease still remains poor[2]. Carcinoembryonic antigen (CEA) is a classic tumor marker for CRC,

and has been used to monitor CRC recurrence and as a prognostic factor for CRC patients. Currently, the serum CEA test is recommended by the American Society of Clinical Oncology[3] and the European Group on Tumor Markers[4] as a prognostic biomarker for recurrent CRC following curative resection. However, the effectiveness of CEA as a preoperative and postoperative marker for CRC remains to be evaluated. In particular, it remains unclear how accurate a negative CEA value is for excluding primary and recurrent CRC, and under what conditions CEA values are inaccurate. Therefore, this study was designed to evaluate the role of serum CEA levels in the diagnosis of primary and recurrent CRC following radical resection.

MATERIALS AND METHODS

Patients

A total of 464 patients with stage I, II, or III CRC were admitted to our hospital between January 1998 and December 2002. Of these patients, 51/464 did not have preoperative serum CEA data available. Therefore, a total of 413 CRC patients were included in this retrospective study.

Surgical procedures

Enrolled patients underwent curative resection for the treatment of CRC. Curative resection was defined as the absence of any gross residual CRC in the surgical bed, in addition to a surgical resection margin that was pathologically negative for tumor invasion. Recurrence in this study included metastasis and local recurrence that was secondary to primary CRC at least 3 mo after radical resection. Recurrent CRC was confirmed by at least one of the following examinations: pathology, computed tomography (CT), magnetic resonance imaging, or X-ray. Of these examinations, a pathologic diagnosis based on biopsy and body-fluid cytological examinations represents the most reliable detection method for CRC. For an imaging-based diagnosis of CRC, successive imaging examinations are required to verify cancer progression. Patient characteristics are summarized in Table 1. The median follow-up time was 69 mo (range, 3-118 mo), during which CRC recurred in 90 patients. For these patients, serum CEA assays were performed within 1 wk of CRC recurrence being confirmed.

Measurement of serum CEA levels

Serum CEA levels in CRC patients were measured using CEA Elecsys analyzers (Roche Diagnostics GmbH, United States) with a reference range of 5.0 ng/mL. CRC patients were then divided into two groups, those with normal serum CEA levels (e.g., $\leq$ 5 ng/mL) and those with elevated serum CEA levels (> 5 ng/mL).

Statistical analysis

All data were analyzed using SPSS, version 11.5 (SPSS Inc., Chicago, IL). A *P*-value less than 0.05 was consid-

Table 1 Parameters of colorectal cancer patients enrolled in this study (*n* = 413)

Variable	*n* (%)
Gender	
Male	270 (65.4)
Female	143 (34.6)
Age (yr)	
< 40	56 (13.6)
40-60	147 (35.6)
> 60	210 (50.8)
Preoperative S-CEA	
$\leq$ 5 ng/mL	260 (63.0)
> 5 ng/mL	153 (37.0)
Location	
Colon	174 (42.1)
Rectum	239 (57.9)
Differentiation	
Well	281 (32.0)
Poor	132 (68.0)
Size (cm)	
$\leq$ 5	275 (66.6)
> 5	134 (32.4)
PT	
T1	8 (1.9)
T2	88 (21.3)
T3	229 (55.4)
T4	88 (21.3)
PN	
N0	245 (59.3)
N1	108 (26.2)
N2	60 (14.5)
Lymphovascular invasion	
Present	23 (5.6)
Absent	390 (94.4)

PT: Pathologic T stage; PN: Pathologic N stage; S-CEA: Serum levels of carcinoembryonic antigen.

ered statistically significant. In addition, a two-sided Pearson χ^2 test and Fisher's exact test were used to analyze the potential correlation between serum levels of CEA and clinicopathologic features of the study subjects. Variables associated with a *P* value less than 0.10 by univariate analysis were applied to a Cox model for multivariate analysis. Disease-free survival (DFS) rates were analyzed using the Kaplan-Meier method and compared using the log-rank test.

RESULTS

For a total of 413 patients that were diagnosed with CRC between January 1998 and December 2002 in our department and were enrolled in this retrospective study, serum levels of CEA were assayed prior to surgical resection. Based on a cutoff value of 5 ng/mL, two patient groups were established. One group was associated with elevated levels of serum CEA (e.g., > 5 ng/mL) (*n* = 153; 37.0%), while the second group was associated with normal levels of serum CEA (e.g., < 5 ng/mL) (*n* = 260; 63%). The stages of CRC associated with these cases included stage I (*n* = 70), IIA (*n* = 140), IIB (*n* = 35), IIIA (*n* = 23), IIIB (*n* = 85), and IIIC (*n* = 60), ac-

Table 2 Correlation between preoperative serum levels of carcinoembryonic antigen levels and clinicopathologic characteristics *n* (%)

Characteristics	Preoperative S-CEA		*P* value
	⩽ 5 ng/mL	> 5 ng/mL	
Gender			
Male	167 (61.9)	103 (38.1)	0.524
Female	93 (65.0)	50 (35.0)	
Age (yr)			
< 40	35 (62.5)	21 (37.5)	0.178
40-60	101 (68.7)	46 (31.3)	
> 60	124 (59.0)	86 (41.0)	
Location			
Colon	106 (60.9)	68 (39.1)	0.223
Rectum	154 (64.4)	85 (35.6)	
Size (cm)			
⩽ 5	188 (68.4)	87 (31.6)	0.002
> 5	70 (52.2)	64 (47.8)	
Differentiation			
Well	176 (62.9)	104 (37.1)	0.997
Poor	83 (62.9)	49 (37.1)	
PT			
T1	8 (100.0)	0 (0.0)	0.005
T2	64 (72.7)	24 (27.3)	
T3	141 (61.8)	87 (38.2)	
T4	46 (52.3)	42 (47.7)	
PN			
N0	162 (66.1)	83 (33.9)	0.260
N1	64 (59.3)	44 (40.7)	
N2	34 (56.7)	26 (43.3)	
Lymphovascular invasion			
Present	11 (47.8)	12 (52.2)	0.122
Absent	249 (63.8)	141 (36.2)	
TNM stage			
Ⅰ	55 (78.6)	15 (21.4)	0.011
Ⅱ	107 (61.1)	68 (38.9)	
Ⅲ	98 (58.3)	70 (41.7)	

PT: Pathologic T stage; PN: Pathologic N stage; TNM: Tumor Node Metastasis; S-CEA: Serum levels of carcinoembryonic antigen.

Table 3 Multivariate analysis of factors for 5-year disease-free survival rates

Factor	Hazards ratio (CI)	*P* value
PT	1.448 (1.081-1.940)	0.013
PN	1.624 (1.264-2.088)	0.000
Preoperative S-CEA	1.663 (1.127-2.455)	0.010
Differentiation	1.347 (0.873-2.079)	0.178
Lymphovascular invasion	1.738 (0.890-3.394)	0.105
Lymph nodes evaluated	1.013 (0.780-1.316)	0.925

PT: Pathologic T stage; PN: Pathologic N stage; CI: Confidence interval; S-CEA: Serum levels of carcinoembryonic antigen.

Figure 1 Disease-free survival curves for patients with stage ⅡA colorectal cancer (A) and stage ⅢB colorectal cancer (B) based on preoperative serum levels of carcinoembryonic antigen.

cording to the 6th International Union Against Cancer (UICC) Tumor Node Metastasis (TNM) staging system[5]. Moreover, elevated serum levels of CEA were detected preoperatively in 21.4% of stage Ⅰ CRC patients, 38.9% of stage Ⅱ CRC patients, and in 41.7% of stage Ⅲ CRC patients, respectively. As a result, preoperative CEA levels were found to correlate with CRC diagnoses according to the UICC TNM staging system ($P = 0.01$). A comparison of preoperative CEA levels with clinicopathological characteristics of the enrolled patients further detected a significant association between serum CEA levels and tumor size and T category (Table 2). However, serum CEA levels did not correlate with patient age, patient gender, tumor location, tumor differentiation, N category, or lymphovascular invasion.

The median follow-up time for this study was 69 mo (range, 3-118 mo), and the 5-year DFS rate was 67% after patients underwent radical resection. Moreover, univariate and multivariate analysis revealed that preoperative serum levels of CEA were a significant independent prognostic factor for 5-year DFS rates (Table 3). The 5-year DFS rate was also found to significantly differ for stage Ⅱ and

stage Ⅲ CRC patients independent of serum CEA levels ($P < 0.05$), yet did not differ for stage Ⅰ CRC patients following radical resection. When stage Ⅱ and stage Ⅲ CRC cases were further subdivided into ⅡA, ⅡB, ⅢA, ⅢB, and ⅢC stages, the 5-year DFS rate for normal and elevated levels of serum CEA patient groups were 84% and 62% for stage ⅡA CRC patients, and 64% and 21% for the stage ⅢB CRC patients, respectively in each case ($P < 0.05$, Figure 1A and B). However, no significant difference in the 5-year DFS rates associated with stage ⅡB, ⅢA, and ⅢC CRC was observed.

Recurrence of CRC was experienced by 90/413 patients, with local recurrence, single CRC metastasis, and multiple CRC metastases occurring in 41/90 (45.6%),

Table 4 Patterns of colorectal cancer recurrence according to serum carcinoembryonic antigen levels *n* (%)

Patterns of CRC recurrence	CEA levels		*P* value
	≤ 5 ng/mL	> 5 ng/mL	
Local relapse	26 (63.4)	15 (36.6)	
Metastasis (single)	11 (33.3)	22 (66.7)	0.007
Metastases (multiple)	4 (25.0)	12 (75.0)	

CEA: Carcinoembryonic antigen; CRC: Colorectal cancer.

Table 5 Correlation between serum carcinoembryonic antigen levels in patients with recurrent colorectal cancer and clinicopathologic characteristics *n* (%)

Clinicopathologic characteristics	S-CEA levels in patients with recurrent CRC		*P* value
	≤ 5 ng/mL	> 5 ng/mL	
Gender			
Male	27 (40.3)	40 (59.7)	0.097
Female	14 (60.9)	9 (39.1)	
Age (yr)			
≤ 40	7 (53.8)	6 (46.2)	0.805
40-60	13 (43.3)	17 (56.7)	
≥ 60	21 (44.7)	26 (55.3)	
Preoperative S-CEA			
≤ 5 ng/mL	31 (67.4)	15 (32.6)	0.001
> 5 ng/mL	10 (22.7)	34 (77.3)	
Location			
Colon	17 (44.7)	21 (55.3)	0.894
Rectum	24 (46.2)	28 (53.8)	
Differentiation			
Well	22 (37.9)	36 (62.1)	0.051
Poor	19 (59.4)	13 (40.6)	
PT			
T1	0 (0.0)	0 (0.0)	0.438
T2	5 (41.7)	7 (58.3)	
T3	19 (40.4)	28 (59.6)	
T4	17 (54.8)	14 (45.2)	
PN			
N0	14 (46.7)	16 (53.3)	0.364
N1	14 (37.8)	23 (62.2)	
N2	13 (56.5)	10 (43.5)	

CRC: Colorectal cancer; PT: Pathologic T stage; PN: Pathologic N stage; S-CEA: Serum levels of carcinoembryonic antigen.

Table 6 Multivariate analysis of parameters for recurrent colorectal cancer patients using the cox proportional hazards model

Parameter evaluated	Hazards ratio (CI)	*P* value
Gender	0.49 (0.151-1.607)	0.241
Differentiation	0.42 (0.142-1.245)	0.118
Preoperative S-CEA	0.27 (0.094-0.767)	0.014
Recurrence pattern	0.34 (0.119-0.950)	0.040

CI: Confidence interval; S-CEA: Serum levels of carcinoembryonic antigen.

surgery in 32.6% of cases (Table 5). Univariate and multivariate analysis also revealed that preoperative serum levels of CEA and recurrence patterns were significantly associated with serum levels of CEA detected during recurrence (Table 6).

DISCUSSION

Since Gold *et al*[6] first described and characterized CEA in 1965, it has become the one of the most widely known tumor markers for gastrointestinal tract diseases, especially for CRC. However, although 90% of CRCs produce CEA[7], elevated serum levels of CEA are not often detected at the time of diagnosis. In this study, normal serum levels of CEA (e.g., < 5 ng/mL) were detected in 67% of the CRC patients assayed, and in 79% of stage I CRC patients. While a correlation between stage of CRC and preoperative CEA levels has previously been observed, a low sensitivity is associated with serum CEA assays in the detection of early stage CRC[8-10]. Accordingly, the usefulness of serum CEA assays for screening of CRC is limited. Despite this, a semi-quantitative relationship between CEA levels and tumor volume has previously been described[11], suggesting that elevated serum levels of CEA detected preoperatively may indicate a larger tumor burden. In the present study, preoperative levels of serum CEA were found to be significantly associated with tumor size and T category, but not with N category or tumor differentiation. Moreover, preoperative CEA levels also correlated with stage of disease, while providing a prognostic determinant of survival. These results are consistent with other studies[12-14], and also confirmed that elevated levels of serum CEA represent an independent prognostic factor for 5-year DFS, especially for cases of stage ⅡA and ⅢB CRC.

In colon cancer, CEA modulates intercellular adhesion, functions as a promoter of cellular aggregation, regulates the innate immune system, and mediates signal transduction[15-17]. Accordingly, it is hypothesized that CEA plays an important role in tumor invasion and metastasis. In this study, the 5-year DFS rate of stage Ⅱ CRC patients with elevated levels of serum CEA were compared with stage Ⅲ CRC patients with normal levels of serum CEA, and no significant difference was found (data not shown). This finding is consistent with another study[18], and suggests that a diagnosis of CRC accompanied by elevated levels of serum CEA may be an indica-

49/90 (54.4%), and 16/90 (17.8%) patients, respectively. The types of metastasis detected included hepatic (*n* = 17), pulmonary (*n* = 10), osseous (*n* = 7), renal (*n* = 2), adrenal (*n* = 3), distal lymphatic (*n* = 2), brain (*n* = 1), and spinal (*n* = 1). Serum CEA levels were found to be higher in patients with CRC metastases compared to patients with local recurrent CRC (*P* < 0.05). Moreover, the percentage of patients with elevated CEA levels and local recurrence was less than that of CRC patients with elevated CEA levels and single or multiple metastases (36.6% *vs* 66.7% and 75.0%, respectively) (*P* < 0.05, Table 4). Patients with a history of elevated CEA levels prior to surgery were also associated with elevated CEA levels directly prior to surgery in 77.3% of cases, whereas patients with no prior history of elevated CEA levels exhibited elevated levels of CEA levels directly prior to

tor for tumor restaging even after surgery. Furthermore, it has been shown that genetic vaccines targeting CEA may be a feasible strategy for the treatment of CRC[19]. For example, Ogata *et al*[20] observed that stage II CRC patients with elevated levels of CEA may be candidates for adjuvant chemotherapy following curative resection.

CRC recurrence has been reported for 30%-40% of patients who undergo curative resection. During the follow-up period of surgical resection, CEA monitoring is typically performed. However, the accuracy and efficacy of CEA monitoring is not always consistent. For example, in the present study, only 54.5% of patients experiencing recurrence had elevated serum levels of CEA. Moreover, these results are consistent with previously reported findings[21]. Typically, elevated levels of CEA detected postoperatively have a high probability of indicating tumor recurrence, while normal levels of CEA detected postoperatively are not useful for excluding the probability of recurrence[22,23]. Therefore, the need for monitoring CEA levels in patients who initially exhibit normal levels of CEA remains to be determined[24]. In the present study, according to the preoperative CEA levels assayed, 77% of recurrent CRC patients had elevated CEA levels, while 32% had normal CEA levels. These results indicate that normal CEA levels may be associated with the relatively early stages of tumor progression, and also with the presence of a non-CEA producing tumor. For example, production of CEA may be reduced in poorly differentiated adenocarcinomas. Furthermore, some studies[25,26] have reported an inverse relationship between tumor grade and CEA levels among patients with nodal metastases and unresectable disease.

Another consideration is the rate of rise for CEA levels that can vary depending on the site of recurrence. It has previously been proposed that monitoring of serum CEA levels is useful for the detection of liver metastases, yet is not useful for the detection of local recurrence or other types of metastasis[27]. In the present study, patients with CRC metastasis, especially multiple metastases, were associated with higher CEA levels, whereas those with local recurrent CRC had a lower CEA level during recurrence (75.0% *vs* 36.6%, respectively, *P* < 0.05). In combination, these results suggest that CEA alone should not determine whether "second-look" surgeries are performed, or whether CT scan or other imaging tools should be required to identify precise sites of recurrence.

As a retrospective study, the limitations associated with this work include the absence of a standard adjuvant therapy protocol and monitoring strategy. For example, CRC monitoring was not at regular time intervals, resulting in a sensitivity bias. In comparison, the cut-off values used to determine elevated CEA levels in other studies have ranged from 3-15 ng/mL[28-30], thereby affecting the sensitivity of serum CEA assays for tumor detection. Furthermore, since CEA levels were found to be associated with T stage and tumor size in the present study, additional large-scale studies are needed to establish the specific cut-off value needed, according to different tu-

mor burden volumes, in order to facilitate the detection of primary and recurrent CRC.

Currently, an ideal tumor marker for CRC is not available[31]. For example, although CEA is a well-known tumor marker for CRC, the detection of serum CEA levels has not proven to be sufficiently sensitive for detecting primary CRC, especially early stage CRC. However, preoperative serum levels have been found to be an independent prognostic factor for patients with CRC following curative resection. Moreover, CRC patients with normal serum levels of CEA have a higher probability of maintaining these levels during CRC recurrence, especially during local recurrence compared with metastasis. Therefore, monitoring of serum CEA levels can facilitate the detection of primary and recurrent CRC; however, this assay must be complemented by other clinical and laboratory assessments.

COMMENTS

Background
Although detection of serum carcinoembryonic antigen (CEA) is widely used to monitor recurrence following curative resection for colorectal cancer (CRC), the sensitivity associated with this readout is not ideal. Therefore, it is important that factors associated with a negative CEA test, when recurrence has been confirmed, be further studied.

Research frontiers
The presence of elevated serum levels of CEA prior to surgical resection for CRC has previously been identified as a prognostic factor for CRC, and therefore, has been well studied. For these patients, postoperative serum CEA surveillance is effective for detecting recurrence. However, for patients who initially present with normal levels of CEA, the need to further monitor CEA levels remains controversial

Innovations and breakthroughs
In this study, CRC patients with normal CEA levels prior to operation were more likely to maintain these levels when recurrence occurred, especially in cases of local recurrence compared with metastasis.

Applications
The findings of this study provide further insight into CRC monitoring strategies, especially for patients with normal CEA levels prior to surgical resection.

Peer review
This is a well designed study, which formally needs few revisions.

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BRIEF ARTICLE

Stress-induced intestinal necrosis resulting from severe trauma of an earthquake

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Abstract

AIM: To investigate the possible reasons and suggest therapeutic plan of stress-induced intestinal necrosis resulting from the severe trauma.

METHODS: Three patients in our study were trapped inside collapsed structures for 22, 21 and 37 h, respectively. The patients underwent 3-4 operations after sustaining their injuries. Mechanical ventilation, intermittent hemodialysis and other treatments were also provided. The patients showed signs of peritoneal irritation on postoperative days 10-38. Small intestinal necrosis was confirmed by emergency laparotomy, and for each patient, part of the small bowel was removed.

RESULTS: Two patients who all performed 3 operations died of respiratory complications on the first and second postoperative days respectively. The third patient who performed 4 operations was discharged and made a full recovery. Three patients had the following common characteristics: (1) Multiple severe trauma events with no direct penetrating gastrointestinal injury; (2) Multiple surgeries with impaired renal function and intermittent hemodialysis treatment; (3) Progressive abdominal pain and tenderness, and peritoneal irritation was present on post-traumatic days 10-38; (4) Abdominal operations confirmed segment ulcer, necrosis of the small intestine, hyperplasia and stiffness of the intestinal wall; and (5) Pathological examinations suggested submucosal hemorrhage, necrosis, fibrosis and hyalinization of the vascular wall. Pathological examinations of all 3 patients suggested intestinal necrosis with fistulas.

CONCLUSION: Intestinal necrosis is strongly associated with stress from trauma and post-traumatic complications; timely exploratory laparotomy maybe an effective method for preventing and treating stress-induced intestinal necrosis.

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INTRODUCTION

Intestinal necrosis is a common condition that is fre-

quently seen in clinical practice[1-6]. It is generally associated with mesenteric thrombosis or bacterial infection after eating contaminated foods[5,6]. However, few cases of stress-induced intestinal necrosis have been reported. Although animal studies have confirmed that stress can cause intestinal mucosal barrier disturbances[7,8], stress-induced intestinal necrosis has not been confirmed. We analyzed the clinical data of 3210 patients who were admitted to our hospital after the Wenchuan earthquake on May 12, 2008. In the laparotomy examinations of 3 patients with no penetrating abdominal trauma, intestinal necrosis was confirmed on postoperative days 10-38. Two of these patients died, while 1 survived and made a full recovery. For these 3 cases, we made the diagnosis of "stress-induced intestinal necrosis" after repeated careful consideration.

MATERIALS AND METHODS

The 3 male patients were 27, 36 and 42 years old, respectively (average age of 35 years). They were trapped inside collapsed structures for 22, 21 and 37 h, respectively (average time of 26.3 h). They were admitted into our hospital at 37, 44 and 39 h, respectively, after trapped in the buildings. Their primary earthquake-related traumatic injuries mainly occurred on the head and extremities, including 1 case with a left thorax crush injury. The physician specialists in various departments of our hospital consulted and discussed the cases with experts from The Liberation Army General Hospital of Beijing immediately after the patients' admission. Treatment plans were designed individually by a multi-disciplinary team. The patients underwent 4, 3 and 3 surgeries, respectively, according to their clinical features. The final operation for each patient, which was a small bowel resection, was performed on postoperative days 38, 21 and 10, respectively. Antibiotic therapy, intravenous fluid hydration, mechanical ventilation and intermittent hemodialysis were administered to all 3 patients after admission.

Representative case report

A 27-year-old healthy male patient with a relatively healthy medical history had multiple body injuries due to high-force impacts from a building collapse in the Wenchuan earthquake on May 12, 2008. He was rescued after being trapped in building debris for 22 h. He was admitted to our hospital on May 14. He remained conscious and presented with multiple contusions on the head and both lower extremities at the time of admission. Neither of his legs could be moved freely, and both were significantly swollen. The pulse of the bilateral dorsalis pedis was absent. The patient's toes presented as dark purple with poor blood circulation. A physical examination showed no signs of abdominal trauma. At the time of admission, emergency decompression surgery was performed on the osteofascial compartment of the left lower extremities under local anesthesia. We suspected that the patient had acute renal failure due

Figure 1 Anesthesia was administrated to the patient who underwent amputation of the bilateral extremities. The surgical area was sterilized in preparation for the operation.

to a lack of urination, and hemodialysis was administrated. On May 16, the patient underwent emergency amputation of both legs at the mid-thigh. On May 20, the patient experienced abdominal distension with diarrhea; blood appeared in a stool sample. On May 24, the patient developed worse abdominal distention, mild tenderness around the belly button and no rebound tenderness or muscle tension. Bowel sounds were absent. An ultrasound test revealed intestinal expansion and a small amount of peritoneal fluid. On May 26, jejunum drainage was placed under gastroscopy. The drainage output was 1000 mL, and the abdominal distension was significantly relieved. On May 30, the patient developed infections in right lower extremitie. A second amputation operation was performed on his right thigh. On June 3, thoracentesis was performed on his left chest cavity due to significantly increased pleural effusion. On June 7, the patient developed dark bloody stool that occurred 3-4 times per day. The patient's urine output gradually returned to normal by intermittent hemodialysis. On June 19, the patient developed worsening abdominal pain and tenderness, and a mass was noted around the middle and lower abdomen. Peritoneal irritation was obviously present. On emergency abdominal exploration (Figure 1), significant intestinal adhesion was noted. The intestine approximately 210 cm below the Treitz ligament and 50 cm above the ileocecal valve was expanded, thickened and twisted into a mass, with a large amount of inflammatory exudate. Intestinal adhesion, necrosis and perforation with fistula were noted (Figure 2). The proximal jejunum was expanded and thickening. The patient underwent small bowel resection, which revealed scattered erosion with hemorrhage in the intestinal mucosa. The postoperative pathology report included hemorrhagic enteritis and intestinal adhesion with fistula formation (Figure 3). The patient experienced dark bloody or tarry stools 2 wk after surgery (approximately 200-800 g/d). Antihemorrhagic, antacid therapy and nutritional support were administrated. Eventually, the patient was discharged and made a full recovery.

Figure 2 The damaged small intestine was adhered into a mass with a fistula. Thickness and hyperplasia were noted on the small intestinal wall after the part of damaged intestine was cleaned.

Figure 3 Large necrotic lesions along with macrophage infiltration were observed in the intestinal mucosa. Collagen fiber hyperplasia was also present on the submucosal membrane.

RESULTS

Two patients from this study died of respiratory failure on the first and second postoperative day (post-traumatic days 22 and 12, respectively). One patient survived. Our patients had the following common characteristics: (1) Multiple severe trauma events with no direct penetrating gastrointestinal injury; (2) Multiple surgeries with impaired renal function and intermittent hemodialysis treatment; (3) Progressive abdominal pain and tenderness, and peritoneal irritation was present on post-traumatic days 10-38; (4) Abdominal operations confirmed segment ulcer, necrosis of the small intestine, hyperplasia and stiffness of the intestinal wall; and (5) Pathological examinations suggested submucosal hemorrhage, necrosis, fibrosis and hyalinization of the vascular wall.

DISCUSSION

Diagnosis of intestinal necrosis: Stress-induced intestinal necrosis

How did we make the diagnosis of intestinal necrosis in these patients? We reviewed the relevant literature and found no related cases. However, cases of stress-induced intestinal injury were relatively common[9-11]. Lu *et al*[10] studied the stress response in rats under heat conditions.

They found severe intestinal mucosal damage associated with changes in gene expression that were related to stress-induced immune regulation in the rat small intestine. Smith *et al*[11] studied the stress response to early weaning in porcine intestines and found that early weaning in pigs can induce stress and lead to impaired mucosal barrier function. The response to trauma, as a comprehensive stress reaction, can cause stress-induced damage to multiple organs in the body[12,13]. However, stress-induced intestinal necrosis has not been reported previously. There are many reports of gastrointestinal feeding tubes leading to partial necrosis of the small intestine, which were thought to be the result of bacterial infections[14,15].

In the 3 patients in our study, no gastrointestinal feeding tubes were placed. After careful consideration, we made the diagnosis of "stress-induced intestine necrosis". The differential diagnosis of "acute intestinal necrosis" includes the following: (1) "Stress-induced intestine necrosis" occurs in severe trauma or multiple surgeries, while "acute intestinal necrosis" occurs for unknown reasons and may be related to contaminated food[16]; (2) "Stress-induced intestinal necrosis" has a relatively slow onset, as the 3 patients in this study had signs of peritoneal irritation on post-trauma days 10-38; "acute intestinal necrosis" develops early, usually within

5 d; (3) "Stress-induced intestinal necrosis" has no obvious abdominal pain and mainly presents as abdominal distention with no signs of peritoneal irritation, while "acute intestinal necrosis" presents with acute abdominal pain and unbearable and obvious signs of peritoneal irritation; (4) With regard to intraoperative findings, "stress-induced intestinal necrosis" usually has intestinal epithelial hyperplasia, segmental ulceration induced necrosis and fistula formation, while "acute intestinal necrosis" has segmental necrosis without intestinal wall thickening; and (5) With regard to pathological results, "stress-induced intestinal necrosis" has submucosal necrosis, hyperplasia, fibrosis and a large number of macrophages and cells with hemosiderin within the tissue, while "acute intestinal necrosis" has fibrin deposition within the small arteries, intestinal hemorrhage and necrosis[17].

Reasons for intestinal necrosis after trauma

We believe that intestinal necrosis is associated with a continuous high level of stress due to primary turuma and traumatic complications. Under stress: (1) Intestinal mucosal permeability increases, which results in increases in peritoneal inflammatory exudate[18]; (2) Intestinal bacteria can translocate peritoneally and worsen peritoneal infections[19-21]; (3) The intracellular space within the endothelium of mesenteric vessels increases, allowing blood cells and plasma to leak through the connective tissue and vessel lamina, leading to vascular hyalinization, sclerosis and fibrosis; (4) As the response to stress continues, the sympathetic adrenergic medullary system is highly excited, which can lead to intestinal vasoconstriction and a reduction in the blood supply[22]; (5) As arteriosclerosis and contraction of the mesenteric vessels continues, the blood supply continues to decrease, resulting in hypoxia of the mucosal tissue, acidosis, mucosal necrosis and sloughing, and ulcers; and (6) Another explanation is that stress induces vagal inactivation and consequently suppresses the "cholinergic anti-inflammatory pathway" which might lead to intestinal injury ,even intestinal necrosis[23].

The 3 patients in this study suffered from long periods of crush injuries followed by several surgeries and intermittent hemodialysis. Their bodies were in a state of continuous stress. The long-term reduction in the mesenteric blood supply led to intestinal necrosis.

Treatment of stress-induced intestinal necrosis

Relative to acute intestinal necrosis, stress-induced intestinal necrosis is a slow progressive process that is difficult to identify. In the Wenchuan earthquake, we only made diagnoses of stress-induced intestinal necrosis for 3 cases. Two of these patients died, and 1 survived, indicating the difficulty of treating this condition. Due to the small number of cases analyzed, our treatment experience is limited, but the following recommendations can be made. First, the primary trauma should be controlled as soon as possible. Stress was continuously present in part because the primary injury was not treated directly. In the representative case, after both legs were amputated, the infection of the amputated surface was not well controlled, resulting in a second right leg amputation. In addition, intermittent dialysis after renal failure could cause intestinal ischemia-reperfusion injury. Second, early administration of appropriate vasodilator could improve intestinal microcirculation. Third, vasoconstrictors should be used as little as possible. As the blood pressure drops, the application of a vasoconstrictor can increase the blood supply of vital organs such as the heart and brain, but it can reduce the intestinal blood supply. Fourth, intestinal flora should be adjusted appropriately. Many studies indicate that in cases of trauma or weakened immune systems, the intestinal flora can translocate, leading to infections in other parts of the body. A large amount of inflammatory secretions were found in the abdominal cavity of all 3 patients in this study, which may be directly related to intestinal flora that translocated peritoneally. Fifth, the timing of surgery is a key factor. All 3 patients in this study underwent laparotomies after they showed signs of peritoneal irritation. As the results showed, the optimal timing might be prior to this point. We believe that the late diagnosis of intestinal necrosis occurred for the following reasons: (1) Stress-induced intestinal necrosis is a relatively slow process; (2) Intestinal ischemia will inevitably lead to inflammatory exudate. At the same time, the omentum and surrounding intestine can wrap around the injured intestinal regions; and (3) It is only after multiple necroses occur and inflammatory substances cannot be contained that the peritoneum will be stimulated, and signs of peritoneal irritation will be presented. Therefore, the optimal timing of surgery is before the signs of peritoneal irritation. Frequent abdominal ultrasounds and abdominal biopsies can help in determining the optimal timing of surgery.

Early signs/warnings/precautions

During intestinal stress-induced injury, fatty acid binding protein (FABP) is widely recognized as a specific marker of intestinal damage[24,25]. FABP is released by epithelial cells of the intestinal mucosa into the circulation following mucosal damage. Studies have shown that the plasma concentration of FABP gradually increases with the severity of shock. Pathological examinations have suggested that the intestinal mucosal damage was becoming progressively worse[26].

Therefore, we could use FABP as a routine test marker for patients with severe trauma. The increased plasma levels of FABP, combined with other abdominal physical signs and ultrasound results, could aid in early abdominal exploration and facilitate the early diagnosis of this condition.

COMMENTS

Background

Stress-induced intestinal injury is generally associated with damage to the intestinal mucosal barrier. This type of injury has been confirmed in many animal experiments, but few studies have reported stress-induced intestinal necrosis.

Research frontiers
There are many reports of partial of the small intestinal necrosis, which were thought to be the results of mesenteric thrombosis or bacterial infection. However, few authors have reported stress-induced intestinal necrosis.

Innovations and breakthroughs
In this paper, the authors investigated the possible reasons and suggested therapeutic plan of stress-induced intestinal necrosis resulting from the severe trauma of an earthquake. Concolund: Stress-induced intestinal necrosis is strongly associated with high level of stress from trauma and post-traumatic complications; For the therapeutic strategy, the primary trauma should be controlled as soon as possible, and timely exploratory laparotomy maybe an effective method for preventing and treating stress-induced intestinal necrosis.

Applications
The patients suffered stress-induced intestinal necrosis should be rarely identified. However, this disease is very dangerous, and even result in death. This paper will offer an alert and instructions for preventing and treating stress-induced intestinal necrosis due to trauma.

Terminology
Fatty acid binding protein is widely recognized as a specific marker of intestinal damage, and which is released by epithelial cells of the intestinal mucosa into the circulation following mucosal damage.

Peer review
In this paper, the authors reported 3 patients who suffered Wenchuan earthquake, had no direct abdominal trauma, and presented small intestinal necrosis diagnosed as "stress-induced intestinal necrosis". This is an interesting paper since, effectively, few data have been published in this domain. And this paper may offer an alert and instructions for preventing and treating stress-induced intestinal necrosis due to trauma.

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World Journal of Gastroenterology

BRIEF ARTICLE

Emodin promoted pancreatic claudin-5 and occludin expression in experimental acute pancreatitis rats

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Abstract

AIM: To investigate the effect of emodin on pancreatic claudin-5 and occludin expression, and pancreatic paracellular permeability in acute pancreatitis (AP).

METHODS: Experimental pancreatitis was induced by retrograde injection of 5% sodium taurocholate into the biliopancreatic duct. Emodin was injected *via* the external jugular vein 0 or 6 h after induction of AP. Rats from sham operation and AP groups were injected with normal saline at the same time. Samples of pancreas were obtained 6 or 12 h after drug administration. Pancreatic morphology was examined with hematoxylin and eosin staining. Pancreatic edema was estimated by measuring tissue water content. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 level were measured by enzyme-linked immunosorbent assay. Pancreatic paracellular permeability was assessed by tissue dye extravasation. Expression of pancreatic claudin-5 and occludin was examined by immunohistology, quantitative real-time reverse transcriptase polymerase chain reaction and western blotting.

RESULTS: Pancreatic TNF-α and IL-6 levels, wet/dry ratio, dye extravasation, and histological score were significantly elevated at 3, 6 and 12 h following sodium taurocholate infusion; treatment with emodin prevented these changes at all time points. Immunostaining of claudin-5 and occludin was detected in rat pancreas, which was distributed in pancreatic acinar cells, ductal cells and vascular endothelial cells, respectively. Sodium taurocholate infusion significantly decreased pancreatic claudin-5 and occludin mRNA and protein levels at 3, 6 and 12 h, and that could be promoted by intravenous administration of emodin at all time points.

CONCLUSION: These results demonstrate that emodin could promote pancreatic claudin-5 and occludin expression, and reduce pancreatic paracellular permeability.

Xia XM, Li BK, Xing SM, Ruan HL. Emodin promoted pancreatic claudin-5 and occludin expression in experimental acute pancreatitis rats. *World J Gastroenterol* 2012; 18(17): 2132-2139 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i17/2132.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i17.2132

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease char-

acterized by interstitial edema, acinar necrosis, hemorrhage, and inflammatory infiltration in the pancreas[1]. Increased paracellular permeability and loss of barrier function in pancreas have been demonstrated at early stages of AP[2-4], but the molecular basis for these phenomena is poorly understood.

Tight junctions, the major apical structures in epithelium and endothelium, have been recently reported to play important roles in barrier function by forming cell-to-cell contacts and sealing paracellular pathway[5,6]. Tight junctions comprise the integral transmembrane proteins occludin, junctional adhesion molecules, and members of the claudin multigene family[4-7]. In mammals, the claudin family of 20-24 kDa integral membrane proteins includes at least 24 members; most of them have been shown to control the permeability of the paracellular pathway[6-8]. Sharing similar membrane location with claudins, occludin also plays an important role in maintaining epithelial and endothelial barriers[9]. Previous studies have demonstrated that claudin-1-5 and occludin are expressed in the pancreas[3,10-12]. Schmitt *et al*[3] have reported that claudin-1 and occludin expression in pancreas is significantly decreased in caerulein-induced AP, suggesting a possible role of tight junctions disruption in interstitial edema formation.

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), an anthraquinone derivative from the Chinese herb *Radix et Rhizoma Rhei*, has been reported to inhibit production of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1[13,14]. Our previous study has demonstrated that emodin significantly reduces serum amylase and inflammatory cytokines and attenuates pancreatic damage in AP rats[15]. However, the effects of emodin on claudin and occludin expression, as well as pancreatic paracellular permeability remain largely undefined.

We have previously found that 6 h after duct infusion of sodium taurocholate, pancreatic claudin-1 and claudin-4 were slightly elevated, claudin-5 and occludin were significantly decreased, whereas claudin-2 and claudin-3 remained unchanged (data not shown). Thus, in the present study, we assessed the effects of emodin on claudin-5 and occludin expression. Time course of pancreatic paracellular permeability, edema, and cytokines were also determined.

MATERIALS AND METHODS

Reagents
All chemicals were purchased from Sigma (St. Louis, MO, United States) unless otherwise indicated. TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Jingmei Biotech (Beijing, China). TRIzol kit and SYBR Green SuperMix-UDG were purchased from Invitrogen (Carlsbad, CA, United States). A first-strand cDNA synthesis kit was purchased from Fermentas (Burlington, Ont., Canada). Antibodies for claudin-5 and occludin were obtained from Zymed

Laboratories (South San Francisco, CA, United States). The Power Vision Two-Step Histostaining Reagent was purchased from ImmunoVision Technologies (Norwell, MA, United States). Glyceraldehyde phosphate dehydrogenase (GAPDH) antibody was purchased from Abcam (Cambridge, United Kingdom). Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Kangchen Biotech (Shanghai, China). Immobilon western chemiluminescent HRP substrate was purchased from Millipore (Boston, MA, United States).

Experimental model
Adult male Sprague-Dawley rats (200-250 g body weight) were obtained from the Animal Facility of Anhui Medical University (Hefei, China). Animals were housed under controlled temperature, humidity and day-night cycles, with free access to standard laboratory feed and water. The Animal Studies Ethics Committee of Anhui Medical University approved all of the experiments.

AP was induced as described by Pereda *et al*[16]. Briefly, animals were anesthetized with intraperitoneal administration of ketamine (80 mg/kg body weight) and acepromazine (2.5 mg/kg body weight). The biliopancreatic duct was cannulated through the duodenum, and the hepatic duct was closed by a small bulldog clamp. Pancreatitis was induced by retrograde injection into the biliopancreatic duct of 5% sodium taurocholate in a volume of 1 mL/kg body weight, at a constant infusion pressure of 20 mmHg. Presenting as controls, sham group received retrograde infusion of sterile saline.

Study design
AP rats were randomly allocated into two groups: the model group and emodin group (2.5 mg/kg body weight). Emodin was injected *via* the external jugular vein immediately after duct infusion of sodium taurocholate. Both the sham group and model group were injected with normal saline of equivalent volume. Samples were obtained 3, 6 and 12 h after duct infusion. For animals that were euthanized at the 12-h time point, a second administration of emodin or saline was adopted, 6 h after duct infusion of sodium taurocholate.

Samples of pancreas were obtained at 3, 6 and 12 h after intraductal infusion, immediately frozen and maintained at -80 ℃ until assayed. Blood samples were obtained from the inferior cava vein by direct puncture. For histological examination, the central body of the pancreas was fixed in 4% neutral phosphate-buffered formalin and then embedded in paraffin wax. Serum amylase activity was measured to confirm the appropriate induction of pancreatitis.

An additional experiment was adopted to assess the effect of emodin on pancreatic dye extravasation (marker of paracellular permeability). Animals were distributed in the same groups as in the previous series.

Histological examination
Rat pancreas was washed in phosphate buffered saline

Table 1 Histological scoring for acute pancreatitis

Condition	Score	Description
Edema	0	Absent
	1	Diffuse expansion of interlobular septa
	2	1 + diffuse expansion of interlobular septa
	3	2 + diffuse expansion of interlobular septa
Inflammation (%)	0	Absent
	1	In parenchyma (< 50 of lobules)
	2	In parenchyma (51-75 of lobules)
	3	In parenchyma (> 75 of lobules)
Vacuolization (%)	0	Absent
	1	Focal (5-20)
	2	Diffuse (21-50)
	3	Severe (> 50)

(PBS), fixed in 10% neutral-buffered formalin, and embedded in paraffin wax. Five-micrometer sections were deparaffinized with xylene, stained with hematoxylin and eosin, and examined by two experienced pathologists in blinded fashion. Pancreatic damage was scored using a grading system described by Ryan *et al*[17]. The grading was based on the number of acinar cell ghosts, the presence of vacuolization, interstitial edema and interstitial inflammation, and to what extent these characteristics affected the pancreas (0 being normal and 3 being severe), giving a maximum score of 12 (Table 1).

Measurement of pancreatic edema and cytokines

The extent of pancreatic edema was estimated by measuring tissue water content. Freshly obtained blotted samples of pancreas were weighed on aluminum foil, dried for 24 h at 95 ℃, and reweighed. The difference between the wet and dry tissue weights was calculated and expressed as wet/dry ratio.

Pancreatic TNF-α and IL-6 were examined using a sandwich ELISA according to the manufacturer's instructions. The tissue homogenate ELISA was corrected by the concentration of protein, and expressed as the content per protein of the tissue (pg/mg protein).

Measurement of paracellular permeability

Paracellular permeability of the pancreas was evaluated by the measurement of Evans blue extravasation[18]. Briefly, Evans blue (20 mg/kg) was injected into the jugular vein of rats, 30 min before duct infusion. Samples of pancreas were obtained 3, 6 and 12 h after duct infusion. A portion of the splenic segment was sectioned and immersed in formamide solution, and homogenized for 2 min. After incubation at room temperature for 24 h, the suspension was centrifuged at 4000 *g* for 30 min. The quantity of dye extracted was determined spectrophotometrically at 620 nm and calculated from a standard curve established with known amounts of Evans blue. Results were corrected by the wet/dry ratio of the pancreas and expressed as the dye content per dry weight of the pancreatic tissue (μg/g tissue).

Western blotting

Western blotting was performed as described by Hi-

etaranta *et al*[19]. From each sample, 20 μg total protein was separated on 4%-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked in blocking solution, incubated overnight with primary antibodies, and developed with an HRP-conjugated secondary antibody (1:1000 dilution). Dilutions for primary antibody were as follows: claudin-5, 1:100; and occludin, 1:300. The immune complexes were then visualized using chemiluminescent HRP substrate and X-ray film. Additional immunoblots were performed using GAPDH antibody as the primary antibody to evaluate equal loading.

Immunohistological analysis

Pancreas sections (4 μm) were dewaxed in graded alcohols, and finally washed in tap water. Endogenous peroxidase activity was blocked by 3% (v/v) H_2O_2, and the antigen was retrieved by microwave in 0.01 mol/L citrate buffer. Sections were then washed in PBS (0.1 mol/L). Mouse anti-rat claudin-5, and rabbit anti-rat occludin polyclonal antibodies were applied at 1:100 and incubated overnight at 4 ℃. Sections were washed four times in PBS for 20 min. The Power Vision Two-Step Histostaining Reagent was used for detection. All sections were developed using diaminobenzidine, and subsequently counterstained with hematoxylin.

Quantitative real-time reverse transcription polymerase chain reaction analysis

Total RNA was extracted using TRIzol Kit and converted to first-strand cDNA according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green SuperMix-UDG in Prism 7000 Q real-time PCR detection system (Applied Biosystems, Foster City, CA, United States). The primer sequences used for PCR were as follows: claudin-5 (forward 5'-TACTCAGCACCAAGGCGAAC-CAC-3', reverse 5'-GCGGCTT CCCACATCG-GTC-3'), occludin (forward 5'-AGTACATGGCTGCTGCTGAT G-3', reverse 5'-CCCACCATCCTCTTGAT GTGT-3'), GAPDH (forward 5'-CA GTGCCAGCC-TCGTCTCA-TA-3', reverse 5'-TGCCGTGGGTAGAGTCAT A-3'). Amplification was performed with use of the following cycles: 50 ℃ for 2 min (UDG incubation), 95 ℃ for 2 min, followed by 40 cycles of denaturing at 95 ℃ for 15 s and annealing at 60 ℃ for 30 s. All reactions were performed in triplicate. Melting curve analysis was performed to ensure the specificity of quantitative PCR. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method described by Livak *et al*[20], where GAPDH was used as reference gene.

Statistical analysis

Results are presented as mean ± SE. One-way repeated-measures analysis of variance (followed by multiple pairwise comparisons using Student-Newman-Keuls method) was used for the analysis of differences between the

Figure 1 Effects of emodin on pancreatic injury in sham group (A), pancreatitis group (B), emodin group (C) and (D) histological score. (Original magnification, × 200). Six rats were studied in each experimental group at each time point. Results are mean ± SD. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* pancreatitis group.

experimental and control groups. All statistical analysis were carried out using SPSS for Windows version 11.5, with statistical significance set at $P < 0.05$.

RESULTS

Effects of emodin on pancreatic paracellular permeability, edema and cytokines in acute pancreatitis rats

Histological sections from representative pancreas are shown in Figure 1. Pancreatic damage was characterized by leukocyte infiltrate, acinar cell necrosis, hemorrhage and fat necrosis (Figure 1B), and the histological score was significantly elevated as compared with the sham operation group at all time points (Figure 1D). Treatment with emodin obviously ameliorated pancreatic damage (Figure 1C), thus decreasing pancreatic pathological scores.

Time course of pancreatic TNF-α and IL-6 was also examined. Pancreatic TNF-α and IL-6 levels were significantly elevated at 3, 6 and 12 h following sodium taurocholate infusion, and treatment with emodin significantly reduced pancreatic TNF-α and IL-6 level at all time points (Figure 2A and B).

Pancreatic edema was evaluated by measuring the pancreatic water content, expressed as wet/dry ratio. As shown in Figure 2C, pancreatic wet/dry ratio was significantly elevated at 3, 6 and 12 h following sodium taurocholate infusion; emodin treatment significantly decreased pancreatic wet/dry ratio at all time points.

Pancreatic dye extravasation, as a marker of paracellular permeability, was examined in the present study.

Figure 2D showed that pancreatic dye extravasation was significantly elevated at 3, 6 and 12 h after induction of AP; emodin treatment significantly inhibited pancreatic dye extravasation at all time points. These findings indicated that emodin could reduce pancreatic paracellular permeability.

Effects of emodin on pancreatic claudin-5 and occludin expression in acute pancreatitis rats

We further evaluated the effect of emodin on pancreatic claudin-5 and occludin expression in sodium taurocholate induced AP rats. Immunolocalization of claudin-5 and occludin in pancreas was investigated using immunohistochemical staining. In sham rats, moderate claudin-5 immunostaining was detected in pancreatic acinar cells and vascular endothelial cells (Figure 3A), and intense occludin immunostaining was detected in pancreatic acinar cells, ductal cells and vascular endothelial cells (Figure 3D). Duct infusion of sodium taurocholate markedly decreased the immunostaining of claudin-5 and occludin (Figure 3B and E), and the immunostaining was enhanced when treated with emodin (Figure 3C and F).

Claudin-5 and occludin protein levels in pancreas were evaluated using western blotting. As shown in Figure 4, sodium taurocholate infusion significantly decreased pancreatic claudin-5 and occludin levels at 3, 6 and 12 h, as compared with sham rats; treatment with emodin markedly arrested the decline at all time points.

Kinetics of claudin-5 and occludin mRNA expression in pancreas was examined using quantitative real-time

Figure 2 Effects of emodin on pancreatic (A) tumor necrosis factor-α, (B) interleukin-6, (C) edema and (D) dye extravasation. Six rats were studied in each experimental group at each time point. Results are mean ± SD. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* pancreatitis group. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.

Figure 3 Immunohistochemical staining of claudin-5 (A-C) and occludin (D-F) in sham group (A, D), pancreatitis group (B, E) and emodin group (C, F) (original magnification, × 200).

reverse transcription (RT)-PCR analysis. Sodium taurocholate infusion significantly downregulated pancreatic claudin-5 and occludin mRNA expression at 3, 6 and 12 h,

and that could be upregulated by intravenous administration of emodin at all time points (Figure 5). Results from present study demonstrated that emodin promoted pan-

Figure 4 Effects of emodin on claudin-5 (A, C) and occludin (B, C) protein levels in rats. Six rats were studied in each experimental group at each time point. Results are expressed as mean ± SD. [a]*P* < 0.05 *vs* sham group; [c]*P* < 0.05 *vs* pancreatitis group. GAPDH: Glyceraldehyde phosphate dehydrogenase.

Figure 5 Effects of emodin on claudin-5 (A) and occludin (B) mRNA levels in rats. Six rats were studied in each experimental group at each time point. Results are expressed as mean ± SD. [a]*P* < 0.05 *vs* sham group; [c]*P* < 0.05 *vs* pancreatitis group. GAPDH: Glyceraldehyde phosphate dehydrogenase.

creatic claudin-5 and occludin expression at the level of mRNA transcription and protein synthesis.

DISCUSSION

The present study investigated the kinetic expression of claudin-5 and occludin in sodium-taurocholate-induced AP, and identified the effects of emodin on pancreatic claudin-5 and occludin expression, as well as pancreatic paracellular permeability in AP rats.

The fundamental functions of epithelia and endothelia in pancreas are to separate distinct compartments and regulate the exchange of small solutes and other substances between them[4]. Increased paracellular permeability may allow noxious contents from the luminal ductal system to enter the interstitium of the pancreatic gland, which results in local inflammation and early edema formation in AP[3]. Lerch *et al*[21] have pointed out that the sealing junctions of the interstitial space in the pancreas are the tight junctions and their proteins. They play crucial roles in the barrier function by constituting tight junction strands and by regulating the tightness of the paracellular pathway[6].

Previous studies have reported that claudin-5 is expressed in the pancreas and localized to the tight junctions[10,22,23]. The role of claudin-5 in barrier function has been investigated in several inflammatory models. Decreased expression and redistribution of claudin-5 is found in acute colitis[24]. Downregulation of claudin-5 has also been demonstrated in experimental autoimmune encephalomyelitis, correlated with breakdown of the blood-brain barrier; recombinant claudin-5 protected brain microvascular endothelial cell cultures from vascular-endothelial-growth-factor-induced increase in paracellular permeability, showing claudin-5 to be a key determinant at the blood-brain barrier[25]. However, in experimental AP, Meriläinen *et al*[26] have reported that the expression of claudin-5 is not changed during pancreatitis. Whether claudin-5 plays a role in pancreatic paracellular permeability therefore needs further investigation.

Occludin shares very similar membrane location with claudins. Based on the staining feature of claudins and occludin along endothelial cell borders in and outside the central nervous system, Persidsky *et al*[8] have speculated that claudins formed the primary make-up of the tight junctions, and occludin further enhances tight junction tightness. Tai *et al*[9] have previously reported that increased paracellular permeability is associated with a specific decrease in occludin, both at the protein and mRNA levels, in hCMEC/D3 cells. Drugs prevent downregulation of occluding, which could decrease paracellular permeability, suggesting an important role of occludin in the blood-brain barrier. In caerulein-

induced AP rats, the disintegration of occludin precedes the increase of serum lipase and amylase, accompanied by increased paracellular permeability, indicating a possible role of occludin in pancreatic barrier function[3].

In the present study, we identified the localization of claudin-5 and occludin in rat pancreas. In normal rats, intense occludin immunostaining was detected in pancreatic acinar cells, ductal cells and vascular endothelial cells, and this was consistent with results from Schmitt *et al*[3] and Borka *et al*[11]. In addition, we also detected moderate claudin-5 immunostaining in pancreatic acinar cells and vascular endothelial cells. Using RT-PCR and Western blotting, our present study confirmed the downregulation of occludin in pancreas of AP rats, which was in keeping with the results of Schmitt *et al*[3]. Our present study also identified the decrease of claudin-5 expression in pancreas of AP rats. It was found that the increase of pancreatic edema and paracellular permeability (marked by extravasation of Evans blue) was accompanied by a decrease of pancreatic claudin-5 and occludin. As demonstrated in previous studies in which paracellular permeability was associated with changes of occludin and claudin multigenes[4-7,23-25], our results suggested possible roles of claudin-5 and occludin in pancreatic barrier function.

Our present study also investigated the effects of emodin on pancreatic edema and paracellular permeability, as well as pancreatic claudin-5 and occludin expression in AP rats. Emodin significantly increased pancreatic claudin-5 and occludin expression at the level of mRNA transcription and protein synthesis, accompanied with decreased pancreatic edema and paracellular permeability. Based on results from previous and present studies, we speculate that the amelioration of pancreatic damage by emodin may contribute, in part at least, to the promotion of claudin-5 and occludin expression.

Disruption of tight junctions results in leakage of amylase and lipase, which could increase production of proinflammatory cytokines; cytokines further impair epithelial barrier function by regulation of expression of the barrier-builders such as the claudin family and occludin[27-29]. In agreement, our present study showed that elevated pancreatic TNF-α and IL-6 levels were paralleled with increased paracellular permeability and decreased expression of claudin-5 and occludin. Emodin could reduce pancreatic TNF-α and IL-6 levels, decrease paracellular permeability, and promote claudin-5 and occludin expression in AP rats, thus it plays an important role in pancreas protection.

In conclusion, our results demonstrate that emodin treatment could ameliorate pancreatic inflammation and edema, reduce paracellular permeability, and promote pancreatic claudin-5 and occludin expression. The decrease of pancreatic paracellular permeability by emodin may contribute, in part at least, to the promotion of claudin-5 and occludin expression.

COMMENTS

Background
Increased paracellular permeability and loss of barrier function in pancreas have been demonstrated at early stages of acute pancreatitis (AP), but the molecular basis for these phenomena is poorly understood.

Research frontiers
Claudin and occludin, the major components of tight junctions in epithelium and endothelium, have been reported to play important roles in barrier function by sealing paracellular pathway. Emodin, an anthraquinone derivative from the Chinese herb Radix et Rhizoma Rhei, has been used for anti-inflammatory purposes. Weather emodin has effects on pancreatic tight junction expression and pancreatic paracellular permeability has not been defined.

Innovations and breakthroughs
A recent report has demonstrated that claudin-1 and occludin expression in pancreas is significantly decreased in caerulein-induced AP, suggesting a possible role of tight junction disruption in interstitial edema formation. This is the first study to report that decreased pancreatic claudin-5 and occludin expression is parallel with increased pancreatic edema and paracellular permeability. Emodin can promote pancreatic claudin-5 and occludin expression, decrease pancreatic paracellular permeability, and inhibit pancreatic inflammation.

Applications
The results of this study may improve the understanding of the pathogenesis of AP, and also provide evidence for emodin in treatment of AP.

Peer review
This is an observational study representing an incremental advance in treatment of acute pancreatitis with emodin. The discussion is adequately developed and focused on the experimental results.

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CASE REPORT

Lymphomatoidgastropathy mimicking extranodal NK/T cell lymphoma, nasal type: A case report

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Abstract

Extranodal natural killer (NK)/T-cell lymphoma, nasal type, exhibits aggressive tumor behavior and carries a poor prognosis. Recently, lymphomatoid gastropathy with NK/T cell infiltration into gastric mucosa has been recognized as a pseudo-malignant disease which regresses without treatment. Because the conventional immunohistochemical criteria of lymphomatoid gastropathy is similar to that of extranodal NK/T-cell lymphoma nasal type, it is difficult to distinguish between the two conditions by histopathological evaluation only. Here, we report a rare case of lymphomatoid gastropathy in a 57-year-old female. Gastroendoscopy on routine check-up revealed elevated reddish lesions < 1 cm in diameter in the gastric fornix and body. Although repeat endoscopies at 1 and 6 mo later revealed no gastric lesions at any locations without any treatments, at 12 mo later gastric lymphomatoid lesions recurred at gastric fornix and body. Histological examination of endoscopic biopsy specimens at 12 mo showed atypical NK cell infiltration with CD3$^+$, CD4$^-$, CD5$^-$, CD7$^+$, CD8$^-$, CD20$^-$, CD30$^-$, CD56$^+$, CD79a$^-$ and T-cell-restricted intracellular antigen-1$^+$ into gastric mucosa. After treatment for *Helicobacter pylori* (*H. pylori*) eradication, the lesions disappeared in all locations of the gastric fornix and body over the subsequent 12 mo. Here, we report a case of *H. pylori*-positive lymphomatoid gastropathy with massive NK-cell proliferation, and also review the literature concerning newly identified lymphomatoid gastropathy based on comparison of extra nodal NK/T-cell lymphoma nasal type. In any case, these lesions are evaluated with biopsy specimens, the possibility of this benign entity should be considered, and excessive treatment should be carefully avoided. Close follow-up for this case of lymphomatoid gastropathy is necessary to exclude any underlying malignancy.

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INTRODUCTION

Extranodal natural killer (NK)/T-cell lymphoma, nasal

type, has the distinctive morphologic features of an angiocentric and angiodestructive growth pattern with frequent necrosis and apoptosis[1]. NK/T cell lymphoma frequently presents as a disease of infiltrative and ulcerative lesions around the nasal cavity, "nasal" and other midline structures, such as skin, gastrointestinal tract, salivary gland and testis, "nasal type"[2]. Characteristic immunohistochemical findings include CD56^{+} (NK cell marker), sCD3^{-}, cCD3^{+}, and Epstein-Barr virus (EBV)[+] *in situ* hybridization[3]. Long-term outcomes of NK/T cell lymphoma are generally poor due to frequent systemic relapses, and only 40% of patients survive longer than 5 years[4]. Primary NK/T cell lymphoma nasal type in the stomach is rare, and the etiology, pathogenesis, and clinical characteristics are unclear[5,6].

Recently, several cases of NK-cell proliferation in gastric mucosa were reported as lymphomatoid gastropathy or NK-cell enteropathy. Newly identified lymphomatoid gastropathy has been characterized as self-limited pseudomalignant NK-cell proliferation in gastric mucosa, and to have a good prognosis irrespective of a good prognosis even when left untreated. Histological findings reveal diffuse infiltrations of medium-sized to large atypical NK/T cells in the lamina propria and glandular epithelium. The cells were CD2^{+/-}, sCD3^{-}, cCD3^{+}, CD4^{-}, CD5^{-}, CD7^{+}, CD8^{-}, CD16^{-}, CD20^{-}, CD45^{+}, CD56^{+}, CD117^{-}, CD158a^{-}, CD161^{-} and granzyme B^{+}. Previously, most cases of lymphomatoid gastropathy were expected to be diagnosed as extranodal NK/T-cell lymphoma nasal type, because of their similar histopathologic findings, and to be treated with chemotherapy, surgery or both[7,8].

Here, we report a case of *Helicobacter pylori* (*H. pylori*)-positive lymphomatoid gastropathy with massive NK-cell proliferation in the stomach, and also review the literature concerning newly identified lymphomatoid gastropathy based on comparison of extra nodal NK/T-cell lymphoma nasal type.

CASE REPORT

A 57-year-old Japanese female without symptoms such as epigastric discomfort, nausea or heart burn showed an erythematous dish-like elevated lesion less than 1 cm in diameter in the greater curvature of the lower body of the stomach and atrophic gastritis with *H. pylori* infection at check-up gastroendoscopy (Figure 1A and B). However, histological findings were no atypical lymphoid cell infiltrations or atypical glands of the gastric mucosa. Follow-up at 1 and 6 mo showed that the elevated erythematous lesion had resolved without treatment (Figure 1C and D).

Twelve months later, repeated endoscopy revealed a similar erythematous elevated lesion < 1 cm in diameter in the anterior wall of the middle body and an erythematous lesion in the fornix (Figure 1E-H). Histological examination of biopsy specimens of the two lesions showed massive atypical medium- to large-sized NK lymphocyte infiltrations with slightly irregular nuclear contours, a dispersed chromatin pattern, and clear cyto-

plasm (Figure 2A and B). Immunohistochemical stains of NK cells showed CD3^{+}, CD4^{-}, CD5^{-}, CD7^{+}, CD8^{-}, CD20^{-}, CD30^{-}, CD56^{+}, CD79a^{-} (Figure 2C-I). Cytotoxic molecule-associated proteins of T-cell restricted intracellular antigen-1 (TIA-1) and granzyme B were both positive (Figure 2J and K). *In situ* hybridization for EBV-encoded RNA was negative(Figure 2L). There was no evidence of the involvement of tumor cells in peripheral blood or bone marrow, or of the involvement of small intestine, colon or other organs by computed tomography and positron emission tomography.

A diagnosis of extranodal NK/T-cell lymphoma nasal type was initially considered based on the atypical NK/T-cell infiltrations into gastric mucosa. However, owing to the negative hematological evaluation for EBV infection, including Epstein-Barr anti-viral capsid antigen immunoglobulin M (< 10 times) and anti-Epstein-Barr nuclear antigen (< 10 times), lack of any evidence of the involvement of other organs, stage IE according to the Ann Arbor classification, and lack of aggressive tumor behavior during observation period, the diagnosis was changed to lymphomatoid gastropathy. The patient was not treated with chemotherapy or gastrectomy but rather *H. pylori* eradication therapy consisting of rabeprazole 10 mg bid, clarithromycin 200 mg bid and amoxicillin 750 mg bid for 7 d. After eradication, no further manifestation of lymphomatoid gastropathy occurred endoscopically and pathologically during 12 mo of follow-up.

DISCUSSION

CD16/CD56^{+} NK cells are a subset of lymphocytes which are associated with innate immunity and cytotoxic function against viruses and tumor cells in peripheral blood, lymphoid tissue, spleen and extranodal sites, such as gastrointestinal mucosa[9]. Nevertheless, little is known about the presence and function of these or other NK cells in gastric mucosa. Here, we reported a rare case of self-limited lymphomatoid gastropathy mimicking extranodal NK/T-cell lymphoma, nasal type, in the stomach. Microscopic observation showed sheets of large peculiar cells with indented nuclei and clear cytoplasm with eosinophilic granules. Immunohistochemical analysis of these atypical cells showed CD3^{+}, CD4^{-}, CD5^{-}, CD7^{+}, CD8^{-}, CD20^{-}, CD30^{-}, CD56^{+}, CD79a^{-}, TIA-1^{+} and granzyme B^{+}. In general, although NK cells in gastric mucosa have no cytotoxic function and low levels of TIA-1 and Granzyme B[10], the relatively high TIA-1 and Granzyme B expression of gastric mucosal NK cell infiltrates in this case suggested that these cells did in fact have a cytotoxic function in this patient, most probably in responding to local inflammation or autoimmunity.

The most important differential diagnosis of lymphomatoid gastropathy is to distinguish it from extranodal NK/T cell lymphoma, nasal type, in stomach. In the present case, a diagnosis of "extranodal NK/T cell lymphoma nasal type" was suspected from the immunohistochemical finding of a strong expression of CD56

Figure 1 Gastroendoscopy revealed an erythematous dish-like elevated lesion in the greater curvature of the lower body at check-up (A and B), and at one (C) and six months later (D); Twelve months later, endoscopy revealed a similar lesion in the anterior wall of the middlebody (E and F), and an erythematous lesion in the fornix (G and H).

Figure 2 Histological examination showed massive atypical medium- to large-sized natural killer lymphocyte infiltrations with slightly irregular nuclear contours. A dispersed chromatin pattern and clear cytoplasm in the gastric mucosa, × 100 (A) and × 400 (B); Immunohistochemical stains showed CD3+ (C), CD4- (D), CD5- (E), CD7+ (F), CD8- (G), CD20- (H), CD56+ (I), T-cell restricted intracellular antigen-1+ (J), granzyme B+ (K) and Epstein-Barr virus-encoded RNA *in-situ* hybridization (L).

and CD3. Extranodal NK/T-cell lymphoma, nasal type, is rarely seen in Western countries but is relatively common in Asia and Central-South American countries[7,11,12], where it accounts for < 2% of all newly diagnosed lymphoma in Japan, 6% in Hong Kong, 8% in Korea, and 5% in Taiwan[13-16]. Histologically, the lymphoma often shows an angiocentric and angiodestructive infiltrate of atypical lymphocytes leading to extensive necrosis. The differential diagnosis of gastrointestinal NK-cell and T-cell lymphomas includes enteropathy-associated T-cell

Table 1 Characteristics of cases of lymphomatoid gastropathy in the stomach and duodenum

Patient	Ref	Age/sex	Symptom	*H. pylori*	Location	Endoscopic findings	Follow-up
1	[7]	52/M	UN	-	Stomach	UN	A (145)
2	[7]	58/M	UN	+	Stomach	UN	A (50)
3	[7]	51/M	UN	+	Stomach	UN	A (60)
4	[7]	50/F	UN	+	Stomach	UN	A (46)
5	[7]	55/M	UN	+	Stomach	UN	A (33)
6	[7]	46/M	UN	+	Stomach	UN	A (60)
7	[7]	65/F	UN	+	Stomach	UN	A (56)
8	[7]	56/F	UN	+	Stomach	UN	A (29)
9	[7]	59/F	UN	+	Stomach	UN	A (18)
10	[7]	75/F	UN	+	Stomach	UN	A (12)
11	[8]	31/M	NA	UN	Stomach, small intestine, colon	Superficial erythematous lesion	A/P (84)
12	[8]	27/F	Abd pain	UN	Stomach	Multiple, superficial ulcer	A/P (23)
13	[8]	53/M	NA	UN	Stomach, duodenum	Gastric lesion	A/P (30)
14	[8]	46/F	+	UN	Duodenum, colon	Superficial ulcer	A/P (36)
15	[8]	61/F	+	UN	Duodenum, colon	Multiple, ulcers	A/P (120)
This case		57/F	NA	+	Stomach	Multiple, erythematous dish-like elevated lesions	A (16)

H. pylori: *Helicobacter pylori*; M: Male; F: Female; A/P: Alive with persistent disease but without progression; A: Alive; UN: Unknown; NA: Not available.

Table 2 Immunophenotypic findings in cases of lymphomatoid gastropathy in the stomach and duodenum

Patient	Ref	cCD3	CD56	TIA/GRZB	CD7	CD5	CD4/CD8	CD20	EBER
1	[7]	+	+	+	+	-	NA	-	-
2	[7]	+	+	+	+	-	-	-	-
3	[7]	+	+	+	+	-	-	-	-
4	[7]	+	+	+	+	-	-	-	-
5	[7]	+	+	+	+	-	-	-	-
6	[7]	+	+	+	+	-	-	-	-
7	[7]	+	+	NA	+	-	-	-	-
8	[7]	+	+	+	+	-	-	-	-
9	[7]	+	+	+	+	-	-	-	-
10	[7]	+	+	+	+	-	-	-	-
11	[8]	+	+	+	+	-	-	-	-
12	[8]	+	+	NA	+	-	-	-	-
13	[8]	+	+	+	+	-	-	-	-
14	[8]	+	+	+	+	-	-	-	-
15	[8]	+	+	+	+	-	-	-	-
This case		+	+	+	+	-	-	-	-

cCD3: Cytoplasmic CD3; TIA: T-cell restricted intracellular antigen; GRZB: Granzyme B; EBER: Epstein-Barr virus-encoded RNA; +: Positive; -: Negative; NA: Not available.

lymphoma, T-cell lymphoma, and more rarely anaplastic large cell lymphoma[17]. Moreover, histopathological diagnosis for several reactive or borderline lesions is also required, including infectious mononucleosis, drug-induced lymphadenitis, and histiocytic/subacute necrotizing lymphadenitis; these lesions histopathologically mimic lymphoma and are occasionally misdiagnosed as malignancy.

The differentiation of extranodal NK/T-cell lymphoma nasal type and lymphomatoid gastropathy by histological findings only is difficult. The differential diagnosis of the two diseases is considered to be as follows. First, the stomach is not a common site of origin of extranodal NK/T-cell lymphoma, nasal type, and most cases of extranodal NK/T-cell lymphoma and NK-cell enteropathy in the stomach are not limited to the stomach at the time the condition is diagnosed[5,18-20]. Previous cases of lymphomatoid gastropathy in the stomach and duodenum extended further down into the gastrointestinal tract, including as far as the colon (Table 1)[5,8]. Second, although some cases of lymphomatoid gastropathy showed necrosis, none showed angiocentric or angiodestructive growth patterns, or prominent apoptotic bodies, which are common features of extranodal NK/T-cell lymphoma, nasal type[7]. Our present case also showed no angiocentric or angiodestructive growth patterns. Third, Epstein-Barr virus-encoded RNA *in situ* hybridization, which is almost always positive in NK/T-cell lymphoma, nasal type, is consistently negative

in lymphomatoid gastropathy (Table 2)[3,21].

NK cells function as cytokine-producing effectors and can act as regulatory cells during inflammation and influence subsequent adaptive immune responses[22]. In acute/chronic inflammation or autoimmune reactions, localization of NK cells has been observed at variousanatomic sites, including skin and gastrointestinal tract[9]. *H. pylori* infection is characterized by marked neutrophil, lymphocyte, monocyte and plasma cell infiltration of gastric mucosa[23]. Chronic *H. pylori* gastric mucosal infection leads to chronic gastritis with severe inflammatory cell infiltration, which results in progressive gastric mucosal atrophy and intestinal metaplasia with higher potential for the development of gastric tumors[24,25]. Mucosa-associated lymphoid tissue (MALT) lymphoma (70%-80%) is well known to be caused by chronic *H. pylori* infection into gastric mucosa and after eradication therapy *H. pylori*-positive MALT lymphoma regresses. Therefore, we have lead to the hypothesis that the pathogenesis of lymphomatoid gastropathy is associated with the gastric mucosal inflammation produced by chronic *H. pylori* infection. Takeuchi *et al*[7] reported that 90% cases of lymphomatoid gastropathy were positive for *H pylori* infection and that lymphomatoid gastropathy in several patients receiving eradication therapy regressed during follow-up observation. In our case, no further manifestation of lymphomatoid gastropathy was seen for 12 mo after *H. pylori* eradication. However, other patients have also shown complete resolution without treatment for *H. pylori* eradication[7,8]. Although lymphomatoid gastropathy may be related with *H pylori* infection, a better understanding of lymphomatoid gastropathy and its relationship with *H pylori* infection awaits further study.

As shown in Table 1, endoscopic characteristics may include raised ulcers or reddish and congestive flat eleva-

tions with a shallow depression[7]. In all cases, multiple lesions of reddish flat elevations were seen. While some cases may resemble early gastric carcinoma, the endoscopic characteristics of lymphomatoid gastropathy are not clearly understood.

In conclusion, we experienced the rare case of lymphomatoid gastropathy, in which eradication treatment for *H. pylori* appeared to be effective. Differentiation of extranodal NK/T-cell lymphoma, nasal type and lymphomatoid gastropathy is difficult, and biological and endoscopic characteristics, prognosis and treatment of lymphomatoid gastropathy are unclear. Therefore, it will be better to clarify those characteristics by further case studies or basic research in feature. In any case, at the time these lesions are evaluated with biopsy specimens, the possibility of this benign entity should be closely considered, and excessive treatment should be carefully avoided. In finally, close follow-up for this case of lymphomatoid gastropathy is necessary to exclude any underlying malignancy, because nobody knows etiology of this NK-cell lymphomatoid gastropathy.

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CASE REPORT

Small intestinal hemolymphangioma with bleeding: A case report

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INTRODUCTION

Hemolymphangiomas are rare benign tumors that appear to arise from congenital malformations of the vascular system. The formation of these tumors may be explained by obstruction of venolymphatic communication, dysembrioplastic vascular tissue and systemic circulation[1]. Hemolymphangiomas most commonly present as cystic or cavernous lesions.

Herein, we report a case of hemolymphangioma of the small intestine with bleeding.

CASE REPORT

The patient was a 57-year-old woman who complained of recurrent melena for more than 2 mo. The complete blood count showed severe anemia, and stool occult blood (OB) was positive. Gastroscopy showed chronic superficial gastritis with erosion and duodenal erosion. Enteroscopy showed a gray mass with ulcers and erosion in the small intestine 30 cm distal to the flexor tendon. The mass was ill-defined, and the size was approximately 5.0 cm × 4.0 cm (Figure 1). Pathological analysis showed

Abstract

Small intestinal hemolymphangioma is a very rare benign tumor. There was only one report of a hemolymphangioma of the pancreas invading to the duodenum until March 2011. Here we describe the first case of small intestinal hemolymphangioma with bleeding in a 57-year-old woman. She presented with persistent gastrointestinal bleeding and endoscopy revealed a small intestinal tumor. Partial resection of the small intestine was thus performed and the final pathological diagnosis was hemolymphangioma. We also highlight the difficultly in making an accurate preoperative diagnosis in spite of modern imaging techniques. To arrive at a definitive diagnosis and exclude malignancy, partial resection of the small intestine was considered to be the required treatment.

Figure 1 Enteroscopy revealed a mass at the small intestine (arrow).

Figure 2 Histological analysis revealed a benign soft tissue mass (arrow) consisting of lymphatic and blood vessels (hematoxylin and eosin, × 100).

an intrinsic layer of dilated lymphatic vessels, a small amount of interstitial neutrophil, eosinophil, plasma cell infiltration. After admission, the stool OB continued to be positive, and the anemia was not corrected. Partial resection of the small intestine was thus performed. The small intestinal tumor was soft, and the final pathological diagnosis was a hemolymphangioma (Figure 2). The patient was discharged 7 d after the successful surgical resection of the tumor. At the annual follow-up, no recurrence was observed, and the patient is currently enjoying normal life.

DISCUSSION

Lymphangiomas are a heterogeneous group of vascular malformations that are composed of cystically dilated lymphatics. These malformations can occur at any age and may involve any part of the body; however, 90% occur in children who are less than 2 years of age and involve the head and neck. These lesions are rarely found in adult patients. Those benign malformations are classified into four categories[2]: Capillary lymphangioma, cavernous lymphangioma, cystic lymphangioma (hygroma) and hemolymphangioma (a combination of hemangioma and lymphangioma).

The incidence of hemolymphangiomas varies from 1.2 to 2.8 per 1000 newborns[3], and both genders are equally affected. The diagnosis in most cases (90%) is made before the age of two[4], 60% of those patients display symptoms at the time of birth. Hemolymphangioma of the small intestine is an uncommon and benign tumor. In a literature review of studies published until March, 2011 (PubMed), there was only one report of a hemolymphangioma of the pancreas invading to the duodenum[4]. However, there are no reports that describe small intestinal hemolymphangioma with bleeding.

The clinical onset of hemolymphangiomas can vary from a slowly growing cyst over a period of years to an aggressively enlarging but non-invasive tumor. Their size varies based on the anatomical location and relationship to the neighboring tissues. Small tumors are usually superficial, whereas the larger ones are located in deeper layers and have a cystic texture. The most common complications are spontaneous or traumatic hemor-

rhage, rupture and infection. On physical examination, the tumors are usually palpated as soft and compressible masses. Histologically, hemolymphangiomas consist of blood vessels and lymphatic channels.

Surgical resection appears to be the most effective treatment for hemolymphangioma, especially when the tumor increases in size and applies pressure on the surrounding tissues. Surgeons usually perform complete removal of the tumor with the surrounding organs that may be potentially invaded, because there is a possibility of recurrence and invasion of surrounding organs[5]. The recurrence rates vary depending on the complexity of the tumors, the anatomical location and the adequacy of the excision. However, lesions that have been completely excised present 10%-27% recurrence, while 50%-100% of partially resected tumors may recur. Partial or incomplete tumor removal may also be associated with complications such as infection, fistula, and hemorrhage[6].

In conclusion, our report describes on a patient with gastrointestinal bleeding due to hemolymphangioma. Surgical resection is the most effective treatment, and the case was associated with a good prognosis. Despite its low frequency, this disease should be considered when gastrointestinal bleeding is observed.

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MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
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United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
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February 23, 2012
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Oesophagus: Everything you need
to know
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Canadian Digestive Diseases Week
2012
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March 1-3, 2012
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March 7-10, 2012
Society of American Gastrointestinal
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World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
United States

April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

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Name of journal

World Journal of Gastroenterology

Instructions to authors

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All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

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1　**Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224   DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2　**Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3   **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4   **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462   PMCID:2516377   DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5   **Vallancien G**, Emberton M, Harving N, van Moorse-laar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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]

Volume with supplement

7   **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatrip-tan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8   **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious dis-eases. 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., as-signee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as υ (in Greek), sample number as n (in italics), and probability as P (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formal-dehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.
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World Journal of Gastroenterology

Contents

Weekly Volume 18 Number 18 May 14, 2012

Contents

Contents

ABOUT COVER

Takeuchi K. Pathogenesis of NSAID-induced gastric damage: Importance of cyclooxygenase inhibition and gastric hypermotility.
World J Gastroenterol 2012; 18(18): 2147-2160
http://www.wjgnet.com/1007-9327/full/v18/ i18/2147.htm

AIM AND SCOPE

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BRIEF ARTICLE

Endoclips *vs* large or small-volume epinephrine in peptic ulcer recurrent bleeding

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Abstract

AIM: To compare the recurrent bleeding after endoscopic injection of different epinephrine volumes with hemoclips in patients with bleeding peptic ulcer.

METHODS: Between January 2005 and December 2009, 150 patients with gastric or duodenal bleeding ulcer with major stigmata of hemorrhage and nonbleeding visible vessel in an ulcer bed (Forrest Ⅱa) were included in the study. Patients were randomized to receive a small-volume epinephrine group (15 to 25 mL injection group; Group 1, *n* = 50), a large-volume epinephrine group (30 to 40 mL injection group; Group 2, *n* = 50) and a hemoclip group (Group 3, *n* = 50). The rate of recurrent bleeding, as the primary outcome, was compared between the groups of patients included in the study. Secondary outcomes compared between the groups were primary hemostasis rate, permanent hemostasis, need for emergency surgery, 30 d mortality, bleeding-related deaths, length of hospital stay and transfusion requirements.

RESULTS: Initial hemostasis was obtained in all patients. The rate of early recurrent bleeding was 30% (15/50) in the small-volume epinephrine group (Group 1) and 16% (8/50) in the large-volume epinephrine group (Group 2) (*P* = 0.09). The rate of recurrent bleeding was 4% (2/50) in the hemoclip group (Group 3); the difference was statistically significant with regard to patients treated with either small-volume or large-volume epinephrine solution (*P* = 0.0005 and *P* = 0.045, respectively). Duration of hospital stay was significantly shorter among patients treated with hemoclips than among patients treated with epinephrine whereas there were no differences in transfusion requirement or even 30 d mortality between the groups.

CONCLUSION: Endoclip is superior to both small and large volume injection of epinephrine in the prevention of recurrent bleeding in patients with peptic ulcer.

INTRODUCTION

Peptic ulcer disease accounts for 50% to 70% of cases

of acute nonvariceal upper gastrointestinal bleeding (UGIB)[1,2]. Initial haemostatic rates of 80% to almost 100% can be achieved with various endoscopic techniques. However, after initial control, bleeding recurs in 10% to 30% of patients[3].

Among various endoscopic techniques a recent International consensus on nonvariceal UGIB recommends combination therapy with clear statement that epinephrine injection alone provides suboptimal efficacy and should be used in combination with another methods[4]. However, several recent studies found injection of a large volume of epinephrine to be superior to injection of a small epinephrine volume with respect to recurrent bleeding from peptic ulcer[5,6]. Since epinephrine injection is effective (initial hemostasis obtained with epinephrine injection range from 85% to 100%), safe, inexpensive and technically easy, the concept of a beneficial effect of large volumes of epinephrine in preventing recurrent ulcer bleeding seems to be very challenging. Therefore, the aim of this prospective study was to compare the rates of recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution (15-25 mL *vs* 30-40 mL) with endoscopic placement of hemoclips in patients with peptic ulcer bleeding. Since it has been suggested that the useful baseline factor for stratification in UGIB trials may be stigmata of hemorrhage in an ulcer, we decided to include in the study only patients presenting with acute UGIB and endoscopically proven gastric or duodenal ulcer with visible vessel in an ulcer bed (Forrest Ⅱa)[7].

MATERIALS AND METHODS

Between January 2005 and December 2009, consecutive patients presenting with acute UGIB were considered for this study. These patients were referred to the Emergency Unit of the Department of Internal Medicine at the "Sestre milosrdnice" University Hospital, Zagreb, Croatia and then if necessary hospitalized at the Interventional Gastroenterology Unit at the same hospital.

UGIB was suspected if hematemesis, melena or hematochezia were seen and/or bloody nasogastric aspirate was observed. In all those patients upper gastrointestinal endoscopy was performed within 6 h of hospital admission. Patients were included only if emergency endoscopy disclosed a gastric or duodenal bleeding ulcer with major stigmata of hemorrhage ("coffee ground" material or blood in the stomach and/or duodenum) and nonbleeding visible vessel in an ulcer bed (Forrest Ⅱ a)[8]. Exclusion criteria were as follows: major comorbid or terminal illness that made endoscopy hazardous; inability or unwillingness to consent to endoscopy and endoscopic therapy; gastric malignancy; minor stigmata of hemorrhage at endoscopy such as oozing from ulcer borders without a visible vessel, flat-pigmented spots, or clean ulcer base. Patients with gastric and duodenal ulcer with either an actively bleeding vessel (spurting or oozing; Forrest Ⅰ), or adherent clot (Forrest Ⅱb) were also excluded.

Endoscopy was performed with standard upper endoscopes (GIF Q140 and GIF Q160, Olympus Optical Co., Japan). Endotherapy was carried out by the well-trained endoscopists, each with at least five years experience in the treatment of patients with GI bleeding. Endoscopic characteristics, including ulcer localization, ulcer size, and type of stigmata, were recorded (Endobase, Olympus, Japan).

Possible complications of endoscopic treatment and complete study protocol were discussed with patients and their relatives, and written informed consent was obtained before endoscopy and entry into the trial. The ethics committee of our hospital approved the treatment protocol. Randomization of eligible patients was carried out at the time of endoscopy by an individual uninvolved with the procedure who opened sealed numbered envelopes containing treatment assignments generated with a computer randomization program. The treatment group allocation was then communicated to the endoscopist in the endoscopy suite. Patients were randomized to a small-volume epinephrine group (15 to 25 mL injection group; Group 1), a large-volume epinephrine group (30 to 40 mL injection group; Group 2) and a hemoclip group (Group 3). In the small-volume epinephrine group (Group 1) 15 to 25 mL of a 1:10 000 solution of epinephrine was injected around the visible vessel (2-4 mL/injection at 2-3 mm from the visible vessel). In the large-volume epinephrine group (Group 2), 30-40 mL of a 1:10 000 solution of epinephrine was injected around the visible vessel at the ulcer bed as in the small-volume epinephrine group. Mechanical hemostasis was performed with stainless steel hemoclips (Olympus, Japan) as has been previously described[9,10]. During endoscopy and endotherapy, electrocardiographic monitoring was used to detect arrhythmias.

Once hemostasis was achieved the bleeding site was observed for at least 10 min and it was assessed by water irrigation at maximal pressure. Failure of the initial hemostasis has been defined if any hemorrhage occurred immediately (within 10 min) after initial endoscopic hemostasis. In these patients crossing over to the other treatment group was not allowed. In all patients two biopsy specimens were taken from the gastric antrum and body, and the presence of *Helicobacter pylori* (*H. pylori*) infection was assessed by histopathological examination of the specimens. In patients with gastric ulcer in whom recurrent bleeding was not observed, control endoscopy was performed 4 d to 5 d after initial hemostasis and biopsy specimens were obtained from the margins and base of gastric ulcers to exclude malignancy.

After initial endoscopic hemostasis, patients were hospitalized and cared for by a physician who was blinded to the endoscopic treatment that had been delivered. Vital signs were monitored hourly whereas blood counts were observed every 6 h for the first 48 h and every 12 h to 24 h thereafter. All patients were given acid suppressive therapy: pantoprazole 80 mg iv, (bolus) and then 40 mg iv, every 8 h for at least 48 h, followed by 40 mg daily by mouth, or esomeprazole 80mg iv, (bolus) and then 40 mg iv, every 8 h for at least 48 h, followed by 20 mg once a day by mouth. Shock was defined as a systolic blood

pressure of less than 90mmHg with symptoms or signs of organ hypoperfusion.

Recurrent bleeding was defined as one or more signs of ongoing bleeding, including fresh hematemesis or melena, hematochezia, aspiration of fresh blood *via* nasogastric tube, instability of vital signs, and a reduction of Hb by more than 2 g/dL over a 24 h period (early recurrence) or over a 7 d period (late recurrence) after initial stabilization of puls, blood pressure and Hb concentration. If recurrent bleeding was suspected, endoscopy was performed immediately. If "coffee ground" material or blood in the stomach and/or duodenum has been found together with active bleeding or a fresh blood clot in the ulcer base were found, recurrent bleeding was considered confirmed. For ethical reasons, additional endoscopic methods for treatment of recurrent bleeding were discussed with patients and their relatives and therapeutic option in all patients with recurrent bleeding was hemoclip application. Patients in whom endoscopic treatment or retreatment was unsuccessful underwent emergency surgery.

The rate of recurrent bleeding, as the primary outcome, was compared between the groups of patients included in the study. Secondary outcomes compared between the groups were primary hemostasis rate (defined as the absence of hemorrhage occurred immediately after initial endoscopic hemostasis), permanent hemostasis (defined as the absence of recurrent bleeding within the 30 d period after initial or secondary endoscopic hemostasis), need for emergency surgery, 30 d mortality and bleeding-related deaths, length of hospital stay, and transfusion requirements.

Statistical analysis

Base on assumption that injection of a large-volume epinephrine decreased the expected rate of recurrent bleeding from 17.1% after injection of small-volume epinephrine solution to zero, 39 patients would have been needed in each group for a power of 80% and a significance level of 0.05[6].

Continuous data were summarized as mean [95% confidence interval (CI)]. The Student *t* test was used to compare the mean values of continuous variables. The Pearson chi-square test and the Fisher exact test were used when appropriate for the comparison of categorical variables. All analyses were performed with a statistical package (SPSS for Windows, United States). A *P* values less than 0.05 were regarded as statistically significant.

RESULTS

From January 2005 to December 2009, 150 patients were included in this study; they were randomly assigned to receive small-volume (15 to 25 mL) injection of epinephrine (Group 1, *n* = 50), large-volume (30 to 40 mL) injection of epinephrine (Group 2, *n* = 50), and hemoclip (Group 3, *n* = 50). During the same period a total of 1516 patients with UGIB were encountered; of these 47.8% had gastric or duodenal bleeding ulcer, 41.2% had non-ulcer lesions

Table 1 Clinical and endoscopic characteristics of the patients at study entry *n* (%)

	Group 1 (*n* = 50)	Group 2 (*n* = 50)	Group 3 (*n* = 50)
Age (yr)	68 (40-96)	61 (30-92)	67 (40-94)
Gender (M/F)	31/19	33/17	34/16
Location of ulcer			
Stomach	26 (52)	23 (46)	28 (56)
Duodenum	24 (48)	27 (54)	22 (44)
Ulcer size (cm)			
< 2	36 (72)	44 (88)	37 (74)
≥ 2	14 (28)	6 (12)	13 (26)
Gastric content			
Blood	19 (38)	22 (44)	21 (42)
Coffee ground	31 (62)	28 (56)	29 (58)
Shock	4 (8)	2 (4)	1 (2)
Hb level (g/dL)	9.3 (3.9-14.7)	9.0 (3.6-14.2)	9.4 (5.6-14.3)
Comorbid disease	36 (72)	35 (70)	33 (60)
NSAIDs	15 (30)	23 (46)	29 (58)
Alcohol consumption	20 (40)	23 (46)	30 (60)
Smoker's	12 (24)	16 (32)	10 (20)
Previous ulcer disease	14 (28)	10 (20)	11 (22)
Previous ulcer bleeding	12 (24)	10 (20)	8 (16)

Continuous data are expressed as mean (95% CI). NSAIDs: Non-steroidal anti-inflammatory drugs.

Table 2 Clinical outcomes of endoscopic therapy *n* (%)

	Group 1	Group 2	Group 3
Primary outcome			
Early recurrent bleeding	15 (30)	8 (16)	2 (4)[c,b]
Stigmata			
Spurting	4 (26.7)	1 (12.5)	1 (50)
Oozing	5 (33.3)	2 (25)	1 (50)
Visible vessel	6 (40)	5 (62.5)	0
Secondary outcomes			
Initial hemostasis	50 (100)	50 (100)	50 (100)
Permanent hemostasis	44 (88)	46 (92)	48 (96)
Emergency surgery	6 (12)	4 (8)	2 (4)
30-d mortality	3 (6)	0 (0)	4 (8)
Blood transfusion (mL)	1041 (120-1997)	912 (0-2039)	840 (0-1893)
Hospital stays (d)	7.5 (1-14)	7.6 (1-15)	5.7(1-15)[b,d]

Continuous data are expressed as mean (95% CI). [b]*P* < 0.01 *vs* group 1; [c]*P* < 0.05, [d]*P* < 0.01 *vs* group 2. Pearson χ^2 test.

(acute erosive gastropathy, portal hypertensive gastropathy, malignancy, Mallory-Weiss tear, angiomata, Dieulafoy's lesion), 9.1% had esophageal or gastric variceal bleeding, and 1.9% had no source of bleeding (Figure 1). Among the 161 patients with UGIB and endoscopically proven peptic ulcer with visible vessel (Forrest Ⅱa), randomly assigned to receive small-volume or large-volume of epinephrine, or hemoclip, 11 patients were excluded because they refused to participate in the study.

Clinical and endoscopic data obtained for patients included in the study are outlined in Table 1. There were no significant differences between the groups with respect to age, gender, ulcer size and location, positive *H. pylori* status, NSAID or alcohol consumption, shock, bleeding stigmata, history of previous peptic ulcer or peptic ulcer

Figure 1 Patients with upper gastrointestinal bleeding. UGIB: Upper gastrointestinal bleeding.

bleeding, comorbid diseases or hemoglobin and hematocrit levels at admission.

Clinical outcome data are summarized in Table 2. Initial hemostasis was obtained in all patients. In the small-volume epinephrine group (Group 1) the mean volume of epinephrine injected was 19.1 mL (range, 16 to 25 mL) whereas in the large-volume epinephrine group (Group 2) the mean volume of epinephrine injected was 37.9 mL (range, 30 to 40 mL). Among patients endoscopically treated with hemoclips (Group 3), multiple clips (up to three) were needed in majority of cases with a median of 1.6 clips per patient.

The rate of early recurrent bleeding was 30% (15/50) in the small-volume epinephrine group (Group 1) and 16% (8/50) in the large-volume epinephrine group (Group 2); the difference did not reach statistical significance ($P = 0.09$). The rate of recurrent bleeding was 4% (2/50) in the hemoclip group (Group 3); the difference was statistically significant with regard to patients treated with either small-volume or large-volume epinephrine solution ($P = 0.0005$ and $P = 0.045$, respectively). Late recurrent hemorrhage was not observed in our patients. With regard to ulcer location and ulcer size as well, there were no significant differences in the rate of early recurrent bleeding between the groups. Also, there were no differences in transfusion requirement or even 30 d mortality between the groups. However, duration of hospital stay was significantly shorter among patients treated with hemoclips than among patients treated with epinephrine (Table 2). There was no bleeding-related death or procedure-related death. Three patients from Group 1 (causes of death were colon malignancy in one patient, cardiac failure in one patient, and obstructive pulmonary disease

with pneumonia in one patient) and four patients from Group 3 died (pulmonary embolism in two patients and myocardial infarction in one patient). Among patients from Group 2 no one died. One patient in whom large-volume injection of the epinephrine solution was administered (35 mL) required emergent surgery because of a perforation.

Of the 15 patients in the small-volume epinephrine group (Group 1), eight patients in the large-volume epinephrine group (Group 2), and two patients in the hemoclip group (Group 3) who had recurrent bleeding, all were treated with hemoclips. Emergency surgery was performed in all patients in whom re-treatment with hemoclips did not produce hemostasis: six patients from group 1, three patients from Group 2, and two patients from Group 3. Majority of patients in whom emergency surgery has been performed had duodenal ulcer located on the duodenal bulb posterior wall (Group 1, 5/6; Group 2, 2/3; Group 3, 3/3, respectively). Successful permanent hemostasis was not statistically different among groups of patients (Table 2).

Therapeutic efficacy of the small-volume epinephrine *vs* large-volume epinephrine and hemoclips is given in Table 3. Small-volume *vs* large-volume epinephrine was not significant in NNT benefit prediction, although small-volume epinephrine (NNT = 4) and large-volume epinephrine (NNT = 9) showed different significant benefits concerning hemoclip treatment.

There were no procedure-related cardiovascular complications in the three groups. Electrocardiographic monitoring did not record any serious cardiac arrhythmia except of occasional sinus tachycardia and isolated supraventricular extrasystoles observed among all patients

Table 3 Therapeutic efficacy of small-volume and large-volume epinephrine, and hemoclips in reducing recurrent bleeding (95% CI)

Recurrent bleeding rate (%)	RRR (%)	ARR (%)	NNT
Small-volume *vs* large-volume epinephrine	46.6 (-11.2-75.0)	14.0 (-2.6-30.3)	8.0 (37.7-3.3)
Small-volume epinephrine *vs* hemoclips	86.7 (51.7-96.5)	26.0 (12.3-40.4)	4.0 (2.5-8.1)
Large-volume epinephrine *vs* hemoclips	75.0 (9.0-23.0)	12.0 (0.2-25.0)	9.0 (4.0-476.0)

RRR: Relative risk reduction; ARR: Absolute risk reduction; NNT: Number needs to treat.

treated with large-volume epinephrine injection. The number of patients who complained of epigastric pain during and/or immediately after the procedure of endotherapy was significantly higher in the large-volume epinephrine group (34/50) than in the small-volume epinephrine (3/50) or hemoclips (2/50) groups ($P < 0.001$).

DISCUSSION

Emergency endoscopy is accepted as the method of choice in the early identification and treatment of a bleeding peptic ulcer[10]. A variety of endoscopic hemostatic methods have been developed and all were found to be similarly effective[3,11]. Epinephrine as the most commonly used agent for endoscopic injection therapy has been demonstrated to be effective for initial hemostasis but appears less effective in preventing further bleeding than other monotherapies, and definitely is less effective than epinephrine followed by a second modality such as sclerosant or a thermal contact device[4,12]. However, when the analysis was restricted to studies that used routine second-look endoscopy with re-treatment of high-risk stigmata, epinephrine injection was not found to be less effective than other monotherapies or epinephrine followed by second modality[12]. On the other hand, limited data indicate that injection of a large volume epinephrine seems to be superior to injection of a small epinephrine volume with respect to recurrent bleeding[5,6,13]. These studies suggested local tamponade is the major effect in sustained hemostasis and that injection of larger volumes of epinephrine may be beneficial in preventing recurrent bleeding by prolonging the hemostatic effect of mechanical compression. Lin *et al*[5] demonstrated that injection of a large volume (13-20 mL) of epinephrine can reduce the rate of recurrent bleeding in patients with high-risk peptic ulcer and is superior to injection of lesser volumes (5-10 mL) of epinephrine (15.4% *vs* 30.8%). Park *et al*[6] reported that injection of 35 to 45 mL of a epinephrine solution was more effective in preventing recurrent bleeding than an injection of 15 to 25 mL of the same solution (0% *vs* 17.1%). Similar results have been found by Liou *et al*[13,14] demonstrated that injection of a large volume (30 to 40 mL) of epinephrine significantly reducing the rebleeding rate in patients with active bleeding ulcer.

The current study clarifies the low value of endoscopic injection therapy with epinephrine alone in patients with peptic ulcer bleeding showing major stigmata of hemorrhage (patients with endoscopically proven peptic ulcer with a visible vessel in an ulcer bed; Forrest Ⅱa). Disparities in inclusion criteria using Forrest classification, across the majority of studies that demonstrated higher effectiveness of large volume diluted epinephrine injection significantly limit the interpretation of those results. Unlike many mentioned studies, this trial was carried out on adequate patient's sample with clearly predefined groups of patients.

Our results have clearly shown that endoscopic therapy with hemoclip represents safe and effective method, superior to both, small-volume (15 to 25 mL) and large-volume (30 to 40 mL) injection of diluted epinephrine in the prevention of early recurrent bleeding from peptic ulcer. Reduction in recurrent hemorrhage rates observed among our peptic ulcer patients treated with hemoclip method positively affected length of hospital stay, reflecting the possibility of significant cost savings.

It has been postulated that possible mechanisms that underlie hemostasis in response to endoscopic injection of diluted epinephrine are vasoconstriction, vessel compression, and platelet aggregation[15,16]. Among these, mechanical compression of the bleeding vessel is the most important factor with respect to initial hemostasis[6,16]. Therefore, it has been assumed that injection of larger volumes of diluted epinephrine may be beneficial in preventing recurrent peptic ulcer bleeding by prolonging the hemostatic effect of mechanical effect and compression[5,6,14]. Despite the fact that previously mentioned assumption has been indirectly confirmed by several studies demonstrating a significantly lower rate of recurrent peptic ulcer bleeding following large volume epinephrine injection, we strongly believe that even sustained mechanical compression achieved by a larger volumes of diluted epinephrine injection is not sufficiently sustained to produce vessel compression that will last enough to provoke platelet aggregation in a greater extent, that would finally result vessel thrombosis. The results observed in this study indicate that local tamponade observed even after larger volumes of diluted epinephrine injection was not effective as hemoclip for the preventing of recurrent bleeding. This observation strongly suggested that vessel compression, produced by a hemoclip has an important role in the mechanisms involved in vessel occlusion, thus preventing the recurrent bleeding.

To our knowledge, this is the first prospective randomized study comparing the rates of the recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution and mechanical endoscopic method in patients with UGIB and endoscopically proven peptic ulcer with nonbleeding visible vessel in an ulcer bed (Forrest Ⅱa). Our results have confirmed that

endoclip is safe and effective method, pointing out to its superiority to both, small volume and large volume injection of diluted epinephrine in the prevention of early recurrent bleeding from peptic ulcer.

COMMENTS

Background

Peptic ulcer disease accounts for 50% to 70% of cases of acute nonvariceal upper gastrointestinal bleeding (UGIB). Initial haemostatic rates of 80% to almost 100% can be achieved with various endoscopic techniques, but, after initial control, bleeding recurs in 10% to 30% of patients. Several recent studies found injection of a large volume of epinephrine to be superior to injection of a small epinephrine volume with respect to recurrent bleeding from peptic ulcer. Since epinephrine injection is effective (initial hemostasis obtained with epinephrine injection range from 85% to 100%), safe, inexpensive and technically easy, the concept of a beneficial effect of large volumes of epinephrine in preventing recurrent ulcer bleeding seems to be very challenging.

Research frontiers

To compare the rates of recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution (15-25 mL *vs* 30-40 mL) with endoscopic placement of hemoclips in patients with acute peptic ulcer bleeding and endoscopically proven gastric or duodenal ulcers with visible vessel in an ulcer bed (Forrest Ⅱa).

Innovations and breakthroughs

This is the first prospective randomized study comparing the rates of the recurrent bleeding after endoscopic injection of two different volumes of an epinephrine solution and mechanical endoscopic method in patients with UGIB and endoscopically proven peptic ulcer with nonbleeding visible vessel in an ulcer bed (Forrest Ⅱa). Unlike many studies, this trial was carried out on adequate patient's sample with clearly predefined groups of patients, in a unique center. A detailed description is provided to allow other investigators to reproduce or validate.

Applications

The results provide sufficient experimental evidence to draw firm scientific conclusions. The results have clearly shown that endoscopic therapy with hemoclip represents safe and effective method, superior to both, small-volume (15 to 25 mL) and large-volume (30 to 40 mL) injection of diluted epinephrine in the prevention of early recurrent bleeding from peptic ulcer. Reduction in recurrent hemorrhage rates observed among our peptic ulcer patients treated with hemoclip method positively affected length of hospital stay, reflecting the possibility of significant cost savings.

Peer review

This is the paper in which authors compere two most commonly used hemostatic methods in patients with bleeding peptic ulcer. The sample size is adequate, in a unique center. A detailed description is provided to allow other investigators to reproduce or validate. The statistical methods used are appropriate. The results provide sufficient experimental evidence or data to draw firm scientific conclusions. The discussion is well organized and provide systematic theoretical analyses and valuable conclusions.

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BRIEF ARTICLE

Beneficial effects of fucoidan in patients with chronic hepatitis C virus infection

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Abstract

AIM: To evaluate the effects of fucoidan, a complex sulfated polysaccharide extract from marine seaweed, on hepatitis C virus (HCV) RNA load both *in vitro* and *in vivo*.

METHODS: HCV-1b replicon-expressing cells were cultured in the presence of fucoidan obtained from *Cladosiphon okamuranus Tokida* cultivated in Okinawa, Japan, and quantified the level of HCV replication. In an open-label uncontrolled study, 15 patients with chronic hepatitis C, and HCV-related cirrhosis and hepatocellular carcinoma were treated with fucoidan (0.83 g/d) for 12 mo. The clinical symptoms, biochemical tests, and HCV RNA levels were assessed before, during, and after treatment.

RESULTS: Fucoidan dose-dependently inhibited the expression of HCV replicon. At 8-10 mo of treatment with fucoidan, HCV RNA levels were significantly lower relative to the baseline. The same treatment also tended to lower serum alanine aminotransferase levels, and the latter correlated with HCV RNA levels. However, the improved laboratory tests did not translate into significant clinical improvement. Fucoidan had no serious adverse effects.

CONCLUSION: Our findings suggest that fucoidan is safe and useful in the treatment of patients with HCV-related chronic liver diseases. Further controlled clinical trials are needed to confirm the present findings.

INTRODUCTION

Hepatitis C virus (HCV) infection often advances to chronic hepatitis due to the low viral clearance rate, leading to liver cirrhosis (LC) and subsequent development of hepatocellular carcinoma (HCC)[1,2]. The estimated global number of people infected with HCV is 170 million and more than 3.5 million new sufferers are diagnosed annually[3]. Currently, there is no vaccine available

for prevention of HCV infection due to the extreme sequence variability within the HCV genome. The first-line treatment for chronic hepatitis C (CHC) includes the combination of pegylated α-interferon (IFN) and ribavirin, a broad spectrum antiviral drug[4,5]. Although the reported HCV eradication rate by this combination therapy is 75%-90% for genotypes Ⅱ and Ⅲ and 45%-52% for genotypes Ⅰ and Ⅳ[6], these rates are still far from ideal. Because of the high rate of nonresponders among those infected with genotype Ⅰ, the predominant strain in Japan, and because antiviral treatment causes frequent, unpleasant and sometimes serious adverse effects[7], the establishment of a new treatment modality without serious adverse effects is desirable[8].

Considering the prolonged period (20-30 years) required for development of LC and HCC in individuals infected with HCV, progression of the disease might be influenced by nutritional status and diet. Although herbal supplements, including silymarin (an extract of milk thistle), are frequently used by patients with chronic liver diseases[9,10], the available scientific evidence for the beneficial effects of these supplements is limited[11]. However, administration of EH0202, a mixture of four herbal extracts, is reported to induce IFN activity and reduce HCV RNA levels in patients with high viral titers[12]. Furthermore, a recent study reported the hepatoprotective effect of birch bark extract in patients with CHC[13].

Fucoidan is a sulfated polysaccharide extracted from marine brown seaweeds that possess some biological activities including anti-inflammatory properties[14,15]. Sulfated polysaccharides, including fucoidan, are also reported to inhibit the replication of viruses such as herpes simplex virus, Sindbis virus, human immunodeficiency virus, parainfluenza virus type Ⅱ, and dengue virus[16-18]. We have also reported recently that oral administration of fucoidan for 12 mo resulted in 42.4% decrease in the human T-cell leukemia virus type Ⅰ proviral load in patients with human T-cell leukemia virus type Ⅰ -associated neurological disease[19]. Since fucoidan shows no toxicity or irritation in humans, it may be useful also as an anti-HCV agent.

To our knowledge, there are no data on the anti-HCV effect of fucoidan. In the present study, we examined the anti-HCV activity of fucoidan extracted from the marine alga, *Cladosiphon okamuranus Tokida* (*C. okamuranus Tokida*) cultivated in Okinawa, Japan. Our pilot study is the first clinical trial that investigated the effect of fucoidan in patients with HCV-related chronic liver diseases.

MATERIALS AND METHODS

Preparation of fucoidan from seaweed
The unsalted brown seaweed *C. okamuranus Tokida* cultivated in Okinawa, Japan, was suspended in water, 0.57% (w/v) citric acid was added to the solution, and then heated at 90 ℃ for 40 min. The suspension was neutralized with NaOH and cooled to 40 ℃. It was centrifuged at 3500 *g* by decantation centrifugal separator. The su-

pernatant was collected, filtered using Cohlo filter, and concentrated by ultrafiltration (molecular weight cutoff 6000). The extracts were dried by spraydrier. They were composed of carbohydrates (72%), uronic acids (24%), and sulfate (8%). Total carbohydrates were determined by the phenol-H2SO4 method using fucose as the standard. Uronic acids were determined by the carbazole-H2SO4 method using D-glucuronic acid as the standard. The sulfate contents were measured by ion chromatography. The main carbohydrates were fucose. Fucoidan content determined by high-performance liquid chromatography was 83% and the molecular weight was 21-kDa. Fucoidan was dissolved in phosphate-buffered saline at a concentration of 30 mg/mL.

Inhibition assay of HCV replicon cells by fucoidan
Fucoidan was added to Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum of HCV subgenomic replicon cells FLR3-1 (genotype Ⅰ b, Con-1;)[20] at a final concentration of 62.5, 125, 250, 500, 1000, 2000, and 3000 μg/mL. FLR3-1 cells were established from human hepatoma HuH-7 cells[21] by stable transfection with subgenomic selectable RNA in which the encoding HCV structural proteins were replaced by the firefly luciferase gene, the internal ribosome entry site of the *Encephalomyocarditis* virus, and the neomycin phosphotransferase gene[22]. After 72 h-incubation, the cells were washed in phosphate buffered saline and lysed in reporter lysis buffer (Promega, Madison, WI). Lysates were assayed for luciferase activity with the luciferase assay system (Promega) using the instructions provided by the manufacturer. With this HCV subgenome, the efficiency of subgenomic HCV expression could be estimated by measuring luciferase activity in the replicon cells.

Measurement of cell viability
Cell viability was measured using the cell proliferation reagent, WST-8 (Wako Pure Chemicals, Osaka, Japan). This method relies on mitochondrial dehydrogenase cleavage of WST-8 to formazan dye to estimate the level of cell viability. Briefly, FLR3-1 cells were incubated in a 96-well microculture plate. After 24 h incubation, fucoidan was added to the cells at various concentrations. After 72 h culture, WST-8 (5 μL) was added for the last 4 h of incubation and absorbance at 450 nm was measured using an automated microplate reader. WST-8 solution was added to the media-only wells to correct for background.

Patients
Table 1 lists the characteristics of the patients. The subjects included in the study were 15 patients with chronic liver diseases (7 men and 8 women; age, 66.1 ± 11.1 years; mean ± SD, range, 42-86), who visited the Nakasonekazu Medical Clinic. This study was carried out as an open-label study. All patients were infected with HCV genotype Ⅰ b, with a serum viral load in excess of 10^5 copies/mL. Nine patients had been diagnosed with CHC, 4 with HCV-related LC, and 2 with HCV-related cirrhosis and HCC.

Table 1 Characteristics of patients

Patient No.	Age (yr)	Gender	Diagnosis	Previous IFN therapy	Other medications
1	73	F	LC	Not eligible	Glycyrrhizin
2	78	F	LC	Not eligible	Glycyrrhizin
3	49	M	LC	Not eligible	Glycyrrhizin
4	72	M	LC + HCC	Not eligible	Glycyrrhizin
5	66	M	LC + HCC	Not eligible	Glycyrrhizin
6	70	M	LC	Not eligible	None
7	70	F	CHC	Not eligible	Glycyrrhizin
8	86	F	CHC	Not eligible	Glycyrrhizin
9	55	M	CHC	Intolerant	None
10	69	M	CHC	Non-responder	Glycyrrhizin
11	71	M	CHC	Non-responder	Glycyrrhizin
12	68	F	CHC	Non-responder	Glycyrrhizin
13	61	F	CHC	Non-responder	Glycyrrhizin
14	62	F	CHC	Non-responder	None
15	42	F	CHC	Non-responder	None

M: Male; F: Female; LC: Liver cirrhosis; CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; IFN: Interferon.

Eight patients were not eligible for IFN treatment because of LC, complication (depression), or advanced age. Seven patients had received IFN therapy in the past. Six patients were non-responders to IFN and 1 discontinued therapy because of side effect (depression). All patients assessed the tolerability as excellent.

During fucoidan treatment, 11 patients received a glycyrrhizin preparation. Fucoidan (provided by Kanehide Bio Co., Okinawa, Japan) was given orally as capsules containing 166 mg of dry extract from *C. okumuranus Tokida* per capsule in a dose of five capsules daily for 12 mo. Informed consent was obtained from all patients enrolled in the study, after a thorough explanation of the aims, risks, and benefits of this therapy.

Test parameters

The outcome parameters included the course of alanine aminotransferase (ALT), aspartate aminotransferase (AST), quantitative HCV RNA levels, subjective symptoms associated with CHC, LC, and HCC (such as fatigue, abdominal discomfort, depression, and dyspepsia), safety, and compliance. Data on all clinical parameters were documented at each visit. HCV RNA levels were determined using the AMPLICOR GT HCV Monitor test (Roche Diagnostics, Basel, Switzerland), which has a lower limit of quantitation of 0.5 kIU/mL at a linear range up to 850 kIU/mL.

Statistical analysis

Data are expressed as mean ± SD. The results of biochemical tests and HCV RNA levels were compared by the Student's *t* test. A *P* < 0.05 was considered significant.

RESULTS

Fucoidan suppresses HCV replication

To assess the effects of fucoidan on intracellular replica-

Figure 1 Anti-hepatitis C virus effects of fucoidan in hepatitis C virus replicon cells. Luciferase (LUC) activity (a marker of replication level) and cell viability of FLR3-1 cells, which constitutively express hepatitis C virus replicon, were measured in the presence of various concentrations of fucoidan. LUC and WST-8 assays were performed in triplicate. Data are mean ± SD.

tion of the HCV genome, HCV subgenomic replicon cells FLR3-1 were cultured in the presence of various concentrations of fucoidan in the medium. The luciferase activities of the FLR3-1 cells showed a concentration-dependent suppression of replication of HCV replicon by fucoidan. The WST-8 assay showed that fucoidan had negligible effect on cell viability (Figure 1). These results suggest that fucoidan inhibits HCV replication but does not have cytotoxic effects.

Effect of fucoidan therapy on HCV RNA and alanine aminotransferase levels

Changes in HCV RNA and serum ALT levels in patients treated with fucoidan are shown in Figure 2. The mean HCV RNA for the 15 patients was 736 ± 118 kIU/mL (range, 100-850 kIU/mL) before fucoidan therapy. As shown in Figure 2A, fucoidan tended to reduce the mean HCV RNA level with time relative to the baseline, with significant falls registered at 8-10 mo of treatment. However, HCV RNA increased after 11 and 12 mo. Biochemical tests showed that the mean serum ALT level, but not AST, correlated with the mean HCV RNA level, although the decrease in ALT level was not significant relative to the baseline (Figure 2B). Whereas the above changes were not associated with improvement in clinical symptoms in every patient, none of the patients showed progression of LC or adverse events.

DISCUSSION

It is estimated that 170 million people worldwide are infected with HCV[3], and some 2 million (1%) of these reside in Japan[23]. Of the HCC cases in Japan, around 80% are caused by HCV infection. The increase in the number of HCC patients contributes to the increase in total deaths in Japan from HCC. This trend is expected to continue until 2015[23]. The general strategies followed in the treatment of CHC include eradication of HCV and suppression of hepatitis.

Figure 2 Effects of treatment with fucoidan on hepatitis C virus RNA and alanine aminotransferase levels in patients with liver diseases. A: Hepatitis C virus (HCV) RNA levels; B: Serum alanine aminotransferase (ALT) levels. Values are mean ± SD. [b]$P < 0.01$ *vs* pretreatment value.

Sulfated polysaccharides including fucoidan are reported to inhibit the growth of various enveloped viruses[16-18]. Fucoidan is thought to inhibit virus adsorption to the cell surface by binding to the cell surface, with subsequent prevention of cell infection[18]. In addition, fucoidan interacts directly with the envelope glycoprotein on dengue virus type II[17]. In the present study, we demonstrated a novel mechanism of action for fucoidan. Using the HCV replicon system, we demonstrated here that fucoidan inhibits intracellular replication of the HCV genome *in vitro*.

To our knowledge, this is the first clinical study to investigate the effects of fucoidan in patients with liver diseases. The rationale for this study was stimulated by experimental data showing the efficiency of fucoidan in cell cultures. Patients with chronic HCV infection, who were not eligible for, did not respond to, or were intolerant of IFN treatment, were treated for 12 mo with fucoidan at 830 mg/d to investigate the effect of this treatment on HCV RNA level. In Case 6 (baseline HCV RNA 380 kIU/mL), fucoidan treatment successfully eradicated HCV at 9 mo, although HCV RNA was 5 kIU/mL at 10 mo. Thus, fucoidan was effective in lowering HCV RNA level in this study, although its effect was temporary. There was a significant decrease in HCV RNA at month 8, 9 and 10 of fucoidan commencement ($P < 0.01$). However, the level increased later at month 12 to become equivalent to the baseline.

We also measured serum IFNα levels to determine the indirect effect of fucoidan on IFNs, especially whether it increases the antiviral activity of IFNs. However, IFNα could not be detected in the serum of patients treated with fucoidan. Furthermore, fucoidan did not enhance IFNs expression in FLR3-1 replicon cells (data not shown). It has been reported that the protective effect of fucoidan is based on direct inhibition of viral replication and stimulation of both innate and adaptive immune defense functions[24]. We are currently investigating the effect of fucoidan on the host immune system including natural killer cell cytotoxic activity.

Our study has certain limitations. First, the study comprised only a small number of patients, including 6 patients who were known non-responders to IFN therapy. Second, all patients harbored HCV virus genotype I b and 6 had cirrhosis. Thus, at least some patients in this cohort could be classified as likely non-responders to IFN therapy[25,26]. Thus, the selection criteria employed in the present study may have favored a poor response to fucoidan.

The abnormally high levels of ALT tended to decrease temporarily during fucoidan treatment, suggesting a correlation between viral load and indices of hepatic dysfunction. Thus, fucoidan may be effective in the management of HCV-related chronic liver diseases, although long-term clinical improvement was not observed in the present study. Importantly, no adverse events were observed in all patients, similar to the results reported in a previously study on fucoidan[19], suggesting that daily oral administration of fucoidan for 12 mo is safe and tolerable.

There is no doubt that patients who fail to respond to conventional treatments often seek alternative therapies. In conclusion, our study demonstrated that fucoidan from *C. okamuranus Tokida* has HCV replication suppressive effects in a replicon cell system. Furthermore, our relatively small uncontrolled pilot study showed that fucoidan has temporary but beneficial effects on HCV RNA levels in HCV infected patients. The preliminary findings suggest that fucoidan may be a useful health-food additive with antiviral activity to be used in the treatment of chronic liver diseases. To suppress the viral titer as much and for as long as possible, we need to define the daily effective dosage. Further studies on the mechanism of fucoidan-induced HCV inhibition may provide alternative strategies for the design of novel anti-HCV drugs.

COMMENTS

Background

Hepatitis C virus (HCV) is a major cause of chronic liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The standard care

for chronic hepatitis C involves the administration of pegylated α-interferon in combination with the nucleotide analog ribavirin. However, this regimen has limited success rate for genotype Ⅰ and Ⅳ, and unfavorable side effects. Thus, it is important to discover more effective and safer agents to improve the clinical treatment on HCV carriers. Fucoidan, a sulfated polysaccharide, has significant biological activities, such as antiviral and anti-inflammatory effects. Nevertheless, there has been no investigation on the efficacy of fucoidan against HCV infection.

Research frontiers

Natural products have been used for the treatment of various diseases as an alternative to conventional chemical agents. So far, several natural products have been screened for their antiviral effect against various viral infections. Screening of natural potent inhibitors for HCV has also become a research hotspot.

Innovations and breakthroughs

Previous studies have shown the efficacy of natural products against HCV replication in a cell-based HCV replicon system. However, there have been few clinical studies that evaluated the safety and efficacy of these natural products. In the present study, the authors investigated the anti-HCV activity of fucoidan obtained from the *Cladosiphon okamuranus Tokida* cultivated in Okinawa, Japan, both *in vitro* and *in vivo*. This pilot study is the first clinical trial that investigated the effect of fucoidan in patients with HCV-related chronic liver diseases.

Applications

Fucoidan inhibited HCV RNA replication in the HCV replicon assay system. The experimental data on fucoidan efficiency in cell culture stimulated the rationale for clinical study. Oral fucoidan administration resulted in temporary reduction of viral loads of genotype Ⅰ b in patients with chronic HCV infection, who were not eligible for, did not respond to, or were intolerant of interferon therapy. Fucoidan is well tolerated and no serious adverse events were observed in any of the patients. Fucoidan exhibited antiviral properties against HCV both *in vitro* and *in vivo*, and would be expected to become a new strategy for HCV infection. Further controlled clinical trials will be required to confirm the present findings.

Terminology

Fucoidan is a complex sulfated polysaccharide found in the cell walls of several edible brown algae, including *Fucus vesiculosus*. The HCV replicon system replicates a modified HCV genome containing luciferase gene to high levels in human hepatoma cells. The efficacy of subgenomic HCV expression was estimated by measuring luciferase activity in the replicon cells. This system provides a powerful tool for studying virus replication and for screening anti-HCV drugs.

Peer review

The paper studied the effects of fucoidan, a complex sulfated polysaccharide extracted from marine seaweed, on HCV RNA load *in vitro* and *in vivo*. The research is of significance because of the high rate of nonresponders in HCV genotype Ⅰ , which is the predominant strain in Japan. Moreover, antiviral treatment causes frequent, unpleasant and sometimes serious adverse effects, Thus the search for a new treatment modality without serious adverse effects is desirable.

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BRIEF ARTICLE

Plasma levels of acylated ghrelin in patients with functional dyspepsia

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Abstract

AIM: To investigate the relationship between plasma acylated ghrelin levels and the pathophysiology of functional dyspepsia.

METHODS: Twenty-two female patients with functional dyspepsia and twelve healthy volunteers were recruited for the study. The functional dyspepsia patients were each diagnosed based on the Rome Ⅲ criteria. Eligible patients completed a questionnaire concerning the severity of 10 symptoms. Plasma acylated ghrelin levels before and after a meal were determined in the study participants using a commercial human acylated enzyme immunoassay kit; electrogastrograms were performed for 50 min before and after a standardized 10-min meal containing 265 kcal.

RESULTS: There were no significant differences in plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia. However, in patients with functional dyspepsia, there was a negative correlation between fasting plasma acylated ghrelin levels and the sum score of epigastric pain (r = -0.427, P = 0.047) and a positive correlation between the postprandial/fasting plasma acylated ghrelin ratio and the sum score of early satiety (r = 0.428, P =0.047). Additionally, there was a negative correlation between fasting acylated ghrelin plasma levels and fasting normogastria (%) (r = -0.522, P = 0.013). Interestingly, two functional dyspepsia patients showed paradoxically elevated plasma acylated ghrelin levels after the meal.

CONCLUSION: Abnormal plasma acylated ghrelin levels before or after a meal may be related to several of the dyspeptic symptoms seen in patients with functional dyspepsia.

Kim YS, Lee JS, Lee TH, Cho JY, Kim JO, Kim WJ, Kim HG, Jeon SR, Jeong HS. Plasma levels of acylated ghrelin in patients with functional dyspepsia. *World J Gastroenterol* 2012; 18(18): 2231-2237 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i18/2231.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i18.2231

INTRODUCTION

Functional dyspepsia is defined as the presence of dyspeptic symptoms thought to originate in the gastroduodenal region that occur in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms[1,2]. To date, functional dyspepsia has been associated with various physiological abnormalities, including delayed gastric emptying[3], altered antro-duodenal motility[4], impaired gastric accommodation[5], visceral hypersensitivity[6], gastric dysrhythmia[7-10]; functional dyspepsia has also been associated with multiple psychiatric and personal factors, such as somatization[11], depression, anxiety[12], and changes in coping skills[13]. However, a number of studies have failed to find associations between dyspeptic symptoms and the putative pathophysiology of functional dyspepsia in patients. The underlying etiology of functional dyspepsia remains unclear.

Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor, and it has potent growth hormone-releasing activity. Ghrelin is predominately produced by endocrine cells in the oxyntic mucosa of the stomach[14,15]. Ghrelin has two subtypes: a deacylated form and an acylated form[16]. The physiologic functions of ghrelin are pleiotropic as follows. First, ghrelin stimulates food intake. An appetite stimulatory effect is associated with both central regulation and peripheral signals[17,18]. Second, ghrelin regulates gastric acid secretion. Ghrelin acts in the central nervous system to stimulate gastric acid secretion[19]. Third, ghrelin induces the migrating motor complex and promotes gastric emptying. Lastly, ghrelin has also been reported to have a gastroprotective effect in the context of the generation of nitric oxide and prostaglandins[20,21].

Given these diverse functions, ghrelin has been hypothesized to play a role in the pathophysiology of functional dyspepsia. The aim of this study was to investigate the role of acylated ghrelin in the pathophysiology of functional dyspepsia. This study measured plasma acylated ghrelin levels of female subjects, before and after a meal; these data were then compared between females with functional dyspepsia and healthy female volunteers. Also, in patients with functional dyspepsia, we determined the correlation between plasma acylated ghrelin levels, symptom scores, and electrogastrogram (EGG) parameters. In addition, patients with functional dyspepsia were divided into two subgroups according to the Rome Ⅲ criteria: patients with postprandial distress syndrome (PDS) and patients with epigastric pain syndrome (EPS). Differences in plasma acylated ghrelin levels between these two subgroups were also evaluated.

MATERIALS AND METHODS

Study subjects

Female subjects between 18 and 60 years of age were recruited from Sep. 2006 to Jan. 2007 at Soonchunhyang University Hospital, Seoul, South Korea. We recruited healthy volunteers by advertisement. Consecutive patients who were diagnosed with functional dyspepsia were invited to participate in the study. To diagnose functional dyspepsia, patients with dyspeptic symptoms thought to originate in the gastroduodenal region were asked to answer a questionnaire based on the Rome Ⅲ functional dyspepsia criteria after the exclusion of organic disease using endoscopic examination. In addition, eligible patients were asked to complete a questionnaire regarding the severity of 10 symptoms; severity was determined using a self-devised scale of absent (0), mild (1), relevant (2), moderate (3), and severe (4). The 10 symptoms were epigastric pain, epigastric burning, upper abdominal discomfort, nausea, upper abdominal fullness, gastric retention, upper abdominal distention, early satiation, vomiting, and belching. According to the predominant symptom, patients were classified as either PDS or EPS. Patients were examined for *Helicobacter pylori (H. pylori)* infection with a rapid urease test (Pronto Dry, Gastrex Corp., Warsaw, Poland) or the ^{13}C-urea breath test (UBiT-IR 300, Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan). The study protocol was approved by the Soonchunhyang University Hospital Institutional Review Board. Written informed consent was obtained from all participants at the time of enrollment.

Study design

This prospective case-control study was designed to elucidate the role of plasma acylated ghrelin levels in the pathophysiology of functional dyspepsia. The primary endpoint was to investigate the difference in the plasma levels of acylated ghrelin between the two investigated groups. The second endpoint was to evaluate correlations between fasting and postprandial plasma acylated ghrelin levels, symptom severity, the EGG parameters in patients with functional dyspepsia. The tertiary endpoint was to examine whether there were any differences in plasma acylated ghrelin levels between patients with PDS and EPS.

Inclusion and exclusion criteria

To be included in the study, patients had to meet the following inclusion criteria: (1) one or more bothersome dyspeptic symptoms, such as postprandial fullness, early satiation, epigastric pain, and epigastric burning, for the last 3 mo, with symptom onset at least 6 mo prior to diagnosis; (2) the ability to cease all medical treatment that could influence the gastrointestinal motility at least 1 wk prior to the test; and (3) informed written consent.

The following exclusion criteria were utilized: (1) subjects who suffered from structural diseases, such as esophagitis, erosive gastroduodenal lesions or ulcers that could explain symptoms; (2) subjects who suffered from systemic diseases, such as diabetes mellitus or thyroid disease; (3) subjects with a history of peptic ulcers or major abdominal surgery; and (4) subjects who were obese, as defined by a body mass index of over 30.

Plasma acylated ghrelin measurement

Fasting blood samples were taken and analyzed for plasma levels of acylated ghrelin, glucose, insulin and growth

Table 1 Baseline subject characteristics

	Healthy volunteers (*n* = 12)	Patients with functional dyspepsia (*n* = 22)	*P* value
Age (yr)	24 (23-34)	46 (23-59)	0.000[1]
Height (cm)	162 (154-167)	160 (150-175)	0.435[1]
Weight (kg)	52.8 (44-58)	55 (45-70)	0.170[1]
Body mass index (kg/m^2)	20.2 (18.1-22.9)	21.2 (17.6-27.3)	0.135[1]
Growth hormone (ng/mL)	0.8 (0.1-3.3)	0.4 (0.01-14.7)	0.339[1]
Insulin (μIU/mL)	8.3 (2.3-14.6)	8.3 (4.0-14.4)	0.639[1]
Fasting blood sugar (mg/dL)	82 (72-97)	88.5 (70-107)	0.026[1]
Helicobacter pylori infection rate (%)	66.7 (8/12)	36.4 (8/22)	0.151[2]

[1]Analysis by Mann-Whitney *U* test; [2]Analysis by Fisher's exact test.

hormone. Postprandial blood samples were obtained fifty minutes after a 10-min meal and analyzed for postprandial plasma levels of acylated ghrelin. For each sample, whole blood was directly drawn into a centrifuge tube that contained 500 U of aprotinin and 1.25 mg of EDTA - 2Na per 1 mL of whole blood. Blood samples in the tubes were immediately centrifuged at 1500 × *g* for 15 min at 4 ℃. Plasma samples were stored at -80 ℃ for later use after immediately adding 100 μL of 1 mol/L HCl per 1 mL of collected plasma. The commercially available human acylated ghrelin enzyme immunoassay kit (Cayman Chemical Co., Michigan, United States) was used to measure the acylated ghrelin levels.

Electrogastrogram

Study subjects visited the office in the morning, after an overnight fast. Three EGG electrodes were connected to the subject's abdomen according to standard method[9,10]. The EGG electrodes were then attached to the Digitrapper EGG recorder (Medtronic Co. WA, United States). A fasting EGG signal was obtained for fifty minutes. After a 10-min break for a standardized meal, the postprandial EGG was also recorded for fifty minutes. Subjects were given a standardized soft diet that contained a total of 265 kcal, composed of 72% carbohydrate, 16% protein, and 12% fat. Nine parameters were measured, namely, the proportions of bradygastria (%), normogastria (%), and tachygastria (%) during the fasting and postprandial periods, the instability coefficient for both periods and the power ratio. The instability coefficient represents the stability of the slow wave and the power ratio represents the fasting electrical power of the slow wave divided by the postprandial electrical power.

Statistics analysis

SPSS software version 17.0 was used for statistical analyses. The Mann-Whitney *U* test was utilized to compare plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia. Fisher's exact test was used to compare the prevalence of *H. pylori* infection between the two groups. In patients with functional dyspepsia, correlation analysis by the Spearman's rho correlation coefficient was performed to assess the correlations between plasma acylated ghrelin levels, symptom scores and EGG parameters.

RESULTS

Subject characteristics

In total, thirty-four female subjects were enrolled. Twelve women were healthy volunteers and twenty-two were patients suffering from functional dyspepsia. Patient baseline characteristics are summarized in Table 1.

The healthy volunteers were significantly younger compared to the patients with functional dyspepsia (*P* = 0.00). The median fasting blood glucose level was significantly higher in patients with functional dyspepsia compared to healthy volunteers (*P* = 0.03), because two functional dyspepsia patients exhibited glucose intolerance. However, no significant differences were observed between the two groups for median growth hormone and insulin levels and body mass index values.

For the assessment of *H. pylori* infection status, thirty participants were tested by upper endoscopy with the rapid urease test, while the remaining four subjects underwent urea breath tests because they had undergone an upper endoscopy within 6 mo preceding study enrollment. *H. pylori* infection rates were 66.7% (8/12) in healthy volunteers, and 36.4% (8/22) in patients with functional dyspepsia. However, the prevalence of *H. pylori* between the two groups was not statistically significant.

The difference in plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia

In healthy volunteers, the median level of plasma acylated ghrelin was 52.2 pg/mL (1.6-228) during fasting and 32.1 pg/mL (1.1-104.2) postprandially. In patients with functional dyspepsia, the median levels of plasma acylated ghrelin during fasting and postprandially were 56.4 pg/mL (25.6-139.1) and 34.2 pg/mL (12.4-141.2), respectively. Interestingly, two patients with functional dyspepsia exhibited a paradoxical increase in postprandial plasma acylated ghrelin levels (Figure 1).

The association between plasma acylated ghelin levels, symptom scores, and electrogastrogram parameters in twenty patients with functional dyspepsia

Correlations between plasma acylated ghrelin levels and the symptom scores of the 10-investigated symptoms: There was negative correlation between fasting plasma levels of acylated ghrelin and total epigastric pain

A Healthy volunteers (*n* = 12)

B Functional dyspepsia (*n* = 22)

Figure 1 Plasma acylated ghrelin levels before and after a meal in healthy volunteers (A) and in patients with functional dyspepsia (B). Bars represent the median values. Note that two patients with functional dyspepsia (*) showed a paradoxical increase in postprandial plasma acylated ghrelin levels.

scores (r = -0.427, P = 0.047) (Figure 2A). In contrast, we found a positive correlation between the postprandial/fasting acylated ghrelin ratio and the total early satiety scores (r = 0.428, P = 0.047) (Figure 2B).

Correlations between plasma acylated ghrelin levels and the nine electrogastrogram parameters: There was a negative correlation between fasting plasma levels of acylated ghrelin and fasting normogastria (%) (r = -0.522, P = 0.013) (Figure 3).

The difference in plasma acylated ghrelin levels between postprandial distress syndrome and epigastric pain syndrome patients

In the thirteen PDS patients, the median plasma level of acylated ghrelin was 58.31 pg/mL (30.53-139.12) during fasting and 35.66 pg/mL (19.61-112.25) postprandially. In the 9 EPS patients, the median plasma level of acylated ghrelin was 49.79 pg/mL (25.56-95.14) during fasting and 32.78 pg/mL (12.35-141.16) postprandially. The

Figure 2 Relationship between plasma acylated ghrelin levels and dyspeptic symptoms in patients with functional dyspepsia. A: There was a negative correlation between fasting acylated ghrelin levels and epigastric pain scores (r = -0.427, P = 0.047); B: There was a positive correlation between the postprandial/fasting acylated ghrelin ratio and early satiety scores (r = 0.428, P = 0.047).

Figure 3 Relationship between fasting plasma acylated ghrelin levels and electrogastrographic parameters. There was a negative correlation between fasting plasma acylated ghrelin levels and fasting normogastria (%) (r = -0.522, P = 0.013).

differences in plasma acylated ghrelin levels between PDS and EPS patients were not statistically significant (Table 2).

Table 2 Postprandial distress syndrome and epigastric pain syndrome patient characteristics

	Postprandial distress syndrome (*n* = 13)	Epigastric pain syndrome (*n* = 9)	*P* value
Age (yr)	46 (23-59)	44 (37-58)	0.713[1]
Height (cm)	160 (154-175)	161 (150-168)	0.946[1]
Weight (kg)	54 (45 -70)	57 (47-62)	0.841[1]
Body mass index (kg/m^2)	20.7 (17.63-27.34)	22.27 (18.44-26.22)	0.640[1]
Growth hormone (ng/mL)	0.41 (0.03 -9.16)	0.46 (0.01-14.72)	0.404[1]
Insulin (μIU/mL)	8.99 (4.12-14.38)	7.88 (4.00-13.80)	0.664[1]
Fasting blood sugar (mg/dL)	92 (81-107)	86 (70-95)	0.114[1]
Helicobacter pylori infection rate (%)	53.8 (7/13)	11.1 (1/9)	0.074[2]
Fasting plasma levels of acylated ghrelin (pg/mL)	58.31 (30.53-139.12)	49.79 (25.56-95.14)	0.443[1]
Postprandial plasma levels of acylated ghrelin (pg/mL)	35.66 (19.61-112.25)	32.78 (12.35-141.16)	0.616[1]

[1]Analysis by Mann-Whitney *U* test; [2]Analysis by Fisher's exact test.

DISCUSSION

Ghrelin has two subtypes: deacylated ghrelin, which lacks an acyl group at third serine residue, and acylated ghrelin, which has the acyl modification necessary for noctanoic acid hormonal activity[16]. Although deacylated ghrelin circulates in far greater amounts compared to the acylated form and is involved in cell proliferation and adipogenesis, only acylated ghrelin exhibits physiologic activity and can stimulate growth hormone release and food intake[22-25]. Therefore, we focused our investigation on acylated ghrelin.

In a study by Takamori *et al*[26] that focused on two age-matched groups, the authors found that fasting levels of deacylated ghrelin were significantly lower in patients with functional dyspepsia compared to controls; however, they reported that both fasting and postprandial levels of acylated ghrelin and postprandial levels of deacylated ghrelin were similar in the two groups. Consistent with these findings, our study did not show any differences in either fasting or postprandial plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia. These results suggest that plasma acylated ghrelin levels may not be directly associated with the pathophysiology of functional dyspepsia.

Plasma acylated ghrelin levels differ significantly in males and females. A study by Akamizu *et al*[16] showed that fasting levels of acylated, but not deacylated ghrelin, in female subjects were higher compared to males after adjustment for body mass index. For this reason, we limited our study to female participants.

H. pylori infection was more prevalent in the healthy volunteers compared to the patients with functional dyspepsia in our study; however, the difference in prevalence was not statistically significant. Although the prevalence of *H. pylori* infection in Korea has been decreasing from 66.9% in 1998 to 59.6% in 2005[27,28], it is still high even in asymptomatic adult subjects. This could be the reason why *H. pylori* was present more in the healthy volunteers in our study.

In our study, fasting plasma acylated ghrelin levels ranged from 1.61 to 227.98 pg/mL and postprandial plasma acylated ghrelin levels ranged from 1.09 to 141.16 pg/mL. Interestingly, plasma acylated ghrelin levels vary more than 100-fold between the extremes.

Most studies have reported the plasma levels of total ghrelin between 300 and 800 pg/mL[19]. Low total plasma ghrelin levels during fasting are associated with insulin resistance, hypertension, and type 2 diabetes[29]. Plasma ghrelin levels rise before the meal and sharply decline as soon as eating commences[30]. The surge of ghrelin levels before the meal is similar to the increase of gastric acid that occurs in the cephalic phase. Plasma ghrelin levels do not begin to recover until thirty minutes after a meal. This delayed recovery suggests that the mechanism for immediate intragastric inhibition of ghrelin release is not present in the stomach, but is instead associated with feedback inhibition, either *via* the release of an intestinal hormone or by insulin release in conjunction with food intake[31]. Interestingly, in our study, postprandial plasma acylated ghrelin levels were paradoxically higher compared to fasting levels in two individuals. Test repetition confirmed these results. One individual had a fasting ghrelin level of 95.1 pg/mL with a postprandial ghrelin level of 141.2 pg/mL. In the other individual, the fasting and postprandial plasma ghrelin levels were 41.1 pg/mL and 46.4 pg/mL, respectively. Both individuals listed epigastric pain and burning sensations as their main symptoms. These results suggest that an abnormal acylated ghrelin response after a meal may be one of the mechanisms involved in the pathophysiology of functional dyspepsia. Further study including more subjects will be needed to confirm this hypothesis.

A study by Shinomiya *et al*[32] revealed that the fasting levels of plasma acylated ghrelin correlated with subjective symptoms of functional dyspepsia in female patients. Similarly, we observed that plasma acylated ghrelin levels were associated with symptom scores and several EGG parameters. First, there was a negative correlation between fasting plasma acylated ghrelin levels and total epigastric pain scores (*r* = -0.427, *P* = 0.047). Therefore, patients with higher plasma levels of acylated ghrelin before the meal suffered from less epigastric pain. Taking into consideration a previous report showed that basal gastric acid secretion was normal in patients with functional dyspepsia[33],the relationship between higher fasting

plasma levels of acylated ghrelin and decreased epigastric pain appear to result from a gastroprotective effect exerted by ghrelin on the gastric mucosa[19-21]. Second, we found a positive correlation between the ratio of acylated ghrelin level (i.e., the postprandial plasma acylated ghrelin level divided by the fasting plasma acylated ghrelin level) and the total early satiety scores (r = 0.428). Thus, blunted ghrelin decreases after the meal were associated with higher early satiety scores. Our results suggest that abnormal responses following a meal might play a role in the impairment of gastric accommodation. Third, we report a negative correlation between fasting plasma acylated ghrelin levels and fasting normogastria (3 cpm) (%). Accordingly, in patients with functional dyspepsia, higher fasting plasma acylated ghrelin levels were associated with fasting gastric dysrhythmia. Gastric dysrhythmia is reported in 40%-50% of patients with dysmotility-like dyspepsia[7,8]; also,abnormal myoelectric activity of the stomach is associated with dyspeptic symptoms, especially nausea and vomiting[9,10]. Further study will be required to evaluate the relationship between fasting plasma acylated ghrelin levels and fasting gastric dysrhythmia.

A study by Shindo *et al*[34] showed that fasting plasma levels of acylated ghrelin in PDS patients were significantly lower compared to healthy volunteers; these levels also tended to be lower compared to EPS patients. However, in our study, no significant differences were observed in either the fasting or postprandial plasma levels of acylated ghrelin between PDS and EPS patients. Interestingly, the two patients who exhibited paradoxical increases in postprandial plasma acylated ghrelin levels had EPS.

The limitations of our study should be noted. These limitations include the small sample, which permitted only the use of non-parametric tests in the statistical analysis. Also, the study group and the control group were not age-matched. Although plasma ghrelin levels are not known to change with age, age may be important factor for this kind of functional study. Additionally, the high level of *H. pylori* infection in our participants was not ideal. However, *H. pylori* infection has no direct relationship to the diagnostic criteria for functional dyspepsia[2]. Further, although a study by Isomoto *et al*[35] showed that the fasting levels of total ghrelin in *H. pylori*-positive patients were significantly lower compared to *H. pylori*-negative patients, the relationship of the plasma acylated ghrelin levels to *H. pylori* infection status has not been evaluated to date. Another factor that was not taken into account was the phase of the participants' menstrual cycles. This is potentially an important variable to control for because a study from De Souza *et al*[36] found that fasting ghrelin plasma concentrations were at least 85% greater in subjects with exercise-associated amenorrhea. Finally, as approximately one third of ghrelin is produced in extra-intestinal organs such as the pancreas and the hypothalamus[14,15], further studies measuring exclusively gastric ghrelin levels are needed.

In conclusion, although no significant differences in plasma acylated ghrelin levels between healthy volunteers and patients with functional dyspepsia were observed, we found that abnormal plasma acylated ghrelin levels before and after the meal may be related to several dyspeptic symptoms in patients with functional dyspepsia. We also observed a paradoxical increase in postprandial plasma ghrelin levels in two patients with EPS, suggesting that abnormal plasma acylated ghrelin levels might be involved in the pathophysiology of functional dyspepsia. Further study in a larger sample size is needed to elucidate the complicated pathophysiology of functional dyspepsia.

COMMENTS

Background
Dyspepsia occurs in approximately 25 percent of the population each year. The most common cause of dyspepsia is functional dyspepsia. However, the pathophysiology of functional dyspepsia is unclear. As ghrelin, an acylated peptide produced predominantly by the stomach, has a well-established role in increasing appetite and food intake and in stimulating gastric emptying and acid secretion, it may play a role in the pathophysiology of functional dyspepsia.

Research frontiers
Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor, which is present on pituitary cells that secrete growth hormone. However, ghrelin exerts many endocrine and extraendocrine biological activities beyond the control of growth hormone secretion. In this study, the authors demonstrate that abnormal plasma acylated ghrelin levels before and after a meal may be related to several dyspeptic symptoms in patients with functional dyspepsia.

Innovations and breakthroughs
The authors found a negative correlation between fasting plasma levels of acylated ghrelin and epigastric pain scores, and a positive correlation between the postprandial/fasting acylated ghrelin ratio and early satiety scores. Further, they also found a negative correlation between fasting plasma acylated ghrelin levels and fasting normogastria. The results regarding the relationship between plasma ghrelin levels and epigastric pain and early satiety scores are interesting.

Applications
By revealing which symptoms in patients with functional dyspepsia is associated with plasma ghrelin levels, this study may represent a future strategy for the research on the relationship between plasma ghrelin levels and the pathophysiology of functional dyspespsia.

Peer review
The authors examined whether fasting and postprandial plasma acylated ghrelin levels exhibited differences in patients with functional dyspepsia compared to healthy volunteers. In addition, they attempted to demonstrate which dyspeptic symptoms and electrogastrogram parameters in patients with functional dyspepsia correlated with fasting and postprandial levels of plasma acylated ghrelin. Overall, this paper is unique despite several weak points.

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BRIEF ARTICLE

Knowledge levels and attitudes of health care professionals toward patients with hepatitis C infection

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Abstract

AIM: To study knowledge levels and attitudes of health care providers toward patients with hepatitis C virus infection in Guilan, a northern province of Iran.

METHODS: This cross-sectional study was performed on 239 health care professionals from the Razi Hospital, including doctors, nurses, and operating room technicians. The questionnaires consisted of questions on demographic characteristics, knowledge levels, and attitudes toward hepatitis C patients. The questionnaire was tested in a pilot study and validated by Cronbach's alpha coefficient. Data were analyzed using SPSS16 software.

RESULTS: The mean ± SD knowledge score was 17.43 ± 2.65 (from a total of 22). 51.9% of the participants achieved scores higher than the mean. There was a significant relationship between knowledge score and age ($P = 0.001$), gender ($P = 0.0001$), occupational history ($P = 0.0001$), and educational history ($P = 0.027$). There was also a significant relationship between attitude level and age ($P = 0.002$), gender ($P = 0.0001$), occupational history ($P = 0.0001$), and educational history ($P = 0.035$). Physicians were significantly more knowledgeable and showed more positive attitudes. There was a positive correlation between knowledge and attitude scores ($P = 0.02$).

CONCLUSION: Discriminatory attitudes are common among health care providers toward hepatitis C patients. It is therefore necessary to improve their knowledge level and attitude toward this disease.

INTRODUCTION

Hepatitis C is a hepatotropic viral infection caused by hepatitis C virus (HCV), which is a major cause of acute hepatitis and chronic liver disease. It is estimated that 170 million people worldwide (3% of the world population) are chronically infected with HCV and are under the risk of cirrhosis and liver cancer. Chronic HCV infec-

tion is usually slowly progressive. HCV infection leads to chronic hepatitis in 50% to 80% of individuals[1-5]. The increasing global prevalence of this disease puts extra demands on health care services and increases the likelihood that health care workers (HCWs) will care for, or have personal contact with, people with hepatitis C[6]. Occupational exposure from percutaneous injuries is a substantial source of infection by blood borne pathogens among HCWs. However, studies of HCWs exposed to HCV by a needle stick injury (NSI), or any other percutaneous injury, have found that the incidence of anti HCV seroconversion is 1.8% (0%-7%) on average[7]. Physicians, laboratory technicians, nurses, and dialysis unit personnel are the main HCWs at risk. Nurses are the most at risk group because they have close contact with patients and are more likely to be exposed to a NSI[8].

NSIs are common in our country. In a study in Qazvin, 52.9% of nurses had a history of NSI[9]. Patients with hepatitis C have met with discrimination and stigmatization in the work place, by family members and by members of their communities. In addition, they may face discrimination from HCWs[10,11]. The discriminatory practices of HCWs may result from a lack of knowledge and negative attitudes toward these kinds of diseases, which could interfere with their willingness to treat these patients because of a fear of contracting hepatitis C. In a study by Richmond *et al*[6], most health care workers had sufficient knowledge about hepatitis C; however, some of them showed discriminatory attitudes toward patients with hepatitis C. Until now, little research has been conducted on HCW knowledge levels and attitudes toward people with hepatitis C in Iran. We therefore studied HCW knowledge levels and attitudes toward treating people with hepatitis C as a blood borne disease, to investigate how attitudes can be influenced by education and how this can affect their willingness to treat these patients.

MATERIALS AND METHODS

A cross-sectional study was conducted using a questionnaire that was created and standardized by Richmond *et al*[6] and translated into Persian. The questionnaire was considered by a panel of consulting experts and its validity was documented by a pilot study using a random sample ($n = 20$) drawn from the subgroups to be surveyed in the main study. The sample was calculated as 345 health care workers, based on the positive attitude proportion among the random subgroup (32%) and considering the precision of 0.06 and the type one error of 0.05. The questionnaire's validity and reliability were also confirmed by Cronbach's alpha coefficient (alpha = 0.7). We used this specifically designed questionnaire for the study because no other instrument was identified that explored HCW attitudes toward treating people with hepatitis C in the detail that was required for the current research. The questionnaire consisted of four parts and 49 questions. Seven questions obtained demographic characteristics,

22 questions were used to explore knowledge levels, 10 questions determined attitudes, and 10 questions were designed to determine compassion levels toward hepatitis C patients, willingness to treat hepatitis C patients, their self reported fear of contracting hepatitis C, and their self reported behavior toward injecting drug users. The mean of the total score from a possible score of 22, based on 22 questions, was used as the discriminant level. Scores higher than the mean indicated a good knowledge level and lower than the mean indicated a poor knowledge level[12].

Regarding attitude, participants were asked to what extent they agreed or disagreed (using a five-point Likert scale ranging from "strongly agree" to "strongly disagree") with each of the statements. For the 10 statements, total scores that could be achieved ranged from 10 to 50. Scores between 10 and 30 were considered as negative attitudes and scores higher than 30 were considered as positive attitudes[13,14]. The knowledge level, attitude questions, and self-behavior statements are shown in Table 1. Demographic data containing participants' age, sex, working history (years which health care workers had worked for health care service), occupation (doctor, nurse), needle stick injury (NSI) history, education (participation in education classes on NSI), were registered for each participant. Questionnaires were filled by direct interviews, which were performed by a trained general practitioner from the research team.

Statistical analysis
Data were entered into SPSS 16 software and analyzed by descriptive statistics (i.e., mean, SD, frequency) and comparison means (i.e., one way ANOVA, χ^2 test). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Response rate and demographic data
The mean age of the participants was 33.06 ± 7.72 years and the mean working history was 7.51 ± 6.49 years. The overall response rate was 69% (239 of 345 HCWs). There was no significant difference between the age, sex, occupation, and working history of responders and non-responders. Table 2 presents the demographic data. Overall, 52.3% of the participants reported having a history of NSI with hepatitis C patients.

Nurses (54%) were most likely to have a history of NSI. Most of the HCWs (79.1%) reported that they had received information on hepatitis C patients and NSI (although they did not refer to the type of education they had received). Nurses (85.3%) were significantly more likely to have received training on hepatitis C, while 72.2% of the physicians had received training and operating room technicians (40%) were the least likely to have received training ($P = 0.001$).

Hepatitis C knowledge level and education
Overall, HCW level of knowledge was satisfactory and

Table 1 Hepatitis C knowledge level, attitude and self-reported statement questions in the questionnaire

Hepatitis C knowledge questions (response options: true, false and uncertain)
  Hepatitis C is caused by a virus
  Hepatitis C is caused by bacteria
  Hepatitis C can be spread through close personal contact such as kissing
  Hepatitis C can be spread through sharing injecting equipment, such as needles, tourniquets, spoons, filters and swabs
  Hepatitis C can be spread by mosquitoes
  Hepatitis C is spread through blood-to-blood contact
  Having a medical and/or dental procedure performed in the Middle East, South East Asia or the Mediterranean increases a person's chances of contracting hepatitis C
  Hepatitis C is spread through the air in an enclosed environment (e.g., crowded buses and elevators)
  Sexual transmission is a common way hepatitis C is spread
  Some people with hepatitis C were infected through unsterile tattooing
  Some people with hepatitis C were infected through blood transfusions
  People with hepatitis C should be restricted from working in the food industry
  Hepatitis C can lead to cirrhosis
  Hepatitis C is associated with an increased risk of liver cancer
  Hepatitis C is a mutation of hepatitis B
  A person can be infected with hepatitis C and not have any symptoms of the disease
  There is a pharmaceutical treatment available for hepatitis C
  There is a vaccine for hepatitis C
  HIV is easier to catch than hepatitis C
  An individual can have hepatitis C antibodies without being currently infected with the virus
  People with hepatitis C should restrict their alcohol intake
  Once you have had hepatitis C, you cannot catch it again because you are immune
Attitudes and self-reported behavior statements according to theme (response options: strongly agree, agree, uncertain, disagree and strongly disagree)
  Attitudes and self-reported behavior toward the implementation of infection control guidelines
    When receiving health care, patients with hepatitis C (HCV) should be identified for safety reasons
    Patients with HCV should be given the last appointment for the day (ICG)
    Health professionals who are HCV positive should be discouraged from having contact with patients
    All patients should be tested for HCV before they receive health care
    I deliver the same standard of care to patients with HCV as I do for other patients
    I feel that I do not have the skills needed to effectively and safely treat patients with HCV
    Following infection control guidelines will protect me from being infected with HCV at work
    I often use additional infection control precautions when treating patients with HCV
    I would prefer to wear two pairs of gloves when treating a bleeding person with HCV
    The infection control guidelines necessary to treat patients with hepatitis C would be a financial burden on my practice/ward
  Attitudes and self-reported compassion toward people with hepatitis C
  I feel sorry for people who contracted HCV through a blood transfusion
  I feel sorry for people who contracted HCV through HIV drug use
Attitudes and self-reported willingness to treat people with hepatitis C
  I do not like treating people with HCV
  I am willing to treat people with HCV
  I believe my profession should have central role in the treatment of HCV

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; ICG: International crisis group.

Table 2 Health care workers' demographic data

Variable		*n* (%)
Age (yr)	≤ 30	108 (45.2)
	31-40	88 (36.8)
	≥ 41	43 (18.0)
Sex	Male	47 (19.7)
	Female	192 (80.3)
Working history (yr)	≤ 5	117 (49)
	6-10	60 (25.1)
	≥ 11	62 (25.9)
Health care group	Physicians	79 (33.1)
	Nurses	150 (62.8)
	Technicians	10 (4.2)
NSI history	Yes	125 (52.3)
	No	114 (47.7)
Education	Yes	189 (79.1)
	No	50 (20.9)

NSI: Needle stick injury.

the mean knowledge score was 17.43 ± 2.65 from a total score of 22. Scores higher than 17.43 indicated a good knowledge level and lower than 17.43 indicated a poor knowledge level. For example, 95.4% of the participants answered the questions about cirrhosis correctly, 97.5% knew that hepatitis C is contracted through blood contact, 95% knew that non-sterile tattoos are also a method of transmission, and 98.3% of HCWs knew that HCV can be spread through sharing injection equipment, such as needles, tourniquets, spoons, filters, and swabs. However, some deficits were identified in their knowledge level. For example, only 37.7% of HCWs answered correctly to the statement: Sexual contact is a common mode of transmission.

Only 54.8% knew that there are effective treatments for hepatitis C, 35.1% did not know, and 10% were not sure about it.

The mean knowledge score of males was 18.78 ±

Table 3 Health care workers' hepatitis C knowledge scores in association with different variables (mean ± SD)

Variables		n	Mean knowledge score (SD)	P value
Sex	Male	47	18.78 (2.12)	0.0001
	Female	192	17.1 (2.66)	
Age	≤ 30	108	16.75 (2.9)	0.001
	31-40	88	17.93 (2.22)	
	≥ 41	43	18.13 (2.41)	
Working history	≤ 5	117	17.05 (2.97)	0.073
	6-10	60	17.6 (2.91)	
	≥ 11	62	17.98 (2.54)	
Professional group	Physicians	79	19.26 (1.97)	0.0001
	Nurses	150	16.61 (2.51)	
	Technicians	10	15.3 (1.25)	
NSI history	Yes	125	17.28 (2.81)	NS
	No	114	17.59 (2.45)	

NSI: Needle stick injury; NS: Not significant.

Table 4 Association of health care workers' attitudes with different variables

Variables	P value
Sex	0.0001
Age	0.002
Working history	0.002
Professional group	0.0001
NSI history	NS
Education	0.035
Knowledge score	0.0001

NSI: Needle stick injury; NS: Not significant.

2.12 and the mean knowledge score of physicians was 19.26 ± 1.97. Physicians were the most knowledgeable, while technicians were the least knowledgeable group (P = 0.0001). Those who were more than 30 years old were the least knowledgeable group among different professional groups (P = 0.0001) (Table 3). In this study, 55.6% of the HCWs who had received training had a good knowledge score, but only 38% of those who had not received training showed good scores (P = 0.027).

Attitudes and self-reported behaviors

In this sample, 159 HCWs (66.5%) showed negative attitudes (score = 10-30) and 80 HCWs (33.5%) showed positive attitudes (score = 31-50). Males who were over 40 years old and had a working history of more than 10 years were more likely to show positive attitudes toward patients with hepatitis C (P = 0.002 for both) (Table 4). In the physicians group, 55.7% showed positive attitudes and, in the nurses group, 24% showed positive attitudes. Physicians were significantly more positive (P = 0.0001). All of the technicians showed negative attitudes. Those who received training also showed significantly more positive attitudes (P = 0.035).

HCWs who were weak in knowledge were more likely to show negative attitudes and those who were knowledgeable were more likely to show positive attitudes (P =

Figure 1 Pearson correlation between health care workers knowledge levels and attitudes.

0.02. There was a positive correlation between knowledge score and attitude (r = 0.227, Figure 1).

Regarding attitude statements, 95.8% of the HCWs believed that hepatitis C patients should be identified for infection control purposes, 82.8% of the participants indicated that they used additional infection control precautions when they knew patients had hepatitis C; 74.4% were double-gloved when they treated a bleeding person with hepatitis C. 48.5% of the participants indicated that patients with hepatitis C should be given the last appointment of the day.

Compassion toward people with hepatitis C was measured in two statements (Table 1). Among the HCWs, 92.1% felt compassion when hepatitis C was acquired through a blood transfusion, compared with 55.6% when it was contracted through injection drug use. Participants' willingness to care for people with hepatitis C was identified by responses to three questions (Table 1). Among HCWs, 82.8% believed that they liked to treat these patients and 15.5% said that they did not like treating these patients. Regarding attitudes toward intravenous (IV)-drug users, 78.7% showed fear toward IV-drug users, while 77% said that they were worried they might contract a disease from them. 35.6% believed that IV-drug users deserved the disease and 40.2% indicated that they did not want to treat IV-drug users. 26.8% believed that opiates should not be used for pain relief in patients with a history of injection drug use. There was an association between HCW knowledge level and fear of contracting a disease (P = 0.0001) and attitudes toward IV-drug users (P = 0.0001); however, there was no association between knowledge level and willingness to treat these patients. In addition, HCW attitudes toward IV-drug users emerged as a significant issue that affected willingness to treat people with hepatitis C (P = 0.035).

DISCUSSION

The total response rate in the present study was rather

high compared to other studies on this issue[6,11,15]. In the present study, the reasons for not responding included not having enough time and not having enough interest. Some part of not having enough interest may have been because of incomplete knowledge on the issue.

In the current study, 49.4% of physicians and 54% of nurses had a history of NSI and the difference, unlike in Zafar's study[16], was not significant. Vitale *et al*[17] and Wicker *et al*[18] reported a lower rate of NSI history. Perhaps the reason for the high rate of NSI among HCWs was not receiving enough practical education, HCW stress, or carelessness because of an increased workload.

HCWs are currently receiving training about blood borne diseases. In the present study, however, we did not refer to the contents and efficacy of training. Nurses in the current study were most likely to have received training, possibly because of their close contact with these patients and their greater interest in having a correct approach toward them.

In the current study, the mean knowledge level score was acceptable. Physicians were more knowledgeable than other groups. Richmond showed a significant relationship between medical groups and mean knowledge scores: doctors were the most knowledgeable group[6]. In the study by Shehab *et al*[19], the knowledge level of internal medicine residents on hepatitis was suboptimal. The higher knowledge level of physicians was likely because of more advanced and professional education on gastrointestinal and liver diseases.

In the present study, males and HCWs who were more than 40 years old were more knowledgeable. In Richmond's study[6], HCWs who were 30-49 years old were the most knowledgeable and those above 40 were the least knowledgeable. This finding shows that older age and greater experience can be associated with greater knowledge. However, in elders, the efficacy of initial education decreases.

In the present survey, HCWs were insufficiently knowledgeable about the complications of hepatitis C. Sood *et al*[20] showed that more than half of the participants answered correctly to the questions about hepatitis C complications. In a study by Nicklin *et al*[21], half of the personnel indicated cirrhosis was caused by hepatitis C and 37% thought it caused liver cancer. In the present study, the knowledge about modes of transmission was also acceptable. In the studies of D'Souza *et al*[11] and van de Mortel *et al*[22], most of the participants indicated that blood transfusion is a major mode of transmission. However, some deficits were seen in HCW knowledge on sexual contact as a mode of transmission for hepatitis C.

In some prospective studies, in which the effect of HCW training on HCV knowledge was investigated, participants who received training showed significant advances in their knowledge levels[23-25]. Zdanuk *et al*[25] showed the same advances after receiving training. However, D'Souza *et al*[11] and Shehab *et al*[19] indicated that education did not produce any advance in knowledge levels. In the current study, education was introduced as an effective agent for developing HCW knowledge. How-

ever, we should not ignore the role of clinical practice as a form of education. For example, in Richmond's survey, while complementary therapists were the group most likely to have been educated about hepatitis C, they were not the most knowledgeable[6]. In addition, in the current study, nurses were the most likely group to have been educated, but were not the most knowledgeable. Therefore, other factors must influence the knowledge level of HCWs. However, we should not deny the fact that the information presented cannot be effective if it has not been repeated and recorded in the mind. In some studies, it has been suggested that one method for getting better results is active and problem-based learning[6,20].

In the present study, attitude scores were significantly different among different groups ($P = 0.0001$). Physicians were the most positive group towards people with hepatitis C and technicians showed negative attitudes. This may be related to the fact that physicians were the most knowledgeable group and the better attitudes of males and elders reflect this, as these groups are mainly composed of physicians. Perhaps the positive attitudes are not related to age or experience.

Education had a significant influence on developing positive attitudes, which was also noted by van de Mortel[22] and Richmond *et al*[6]. However, it should be considered that HCW attitudes on hepatitis C patients might be influenced by the attitudes of colleagues. A problem associated with consulting colleagues is that the information provided could be inaccurate, outdated, or reflect just subjective clinical experiences about people with hepatitis C.

In the current study, most of the participants indicated willingness to treat patients with hepatitis C, as in the studies by Hu *et al*[26] in dentistry students and a study by van de Mortel[22].

The HCWs' approach toward giving opiates to the IV-drug users for pain relief reflects deficits in their understanding of pain management and drug dependence. This also demonstrates the powerful influence of attitude on their clinical behavior. In the present study, some HCWs said that IV-drug users deserved to contract hepatitis C and this attitude was affected by their knowledge level and influenced their willingness to treat patients with hepatitis C. In addition, Richmond *et al*[6] showed that HCW willingness to treat patients with hepatitis C was significantly under the influence of their belief on injection drug users, rather than their knowledge of hepatitis C. This shows the role of social prejudice on self-reported behavior. Access to health services could be difficult for people with hepatitis C because HCWs believe that they are injection drug users[27].

Finally, as we expected, there was a significant correlation between HCW knowledge levels and attitudes ($P = 0.0001$). This finding was also reported in the studies of Richmond[6], Vitale *et al*[17] and van de Mortel[22]. We suggest that occupational experience and fear of contracting hepatitis C can also influence the willingness to treat people with hepatitis C.

This study had the advantage of direct interviews

with the responders, and not just distributing the questionnaires among them. The response rate in the present study (69%) was higher than some other similar surveys[6,11,15]. However, some limitations should be noted. For example, like other similar studies, self-reported behavior was not validated against actual clinical behavior. Self-reported responses may not reflect responders' actual attitudes[6].

In conclusion, we showed that discriminatory behaviors are common among HCWs towards patients with hepatitis C. Attitudes are directly under the influence of knowledge levels; therefore, it is necessary to increase the level and quality of training among HCWs to prevent discrimination and prejudice towards patients with hepatitis C.

ACKNOWLEDGMENTS

COMMENTS

Background

Hepatitis C is a chronic prevalent liver infection worldwide which is caused by a blood borne pathogen (hepatitis C virus, HCV). Occupational exposure due to percutaneous injuries is a source of infection with this pathogen among health care workers. Because this group are in close contact with the patients with hepatitis C their knowledge and attitude toward the disease and the patients is so critical and can influence on their own health and their behavior toward the patients. This issue has not evaluated enough until now.

Research frontiers

In contacting with hepatitis C patients, prejudice and discrimination is so prevalent. The research hotspot is to evaluate health care workers' knowledge toward hepatitis C and its effect on their attitude toward the patients and this way lowering discrimination against the patients through improving the knowledge level among this critical group.

Innovations and breakthroughs

Until now no survey has investigated the association of knowledge and attitude of health care workers toward patients with hepatitis C in Iran, a country with high prevalence of the disease. In most of the previous surveys, the questionnaires were passively distributed among the participants. But the present study has the advantage of direct interviewing with the responders. Also the response rate in this study was much higher than some other similar investigations.

Applications

The present study showed that health care professionals' attitude and behavior toward hepatitis C patients is directly under the influence of their knowledge and education on hepatitis C. So by increasing education on this issue maybe we can do something on the care given to the patients with hepatitis C.

Terminology

Hepatitis C: A viral liver infection caused by hepatitis C virus which can be transmitted by blood. This kind of hepatitis will usually end to chronic liver disease and cause cirrhosis; Knowledge: Mental perception and clearly realizing the information and learning; Attitude: Mental backgrounds which are achieved by experiences and can influence person's reactions and behaviors toward others and have close association with the personality.

Peer review

This is a good study which gives important information which could be used in improvement of the treatment of the hepatitis C patients. The survey reports the knowledge and attitudes of health care worker (HCW) impacting on the care given to patients with HCV. Their findings reflect on the significant relationship between attitude level and knowledge score, and various demographic factors of HCW. The study is well reported and holds significant value (on a regional level) towards the care of HCV patients.

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BRIEF ARTICLE

Antibiotic resistance and *cagA* gene correlation: A looming crisis of *Helicobacter pylori*

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Abstract

AIM: To determine antibiotic resistance of *Helicobacter pylori* (*H. pylori*) in Pakistan and its correlation with host and pathogen associated factors.

METHODS: A total of 178 strains of *H. pylori* were isolated from gastric biopsies of dyspeptic patients. Susceptibility patterns against first and second-line antibiotics were determined and trends of resistance were analyzed in relation to the sampling period, gastric conditions and *cagA* gene carriage. The effect of *cagA* gene on the acquisition of resistance was investigated by mutant selection assay.

RESULTS: The observations showed that monoresistant strains were prevalent with rates of 89% for metronidazole, 36% for clarithromycin, 37% for amoxicillin, 18.5% for ofloxacin and 12% for tetracycline. Furthermore, clarithromycin resistance was on the rise from 2005 to 2008 (32% *vs* 38%, $P = 0.004$) and it is significantly observed in non ulcerative dyspeptic patients compared to gastritis, gastric ulcer and duodenal ulcer cases (53% *vs* 20%, 18% and 19%, $P = 0.000$). On the contrary, metronidazole and ofloxacin resistance were more common in gastritis and gastric ulcer cases. Distribution analysis and frequencies of resistant mutants *in vitro* correlated with the absence of *cagA* gene with metronidazole and ofloxacin resistance.

CONCLUSION: The study confirms the alarming levels of antibiotic resistance associated with the degree of gastric inflammation and *cagA* gene carriage in *H. pylori* strains.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is among the most widespread infectious agents because of its high colonization rate and persistent nature in its host's stomach. Mostly the colonization is silent but overt damage of gastric mucosa occurs in certain cases leading to the development of gastritis, duodenal ulcer (DU), gastric ulcer (GU), gastric cancer (GC) and mucosa associated lymphoid tissue lymphoma. It is believed that CagA toxin is responsible for the underlying virulence mechanism because of its ability to translocate into gastric epithelial cells. In host cells, it binds with cellular SRC homology 2 domain-containing tyrosine phosphatase (SHP-2) protein and subsequently damage of gastric mucosa.

Triple therapy constituting the combination of a proton pump inhibitor or bismuth citrate and two antibiotics such as amoxicillin (AML), clarithromycin (CLR) or metronidazole (MTZ) is the internationally recommended first-line regime to eradicate *H. pylori* in symptomatic patients[1,2]. However, the combination might be delivered concomitantly, sequentially or in the form of a traditional straight course for 10-15 d to attain the highest cure rate[3]. In case of treatment failure, several other antibiotics such as fluoroquinolones and tetracycline (TE) are used as secondary options[3,4]. The increased rates of CLR and MTZ resistance have further compounded the already challenging treatment strategy within the harsh acidic environment of the stomach. Patient compliance has declined to less than 80% due to resistance against one of the antibiotics used in the first-line regime[5,6].

Pakistan is among the countries with high prevalence of *H. pylori* infection. The organism is not only associated with severe clinical outcomes[7] but is also carried by the healthy population. Empirical treatment has always been in practice without examining whether it matches *in vitro* antibiotics susceptibility testing (AST) or not. As a result, patient compliance has diminished up to 70%-75% in the last decade[8,9]. As most of studies from Pakistan are based on the outcome of therapy, information is scanty on the status of AST profiles of local isolates. Previous attempts, some of which are based on alternate AST methods, provide limited information[10,11], while no data is available on AST profiles for second-line options.

The paucity of information on the issue and the key role it plays in controlling *H. pylori* infection led us to conduct the present investigation that not only provides a detailed AST profile of local *H. pylori* isolates against first- and second-line regimes but also analyzes their distribution in various groups of patients. The study also contributes toward the better understanding of the role of *cagA* gene in the evolution of resistance.

MATERIALS AND METHODS

Patients and sampling

A total of 178 *H. pylori* strains isolated from gastroduodenal biopsies were included in this study. Biopsy samples were taken from symptomatic patients (*n* = 450) who underwent gastroduodenal endoscopy at Medical Unit Ⅱ, Civil Hospital, Dow University of Health Sciences, Karachi, from March 2005 to November 2008. They were grouped as non ulcerative dyspepsia (NUD), gastritis, GU and DU on the basis of endoscopic findings. Patients with previous treatment history for *H. pylori* infection and/ or GC were excluded. Samples were collected in 20% sterile glucose solution, transported in ice, and processed within two hours of collection. The study was conducted upon approval from the ethical review board of the University of Karachi, Pakistan.

Molecular diagnosis

Genomic DNAs were extracted from crushed tissue samples by SDS-PK method[12] (Khan, 2006 No. 354). Molecular diagnosis for the presence of *H. pylori* was conducted by polymerase chain reaction (PCR) targeting the *16SrRNA* gene as described previously[13]. Samples that were found positive with the *16SrRNA* gene of *H. pylori* were further examined for the presence of the *cagA* gene by PCR using primers designed for the entire 3' repeat conserved region. Amplification was performed at 35 cycles of 95 ℃ for 1 min, 52 ℃ for 1 min, and 72 ℃ for 1 min with a final extension of 7 min at 72 ℃[14]. A segment of human β-globulin gene was amplified as the internal control.

Isolation and identification of Helicobacter pylori strains

For the isolation of *H. pylori,* biopsy samples were crushed with the help of a sterile disposable tissue homogenizer and inoculated on Columbia Blood Agar (Oxoid, United Kingdom) containing 7% laked horse blood (Oxoid, United Kingdom) and *H. pylori* selective supplement Dent (Oxoid, United Kingdom). Plates were incubated at 37 ℃ for 5 d under microaerophilic conditions using a Campygen gas generating kit (Oxoid, United Kingdom). Suspicious small dew drop colonies were subjected for morphological and biochemical identification.

For further confirmation, genomic DNA was extracted and subjected to PCR analysis for the *16SrRNA* gene of *H. pylori* per the above-mentioned protocol.

Determination of minimum inhibitory concentrations of antibiotics

Susceptibility patterns of *H. pylori* isolates was determined against a battery of antibiotics including MTZ, AML, CLR, DA, TE, ofloxacin (OFX) and erythromycin (E). Various concentrations of antibiotics were added to Mueller-Hinton agar containing 5% old sheep blood (SBMHA). Bacterial suspensions were prepared in sterile phosphate buffered saline (PBS) with density equivalent to 3 McFarland's turbidity standard. Ten microliters of each strain was spotted on the plates and incubated for 72 h under microaerophilic conditions. The lowest concentration of antibiotic able to inhibit visible bacterial growth was considered as minimum inhibitory concentrations (MIC). Results were interpreted according to standard criteria[15].

Figure 1 Prevalence of *Helicobacter pylori* resistance to first-line drugs. Dotted box represents the distribution of resistant strains at different minimum inhibitory concentrations (MICs). MIC breakpoints were ≥ 8 μg/mL (A), ≥ 1 μg/mL (B) and ≥ 2 μg/mL (C). S: Sensitive; R: Resistant.

Mutant selection assay

To determine the role of *cagA* gene in the emergence of resistance, two *cagA*+ and two *cagA* strains were subjected to mutant selection assay. A total of 1.2×10^{6}-1.5×10^{6} colony forming units (CFU) of bacterial strains suspended in PBS was spread over SB-MHA plates contain-

ing varying concentrations of antibiotics and incubated for 72 h at 37 ℃ under microaerophilic conditions. Frequency of resistant mutants was determined as the CFU of each strain grown on antibiotic supplemented plates divided by the starting inocula[16].

Statistical analysis

Statistical analyses were performed by PASW statistics 18 (SPSS Inc., Chicago, IL, United States). Pearson's χ^2 test was applied to compare categorical data. Linear regression was applied to correlate the frequency of resistance with *cagA* gene. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Antibiotic susceptibility profile

Out of 450 dyspeptic patients, 201 (45%) were found positive for *H. pylori* by PCR and 178 (40%) by culture. AST profile of 178 *H. pylori* strains revealed high levels of resistance against the first-line regime. A total of 149 (84%) were found to be resistant (MIC ≥ 8 μg/mL) to MTZ. As shown in Figure 1A, 34 (19%) strains had MIC 16 μg/mL, 16 (9%) had 32 μg/mL, 27 (15%) had 64 μg/mL, 12 (7%) had 128 μg/mL and 35 (20%) had MIC 256 μg/mL whereas 27 (15%) isolates were inhibited at boarder-line concentration 8 μg/mL. In case of CLR, 64 (36%) strains showed resistance with MICs ≥ 1 μg/mL. The highest MIC 32 μg/mL was observed only in 4 (2.2%) isolates while 25 (14%) had MIC 2 μg/mL (Figure 1B). Sixty six (37%) strains showed resistance to AML with MICs ≥ 2 μg/mL. Surprisingly, 44 (24.7%) strains exhibited MIC 8 μg/mL (Figure 1C).

Emergence of resistance against first-line therapy led us to investigate the susceptibility patterns of antibiotics usually given as second-line treatment options, such as TE, fluoroquinolones and clindamycin (DA). Data analysis shows an evenly distributed pattern of DA activity with MICs ranging from 0.25 to 32 μg/mL (Figure 2A). Resistance to TE was found in 21 (12%) strains. MIC 4 μg/mL was observed in 15 (8.4%) strains and 8 μg/mL in 7(3.9%) strains (Figure 2B). As shown in Figure 2C, a total of 33 (18.5%) strains were resistant to OFX. MIC of OFX was 2 μg/mL and 4 μg/mL for 21 (11.8%) and 12 (6.7%) strains respectively. We further analyzed the rate of multidrug resistance (MDR) in our studied population. A total of 46 (26%) of the isolates were resistant to two antibiotics i.e. MTZ and AML whereas 62 (35%) were resistant to MTZ and CLR. MDR isolates who were resistant to all first-line antibiotics (R-phenotype; MTZ[r]CLR[r]AML[r]) were 20 (11%). Of these 7 (4%) were also resistant to tetracycline (R-phenotype; MTZ[r]CLR[r]AML[r]TE[r]) as shown in Figure 2D.

Correlation of resistance with demographic and disease factors

We next examined whether the proportion of strains showing antibiotic resistance were increased over time or not. For the purpose, the strains were divided into four groups; 2005 (*n* = 24), 2006 (*n* = 55), 2007 (*n* = 73) and

Figure 2 The prevalence of *Helicobacter pylori* resistance to second-line drugs. Dotted box represents the distribution of resistant strains at different minimum inhibitory concentrations (MICs). MIC breakpoints were ≥ 1 μg/mL (A), ≥ 4 μg/mL (B) and ≥ 2 μg/mL (C); D: Distribution of multidrug resistant strains. AML: Amoxicillin; CLR: Clarithromycin; MTZ: Metronidazole; TE: Tetracycline. S: Sensitive; R: Resistant.

2008 (n = 26) according to the year of sample collection. In general, a significantly progressive trend was only observed in CLR resistance from 32% (n = 8) in 2005 to 35% (n = 19) in 2006, 37.5% (n = 27) and 38% (n = 19) in 2008 (P = 0.004). On the contrary, MTZ resistant strains went down by year from 72% (n = 17) in 2005 to 69% (n = 38) in 2006, 68% (n = 50) in 2007 and 65% (n = 17) in 2008. However AML and OFX resistance remained steady with the rates of approximately 37% and 19% throughout the study period whereas overall resistance rates of TE were 10% (n = 2) in 2005, 13% (n = 7) in 2006, 12% (n = 9) in 2007 and 11% (n = 3) in 2008 (Figure 3A).

Comparative analysis was also performed according to the endoscopic findings of each patient from which *H. pylori* strains were isolated. Out of 178, a total of 25 strains were isolated from NUD cases, 89 gastritis cases, 26 GU cases and 38 from DU cases. Our findings indicate that AML and OFX resistance rates were more common in gastritis patients at 48% (n = 43) (P = 0.005) and 22% (n = 20) of cases (P = 0.08) respectively. In contrast, respective resistance rates of AML and OFX were 31%

(n = 8) and 19% (n = 5) in GU and 16% (n = 6) and 21% (n = 8) in DU patients while no OFX resistant strain was found in NUD group. In case of MTZ, resistance rate was significantly higher among the patients with damaged mucosa such as gastritis (90%), GU (100%) and DU (92%) compared with NUD cases (70%) (P = 0.001). CLR resistance was observed in 53% (n = 13) of NUD, 20% (n = 18) gastritis, 18% (n = 4) GU and 19% (n = 5) of DU cases (P = 0.000) as shown in Figure 3B.

Correlation of drug resistance with cagA gene

The *cagA* genotypes of *H. pylori* usually correlate with the severity of disease; therefore, we determined the relationship of *cagA* gene with the susceptibility profile in local isolates. In this study 83 out of 178 (47%) *H. pylori* strains carried the *cagA* gene. The percentage of *cagA*+ strains was 49% (n = 44) among patients with gastritis, 69% (n = 18) GU and 60% (n = 23) DU whereas only 8% (n = 2) of the strains isolated from NUD cases carried this gene. Due to the low frequency of *CagA*+ strains in NUD cases, further analysis was only based only on a total of 153 strains which were isolated from gastritis, GU and DU

Figure 3 The trend analysis antimicrobial drug resistance in *Helicobacter pylori* strains (*n* = 178). A: From 2005 to 2008, significant increase in clarithromycin resistance was observed by linear regression (*P* = 0.004); B: Correlation of drug resistant strains in various groups of patients. Significant distribution of metronidazole (*P* = 0.001), clarithromycin (*P* < 0.000) and amoxicillin (*P* = 0.005) resistance was observed by Pearson's χ^2 test. NUD: Non ulcerative dyspepsia; GU: Gastric ulcer; DU: Duodenal ulcer.

Figure 4 The correlation of antimicrobial drug resistance with *cagA* gene. A: Rate of drug resistance in *Helicobacter pylori* (*H. pylori*) strains carrying (*n* = 81) and devoid of (*n* = 72) of *cagA* gene. Statistical differences were observed by Pearson's χ^2 test. To determine the effect of cagA carriage on the development of resistance, cagA[+] (*n* = 2) and cagA[-] (*n* = 2) *H. pylori* strains were exposed to the increasing concentrations of metronidazole (B) clarithromycin (C) and ofloxacin (D). Bacterial growth was monitored at different concentration of antibiotic and frequencies of resistant mutants were determined as the colony forming units of *H. pylori* strain divided by the starting inocula.

cases. Of these, 81 (53%) were *cagA*[+] and 72 (47%) were *cagA*[-]. Analysis of the drug resistance indicates the lower prevalence of OFX (11%, *n* = 9), MTZ (83%, *n* = 67) and AML (25%, *n* = 20) resistance in *cagA*[+] strains compared with 22% (*n* = 16), 100% (*n* = 72) and 32% (*n* = 23) in *cagA*[-] strains respectively (Figure 4A). In contrast, CLR resistance was more prevalent in *cagA*[+] strains (43%, *n* = 35) than in *cagA*[-] (33%, *n* = 24). The analysis indicates a possible link between *cagA* gene and the development of drug resistance.

To determine whether the rate of acquisition of antibiotic resistance varies between *cagA*[+] and *cagA*[-] strains, we exposed selected strains to increasing concentrations of MTZ, OFX and CLR. Bacterial growth was monitored at each concentration of antibiotics and frequency of resistant mutants was determined as the CFU of *H. pylori* strain divided by the starting inocula. We observed that *cagA*[-] strains were able to mutate more frequently under the selective pressure of MTZ since they were able maintain their frequencies even after the exposure of 6 × MIC of MTZ (r^2 0.9966, *P* = 0.0374). In contrast, more than 1 $\log_{10}$ decrease in bacterial growth was observed in *cagA*[+] strains with increasing concentrations of MTZ (Figure 4B). Similarly *cagA*[-] strains maintained their frequency in the increasing concentration of OFX (r^2 = 0.9966, *P* = 0.0374) whereas a sharp decline was observed in the development of resistant mutants of *cagA*[+] strains (Figure 4D). However, no significant difference was observed in the case of clarithromycin (Figure 4C).

DISCUSSION

H. pylori is often neglected for antimicrobial susceptibility testing because of its complex growth requirement and low recovery rate by bacterial culture. Increasing reports of treatment failure necessitate surveillance studies to analyze the trend of drug resistance especially in developing countries where MDR is quite common in other bacterial species. To determine the trend of antibiotic resistance in Pakistan, we conducted a 4-year longitudinal study comprised of 178 *H. pylori* strains. AST profile revealed high levels of resistance against the first-line regime including MTZ (84%), CLR (36%) and AML (37%). Our results and those from recently published papers from other countries show comparable prevalence rates; for example 33% resistance to AML was observed in the United States[17] whereas MTZ and CLR resistance rates were 31% and 33% in Ireland[18], 61% and 26% in France[19], 48% and 28% in Saudi Arabia[20], 80% and 45% in India[21], and 77% and 15% in Bangladesh respectively[22]. Moreover the trends of CLR and MTZ resistance were also in agreement with previously published reports[6,23]. In contrast with our observations, available data indicate a low occurrence of such strains in southeast Asian countries such as Malaysia and Taiwan where *H. pylori* is endemic[6,24]. However, the distinct genotypic nature of Southeast Asian strains provides a possible explanation for the differences in resistance profiles compared with rest of the world, including Pakistan.

The global analysis of clinical data clearly indicates that drug resistance to AML, MTZ and CLR has a central role in poor patient compliance to "gold standard" triple therapy to *H. pylori* infection, especially in the case of CLR if the point mutations in peptidyltransferase of 23S *rRNA* gene are responsible to phenotypic behavior[25]. Therefore, Maastricht Ⅲ consensus guidelines proposed not to provide CLR based empirical therapy if primary resistance rates are more than 15%-20% in the respective territory[4]. The present study clearly indicates the upward trend in the primary resistance to CLR in our population with an average of 36% in mono-resistance and 22% in MDR (*R-phenotype*; MTZ[r]CLR[r]AML[r]) strains which provides an possible reason for the poor patient compliance (up to 70%-75%) with CLR based therapies in Pakistan as reported earlier.[8,9] Although MDR strains were equally present in our studied population when compared with the rates in other countries[26,27], they were less prevalent than mono-resistant strains.

Fluroquinolones such as ofloxacin or ciprofloxacin and tetracycline are usually considered as second-line therapy for *H. pylori* infection. In this study, the prevalence of TE resistance was comparable to that of other countries, however resistance to OFX was at a higher level than that seen other countries[18,28]. Mutations in the *gyrA* gene that are responsible for fluoroquinolone resistance have been directly linked with the failure of *H. pylori* eradication[29] therefore the higher rate of OFX resistance is alarming. These antibiotics are generally used to treat gastrointestinal infections in Pakistan; consequently, the resistance occurs in other Gram negative bacteria such as *Salmonella, Shigella* and *Escherichia coli*[30] and therefore the transmission of resistance in *H. pylori* can be anticipated. To combat the situation, broad-spectrum fluoroquinolones such as levofloxacin have been introduced; however, the development of resistance and intense side effects hamper its wide use despite its better compliance rate[31].

Genotypic differences of *H. pylori* directly influence the pathogenesis of infection. Such effects have been widely evidenced with the *cagA* gene carriage however the exact mechanism remains elusive. In this study, the differential prevalence of MTZ and OFX resistance in *cagA*[+] and *cagA*[-] strains clearly indicate the absence of *cagA* gene contributes in the acquisition of resistance which was further evidenced by the differential frequencies of resistant mutants developed with the increasing amount of each antibiotic as previously observed by Taneike *et al*[16] previously. The underlying phenomenon is usually explained by the ability of *cagA*[+] strains to cause intense inflammation which might increase the availability of antibiotics at the site of infection and eventually lead to better eradication of infection. In other words, it describes no direct role of *cagA* gene in antibiotic resistance. However, undermining the hypothesis, we observed that drug sensitive strains were more prevalent in NUD cases, despite of the absence of *cagA* gene, compared to those patients with damaged gastric mucosa. Taken together, the present study suggests that *cagA* gene and the degree

of tissue damage might be two independent factors that affect the drug susceptibility of *H. pylori*.

In summary, we observed that the magnitude of drug resistance in *H. pylori* strains is alarming in Pakistan. The degrees of gastric inflammation and bacterial genotypes are independently implicated in the development of resistance. The study reaffirms the need for both the continuous surveillance for drug resistance and the development of effective prevention and treatment strategies at national and regional levels.

ACKNOWLEDGMENTS

COMMENTS

Background
Eradication of *Helicobacter pylori* (*H. pylori*) is directly associated with symptomatic relief in the patients of gastroduodenal diseases. However, the failure of combination therapy containing two antibiotics and a proton pump inhibitor often results because of antibiotic resistance.

Research frontiers
Pattern of antibiotic resistance in *H. pylori* varies in different settings. However it is yet to determine that how host and pathogenic factors affect the prevalence of resistance.

Innovations and breakthroughs
This study indicates the alarming level of antibiotic resistance among Pakistani strains of *H. pylori* especially the magnitude of clarithromycin resistance is on rise and more commonly observed in non ulcerative dyspeptic patients. We further describe that *cagA* gene carriage and the degree of gastric inflammation are two independent factors affecting the metronizadole and ofloxacin resistance in *H. pylori*.

Applications
It is important to conduct continuous surveillance of antibiotic resistance in *H. pylori*. This study helps to comprehend antibiotic resistance pattern in *H. pylori* that facilitate to developing effective treatment strategy in different groups of patients.

Terminology
Non ulcerative dyspepsia (NUD) is defined as presence of upper gastrointestinal tract symptoms such as stomachache, indigestion and vomiting in patients who did not have damaged gastric mucosa.

Peer review
Overall, the study was well carried out and generally well written. However there are a few areas that needs further clarification mainly in the results section.

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BRIEF ARTICLE

CD74 and macrophage migration inhibitory factor as therapeutic targets in gastric cancer

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Abstract

AIM: To investigate the relationship and molecular features of CD74/macrophage migration inhibitory factor (MIF)/Toll-like receptor 4 (TLR4) in gastric cancer.

METHODS: CD74, MIF and TLR4 expression in the paraffin-embedded sections of gastric cancer from 120 patients were detected by immunohistochemical staining. Knock down of CD74 expression in gastric cancer cell line MKN-45 was performed by lentivirus transduction and detected by Western blotting. MKN-45 cell proliferation assay under the stimulants was measured by the cell counting kit 8 (CCK8) assay and MIF concentration in the culture medium was detected by enzyme-linked immunosorbent assay. Surface staining of CD74 in the MKN-45 cell line under the stimulation of lipopolysaccharide (LPS) was measured by flow cytometry. MIF, CD74 and TLR4 co-localization in the MKN-45 cell line was performed by the immunoprecipitation.

RESULTS: CD74, MIF and TLR4 were found to be expressed in gastric cancer and increased significantly in the advanced stage, and were also associated with lymph node metastasis. Correlation analysis revealed that CD74 was positively correlated with MIF ($r = 0.2367$, $P < 0.01$) and both proteins were also associated with TLR4 ($r = 0.4414$, $r = 0.5001$, respectively, $P < 0.01$). LPS can significantly promote MKN-45 cell proliferation (3.027 ± 0.388 *vs* 4.201 ± 0.092, $P < 0.05$), induce MIF production (54.333 ± 2.906 pg/mL *vs* 29.667 ± 3.180 pg/mL, $P < 0.01$) and cell surface expression of CD74 ($75.6\% \pm 4.046\%$ *vs* $9.4\% \pm 0.964\%$, $P < 0.01$) at LPS concentration of 1 μg/mL compared to medium control. Knockdown of CD74 or using anti-CD74 and MIF antagonist ISO-1 significantly reduced LPS-induced MKN-45 cell proliferation (4.201 ± 0.092 *vs* 3.337 ± 0.087, 4.534 ± 0.222 *vs* 3.368 ± 0.290, 4.058 ± 0.292 *vs* 2.934 ± 0.197, respectively, $P < 0.01$). MIF, CD74 and TLR4 could co-localize in the MKN-45 cell line.

CONCLUSION: Upregulation of MIF, CD74 and TLR4 are associated with increasing clinical stage and provide an opportunity as novel gastric cancer chemoprevention and/or treatment strategy.

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Zheng YX, Yang M, Rong TT, Yuan XL, Ma YH, Wang ZH, Shen LS, Cui L. CD74 and macrophage migration inhibitory fac-

INTRODUCTION

CD74 is a transmembrane glycoprotein that associates with MHC Ⅱ, and is an important chaperone that regulates antigen presentation for the immune response. CD74 is expressed at high levels by antigen-presenting cells (APCs), including B cells, monocytes, macrophages and dendritic cells in normal tissues[1,2]. Although cell surface expression of CD74 is low in many cell types, rapid internalization with concomitant re-expression at the cell surface provides a steady state level of CD74-MHC Ⅱ complex at the cell surface that is sufficient for biological function[3]. More recently, CD74 expression has been examined in cell types other than APCs, such as epithelial cells, and is particularly important in the complex immunological mechanisms and in the link between chronic inflammation and carcinogenesis in the gastrointestinal tract[4]. Substantial evidence has demonstrated that CD74 protein is upregulated in cancer cells, indicating its role in tumorigenesis and angiogenesis[5]. The contribution of CD74 to carcinogenesis is multifaceted. High levels of CD74 expression associated with class Ⅱ MHC expression might prevent tumor antigen presentation by blocking the peptide binding cleft and preventing antigenic peptide binding for presentation to T cells, rendering tumors less immunogenic[6]. In addition, CD74 is the receptor for macrophage migration inhibitory factor (MIF), which, when bound to CD74, initiates survival pathways and cell proliferation[7,8] and facilitates adhesion of *Helicobacter pylori* to gastric epithelial cells (GECs)[9,10].

MIF is an upstream activator of innate immunity that regulates subsequent adaptive responses. In addition to its roles in inflammation and immunity, recent studies have shown that MIF contributes to tumorigenesis. MIF is overexpressed in several tumors including breast cancer, gastric cancer, lung cancer, hepatocellular carcinoma, and cervical cancer[11-15]. MIF binding to CD74 might contribute to carcinogenesis in chronic conditions through the upregulation of proinflammatory cytokines, including interleukin (IL)-8, which upregulates CD74 and has its own mechanisms leading to increased proliferation, tumor growth, and angiogenesis[16]. MIF binding to CD74 affects proliferation and cell cycle events, including antagonism of p53, inhibition of retinoblastoma function, and activation of Akt[17]. This combination of properties suggests that MIF may play a pivotal role in tumor biology.

Pattern-recognition receptors such as Toll-like receptors (TLRs) act as sensors that detect microbial infections and induce a proinflammatory response[18]. TLRs are a family of mammalian homologs of the *Drosophila* Toll proteins and they recognize pathogen-associated molecular patterns that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. In mammalian systems, TLR4 confers responsiveness to Gram-negative lipopolysaccharide (LPS), induces cyclo-oxygenase (COX)2, and is important for proliferation and apoptosis in response to gastrointestinal injury[19].

In previous studies, it has been reported that CD74 and MIF are upregulated in gastric cancer[12,20]. However, how CD74 and MIF are elevated in gastric cancer remains unclear. The relationship between MIF/CD74/TLR4 expression by the tumor and clinicopathological factors in gastric carcinoma needs to be further demonstrated. In this study, we examined CD74, MIF and TLR4 expression in gastric cancer and analyzed their correlations with clinicopathological factors. Also, we used the gastric cancer epithelial cell line MKN-45 to confirm that, under LPS stimulation, MIF production and surface CD74 expression increased, thus promoting cell proliferation. These results suggest that the MIF/CD74 pathway may greatly induce gastric tumorigenesis in infection.

MATERIALS AND METHODS

Patients, specimens and immunohistochemistry

One hundred and twenty patients with gastric cancer, who underwent surgery at Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine, China, were included in this study. Prior to sample collection, appropriate permission was granted from the research ethical committee of Xinhua Hospital. The surgical specimens were fixed in formalin and embedded in paraffin before they were archived. For immunohistochemical staining, paraffin-embedded sections were deparaffinized in xylene and hydrated in 95%, 85%, 75% and 50% ethanol sequentially. Antigens were retrieved by heating for 15 min with 10 mmol citrate buffer (pH 6.0) in a microwave oven. The sections were incubated with 3% hydrogen peroxide to quench endogenous tissue peroxidase activity, and normal goat serum was used as the blocking agent (DakoCytomation, Glostrup, Denmark). The sections were then incubated with CD74 monoclonal antibody (mAb) (1/200 dilution; clone LN2; BD Pharmingen) or MIF Ab (1/100 dilution; clone 2A10-4D3; Sigma-Aldrich) or TLR4 antibody (1/100 dilution; clone 76B357.1; Abcam) at 4 ℃ overnight. Affinity-purified goat anti-mouse IgG conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as secondary antibody. The sections were developed using the liquid diaminobenzidine-substrate chromogen system (DakoCytomation). CD74, MIF and TLR4 expression was separately assessed by two observers who were blinded to the clinical data. CD74 expression was evaluated based on Ishigami's classification[12], by which, according to the percentage of positive cells, cases were divided into two groups: negative, CD74-positive cells < 10%, and positive, CD74-positive cells ≥ 10%. MIF and TLR4 staining was evaluated as follows: -: undetectable;

+: weakly positive; ++: moderately positive; and +++: strongly positive.

Cell culture

The gastric epithelial cell line MKN-45 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) in 5% CO_2 at 37 ℃.

CD74 shRNA and cell sorting

Four different shRNA sequences for CD74 (NM_00 1025158.1) were purchased (GeneChem, Shanghai, China). These sequences were inserted into the pGCSIL-green fluorescent protein (GFP) plasmid (Takara Bio Inc, Otsu, Shiga, Japan) and transformed in *Escherichia coli* for propagation. Purified shRNA plasmids were then used to generate lentiviral particles by expressing them together with a gag-pol-env-encoding plasmid in a HEK293T packaging cell line. Centrifuged cell culture supernatants that contained lentivirus particles were used to infect MKN-45 cells. On day 5, GFP^{+} cells were sorted by FACSAria (BD Biosciences, NJ, USA) to > 98% purity. Cells that had been infected with the lentiviral shRNA that gave rise to the strongest CD74 knockdown (target sequence: GCAT-GAAGCTTCCCAAGCCTC) were cultured and used for further experiments.

Flow cytometry

For surface staining of CD74 in the MKN-45 cell line, cells were harvested and washed with PBS supplemented with 2% FBS. Mouse anti-human FITC-conjugated CD74 and isotype control (BD Biosciences) were used and cultured at 4 ℃ for 30 min, after two washes and detected by flow cytometry (BD Biosciences).

Immunoprecipitation

Two nanograms of recombinant MIF (rMIF) (R&D Systems, Mineapolis, MN, USA) was added to MKN-45 cell lysates, which were rotated for 2 h at 4 ℃. Lysate mixtures were precleared with protein A/G beads (GE Healthcare, Pittsburgh, PA, USA) for 2 h at 4 ℃. MIF was immunoprecipitated using protein A/G beads that were preincubated with anti-MIF mAb (R&D Systems) for 2 h at room temperature. After washing, beads were incubated with the lysate mixture of MIF and cell lysates. Beads were then washed four times and the bound material was eluted for immunoblotting.

Immunoblot analysis

GFP[+] MKN-45 cell lysates or eluted antigens were subjected to 10% SDS-PAGE. Immunoblot analysis was performed by transfer of proteins onto nitrocellulose membranes (Schleicher and Schuell Microscience, Dassel, Germany) using a mini Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA). After 2 h blocking, the membranes were incubated overnight at 4 ℃ with anti-human CD74

(clone EPR4064; Origene, Rockville, MD, USA), anti-human TLR4 (clone 76B357.1; Abcam) specific antibody, and β-actin antibody (Sigma-Aldrich, St Louis, MO, USA). After washing, subsequent incubation with appropriate horseradish-peroxidase-conjugated secondary Antibodies for 1 h at room temperature, and extensive washing, signals were visualized by ECL substrate (Pierce Chemical, Rockford, IL, USA).

LPS and MIF stimulation and proliferation assay

Approximately 10^4 cells/well were grown in 96-well microtiter plates and incubated overnight in 200 μL culture medium. Cells were starved without FCS overnight at 80%-90% confluence and then treated with recombinant human MIF (R&D Systems) and LPS (Sigma-Aldrich) at different concentrations, with or without 2 h pretreatment with ISO-1 [(S,R)-3-(4-hydroxyphenyl)-4,5- dihydro-5-isoxazole acetic acid methyl ester] at 100 nmol (Cal-Biochem, Darmstadt, Germany), or anti-CD74 5 μg/mL (C-16; Santa Cruz Biotechnology) and isotype control (BD Biosciences). Cells without any treatment were used as controls. After 24 h culture, OD was measured using the microplate computer software (Bio-Rad Laboratories) according to the protocol of the CCK8 assay kit (Dojindo, Kumamoto, Japan).

MIF enzyme-linked immunosorbent assay

MKN-45 cells were cultured in 96-well plates and stimulated with the LPS at different concentrations for 24 h. Supernatants from wells were used to quantitate the production of MIF by enzyme-linked immunosorbent assay. The MIF enzyme-linked immunosorbent assay kit was obtained from R&D Systems, and assays were performed according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± SD. Comparison of any two groups was performed by Student's *t* test, and one-way ANOVA was performed for multiple comparisons. CD74, MIF and TLR4 protein expression related to clinicopathological parameters was tested using the Mann-Whitney *U* test and Kruskal-Wallis ANOVA. The relationship between immunohistochemistry scores for CD74, MIF and TLR4 was explored using Spearman's correlation coefficient. Statistical significance was assumed if the *P* value was < 0.05. All analyses were performed using SPSS v14.0.

RESULTS

Overexpression of CD74, MIF and TLR4 in gastric cancer

We routinely collected tissue specimens from patients undergoing surgical operation of known gastric cancer and cut 4-μm-thick sections to stain for the presence of CD74, MIF and TLR4 in the adjacent sections. We stained a total of 120 specimens. CD74, MIF and TLR4 immunoreactivity was identified on the surface of the tumor cells. Some populations of tumor-infiltrating lymphocytes were

CD74   MIF   TLR4

Figure 1 **Representative sections show CD74, migration inhibitory factor and toll-like receptor 4 staining pattern in gastric cancer in each clinical stage.** Gastric tumor sections stained for CD74 (A, D, G and J), MIF (B, E,H and K), toll-like receptor 4 (TLR4) (C, F, I and L) in each clinical stage, and CD74, Migration inhibitory factor (MIF) and TLR4 staining for the same stage are from the same patient, demonstrating that MIF and its receptor CD74 and TLR4 are expressed in close proximity in the tumor microenvironment. Original magnifications: 200 ×.

also immunopositive for those markers (Figure 1). Positive antigen expression of CD74 was observed in 100 of 120 specimens (81%), with the overwhelming majority of CD74-positive specimens with localization of the marker to the apical and perinuclear region of the cytoplasm (Figure 1A, D, G and J and Table 1). There was no difference in CD74 scores between adenocarcinoma from patients aged above or below 60 years (Table 1, $P = 0.5969$). Also, there was no difference between male and female patients (Table 1, $P = 0.9910$) and cell differentiation ($P = 0.3565$). However, a significant difference in CD74 scores between adenocarcinoma with different clinical stage was observed (Table 1, $P = 0.0141$) and lymph node metastasis ($P = 0.0158$).

Immunohistochemical staining also showed that MIF and TLR4 were primarily localized in the cytoplasm and occasionally on the membrane or nuclei of GECs (Figure 1). The positive staining of MIF and TLR4 was observed in 97 (81%) and 99 (83%) respectively of 120 gastric cancer, and the representative example of positive staining in each stage was shown in Figure 1. Like the CD74 staining, there was no difference in age, sex, and cell differentiation, but there were significant in clinical stage and lymph node metastasis (Table 1).

TLR4 and its correlation with CD74 or MIF in gastric cancer

The function of cell surface CD74 as a receptor for MIF provided the rationale for dual analysis of CD74 and MIF immunoreactivity in gastric cancer. A combined MIF and CD74 epithelial score might have a higher predictive value than either parameter alone. Table 2 shows the distribution of CD74 and MIF epithelial staining. There was a significant correlation between MIF and CD74 epithelial scores in individual adenocarcinomas ($r = 0.2367$, $P < 0.01$). TLR4 engagement by ligands such as bacterial LPS leads to proinflammatory cytokine production. Furthermore, from the correlation analysis, we observed that TLR4 had a significant correlation with CD74 ($r = 0.4414$, $P < 0.01$) and MIF ($r = 0.5501$, $P < 0.01$) (Table 2), which suggests that chronic inflammation might have an important association with gastric carcinogenesis.

LPS induces MIF production and surface CD74 expression in gastric cancer cell line

As with immunohistochemical staining, TLR4, CD74 and MIF were highly correlated with the tumor stage and lymph node metastasis, thus, we sought to determine MIF production or CD74 expression by GECs in response to LPS stimulation. Gastric epithelial cell line

Table 1 Correlation of migration inhibitory factor, CD74 and toll-like receptor 4 expression with clinicopathological variables in gastric cancer *n* (%)

Variables	No.	CD74 expression		P	MIF expression		P	TLR4 expression		P
	Cases	Positive	Negative		Positive	Negative		Positive	Negative	
Age (yr)				0.5959			0.8612			0.6421
< 60	46	40 (87)	6 (13)		38 (83)	8 (17)		38 (83)	8 (17)	
> 60	74	60 (81)	14 (19)		59 (80)	15 (20)		61 (82)	13 (18)	
Sex				0.9910			0.5817			0.6358
Male	75	61 (81)	14 (19)		60 (80)	15 (20)		60 (80)	15 (20)	
Female	45	39 (87)	6 (13)		37 (82)	8 (18)		39 (87)	6 (13)	
Histological type				0.3565			0.8440			0.2172
Well	20	16 (80)	4 (20)		16 (80)	4 (20)		17 (85)	3 (15)	
Moderate	40	30 (75)	10 (25)		33 (83)	7 (17)		30 (75)	10 (25)	
Poor	60	54 (90)	6 (10)		48 (80)	12 (20)		52 (87)	18 (13)	
TNM stage				0.0141			0.0281			0.0153
I	26	16 (62)	10 (38)		17 (65)	9 (35)		17 (50)	9 (50)	
II	28	20 (71)	8 (29)		22 (79)	6 (21)		20 (57)	8 (43)	
III	33	29 (88)	4 (12)		28 (85)	5 (15)		30 (67)	3 (33)	
IV	33	31 (94)	2 (6)		30 (91)	3 (19)		32 (85)	1 (15)	
Lymph node metastasis				0.0158			0.0251			0.0152
Negative	50	37 (74)	13 (26)		36 (72)	14 (28)		33 (66)	17 (34)	
Positive	70	63 (90)	7 (10)		61 (87)	9 (13)		66 (94)	4 (6)	

MIF: Migration inhibitory factor; TLR4: Toll-like receptor 4.

Table 2 Correlation analysis of CD74, migration inhibitory factor and toll-like receptor 4 epithelial staining in 120 human gastric cancer patients

	MIF expression			r	P		TLR4 expression			r	P		TLR4 expression			r	P
CD74	(+)	(-)	Total	0.2367	< 0.01	CD74	(+)	(-)	Total	0.4414	< 0.01	MIF	(+)	(-)	Total	0.5501	< 0.01
(+)	85	15	100			(+)	90	10	100			(+)	89	8	97		
(-)	12	8	20			(-)	9	11	20			(-)	10	13	23		
Total	97	23	120			Total	99	21	120			Total	99	21	120		

MIF: Migration inhibitory factor; TLR4: Toll-like receptor 4.

MKN-45 was cultured in 96-well plates and stimulated with LPS (0.1, 0.5, 1 and 5 μg/mL) for 24 h. LPS significantly induced MIF production (54.333 ± 2.906 pg/mL *vs* 29.667 ± 3.180 pg/mL, *P* < 0.01) at a concentration of 1 μg/mL (Figure 2A), suggesting that under conditions of inflammation, such as Gram-negative infection, MIF can be induced. MKN-45 cell line expressed high amounts of TLR4, but LPS stimulation did not significantly induce TLR4 expression (Figure 2B). Although immunohistochemistry confirmed the presence of MIF receptor in gastric tumors, for extracellular MIF signaling to be mediated by CD74 *in vivo*, it must be present on the cell surface. Therefore, we analyzed the MKN-45 cell line with LPS stimulation, to determine whether surface expression of CD74 was present. We detected CD74 by flow cytometry and revealed a detectable but low level of surface CD74 expression. Stimulation with 1 μg/mL LPS for 24 h increased surface expression of CD74 from the basal level of 9.4% ± 0.964% to 75.6 ± 4.046% (*P* < 0.01) (Figure 2C), suggesting that surface CD74 expression by GECs is dependent on LPS stimulation. LPS stimulation can greatly induce MIF and surface CD74 expression and enhance the MIF/CD74 pathway.

LPS induces MIF and CD74 expression increases GEC proliferation

Various reports have shown that MIF or LPS increases proliferation of some cell types[21,22]. We investigated the ability of MIF or LPS to induce proliferation of GECs. rMIF or LPS were incubated with MKN-45 cells for 24 h. Proliferation was measured by nonradioactive cell proliferation colorimetric assay, as used in several recent studies[23]. Standard curves of known numbers of cells were run with each assay to extrapolate cell number from treated samples. As seen in Figure 3B and C, MKN-45 cell proliferation was significantly increased when stimulated with LPS (3.027 ± 0.388 *vs* 4.201 ± 0.092, *P* < 0.01) or MIF (3.160 ± 0.054 *vs* 4.856 ± 0.068, *P* < 0.05) at 1 μg/mL compared with medium control.

To investigate the role of CD74 in the observed proliferation, we used lentivirus shRNA that targeted CD74. Figure 3A shows that the transduction efficiency of MKN-45 cells between the control and CD74 shRNAs was equal, and after sorting, the GFP^{+} cells reached 98%. Western blotting showed that CD74 expression was strongly knocked down (Figure 3B). When CD74 expression was knocked down, the proliferation of MKN-45 cells

Figure 2 Lipopolysaccharide stimulation induced migration inhibitory factor and surface CD74 expression in gastric cancer cell line MKN-45. A: MKN-45 cell line was stimulated with lipopolysaccharide (LPS) at the indicated concentration respectively for 24 h, the supernatants were collected and migration inhibitory factor (MIF) concentration was measured by enzyme-linked immunosorbent assay; B and C: MKN-45 cell line was stimulated with or without LPS (1 μg/mL) for 24 h, toll-like receptor 4 (TLR4) (B) and CD74 surface expression was detected by flow cytometry (C, left panel), the mean values of CD74-positive cells were compared between the medium and condition group (right panel). [b]*P* < 0.01 *vs* medium group.

after stimulation by LPS or MIF was greatly inhibited (4.201 ± 0.092 *vs* 3.337 ± 0.087, 4.856 ± 0.068 *vs* 3.160 ± 0.054, respectively, *P* < 0.01) (Figure 3C and D). The same effect was observed when anti-CD74 blocking antibodies were incubated with cells at 2 h before addition of LPS (4.534 ± 0.222 *vs* 3.368 ± 0.290, *P* < 0.01), (Figure 3E). Notably, after anti-CD74 treatment, proliferation levels were decreased to levels similar to those of untreated cells. To investigate further the role of MIF in LPS-induced GEC proliferation, using the MIF specific inhibitor ISO-1, MKN-45 cell proliferation was greatly inhibited (4.058 ± 0.292 *vs* 2.934 ± 0.197, *P* < 0.01) (Figure 3F). These data suggest that LPS stimulated GEC proliferation through the MIF/CD74 pathway.

TLR4 and MIF/CD74 co-localization

CD74 has been suggested to act as a receptor for MIF in several studies. We have shown that GECs express large amounts of CD74, which is upregulated under inflammatory conditions. Consequently, we examined the role of CD74 as a receptor for MIF on GEC by immunoprecipitation and western blotting. rMIF was incubated with MKN-45 cell lysates. MIF was immunoprecipitated by the MIF antibody along with GEC proteins bound to it. Western blotting using anti-CD74 mAb revealed that CD74 was co-precipitated with MIF, and TLR4 was co-precipitated with MIF (Figure 4). These results suggest that TLR4/CD74/MIF can form a complex to promote cell proliferation.

DISCUSSION

Recent data have expanded the concept that inflammation is a critical component of tumor progression. Many cancers arise from chronic irritation and inflammation. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration[24,25]. TLRs are evolutionarily conserved transmembrane molecules that help the immune system to recognize pathogen-associated molecular patterns, and TLR4 sensitizes immune cells to bacterial LPS. When stimulated by LPS, many intracellular signaling pathways are activated, and lead to the generation of nuclear factor-κB, which in turn promotes proinflammatory cytokine production and release[26]. The unique biological activities of MIF have the potential to contribute to an *in vivo* microenvironment favoring tumor growth and invasiveness. These functional activities include: tumor suppressor downregulation, COX-2 and prostaglandin E2 upregulation, and potent induction of angiogenesis[27,28]. Recent evidence has suggested another important role for the CD74 molecule in the activation of cell survival pathways. CD74 is a cell receptor for the proinflammatory cytokine, MIF. Although CD74 itself is able to bind MIF, when bound to surface expressed CD44, the CD74-CD44 complex is able to initiate several survival pathways, including the extracellular signal-regulated kinase-1/2-mitogen-activated protein kinase

Figure 3 Lipopolysaccharide stimulation induced migration inhibitory factor and surface CD74 expression in gastric cancer cell line MKN45. A: MKN-45 cells were transduced with control or CD74-specific shRNA, and the percentage of GFP+ cells is shown before or after flow cytometry sorting; B: CD74 expression was measured by western blotting when the MKN-45 cells were infected by control or CD74-specific shRNA; C and D: MKN-45 cells were knocked down for CD74 and stimulated with lipopolysaccharide (LPS) (C) or migration inhibitory factor (MIF) (D) for 24 h; cell proliferation was measured by CCK8; E and F: MKN-45 cells were stimulated with LPS at 1 μg/mL, and blocked with MIF antagonist ISO-1 (E) or CD74 antibody (F) for 24 h, and cell proliferation was measured by CCK8. GFP: Green fluorescent protein.[a]*P* < 0.05, [b]*P* < 0.01.

signaling cascade, and to stimulate cell proliferation by enhanced expression of cyclins and other regulatory factors[29].

It has been reported that CD74 surface expression is increased under inflammatory conditions and during *H. pylori* infection, and the bacterium can also use CD74 as a point of attachment to GECs[30]. The dramatic increase in CD74 expression during infection and the high turnover rate of CD74 suggests that both MIF and *H. pylori* can use CD74 as a receptor. Our data demonstrate that in gastric cancer, TLR4 expression is increased and has a strong association with disease stage and lymph node metastasis. GEC proliferation was significantly in-

creased by LPS stimulation, suggesting that gastric cancer is strongly correlated with inflammation. Similarly, rMIF induced proliferation of GECs in a dose-dependent manner. Proliferation was decreased when CD74 was blocked by knockdown of CD74 gene or with antibodies or MIF was blocked by the antagonist ISO-1.

Immunohistochemical staining showed that CD74, MIF and TLR4 has a strong association with cancer stage, suggesting that CD74, MIF and TLR4 have a role in tumor progression. Ishigami *et al*[12] have reported that CD74 expression in gastric cancer is a useful prognostic marker and is correlated with surgical outcome. McClelland *et al*[13] have observed coexpression of CD74 in close proxim-

Figure 4 Migration inhibitory factor binds to CD74 and toll-like receptor 4 on gastric epithelial cells. r-Migration inhibitory factor (MIF) was mixed with MKN-45 cell lysates and immunoprecipitated with anti-MIF with bound cell proteins. Western blotting analysis with anti-CD74 and anti-toll-like receptor 4 (TLR4). MKN-45 lysates were run as a control in the right lane, and MKN-45 cell lysates immunoprecipitated with isotype control antibody were run in the left lane.

ity to the ligand MIF in non-small cell lung cancer, and have found that coexpression is associated with higher levels of CXC chemokines. In the current study, we also found positive correlation between MIF and CD74 and TLR4 in gastric cancer through correlation analysis. We further showed that CD74, MIF and TLR4 could form a complex, and under LPS stimulation, greatly induced cell proliferation. These findings suggest that TLR4, MIF and CD74 overexpression may be related to the pathogenesis of gastric cancer, and they could become promising therapeutic targets.

In summary, our study demonstrated the positive correlation of CD74/MIF/TLR4 in gastric cancer, suggesting that inflammation, as induced by LPS stimulation, can enhance the CD74/MIF pathway, promoting GEC proliferation and gastric carcinogenesis. Blocking of CD74 or MIF may provide a novel strategy for gastric cancer chemoprevention and/or treatment.

ACKNOWLEDGMENTS

COMMENTS

Background
CD74 is an important chaperone of MHC Ⅱ that regulates antigen presentation for the immune response. It is also expressed on epithelial cells, and is particularly important in the complex immunological mechanisms and in the link between chronic inflammation and carcinogenesis in the gastrointestinal tract. Macrophage migration inhibitory factor (MIF) binding to CD74 might contribute to carcinogenesis in chronic conditions, leading to increased proliferation, tumor growth, and angiogenesis. Toll-like receptor 4 (TLR4) confers responsiveness to Gram-negative lipopolysaccharide (LPS), and is important for proliferation and apoptosis in response to gastrointestinal injury.

Research frontiers
Recent data have expanded the concept that inflammation is a critical component of tumor progression. In a previous study, it has been reported that CD74 and MIF are upregulated in the gastric cancer. However, how CD74 and MIF are elevated in gastric cancer remains unclear. The relationship between MIF/CD74/TLR4 expression by the tumor and clinicopathological factors in gastric carcinoma needs to be further investigated.

Innovations and breakthroughs
In this study, CD74, MIF and TLR4 were found to be expressed in gastric cancer and increased significantly in the advanced stage; they were also associated with lymph node metastasis. Correlation analysis revealed that CD74 was positively correlated with MIF and both proteins were also associated with TLR4. LPS can significantly promote MKN-45 gastric cancer cell proliferation, and induce MIF production and cell surface expression of CD74. Knockdown of CD74 or using anti-CD74 and MIF antagonist ISO-1 significantly reduces LPS-induced MKN-45 cell proliferation. MIF, CD74 and TLR4 can co-localize in MKN-45 cells.

Applications
The study demonstrates the positive correlation of CD74/MIF/TLR4 in gastric cancer, suggesting that inflammation, as caused by LPS stimulation, can enhance the CD74/MIF pathway, promoting gastric epithelial cell proliferation and gastric carcinogenesis. Blocking of CD74 or MIF may provide a novel strategy for gastric cancer chemoprevention and/or treatment.

Terminology
CD74, also known as the invariant chain, participates in several key processes of the immune system, including antigen presentation, B-cell differentiation and inflammatory signaling. Recently, studies have revealed that CD74 is a receptor for macrophage MIF and is upregulated in inflammation, which has the potential to contribute to an *in vivo* microenvironment favoring tumor growth and invasiveness. As a participant in several immunological processes and an indicator of disease in some conditions, CD74 has potential as a therapeutic target.

Peer review
In this study, the authors demonstrated the positive correlation between CD74, MIF and TLR4 in gastric cancer and their association with clinicopathological factors. They revealed that LPS stimulation induced gastric cancer cell proliferation through enhanced MIF production and CD74 expression, and that knockdown of CD74 or using anti-CD74 antibody and MIF antagonist could reduce LPS-induced MKN-45 cell proliferation. This study certainly provides a novel mechanism of gastric carcinogenesis associated with the CD74/MIF pathway. The results suggest that CD74 and MIF could be novel therapeutic and chemopreventive targets in gastric cancer treatment.

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BRIEF ARTICLE

Preventive effects of geranylgeranylacetone on rat ethanol-induced gastritis

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Abstract

AIM: To establish a rat ethanol gastritis model, we evaluated the effects of ethanol on gastric mucosa and studied the preventive effects of geranylgeranylacetone on ethanol-induced chronic gastritis.

METHODS: One hundred male Sprague-Dawley rats were randomly divided into 4 equal groups: normal control group, undergoing gastric perfusion of normal saline (NS) by gastrogavage; model control group and 2 model therapy groups that underwent gastric perfusion with ethanol (distillate spirits with 56% ethanol content) by gastrogavage for 4 wk. Low or high doses of geranylgeranylacetone were added 1 h before ethanol perfusion in the 2 model therapy groups, while the same amount of NS, instead of geranylgeranylacetone was used in that model control group. The rats were then sacrificed and stomachs were removed. The injury level of the gastric mucosa was observed by light and electron microscopy, and the levels of prostaglandin 2 (PGE2), endothelin-1 (ET-1) and nitric oxide (NO) were measured by radioimmunoassay and the Griess method.

RESULTS: The gastric mucosal epidermal damage score (EDS; 4.5) and ulcer index (UI; 12.0) of the model control group were significantly higher than that of the normal control group (0 and 0 respectively, all *P* = 0.000). The gastric mucosal EDS and UI of the 2 model therapy groups (EDS: 2.5 and 2.0; UI: 3.5 and 3.0) were significantly lower than that of the model control group (all *P* < 0.01). There was no statistically significant difference between the low-dose and high-dose model therapy groups. The expression value of plasma ET-1 of the model control group was higher than that of the normal control group (*P* < 0.01) and the 2 model therapy groups (all *P* < 0.01). The expression values of gastric mucosal PGE2 and serum NO of the model control group were lower than those of the normal control group (all *P* < 0.05) and the 2 model therapy groups (all *P* < 0.05). The thickness of the gastric mucous layerand the hexosamine content in the model control group were significantly lower than that in the normal control group (all *P* < 0.01) and the 2 model therapy groups (all *P* < 0.05). Scanning and transmission electron microscopy observation showed that in the model control group, the epithelial junctions were vague, the intercellular joints disappeared and damage of the intracellular organelles were significantly worse than those in the normal control group. However, in the 2 model therapy groups, damage to the intercellular joints and organelles was ameliorate relative to the model control group.

CONCLUSION: Administration of geranylgeranylacetone was correlated with a more favorable pattern of gastric mucosa damage after ethanol perfusion. The mechanism could be related to regulation of ET-1, NO and PGE2.

INTRODUCTION

The liver is a main organ for ethanol metabolism. The gastrointestinal tract is also involved in the ethanol metabolic process. Research[1] has reported that binge drinking or long-term drinking can cause acute or chronic gastric mucosal injury. Ethanol can be converted into acetaldehyde in first-pass metabolism in the gastrointestinal tract, which may have a carcinogenic affect on the gastrointestinal tract through local toxic effects. Geranylgeranylacetone is a derivative of terpenes. Studies have shown that geranylgeranylacetone has a therapeutic effect on chronic gastritis, digestive ulcers and portal hypertensive gastropathy[2-5]. There have been few studies on the repair mechanisms of gastric mucosal damage caused by ethanol. By using a rat ethanol gastritis model, we aimed to study the effect and mechanisms of geranylgeranylacetone on repairing gastric mucosa by observing the histological and ultrastructure changes and detecting the expression levels of plasma endothelin-1 (ET-1), serum nitric oxide (NO) and gastric mucosal prostaglandin 2 (PGE2) in this research.

MATERIALS AND METHODS

Materials

Adult male Sprague-Dawley (SD) rats, 8 wk old, weighing 200 ± 50 g, were purchased from the Animal Center of Zhejiang University of Traditional Chinese Medicine. They were fed in a specific pathogen free environment with 12 h of light a day with unlimited drinking water. Geranylgeranylacetone was manufactured by Japan Eisai Co., Ltd., and packed by the Suzhou Eisai Pharmaceutical Co. A kind of liquor (Trade name: Red Star Erguotou, with 56% ethanol, manufactured by a general Beijing brewing factory) was used for gastrogavage to establish an ethanol-induced gastritis model in rats. The ET-1 radioimmunoassay determination kit was purchased from Beijing North Institute of Biotechnology Technology. The PGE2 radioimmunoassay kit was purchased from the Beijing Huaying Biotechnology Research Institute.

Animal treatments

Groups: One hundred adult male SD rats were randomly divided into four groups, including the normal control group, the model control group, the low-dose model therapy group (50 mg/kg) and the high-dose model therapy group (200 mg/kg). The ethanol gastritis model was built by following an established procedure[6]. Red Star Erguotou liquor with 56% ethanol content was used for feeding the laboratory rats by gastrogavage. On every Tuesday and Friday, the rats were fed with ethanol after fasting for 12 h (food was removed on every Monday and Thursday at 9 pm, and gastrogavage was performed on the next day at 9 am). The dose of ethanol was 8 g/kg body weight. The conversion formula was: weight of ethanol (A) = liquor volume (mL) × ethanol content (vol/vol) × ethanol density (8 g/kg). The normal control group received the same amount of normal saline instead of ethanol. All laboratory rats were administered treatment for 4 wk. In the model therapy group, geranylgeranylacetone was dissolved in pure water and was administered by gastrogavage 1 h before ethanol feeding each time. In the normal control and model control groups, only normal saline, instead of geranylgeranylacetone was administered.

Specimen collection: Observations were made of the reactivity, activity and death of rats in each group during the experiment. The animals were killed by cervical dislocation after administration of an overdose of sodium pentobarbital on the 4th weekend of the experiment. The abdomen was opeded immediately. The whole stomach was cut and removed 1.5 cm away from the cardia and the pylorus. Dissection was done along the greater curvature for general specimen observation. The obviously damaged gastric mucosa specimen was rinsed in cold saline solution. Then, the specimen was placed in formaldehyde and glutaraldehyde, and stored in liquid nitrogen solution for later observation. All operations of all specimens were assigned to the same experienced professional laboratory personnel. All animal studies were approved by the Animal Care and Use Committee of Zhejiang University in accordance with the Chinese guidelines for the care and use of laboratory animals.

Determination of gastric mucosal injury index: The length and the width of the injured gastric mucosa region were measured with a vernier caliper. The gastric mucosa ulcer index (UI) was determined according to the Guth standard[7]: spot erosion was recorded as 1 point, erosion length < 1 mm was recorded as 2 points, 1-2 mm was recorded as 3 points, 2-3 mm was recorded as 4 points, and > 3 mm was recorded as 5 points, the score doubled if the erosion width was > 1 mm.

Determination of the thickness of the gastric mucous layer and the mucus glycoprotein content: The thickness of the gastric mucous layer in each group was measured by converted fluorescence microscopy with a thick smear method (using an ink staining method to enhance the contrast). The thickness of the gastric mucous gel layer was detected by measuring the thickness of the

centric bright area with a micrometer eyepiece. Detection of the levels of hexosamine (the main component of mucus glycoprotein) was performed by colorimetric assay using a spectrophotometer.

Histopathology: Four percent formalin-fixed gastric mucosa tissues were embedded by paraffin after gradient dehydration, 4 μm serial sections were obtained and HE staining was performed. We used the epithelial damage score (EDS) to rate morphological changes in the gastric mucosa under light microscopy: normal gastric mucosa was is recorded as 1 point, mucosal epithelial cell damage was recorded as 2 points, damage involving the glandular cells was recorded as 3 points, and mucosal erosion, bleeding or ulceration was recorded as 4 points. We observed a 1 cm length in each slice under light microscopy, and then calculated the cumulative score for each slice. Light microscopy was used to evaluate the degree of gastric damage, which was performed by two pathologists who were unaware of the treatment.

Observation of the ultrastructure of the gastric mucosa: We took 5 mm × 5 mm specimens close to the gastric antrum. According to the electron microscopy procedure, specimens were double fixed by 2.5% glutaraldehyde and 2% osmium tetroxide and subjected to conventional ethanol dehydration, and iso-amyl acetate transition. Critical point drying was carried out on a HCP-2 type critical point drying apparatus. The sample was stuck and a gilded target alloy was placed on the specimens using an IB-5 ion sputter coater. Specimens were observed on the sample stage of the electron microscope. We observed the cell morphology of the gastric mucosa, the junctions of the gastric mucosa epithelial cells, the shape of the gastric pit, and the ultrastructural changes of the intracellular mitochondria as well as the Golgi apparatus and other organelles in all of the laboratory rats by scanning electron microscopy and transmission electron microscopy.

Detection of the levels of serum nitric oxide, plasma endothelin-1, and gastric mucosal prostaglandin 2: We drawed 2 mL of blood from the abdominal aortic vein and injected it into the anticoagulant tube containing 30 μL 10% ethylenediaminetetraacetic acid disodium and 800 U aprotinin and mixed it well. We carried out centrifugal separation of the plasma at 4 ℃ at 3000 r/min for 30 min. Detection of plasma ET-1 levels and gastric mucosal PGE_2 content was carried out by a radioimmunoassay method with strict attention to the instructions. Detection of serum NO levels was carried out by a chemiluminescence method (according to the Griess method).

Statistical analysis
All values are expressed as mean ± SE. The Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

RESULTS

Observation of the general situation of rats
In the model control group, 3 of 25 rats died and most of the others were listless, had a bad appetite, and were unresponsive to external stimuli. In the normal control group and the model therapy groups, most of the rats had alert consciousness, normal appetites, agile responses to outside stimuli, and no deaths. The differences between the model control group and the other groups were significant.

The gastric mucosa ulcer index and the gastric epithelial damage score
In the normal control group, there were only 2 rats (2/25, 8%) that had punctate erosion on the gastric body mucosa. The mucosa of the others was not damaged and the UI was 0. In the model control group, the erosion and ulcers on the gastric mucosa were obvious and the median ulcer index was 12.0, which was significantly different ($P < 0.01$) from the normal control group. The gastric mucosal damage of the low-dose and high-dose model therapy groups was significantly reduced and the UIs were decreased (3.5 and 3.0, respectively). The results were statistically significant difference ($P < 0.01$) compared with the model control group. But there was no statistically significant difference between the low-dose and high-dose model therapy groups($P > 0.05$). In the model control group, the EDS values (median 4.5) were higher than that in the normal control group (median 0, $P < 0.01$). However, in the low-dose and high-dose therapy groups, the EDS values (median 2.5 and 2.0 respectively) were lower than the model control group (median 4.5, $P < 0.05$). The EDS values of the low-dose and the high-dose model therapy groups were not statistically significantly different ($P > 0.05$) (Figure 1 and Table 1).

Histological changes
In the control group, the gastric mucosa was smooth, the layers of the gastric mucosa had clear boundaries under high-power microscopy, and there was no significant inflammatory cell infiltration and edema. In the model control groups, the gastric mucosal surface was uneven with erosion, ulcers and bleeding, and under high-power microscopy the gastric mucosa were congested and had edema. With telangiectasia, the surface mucus layers were damaged and the submucosal gastric glands were incomplete. In the model therapy groups, the gastric mucosal injuries were obviously slighter than the model control group. In the model control group, there was only scattered mucosal damage and local congestion. Under high-power microscopy, the gastric surface mucus layer in the model therapy groups was basically intact and the submucosal layers were slight congested and had less inflammatory cell infiltration, especially in the high-dose model therapy groups (Figure 2).

Figure 1 The gross anatomy of gastric mucosa in different groups. A: Gastric mucosa of normal control group was non-destructive; B Gastric mucosa of model control group showed erosion and ulcer; C: The gastric mucosa damage of low-dose model therapy group was relatively reduced according to model control group; D: The gastric mucosa damage of high-dose model therapy group was significantly reduced according to model control group.

Ultrastructural changes

Ultrastructural changes under scanning electron microscopy: In the normal control group, the gastric mucosa epithelial cells were closely joined and were ring-wise arranged around the gastric gland openings. The gastric pits were clear with ordered cells (according to the arrow). The model control group showed extensive gastric epithelial cell loss, disappearance of gastric pits, and revealed the glandular epithelium (according to the arrow). In the low-dose and high-dose model therapy groups, the gastric epithelial cells showed a basically complete structure and a small amount of ruptured epithelial cells (according to the arrow) (Figure 3).

Ultrastructural changes under transmission electron microscopy: In the normal control group, the gastric mucosal organelles had integrated structures with no degeneration, and the microvillis were arranged in neat rows with no loss. The model control group showed widened cell gaps, vague intercellular junctions, sparse and deciduous microvillis, and swollen mitochondria and endoplasmic reticulum. In the model therapy groups, the cells were arranged in neat rows and the intercellular junctions were clear. The structures of the mitochondria and endoplasmic reticulum were clear with mild swelling (Figure 4).

The thickness of the gastric mucous layer and hexosamine assay results

The thickness of the gastric mucous layer and the contents of the hexosamine in the model control group were significantly lower than the normal control group ($P < 0.01$). While the thickness of the gastric mucous layer and the contents of the hexosamine in the model therapy groups were lower than the normal control group, they obviously were higher than the model control group, and the differences was statistically significant ($P < 0.05$) (Table 2).

Detection of plasma endothelin-1, serum nitric oxide and gastric prostaglandin 2 levels

Plasma endothelin-1 levels: Compared with the normal control group, the levels of plasma ET-1 were significantly higher in the model control group ($P < 0.01$) and were significantly lower in the high-dose model therapy group ($P < 0.05$). While the levels of plasma ET-1 in the model therapy groups (including both the high-dose and low-dose groups) were significantly decreased compared to the model control group ($P < 0.01$) (Table 2).

Serum nitric oxide levels and gastric mucosal prostaglandin 2 levels: Compared with the normal control group, the levels of serum NO and gastric mucosal PGE₂ were significantly decreased in the model control group ($P < 0.05$). In both model therapy groups, the content was significantly higher than in the model control group ($P < 0.05$), and this was especially the case in the high-dose model therapy group (Table 2).

DISCUSSION

The integrity of the gastric mucosa depends on the protec-

Table 1 A comparison of the injury of gastric mucosa in different groups

Group	The gastric mucosa ulcer index		The epithelium damage scores	
	Median	Interquartile range	Median	Interquartile range
Normal control group	0.00	0.5	0.00	1
Model control group	12.00[d]	4.5	4.5[d]	2
Low-dose model therapy group	3.50[b,d]	1.5	2.5[a,d]	1
High-dose model therapy group	3.00[b,d]	1.5	2.0[a,d]	1

[a]$P< 0.05$, [b]$P < 0.01$ *vs* the model control groups; [d]$P< 0.01$ *vs* normal control groups.

Table 2 Comparison of the thickness of gastric mucous layer, the content of hexosamine, the content of plasma endothelin-1, serum nitric oxide and gastric mucosal prostaglandin 2 in different groups

Group	The thickness of gastric mucous layer (μm)	The content of hexosamine (mg/g protein)	Plasma endothelin-1 (pg/mL)	Serum NO (μmol/L)	Gastric mucosal PGE$_2$ (pg/mg)
Normal control group	86.25 ± 3.21	65.57 ± 3.85	52.19 ± 2.82	30.20 ± 2.39	298.7 ± 9.28
Model control group	66.18 ± 5.11[b]	21.51 ± 4.54[b]	74.65 ± 8.84[b]	17.6 ± 3.37[a]	163.2 ± 8.84[a]
Low-dose geranylgeranylacetone	79.43 ± 6.67[a,c]	31.78 ± 5.78[a,c]	35.98 ± 4.78[a,d]	50.60 ± 10.68[c]	205.7 ± 10.39[c]
High-dose geranylgeranylacetone	81.34 ± 5.98[a,c]	37.78 ± 4.98[a,c]	26.87 ± 4.87[a,d]	69.10 ± 9.56[c]	265.5 ± 13.39[c]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* normal control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* model control group. NO: Nitric oxide; PGE2: Prostaglandin 2.

Figure 2 Histological changes of different groups. A: Normal control group: gastric mucosa was smooth, with clear boundaries, and no significant inflammatory cell infiltration and edema (HE staining × 200); B: Model control group: the gastric mucosa surface was uneven with erosion, ulcer bleeding, edema and telangiectasia, the submucosal gastric glands was incompleted (HE staining × 200); C: Geranylgeranylacetone treated group: the gastric surface mucus layer was basic intact, with scattered mucosal damage, local congestion and edema, fewer inflammatory cell infiltration (HE staining × 200).

tion of the gastric mucosal barrier (including, for example, the mucus-bicarbonate barrier and mucosal microcirculation) and can be damaged by a variety of factors (internal or external) with the production of a number of inflammatory mediators and cytokines, resulting in secondary mucosal damage[8-10]. Of the damaging factors, ethanol is an important external factor. With both hydrophobic alkyl and hydrophilic hydroxyl in its molecular stucture, ethanol can damage the gastric mucosal barrier defense system, diminish the capacity of the gastric mucosa to defend the invasion of gastric acid, bile and many digestive enzymes, causing mucosal edema, erosion, hemorrhage and necrosis. In this study, excessive ethanol intake can cause laboratory rats to become apathetic, lose appetites, have slow responses, and have increased mortality. In our study, damage of the gastric mucosa in the model control group, including the general view, microscopic structures and ultrastructure changes, was significantly serious compared to the normal control group.

The gastric mucous layer is the first defensive line of the gastric mucosa to against external stimuli. Studies[11-13] have shown that changes in thickness and content; i.e., mucous glycoprotein (hexosamine) can reflect the anti-invasive ability of the gastric mucous. The results of this study showed that excessive ethanol intake can significantly reduce the thickness of the gastric mucous layer, reduce the content of hexosamine in the mucus gel layer, and finally result in the decline of the anti-invasive ability of the gastric mucosa to ethanol and other external attacks.

Studies[14,15] in recent years have shown that the impaired microcirculation is one of the pathological reasons for gastric mucosal barrier damage, which is accompanied by elevated levels of ET-1, and declining levels NO and PGE2 in blood and the gastric mucosa. NO and PGE2 are recognized vasodilator factors *in vivo*, which can inhibit platelet aggregation and thrombosis, accelerate the flow of the gastric mucosal microcirculation, promote the

Figure 3 Ultrastructural changes under scanning electron microscopy. A: Normal control group: Epithelial cells were closely joined and ringwise arranged around the gastric gland openings (arrow), the gastric pits were clear with ordered cells (× 2500); B: Model control group: extensive gastric epithelial cell loss, disappearance of gastric pits (arrow), and revealed glandular epithelium (× 2500); C: Low-dose geranylgeranylacetone treated group: the gastric epithelial cells showed basically complete structure and fewer ruptured epithelial cells (arrow, × 2500); D: High-dose geranylgeranylacetone treated group: the gastric epithelial cells showed relatively perfect structure and fewer ruptured epithelial cells (arrow, × 2500).

Figure 4 Ultrastructural changes under transmission electron microscope. A: Normal control group: the microvillis were arranged in neat rows and with no loss, organelles had integrated structure. intercellular junction were distinct (arrow, x 30 000); B: Model control group: widened cell gaps, vague intercellular junction(according to arrow), sparse and deciduous microvillis, and swelling mitochondria and endoplasmic reticulum (× 30 000); C: Low-dose geranylgeranylacetone treated group: the cells were arranged in neat rows and the intercellular junction were relative clear (arrow × 30 000), the structure of mitochondria and endoplasmic reticulum were mild swelling; D: High-dose geranylgeranylacetone treated group: the cells were arranged in neat rows and the intercellular junction were obvious clear (arrow × 30 000), the structure of mitochondria and endoplasmic reticulum were mild swelling.

secretion of bicarbonate, mediate the adaptive immune protective function, increase protein synthesis and cell renewal, and finally enhance the repair ability of the damaged gastric mucosa. ET-1 is the strongest vasoconstrictor *in vivo*. Lazaratos *et al*[16] reported that after injection of exogenous ET-1 in the rat gastric artery, the gastric mucosa was obviously damaged, while injection of an endothelin receptor antagonist in advance can significantly reduce the gastric mucosal damage. Under physiological conditions, these factors work together to regulate the gastric mucosal microcirculation and maintain homeostasis. However, imbalanced regulation caused by various factors will disorder the gastric mucosal microcirculation, affect the integrity of the gastric mucosa, and thus lead to a variety of gastrointestinal diseases. This study[16] showed that the content of gastric mucosal PGE_2 and serum NO were decreased in the model control group compared with the normal control group, and were negatively correlated with the gastric mucosal UI and EDS. The study also found that the levels of plasma ET-1 in excessive ethanol intake rats were higher than that in the normal control group, and were positively correlated with the UI and EDS. All these results suggested that the role of ethanol in damage to the gastric mucosa and weakening of its ability to repair may be caused by it stimulating the secretion of ET-1, and inhibiting the synthesis and secretion of endogenous NO and PGE_2.

Geranylgeranylacetone

Geranylgeranylacetone (a derivatives of terpenes with a molecular formula of $C_{23}H_{38}O$) has been a widely used gastrointestinal mucosa protective agent in recent years. It can stimulate the synthesis and secretion of macromolecule glycoprotein and phospholipids in the gastric mucus layer and maintain the normal structure and function of the gastric mucus layer. It therefore has a strong role in renovation of various experimental and clinical gastric mucosal lesions. Studies[17,18] have shown that geranylgeranylacetone can stimulate the regenerated gastric mucosal cells to secrete hexosamine and carry out the biosynthesis of gastric mucosal PGE_2. The mechanism may be related to the fact that geranylgeranylacetone changes the fluidity of membrane phospholipids, and increases the production of phospholipase A2, which is an important intermediate product for the PGE_2 and hexosamine. Hexosamine is an essential component of polymer glycoprotein in the gastric mucosa gel layer, and PGE_2 is a local hormone in the gastric mucosa. Studies have reported that PGE_2 is involved in the improvement of gastric mucosal microcirculation, and the continuous secretion of PGE_2 under the external stimulation helps to renovate gastric mucosa lesions[19-21]. Studies[22-24] have also shown that NO takes part in the process of geranylgeranylacetone inducing gastric mucus synthesis, in which NO synthase plays an important role, Meanwhile the synthesis of ET-1 is inhibited. Geranylgeranylacetone was used to pre-treat ethanol gastritis in the rat model in this study. We also showed that geranylgeranylacetone can elevate the serum

NO and gastric mucosal PGE_2 content and decrease the plasma ET-1 content to varying degrees with different dosages. Meanwhile, the gastric anti-invasion ability of ethanol showed a corresponding increase. In conclusion, Administration of GGA was correlated with a more favourable pattern of gastric mucosa damage after alcohol perfusion. The mechanism could be related to regulation of ET-1, NO and PGE_2. The molecular pathways and mechanisms, however, need to be studied further.

COMMENTS

Background
Binge drinking or long-term drinking can cause acute or chronic gastric mucosal injury. Ethanol can be converted into acetaldehyde in the first-pass metabolism in the gastrointestinal tract, which may have a carcinogenic affect on the gastrointestinal tract through local toxic effects. Studies have shown that geranylgeranylacetone has a therapeutic effect on chronic gastritis, digestive ulcers and portal hypertensive gastropathy.

Research frontiers
By establishing rat ethanol gastritis model, the authors evaluated the effects of ethanol on the gastric mucosa and studied the preventive effects of geranylgeranylacetone on ethanol-induced chronic gastritis.

Innovations and breakthroughs
There have been few studies on repair mechanisms for gastric mucosal damage caused by ethanol. Moreover, the effect and mechanisms of geranylgeranylacetone on repairing ethanol-induced gastritis have seldom been evaluated. This study demonstrated the administration of geranylgeranylacetone was correlated with a more favorable pattern of gastric mucosa damage after ethanol perfusion. The mechanism could be related to regulation of prostaglandin 2 (PGE_2), endothelin-1 (ET-1) and nitric oxide (NO).

Applications
The study results suggest that geranylgeranylacetone can protect the rat gastric mucosa from ethanol-induced injury by changing the mobility of the cell membrane phospholipid bilayer, which further promotes the synthesis of endogenous PGE_2 and NO, and inhibits the secretion of ET-1.

Terminology
Geranylgeranylacetone is a derivative of terpenes, which has a therapeutic effect on chronic gastritis, digestive ulcers and portal hypertensive gastropathy. NO and PGE_2 are recognized vasodilator factors *in vivo*, which can inhibit platelet aggregation and thrombosis, accelerate the flow of the gastric mucosal microcirculation, promote the secretion of bicarbonate, mediate the adaptive immune protective function and increase protein synthesis and cell renewal. ET-1 is the strongest vasoconstrictor *in vivo*.

Peer review
It is an interesting study that confirms alcohol can damage the gastric mucosa. The study demonstrated the protective effect of geranylgeranylacetone on alcohol damage to the gastric mucosa and it elucidated the underlying mechanisms of this protective action.

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BRIEF ARTICLE

Role of bone marrow-derived mesenchymal stem cells in a rat model of severe acute pancreatitis

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Abstract

AIM: To investigate the role and potential mechanisms of bone marrow mesenchymal stem cells (MSCs) in severe acute peritonitis (SAP).

METHODS: Pancreatic acinar cells from Sprague Dawley rats were randomly divided into three groups: non-sodium deoxycholate (SDOC) group (non-SODC group), SDOC group, and a MSCs intervention group (i.e., a co-culture system of MSCs and pancreatic acinar cells + SDOC). The cell survival rate, the concentration of malonaldehyde (MDA), the density of superoxide dismutase (SOD), serum amylase (AMS) secretion rate and lactate dehydrogenase (LDH) leakage rate were detected at various time points. In a separate study, Sprague Dawley rats were randomly divided into either an SAP group or an SAP + MSCs group. Serum AMS, MDA and SOD, interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α levels, intestinal mucosa injury scores and proliferating cells of small intestinal mucosa were measured at various time points after injecting either MSCs or saline into rats. In both studies, the protective effect of MSCs was evaluated.

RESULTS: *In vitro*, The cell survival rate of pancreatic acinar cells and the density of SOD were significantly reduced, and the concentration of MDA, AMS secretion rate and LDH leakage rate were significantly increased in the SDOC group compared with the MSCs intervention group and the Non-SDOC group at each time point. *In vivo*, Serum AMS, IL-6, TNF-α and MAD level in the SAP + MSCs group were lower than the SAP group; however serum IL-10 level was higher than the SAP group. Serum SOD level was higher than the SAP group at each time point, whereas a significant between-group difference in SOD level was only noted after 24 h. Intestinal mucosa injury scores was significantly reduced and the proliferating cells of small intestinal mucosa became obvious after injecting MSCs.

CONCLUSION: MSCs can effectively relieve injury to pancreatic acinar cells and small intestinal epithelium, promote the proliferation of enteric epithelium and repair of the mucosa, attenuate systemic inflammation in rats with SAP.

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INTRODUCTION

Acute pancreatitis (AP) is characterized by a rapid onset and disease progression, with high fatality. Severe acute pancreatitis (SAP) is extremely challenging to treat and the mortality rate is approximately 20%-40%[1]. Several studies currently suggest that the pathogenesis of AP involves complicated cascade reactions that start from the activation of pancreatin in pancreatic acinar cells. Pancreatin causes injury to the acinar cells and induces both local and systemic inflammation[2]. Inflammatory factors such as C-reactive protein, tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, nitric oxide (NO) and endothelin (among others) are thought to be involved in both the genesis and progression of AP and play a critical role in the progression from slight acute pancreatitis to severe acute pancreatitis[3]. Intestinal barricade function is significantly injured in SAP permitting bacteria to invade the enteric cavity and allowing endotoxin to enter the circulatory system thereby inducing a systemic inflammatory factor cascade reaction that aggravates the condition[4].

Mesenchymal stem cells (MSCs) are multipotent stem cells. One previous study demonstrated that MSCs had strong immunoregulatory effects and multidirectional differentiation potency[5]. Other recent studies found that MSCs also played a special role in inhibiting inflammatory reactions and promoting tissue repair[6]. For example, Hagiwara *et al*[7] found that rats with renal injury caused by ischemia-reperfusion induced a significant reduction in renal cell apoptosis after the injection of thymidine kinase-expressing MSCs (TK-MSCs). In addition, nitric oxide synthase (NOS) and NO levels were significantly reduced, which significantly inhibited: the infiltration of neutrophils and mononuclear macrophages; reduced the activity of peroxidase; delayed the production of peroxide, phosphorylation of p38 extracellular signal regulated kinase, the expression of TNF-α, and monocyte chemoattractant protein-1 cell adhesion. TK-MSCs also inhibited H_2O_2-induced cell apoptosis and increased Akt phosphorylation and cell activity in the periphery of the renal tubular cells. Tögel *et al*[8] administered MSCs to mice with acute renal failure for 24 h. The proinflammatory cytokines IL-1β, TNF-α, IFN-γ and NOS were all significantly reduced, whereas the anti-inflammatory factors IL-10, β fibroblast growth factor, TGF-α and B cell lymphoma-2 appeared highly expressed. In a pulmonary injured animal model, Iyer *et al*[9] found that MSCs attenuated a self-inflammatory reaction and enhanced the anti-inflammatory reaction by regulating the proliferation, differentiation, and delomorphous nature of immunocytes.

MSCs have also been demonstrated to have therapeutic effects in inflammatory diseases. For example, Imberti *et al*[10] injected MSCs in a cisplatin-induced acute renal injury model in mice and found that the MSCs enhanced mitosis. In addition, the production of insulin-like growth-factor-1 promoted the repair of renal tubules. In the treatment of chronic ischemic cardiomyopathy, MSCs were injected into ligate ramus descendens anterior arteril coronariae sinistrae. They were also injected into acute myocardial infarction regions. MSCs in both cases enhanced the contractile force of the cardiac muscle cells, regulated the contents and composition of collagen fibers in the tissue, and prevented the reconstruction of cardiac ventricles, thereby protecting the basic structure of cardiac muscle[11]. After intravenous injection of MSCs in experimental rats with spinal injury, MSCs assembled and survived in the host injury spinal cord and promoted the neural repair and recovery of nerve function[12]. In yet another study, rats with a radioactive intestinal injury were injected with labeled MSCs and the intestinal chorioepithelium regeneration occurred in the injured intestinal mucosa for 3 d and the radial related regions (e.g., kidney, spleen, stomach) also had MSCs[13]. Finally, after injecting MSCs into rats with an intestinal injury (ischemia/reperfusion), the permeability of the intestine was reduced and the injury to the intestinal villi was attenuated[14]. Together, these data indicate that MSCs can reduce the expression of various inflammatory factors and promote the repair of various tissues and organ injury.

Because the treatment of AP with stem cells has not been studied to date, and based on the ability of MSCs to inhibit inflammatory reactions and promote tissue repair, the purpose of this study was to explore the role, and the possible mechanisms, of MSCs in rats with SAP.

MATERIALS AND METHODS

Animals

Healthy Sprague Dawley rats weighing 200-300 g were provided by Shanghai SLK experimental animal Company [Batch No. SCXK (Shanghai) 2007-0005, China]. The study was approval by the Institutional Animal Care and Use Committee Fujian Medical University. The care and handling of all animals were in accordance with guidelines for animal ethics.

Drugs, reagents and instruments

The following reagents were used in the experiments: sodium deoxycholate (SDOC) , 3-(4,5-dimethylthiazol-2-yl)-2-,5-diphenyltetrazolium bromide (MTT; Sigma), fetal bovine serum (Purpleflower holly leaf, Hangzhou), Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher, United States), ethylenediaminetetraaceticacid (EDTA)-trypsin (Amersco Co., United States), 4,6-diamidino-2-phenylindole (DAPI; Roche, Switzerland), malonaldehyde (MDA), superoxide dismutase (SOD), amylase (AMS) secretion rate and lactate dehydrogenase (LDH) kits (Nanjing Jianchen Scientific Co. Ltd), transwell double layer culture dish (Corning Costar, United States), IL-10 enzyme-linked immunosorbent assay (ELISA) kit for rats, IL-10 ELISA for rats, and TNF-α ELISA kit for rats (all ELISAs from Wuhan Youer Bio-scientific Co., Ltd), and antibody against proliferating cell nuclear antigen (Shanghai Zhuokang Bio-scientific Co., Ltd).

Culture, identification, and labeling of mesenchymal stem cells

Male rats weighting about 200 g were humanely sacrificed by cervical dislocation. The bone marrow was aseptically collected and subsequently cultured using whole marrow differential adherence methods. MSCs were obtained by multiple digestions and passages. The cellular identification of the expression of MSCs surface markers (i.e., CD29, CD34, CD45 and CD90) were detected using flow cytometry. The cells were labeled with DAPI and observed under fluorescence microscope. Third generation MSCs was acquired for subsequent experimentation.

Cell experiments

Pancreatic acinar cells from the rats were separated using the collagenase method[15]. The cells were seeded in Hanks buffer solution containing 10% fetal calf serum at a density of 1×10^6 cells/mL. The purity of the pancreatic acinar cells was > 80% and the survival rate was > 90%). Cells were seeded in 35 mm × 35 mm culture dishes and incubated at 37 ℃ and 55% CO_2 for 2 h. Cell morphology was examined using phase contrast microscopy. The cultured acinar cells were seeded in the underlayer of transwell double-deck culture dishes.

Pancreatic acinar cells were randomly divided into three groups: non-sodium deoxycholate group (non-SODC group), SDOC group, and a MSCs intervention group. In the SDOC group, the pancreatic acinar cells were seeded in the bottom of the transwell double-layer culture dishes and had a final concentration of 50 μmol/L SDOC. In the Non-SDOC group, the pancreatic acinar cells were seeded in the bottom of the transwell double-layer culture dishes and were not cultured with SDOC. In MSCs intervention group, the insert of the transwell plates was inserted into the poles and the third generation MSCs were seeded at a density of 1×10^6 cell/mL. The culture medium in the insert and the six-pole plate were fused, thereby establishing the co-culture system of MSCs and acinar cells. The co-culture medium was LG-DMEM with SDOC at a final concentration of 50 μmol/L. Subsequently, the cells in each group were incubated for 0.5 h, 1 h, 4 h and 10 h. Alterations in cell morphology were examined and cell survival was quantitatively detected by the MTT assay. The cell survival rate was expressed by the percentage in each group using the following equation: 100% × absorbance at 490 in each group/absorbance at 490 in the fresh separated pancreatic acinar culture medium.

The supernatants were also collected and the concentration of MDA was determined using the thiobarbituric acid method. The density of culture serum SOD was also determined using the xanthine oxidase method. The AMS secretion rate and LDH leakage rate of acinar cells were measured by enzyme kinetics methods. The AMS secretion rate was cell supernatant AMS/cell total AMS × 100% and the LDH leakage rate was cell supernatant LDH/cell total LDH × 100%.

Animal experiment

Thirty-six male rats were randomly divided into either the SAP group or the SAP + MSCs group. The SAP model was established by injecting deoxy-STC under the pancreatic capsule[16]. Specifically, following a peritoneal injection of 2.5% thiopental sodium, the pancreas of each rat was sufficiently exposed after entering into the abdomen *via* a median abdominal incision. Next, 1 mL of 3.8% STC was slowly injected into the inferior aspect of capsule using a No. 4 needle from the tail of pancreas, which made the entire pancreas swell. The pancreas was replaced 2 min later and the abdominal cavity was sutured closed routinely. In the SAP + MSCs group, 2 mL of the MSCs cell suspension (containing approximately 1×10^6 cells/mL determined *via* DAPI fluorescence immunity labeling) were injected into the caudal vein. In the SAP group, 2 mL of normal saline was injected. Six mice were randomly collected from both groups 6 h, 24 h and 72 h postinjection. Blood was collected from the apex of the heart and 5 cm of the small intestine (the section from the terminal ileum and extending distally) was obtained. Serum AMS was detected and the concentrations of serum IL-6, IL-10 and TNF-α were determined using ELISAs. Serum MDA concentration was determined using the thiobarbituric acid method, and the concentration of serum SOD was measured *via* the xanthine oxidase method.

The small intestinal tissue was flash frozen, and the number of DAPI positive cells was measured under fluorescence microscopy. Conventional hematoxylin and eosin staining was performed on sections of small intestine and injury to the intestinal mucosa was assessed in six different, randomly selected, high-power fields (original magnification × 400). According to the injury scoring criteria of Chiu's intestinal tissue[17], injury to intestinal mucosa, infiltration of inflammatory cells, and degree of hemorrhage and hyperemia were scored. The proliferating cell nuclear antigen Ki-67 immunohistochemistry staining was performed to note any proliferation of intestinal mucosa cells. Again, six different high-power fields (× 400) were randomly selected and the number positive cells were counted.

Statistical analysis

All data were expressed by mean ± SD. The mono-factor variance analysis was applied for comparisons between groups. A $P < 0.05$ was considered statistically significant, and all analyses were performed using SPSS 13.0.

RESULTS

General morphology of mesenchymal stem cells and the expression of surface markers

Third generation of MSCs were examined under an inverted microscope. The cells assumed a fusiform and swirling colony (Figure 1). As shown in Figure 2, the positive rate of CD29 was 98.6% and the positive rate of CD90 was 99.6%. In contrast, CD34 and CD45 were

Figure 1 Third generation mesenchymal stem cells were spindle-shaped and formed spiral-like colonies (original magnification × 100).

negative (0.56% and 0.89%, respectively), demonstrating that the purity of the MSCs was > 95%.

Morphology of pancreatic acinar cells

After the pancreatic acinar cells were cultured for 2 h, no adherence was noted under the inverted microscope. Instead, the cells assumed a cluster formation and assembled with lumping. The boundary of the cells was clear and the refraction was strong. High density particles containing proenzymes could be seen in the cells (Figure 3).

The cell survival rate of fresh separated pancreatic acinar cell was comparatively high and that in each group was reduced. This reduction was most evident in the SDOC group. The cell survival rate at each time point in the MSCs intervention group was significantly increased compared with the SDOC group (Table 1).

Amylase secretion and lactate dehydrogenase leakage rates

The AMS secretion rate and LDH leakage rate of pancreatic acinar cell in the SDOC group at each time point was significantly higher than the other two groups. The AMS secretion rate and LDH leakage rate in the MSCs intervention group at each time point was significantly reduced compared with the SDOC group (Table 2).

Oxidative stress

MDA and SOD were measured in the supernatants collected from each group (Table 3). In SDOC group, SOD activity significantly reduced and this difference was significant compared with the non-SDOC group ($P < 0.05$). With the extension of SDOC reaction time, the SOD activity in the cell culture supernatants was further reduced, which was also significantly lower than the non-SDOC group at the same time points ($P < 0.05$). However, MDA content in cell culture supernatants was significantly higher in the SDOC group than the non-SDOC group at the corresponding time points ($P < 0.05$). The SOD activity in the MSCs intervention group at each time point was significantly increased compared with the SDOC group, whereas MDA content was significantly lower than the SDOC group ($P < 0.05$).

Figure 2 The expression of mesenchymal stem cells surface markers detected by flow cytometry. The proportion of CD29+ (A) cells was 98.6%, the proportion of CD90+ (B) cells was 99.6%, the proportion of CD45+ (C) cells was 0.89% and CD34+ (D) cells was 0.56%. B: The boundary of the cells.

Figure 3 Separated pancreatic acinar cells (original magnification × 100).

Permanent planting of mesenchymal stem cells in small intestine

Blue fluorescing cells (DAPI positive cells) were observed in sections of small intestinal tissue of rats that were flash frozen in the rats included in the SAP + MSCs group (Figure 4).

Effect of mesenchymal stem cells transplantation on serum amylase levels and oxidative stress

Serum AMS levels of rats in the SAP group at each time point was significantly enhanced compared with those measured in the SAP + MSCs group ($P < 0.05$, Table 4). Serum MDA and SOD levels have been summarized in Table 5. Serum MDA levels tended to initially increase, but were then reduced following the injection of MSCs. Serum MDA levels at each time point in the SAP + MSCs group were significantly lower than in the SAP group ($P < 0.05$). Serum SOD levels in the SAP + MSCs group was higher than in the SAP group, whereas a significant between-group difference in SOD level was only noted after 24 h ($P < 0.01$).

Regulation of mesenchymal stem cells transplantation on inflammatory factors

Serum IL-6, IL-10 and TNF-α levels in the two groups have been summarized in Table 5. Serum IL-10 and TNF-α after MSCs transplantation tended to increase then decrease. IL-6 was persistently elevated and was obvious in the SAP + MSCs group. After MSCs transplantation, serum IL-6 and TNF-α levels were significantly lower than in the SAP group ($P < 0.05$), Further, serum IL-10 was significantly higher in the SAP + MSCs group than the SAP group ($P < 0.05$). After 72 h, each cytokine was not significantly different between the two groups.

Assessment and scoring of intestinal tissues at different time points after mesenchymal stem cells transplantation

Using a conventional light microscope, the intestinal mucosa was clearly damaged in the SAP group. Specifically, the lamina propria was destroyed, the blood capillary network was exposed, there was bulk infiltration of neutrophils, local regions of hemorrhage, there was a depopulation of intestinal villi, and the glands of the lamina propria showed a variable degree of destruction. In contrast, these changes were rarely noted in the SAP + MSCs group. The main changes noted were neutrophil infiltration of the proper layer and engorgement of the capillaries. The Chiu intestinal tissue damage score in the SAP + MSCs group was significantly lower than that in the SAP group after 6 h (36.33 ± 5.72, $P = 0.045$), 24 h (46.33 ± 2.80, $P < 0.05$), and 72 h (26.67 ± 3.08, $P < 0.05$) as described in Table 6.

Cell proliferation in the small intestinal mucosa at different time points following mesenchymal stem cells transplantation

Cellular regeneration in the small intestinal mucosa in the SAP + MSCs group was more obvious than that in the SAP group, which was in accordance with conventional pathology (Figure 5). For 6 h after transplantation, neither of the two groups had any evidence of proliferation. Then the cell proliferation of small intestinal mucosa in the SAP + MSCs group became significant different than the SAP group ($P < 0.05$) as shown in Table 6 and Figure 6.

DISCUSSION

The goal of this study was to explore the role of bone marrow MSCs in a model of SAP to provide a new, practical basis for the intervention of this often fatal disease. The results of this study are supported by a recently published study on the inhibition of inflammation and reduction of acute pancreatitis in rats by human bone marrow-derived clonal mesenchymal stem cells[18]. Other studies have also demonstrated that SAP induces functional disturbances of the intestinal barrier, resulting in the displacement of bacteria in enteric cavity. Endotoxin subsequently enters the blood and induces a systemic inflammatory factors cascade reaction that aggravates the pathogenic condition. In this course, impairment of free radicals is thought to be one of the most important links between endotoxin and inflammatory reaction. Specifically, oxygen-derived free radicals can induce lipid peroxidation of biological membranes, change the activity of proteins and enzymes, and directly assault DNA and injure the mitochondria, *etc.*, thereby elevating oxidative stress levels in cells[19,20].

Previous studies have also demonstrated that MSCs reduce oxygen-derived free radical levels in the body *via* multiple pathways and maintain the stability of membranes. Exogenous MSCs protected vascular endothelial cells to avoid the damage of oxidative stress[21], and relieve the oxidative damage of neuroblastoma[22]. In one study[23], Kallikrein-modified MSCs were transplanted into the renal tissues of rats with ischemic/reperfusion injury. Those MSCs inhibited the infiltration of neutrophils and mononuclear macrophages, reduced the activity of myeloperoxidases, diminished the formation of superoxides, and relieved H_2O_2-induced apoptosis. Another study reported that MSCs reduced amylase and lipase levels in the serum of rats with injury to the pancreas and

Table 1 Measurement of cell survival rate by the 3-(4,5-dimethylthiazol-2-yl)-2-,5-diphenyltetrazolium bromide assay of pancreatic acinar cells at various time points (mean ± SD)

Group	0.5 h	1 h	4 h	10 h
Non-SODC group	93.83% ± 3.13%	89.00% ± 2.83%	81.83% ± 3.06%	75.00% ± 6.54%
SODC group	87.83% ± 6.59%[a]	77.50% ± 9.35%[a]	65.83% ± 8.23%[a]	39.17% ± 8.26%[a]
MSCs intervention group	90.00% ± 3.41%	82.17% ± 7.47%	75.17% ± 5.85%[c]	51.83% ± 6.79%[c]

[a]$P < 0.05$ *vs* the non-sodium deoxycholate (SDOC) group,[c]$P < 0.05$ *vs* the SDOC group. MSCs: Mesenchymal stem cells.

Table 2 Changes in amylase secretion rate and lactate dehydrogenase leakage rate of pancreatic acinar cells at various time points (mean ± SD)

Index	Group	0.5 h	1 h	4 h	10 h
AMS	Non-SDOC group	7.47 ± 0.67	8.97 ± 0.69	20.32 ± 2.00	24.28 ± 2.47
	SDOC group	11.75 ± 2.40[a]	17.23 ± 2.43[a]	40.88 ± 3.61[a]	60.38 ± 4.01[a]
	MSCs intervention group	10.18 ± 1.53	14.48 ± 1.74[c]	29.33 ± 2.16[c]	40.33 ± 4.27[c]
LDH	Non-SDOC group	3.00 ± 0.63	3.47 ± 0.59	13.17 ± 2.86	23.40 ± 2.55
	SDOC group	7.65 ± 1.75[a]	12.00 ± 3.17[a]	39.02 ± 2.38[a]	53.70 ± 6.73[a]
	MSCs intervention group	5.35 ± 1.01[c]	8.33 ± 3.08[c]	27.67 ± 3.39[c]	38.33 ± 3.20[c]

[a]$P < 0.05$ *vs* the non-sodium deoxycholate (SDOC) group; [c]$P < 0.05$ *vs* the SDOC group. MSCs: Mesenchymal stem cells; AMS: Amylase; LDH: Lactate dehydrogenase.

Table 3 Comparison of superoxide dismutase and malonaldehyde levels in pancreatic acinar cell culture supernatants at various time points (mean ± SD)

Index	Group	0.5 h	1 h	4 h	10 h
Superoxide dismutase (U/mL)	Non-SDOC group	194.83 ± 26.48	185.83 ± 37.79	170.00 ± 25.42	165.00 ± 31.72
	SDOC group	116.17 ± 28.85[a]	108.00 ± 41.52[a]	102.00 ± 33.45[a]	90.67 ± 33.55[a]
	MSCs intervention group	125.50 ± 39.20	138.50 ± 42.03	147.67 ± 37.25[c]	139.00 ± 46.22[c]
Malonaldehyde (μmol/L)	Non-SDOC group	3.50 ± 5.84	4.17 ± 0.75	4.33 ± 1.27	4.67 ± 1.21
	SDOC group	4.40 ± 1.33	6.33 ± 1.63[a]	7.33 ± 1.21[a]	8.00 ± 1.10[a]
	MSCs intervention group	3.97 ± 0.89	5.00 ± 1.41	5.33 ± 1.63[c]	5.83 ± 2.04[c]

[a]$P < 0.05$ *vs* the non-sodium deoxycholate (SDOC) group; [c]$P < 0.05$ *vs* the SDOC group.

Figure 4 Transplanted mesenchymal stem cells were stained with 4,6-di-amidino-2-phenylindole in advance, flash-frozen then observed under fluorescence microscope. The blue fluorescent 4,6-diamidino-2-phenylindole-positive cells (mesenchymal stem cells) were noted in the small intestinal tissue.

Table 4 Comparisons of serum amylase levels (U/L) in rats that were or were not treated with mesenchymal stem cells following establishment of an severe acute pancreatitis model (*n* = 36, mean ± SD)

Group	Post-MSCs or saline injection (h)		
	6	24	72
SAP	3753.83 ± 791.65	5344.67 ± 649.63	7762.50 ± 977.30
SAP + MSCs	2671.33 ± 547.57[a]	4235.83 ± 554.57[a]	5615.17 ± 809.30[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the severe acute pancreatitis (SAP) group. MSCs: Mesenchymal stem cells.

repaired the necrotic pancreatic tissue. MSCs might also inhibit inflammation and involve in the reaction by producing some soluble materials[24].

The production of MDA and lipid peroxidation are parallel; therefore, detecting MDA is thought to represent lipid peroxidation. In addition, SOD is a critical free radical scavenger in mammals and its concentration reflects the ability of the body to scavenge oxygen-derived free radicals[25,26]. In this study, serum MDA of rats in the SAP + MSCs group was reduced while SOD level was heightened, indicating that MSCs transplantation could reduce the oxidative stress level of SAP rats, relieve lipid peroxidation, protect the stability of the membranes, improve the

Table 5 Comparison of serum malonaldehyde, superoxide dismutase levels, interleukin-6, interleukin-10 and tumor necrosis factor-α measured at various time points after injecting either mesenchymal stem cells or saline into rats after establishing an severe acute pancreatitis model (*n* = 36, mean ± SD)

Index	Group	Post-mesenchymal stem cells or saline injection (h)		
		6	24	72
Malonaldehyde (nmol/mL)	Severe acute pancreatitis group	4.89 ± 0.97	5.20 ± 1.21	4.43 ± 0.42
	Severe acute pancreatitis + mesenchymal stem cells group	3.68 ± 0.38[a]	3.89 ± 0.59[a]	3.36 ± 0.98[a]
Superoxide dismutase (U/mL)	Severe acute pancreatitis group	43.16 ± 6.94	48.13 ± 3.93	45.83 ± 4.72
	Severe acute pancreatitis + mesenchymal stem cells group	48.05 ± 3.83	61.29 ± 7.81[b]	50.75 ± 7.59
Interleukin-6 (pg/mL)	Severe acute pancreatitis group	107.70 ± 13.08	128.52 ± 8.52	134.06 ± 13.12
	Severe acute pancreatitis + mesenchymal stem cells group	90.16 ± 9.55[a]	107.33 ± 12.13[b]	143.24 ± 12.11
Interleukin-10 (pg/mL)	Severe acute pancreatitis group	31.08 ± 6.64	45.02 ± 4.28	40.11 ± 8.39
	Severe acute pancreatitis + mesenchymal stem cells group	40.84 ± 7.05[a]	52.08 ± 5.79[a]	41.76 ± 3.37
TNF-α (pg/mL)	Severe acute pancreatitis group	106.15 ± 9.01	132.62 ± 8.64	122.42 ± 13.44
	Severe acute pancreatitis + mesenchymal stem cells group	91.47 ± 10.00[a]	119.47 ± 10.83[a]	110.91 ± 9.92

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the severe acute pancreatitis group. TNF-α: Tumor necrosis factor-α.

Table 6 Comparison of intestinal mucosa injury scores (each slice/score) and proliferating cells of small intestinal mucosa (each slice/number) determined at various time points after injecting either mesenchymal stem cells or saline after establishment of an severe acute pancreatitis model (*n* = 36, mean ± SD)

Group	Post-MSCs or saline injection (h)					
	6		24		72	
	Intestinal mucosa injury scores	proliferating cells number	Intestinal mucosa injury scores	proliferating cells number	Intestinal mucosa injury scores	proliferating cells number
SAP group	43.33 ± 4.84	39.50 ± 5.09	52.83 ± 5.27	59.67 ± 6.80	32.17 ± 4.17	81.50 ± 7.89
SAP + MSCs group	36.33 ± 5.72[a]	40.83 ± 5.12	46.33 ± 2.80[a]	68.00 ± 3.22[a]	26.67 ± 3.08[a]	101.00 ± 11.58[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* the severe acute pancreatitis (SAP) group. MSCs: Mesenchymal stem cells.

scavenging ability of oxygen-derived free radicals, relieve oxygen-derived free radical-induced multiple injury, and protect SAP-induced intestinal tissue damage.

After functional damage of the intestinal barrier, the displacement of endotoxin has multiple pathologic and physiologic consequences. For instance, endotoxin induces pyrogenic reactions, activates the complement system, affects mononuclear macrophages and endothelial cells, induces the genesis of endogenous mediators including TNF, IL, oxygen-free radicals, interferons, *etc*.[27], resulting in the aggravation of pathogenic conditions or even death. The displacement of endotoxin impairs intestinal epithelial cells and increases intestinal permeability[25]. MSCs also inhibit multiple immunocytes, such as T lymphocytes[28], secrete inhibitory mediators of inflammation such as IL-4 and IL-10, parasecrete IL-10, HGF, VEGF, reduce apoptosis signals[29], and relieve endotoxin-induced inflammatory reactions[30]. In the current study, mediators of inflammation, including serum IL-6 and TNF-α of rats in the SAP + MSCs group was higher than the SAP group, whereas IL-10 levels (an anti-inflammatory mediator) were lower in the SAP + MSCs group than the SAP group. This result is similar to several previous studies[13,31] indicating that MSCs might have a role in immunosuppression by reducing the expression of inflammatory factors and promoting the expression of anti-inflammatory mediators. The study reported herein also found that the Chiu intestinal tissue injury scores at

6 h, 24 h and 72 h after transplantation were significantly lower in the SAP + MSCs group than the SAP group, and cellular regeneration in the small intestinal mucosa in the SAP + MSCs group was more evident than in the SAP group. Therefore, MSCs appear to relieve the degree of injury to the small intestinal epithelium, promote the repair of enteric epithelium of rats, and maintain the integrity of the barrier of the intestinal mucosa.

Pancreatic acinar cells are the functional unit for the external secretion of the pancreas, which accounts for 80% of pancreatic tissue. SAP is caused by a functional disorder and impairment of pancreatic acinar cells[32]. During the process of SAP, inflammatory mediators, metabolic products of arachidonic acid, and oxygen-derived free radicals might reduce the antioxidative ability of pancreatic cells[33], enhance vascular permeability, and cause tissue thrombosis and hemorrhage, thereby inducing necrosis of the pancreas[34]. Thus, maintaining the function of pancreatic cells has a critical significance in relieving the severity of SAP. In this study, MDA levels in the MSCs intervention group were lower than in the SDOC group; however, SOD levels in the MSCs intervention group were higher than the SDOC group indicating that MSCs could impact the oxidative stress level of pancreatic acinar cells of injury rats inducted by SDOC, abrogate lipid peroxidation, protect the stability of membranes, improve the scavenging ability of free radicals, relieve free radical-induced injury to protect pancreatic acinar cells.

Figure 5 Description of the intestinal pathologic manifestations 6 h after mesenchymal stem cells transplantation. A: Extensive injury of the intestinal mucosa was obvious in the severe acute pancreatitis (SAP) group; B: The dissection of the upper cortex of the intestinal mucosa was noted in the SAP + marrow mesenchymal stem cells (MSCs) group; C, D: Injury to the intestinal mucosa in the SAP (C) and SAP + MSCs groups (D) 24 h after MSCs transplantation were more severe than at 6 h; E, F: Repair of the intestinal mucosa was seen in the SAP (E) and SAP + MSCs groups (F) (HE staining, original magnification × 200).

Figure 6 The immunohistochemical staining of proliferating cell nuclear antigen Ki-067 after mesenchymal stem cells transplantation at 6 h (A, B), 24 h (C, **D) and 72 h (E, F).** Cell proliferation (brown cells) was obvious. The number of stained (brown) cells in the severe acute pancreatitis (SAP) + mesenchymal stem cells group (B, D, F) were significantly higher than the SAP group. Cell numbers gradually increased with time (original magnification × 400).

In conclusion, this study found that MSCs could relieve injury to pancreatic acinar cells in rats with SAP, attenuate inflammation and injury in the small intestinal epithelium, promote the proliferation of enteric epithe-

lium and repair of the mucosa, and maintain the integrity of the intestinal barrier function. Potential mechanisms might involve regulating the oxidative stress levels of rats with SAP, inhibiting the extensive release of mediators of inflammation and cytokines, promoting the secretion of mediators of inflammation, and scavenging oxygen-derived free radicals. The specific mechanisms remain worthy of further study.

COMMENTS

Background

Acute pancreatitis (AP) is characterized by a rapid onset and disease progression with high fatality. Severe acute pancreatitis (SAP) is extremely challenging to treat. Several studies currently suggest that the pathogenesis of AP involves complicated cascade reactions of inflammation. Mesenchymal stem cells (MSCs) are multipotent stem cells which had strong immunoregulatory effects and multidirectional differentiation potency. Recent studies found that MSCs also played a special role in inhibiting inflammatory reactions and promoting tissue repair in various inflammation-based diseases such as kidney disease in ischemia/reperfusion injury, collagen-induced arthritis, and acute renal failure. However, very few studies to date have investigated the potential role of cell therapy for pancreatitis.

Research frontiers

Inflammation plays an important role in the pathology of AP. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 as proinflammatory cytokines are produced mainly during AP. The research hotspot is to explore whether MSCs could reduce the level of inflammatory factors and promote the repair of various tissues and organ injury in AP.

Innovations and breakthroughs

Recent reports have indicated that MSCs can reduce the expression of various inflammatory factors and promote the repair of tissues and organ injury. In the present study the authors found that MSCs can also effectively relieve injury to pancreatic acinar cells and small intestinal epithelium, promote the proliferation of enteric epithelium and repair of the mucosa, attenuate systemic inflammation in rats with SAP.

Applications

The study results suggest that MSCs have a special role in inhibiting inflammatory reactions and promoting tissue repair in rats with SAP that might be developed as a cell therapy for pancreatitis.

Terminology

Severe acute pancreatitis (SAP): SAP is a serous gastrointestinal disorder which caused by a functional disorder and impairment of pancreatic acinar cells. The disease is characterized by a rapid onset and disease progression which has a high fatality. Mesenchymal stem cells (MSCs): MSCs are multipotent stem cells which derived form bone marrow. They have strong immunoregulatory effects and multidirectional differentiation potency.

Peer review

The authous detected the cell survival rate, the concentration of malonaldehyde (MDA), the density of superoxide dismutase (SOD), serum amylase (AMS) secretion rate and lactate dehydrogenase leakage rate in pancreatic acinar cell experiments, and measured serum AMS, MDA and SOD, IL-6, IL-10, and TNF-α levels, intestinal mucosa injury scores and proliferating cells of small intestinal mucosa at various time points in animal experiments. The results are interesting and suggest that MSCs could relieve injury to pancreatic acinar cells in rats with SAP, attenuate inflammation and injury in the small intestinal epithelium, promote the proliferation of enteric epithelium and repair of the mucosa. It is believable that MSCs infusion might be a promising treatment method for AP or SAP.

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BRIEF ARTICLE

Clinical significance of connective tissue growth factor in hepatitis B virus-induced hepatic fibrosis

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Abstract

AIM: To determine the utility of connective tissue growth factor (CCN2/CTGF) for assessing hepatic fibrosis in hepatitis B virus (HBV)-induced chronic liver diseases (CLD-B).

METHODS: Enzyme-linked immunosorbent assay was used to measure CCN2 in sera from 107 patients with chronic hepatitis B (CHB) and 39 patients with HBV-induced active liver cirrhosis and 30 healthy individuals. Liver samples from 31 patients with CHB, 8 patients with HBV-induced liver cirrhosis and 8 HBV carriers with normal liver histology were examined for transforming growth factor β-1 (TGF-β1) or CCN2 mRNA levels by *in situ* hybridization, and computer image analysis was performed to measure integrated optimal density (IOD) of CCN2 mRNA-positive cells in liver tissues. Histological inflammation grading and fibrosis staging were evaluated by H and E staining and Van Gieson's method.

RESULTS: Serum CCN2 concentrations were, respectively, 4.0- or 4.9-fold higher in patients with CHB or active liver cirrhosis as compared to healthy individuals ($P < 0.01$). There was good consistency between the levels of CCN2 in sera and CCN2 mRNA expression in liver tissues ($r = 0.87$, $P < 0.01$). The levels of CCN2 in sera were increased with the enhancement of histological fibrosis staging in patients with CLD-B ($r = 0.85$, $P < 0.01$). Serum CCN2 was a reliable marker for the assessment of liver fibrosis, with areas under the receiver operating characteristic (ROC) curves (AUC) of 0.94 or 0.85 for, respectively, distinguishing normal liver controls from patients with F1 stage liver fibrosis or discriminating between mild and significant fibrosis.

CONCLUSION: Detection of serum CCN2 in patients with CLD-B may have clinical significance for assessment of severity of hepatic fibrosis.

INTRODUCTION

Hepatic fibrosis, characterized by an excessive accumu-

lation of extracellular matrix (ECM) components, is a common feature of many chronic liver diseases (CLD-B) and can ultimately lead to liver cirrhosis[1,2]. Hepatitis B virus (HBV) is a predominant cause of chronic liver disease and presents a high risk of fibrosis progression[3]. While the pathobiology of HBV-induced hepatic fibrosis has not been fully clarified, HBV presents a huge medical challenge because one third of the world's population has been infected and 350 million people are carriers of the virus. Hepatitis B is endemic in China (> 8% prevalence) and has caused epidemics in other parts of Asia and Africa.

Over last two decades, hepatic stellate cells (HSCs) have dominated studies exploring mechanisms of hepatic fibrosis[4]. In response to chronic liver injury, quiescent HSCs become activated myofibroblast-like cells that express α-smooth muscle actin (α-SMA) and produce components of the ECM, including fibrillar collagens[1,2]. This process is driven by a variety of growth factors, cytokines and matricellular proteins. Connective tissue growth factor (CCN2, also known as CTGF) is a secreted matricellular protein that is recognized increasingly as a central player in hepatic fibrosis[5]. Previously, we have shown that CCN2 production and secretion is enhanced by transforming growth factor-β1 (TGF-β1) in rat HSC and exposure of HSCs to CCN2 induces cell adhesion, migration, and proliferation[6,7]. We and others have shown that CCN2 induces expression of α-SMA or type I collagen in HSCs consistent with a role in activation and fibrogenesis[8,9]. In addition, CCN2 also stimulates survival pathways in activated HSCs thereby prolonging their fibrogenic potential[10]. In human or experimental liver fibrosis, CCN2 expression is higher than in normal liver, with strong correlation between hepatic CCN2 production and the degree of liver fibrosis[11].

Given the central role of CCN2 in hepatic fibrosis, we investigated the levels of serum CCN2 of patients with HBV-induced CLD-B, and determined the potential clinical value of hepatic or serum CCN2 levels in diagnosing the severity of HBV-induced hepatic fibrosis.

MATERIALS AND METHODS

Ethics
This work was approved by First Hospital of Jilin University and was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Informed consent was obtained from all patients prior to sample collection.

Clinical data
Serum was collected from 146 patients with CLD-B who were either outpatients or inpatients at First Hospital (Jilin University, Changchun, China) from June 2008 to September 2011. Of the 146 patients, there were 107 cases with chronic hepatitis B (CHB) (69 men and 38 women; mean age: 43.9 years, range: 18-76 years), 39 cases with active liver cirrhosis (27 men and 12 women; mean age:

41.9 years, range: 26-68 years). As determined using the Child-Pugh score to assess the severity of liver cirrhosis, 25 cases were class A and 14 cases were class B.

Serum was collected from 30 healthy individuals (18 men and 12 women; mean age: 33.6 years, range: 19-56 years) for controls. Serum and liver samples were collected from 8 HBV carriers who had histological normal livers (6 men and 2 women; mean age: 28.6 years, range: 19-38 years), and were studied as normal controls for the assessment of liver fibrosis of CLD-B patients. Patients and HBV carriers meet the diagnostic criteria for chronic HBV infection[12].

Liver tissue samples from 39 CLD-B patients and 8 HBV carriers were obtained using a percutaneous needle. The length of each sample was more than 1.5 cm. There were 31 cases with CHB (23 men and 8 women; mean age: 29.8 years, range: 18-50 years) and 8 cases with HBV-induced active liver cirrhosis (6 men and 2 women; mean age: 36.6 years, range: 26-54 years). Importantly, initial studies showed that levels of serum CTGF, Collagen I, Collagen III or aminotransferases in CLD-B patients receiving a liver biopsy were not significantly different as compared to CLD-B patients without a biopsy.

CCN2 enzyme linked immunosorbent assay
Sera were stored at -70 ℃ for 1 to 6 mo before analysis. The level of CCN2 in sera from 146 patients with CLD-B, 8 HBV carriers and 30 healthy individuals were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to manufacturer's protocols (USCN Life Science and Technology Co, TX, United States). Briefly, microtiter wells were pre-coated for 2 h at 37 ℃, with 100 μL of each standard or 1:20 dilutions of sera. The plates were then developed by sequential addition of biotinylated anti-CCN2 antibody, avidin-conjugated horseradish peroxidase and tetramethylbenzidine substrate solution, and the color reaction was measured at 450 nm.

Grading and staging of liver biopsies
Liver tissue samples from 31 CHB patients, 8 patients with HBV-induced liver cirrhosis and 8 HBV carriers were individually fixed, paraffin-embedded and subjected to H and E staining or Van Gieson's method to determining histological inflammation and fibrosis which were scored using the Metavir system. Fibrosis was staged on a 4-point scale (F0: No fibrosis; F1: Minimal fibrosis; F2: Fibrosis with a few septa; F3: Numerous bridging fibrosis without cirrhosis; F4: Cirrhosis or advanced severe fibrosis). F1-F2 was defined as mild fibrosis and F3-F4 as significant fibrosis. Inflammation was graded on a four-point scale from A0, which indicated no inflammatory activity, up to A3, which indicated severe activity.

In situ hybridization
In situ hybridization (ISH) was performed using digoxigenin-labled sense or anti-sense probes for CCN2 or TGF-β1 (*Boster Biotechnology* Co. Ltd. Wuhan, China). In

Figure 1 Production of connective tissue growth factor or transforming growth factor β-1 in fibrous septa of hepatitis B virus-infected livers. Connective tissue growth factor mRNA (A) or protein (B) were detected by *in situ* hybridization (ISH) or immunohistochemistry respectively. transforming growth factor β-1 mRNA (C) was detected by ISH while collagen bundles (D) were stained red using Van Gieson's method. Original magnification, × 200 in A, B, C and D. Examples of positively stained cells or structures in each panel are arrowed.

brief, liver tissue samples from CLD-B patients were formaldehyde-fixed and parafin-embedded. The tissue sections (5 μm) were deparaffinized, rehydrated with PBS, digested with pepsin (30 μg/mL) for 10 min at 37 ℃, fixed in 4% paraformaldehyde in PBS and washed in 3 ×SSC. The samples were pre-hybridized at 40 ℃ for 2 h, and hybridization was performed overnight at 40 ℃ with sense or anti-sense probes. After hybridization, excess probes were removed by sequential washing in twice concentrated (2×) saline-solution citrate buffer (SSC), 0.5×SSC and then 0.2×SSC at 37 ℃ for 2 h. The tissue sections were incubated at 37 ℃ for 1 h with biotinylated mouse anti-digoxigenin, followed by addition of the streptavidin-biotin-peroxidase complex for 20 min. The slides were then developed with 3-amino-9-ethylcarbazole (Boster Biotechnology). Ten random images (original magnification ×400) of each slide underwent computer image analysis using Image-Pro Plus 6.0 software to assess the integrated optimal density (IOD) of CCN2-positive cells in liver tissues.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5 μm) were de-waxed and re-hydrated. Sections were incubated overnight at 4 ℃ with mouse anti-human α-SMA monoclonal antibody (Zhongshan Goldbridge Biotechnology, Beijing, China) or rabbit anti-human CCN2 polyclonal antibody (Santa Cruz, Heidelberg, Germany) or rabbit anti-human

F4/80 polyclonal antibody (Spring Bioscience, United States). Sections were washed in PBS and incubated at room temperature for 10 min with biotinylated *goat anti-mouse* and *rabbit* IgG (Maixin Bio, Fuzhou, China). After washing with PBS, sections were incubated with streptavidin-peroxidase (Maixin Bio, Fuzhou, China) for 10 min and then developed with diaminobenzidine or 3-amino-9-ethylcarbazole.

Statistical analysis

The values reported represent the median [95% confidence interval (CI)] of the measurements. Statistical analysis of the data was performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, United States). The nonparametric Wilcoxon signed ranks test was used for pair-wise comparison of groups and Spearman's rank correlation analysis was used to determine the relationship between two variables. Areas under the receiver operating characteristic (ROC) curves (AUC) were calculated for comparing the accuracy of the CCN2 in sera in different subgroups.

RESULTS

Localization of CCN2 mRNA or protein in HBV-induced chronic liver disease

In normal livers, only mild CCN2 mRNA staining was detected in portal tracts and there was no staining in the

Figure 2 Cellular localization of key fibrotic markers in hepatitis B virus -induced liver fibrosis. α-SMA-positive hepatic stellate cells (HSCs) were not detectable in hepatitis B virus carriers who had normal liver histology (A) but were present in chronic hepatitis B (CHB) liver (B). In CHB liver samples, there was no staining when the *in situ* hybridization probes were omitted (C) but their inclusion demonstrated the presence of either connective tissue growth factor mRNA in activated HSCs (D) or transforming growth factor β-1 mRNA in activated HSCs (E) or Kupffer cells (F). F4/80 antigen-positive Kupffer cells (G). Original magnification, × 400 in A-G. Examples of postively stained cells in each panel are arrowed.

central vein or lobule (data not shown). However, CCN2 mRNA and protein were localized to fibrotic septa in CLD-B patients with hepatic fibrosis (Figure 1A and B) or cirrhosis (data not shown), and the pattern of CCN2 staining was well correlated with the distribution of collagen fibers (Figure 1D). By comparison to normal livers, CHB patients demonstrated strong α-SMA-positive staining in presumptive activated HSC (Figure 2A and B) and these cells also stained strongly for CCN2 mRNA (Figure 2C and D).

Since CCN2 acts downstream of TGF-β1 to drive fibrosis[13], we examined the expression and distribution of TGF-β1 mRNA in the liver of CLD-B patients. TGF-β1 mRNA was detected in the fibrotic septa of patients with hepatic fibrosis (Figure 1C) or cirrhosis (data not

shown) and was localized to activated HSCs in the lobule (Figure 2E) and Kupffer cells (Figure 2F and G) within inflammatory area in the livers of CHB patients.

Serum levels of CCN2 in HBV-induced chronic liver diseases

Since CCN2 is synthesized with a signal peptide and can exist extracellularly in soluble or matrix-associated forms, we examined serum from CLD-B patients for the presence of CCN2 protein by ELISA. As shown in Table 1, serum CCN2 concentrations were, respectively, 4.0- or 4.9-fold higher in patients with CHB or active liver cirrhosis as compared to healthy individuals ($P < 0.01$). There was no difference in the serum CCN2 levels between the HBV carriers and healthy individuals.

Table 1 Serum connective tissue growth factor concentrations in patients with chronic liver diseases (μg/L)

Group	*n*	Median (95% CI)
Healthy control	30	2.2 (1.6-2.8)
HBV carrier	8	2.2 (1.5-2.9)
Chronic hepatitis B	107	8.8 (6.0-12.3)
Active liver cirrhosis	39	10.9 (7.0-14.6)

HBV: Hepatitis B virus.

Table 2 Relationship between fibrosis stage and hepatic or serum connective tissue growth factor content

Fibrosis stage	*n*	Serum CCN2 (μg/L) median (95% CI)	Hepatic CCN2 mRNA (IOD) median (95% CI)
Normal control	8	2.2 (1.5-2.9)	6.0 (3.9-8.8)
F1	11	6.8 (5.0-8.9)	19.4 (12.3-26.4)
F2	9	8.9 (7.1-10.7)	25.6 (13.9-34.8)
F3	11	9.4 (7.3-12.0)	31.9 (19.7-44.6)
F4	8	10.1 (8.2-12.1)	39.6 (25.5-52.8)

Normal Control samples were from hepatitis B virus carriers and had normal liver histology; F1-F2: Mild fibrosis; F3-F4: Significant fibrosis. CCN2: Connective tissue growth factor; IOD: integrated optimal density.

Figure 3 Receiver operating characteristic curve of connective tissue growth factor. Receiver operating characteristic (ROC) curves of connective tissue growth factor distinguishing normal liver controls from patients with F1 stage liver fibrosis (A) or discriminating between mild and significant fibrosis (B) with areas under the ROC curves of 0.94 or 0.85, respectively.

CCN2 production as a function of severity of fibrosis or inflammation

Having shown that hepatic and serum CCN2 concentrations were higher in CLD-B patients than in healthy individuals, we next investigated if there was a correlation between CCN2 and fibrosis stage. As shown in Table 2, serum concentrations and hepatic content of CCN2 increased in proportion to the severity of fibrosis; Spearman's rank correlation analysis showed that correlation coefficients were 0.85 and 0.89 (both $P < 0.01$), respectively. However, the levels of CCN2 in sera were not correlated with the degree of inflammation in CHB patients.

Diagnostic performance of serum CCN2

We further analyzed the diagnostic performance of serum CCN2 for assessing liver fibrosis using the ROC curves. Calculation of the areas under ROC curves (AUC) showed that serum CCN2 could be used to distinguish either normal liver controls from patients with F1 stage liver fibrosis (AUC = 0.94) or mild fibrosis (F1/F2) from significant fibrosis (F3/F4) (AUC = 0. 85) (Figure 3).

DISCUSSION

Chronic HBV infection can cause hepatic fibrosis and eventually cirrhosis. Over the last few years, HBV infection has been studied extensively *in vitro* with the finding that expression of the HBV X protein (HBx) in hepatocytes results in paracrine activation and proliferation of human or rat HSC resulting in their increased expression of collagen I , CCN2, α-SMA, matrix metalloproteinase-2, or TGF-β[14,15]. Although hepatocytes serve as a suitable host for HBV and permit viral replication and antigen production, HBV can also transiently infect and replicate in human HSCs which directly increases the production of collagen type in the cells[3,16]. Collectively, these findings have shown that activation of fibrogenic pathways in HSC following HBV infection of either hepatocytes or HSC is a key event in HBV-mediated hepatic fibrosis. In this regard, CCN2 has emerged as a potential key fibrogenic mediator in response to HBV in as much as CCN2 supports HSC activation, promotes HSC proliferation and survival, and acts downstream of TGF-β to drive HSC collagen production[6,7,10,13,17]. In this study, we showed that CCN2 mRNA and protein were expressed at high levels by myofibroblasts (including presumptive activated HSC) in fibrotic septa in CLD-B patients with hepatic fibrosis or cirrhosis, and that increased levels of hepatic or circulating CCN2 were associated with severe fibrosis. This result is consistent with previous observations by others[11].

Activation of Kupffer cells, the resident macrophage population of the liver, serves as a central determinant of the liver's response to injury and repair, and the resulting inflammatory reaction is an important prerequisite for HSC activation and progression to hepatic fibrosis[18,19]. Macrophage-derived TGF-β1 has been identified as a potential paracrine stimulator of HSC activation[20] and addition of TGF-β antibodies to Kupffer cell conditioned medium inhibits its ability to induce expression of

CCN2, collagen I and TIMP-1 when added to cultured HSC[21]. In the present study, both TGF-β1 and CCN2 mRNA were detected in presumptive activated HSCs, while TGF-β1 mRNA alone was detected in Kupffer cells within inflamed areas of livers from CHB patients. These data support the notion that TGF-β1 upregulates CCN2 production in HSCs *via* paracrine and autocrine pathways, and further enhance the effects of CCN2 during fibrogenesis. This is supported by *in vitro* studies showing that CCN2 is a downstream mediator of TGF-β1-induced collagen I production in human HSCs[16].

CCN2 is a secreted protein that has been detected in several human body fluids including serum, cerebrospinal fluid, follicular fluid, uterine fluid, or urine[22]. This has led to examination of the potential utility of evaluating CCN2 concentrations in highly accessible fluids such as serum or plasma for non-invasive diagnostic evaluation of the extent or severity of various fibrotic pathologies. Thus, serum levels of CCN2 have been shown to be correlated with the extent of systemic skin sclerosis and severity of pulmonary fibrosis in human subjects[23] and to serve as a biomarker of progressive kidney fibrosis in chronic allograft nephropathy in a clinical and experimental study[24]. Studies on circulating CCN2 levels in assessment of hepatic fibrosis have just begun to gain momentum and are founded on the well documented over-expression of CCN2 in fibrotic livers due to its production by multiple cell types (including HSC, hepatocytes, biliary epithelial cells). An early study reported as association between elevated CCN2 serum levels and progression of hepatic fibrosis in biliary atresia[25] while a more recent investigation demonstrated significantly elevated serum levels of CCN2 in patients with chronic hepatitis and cirrhosis that were well correlated with the progression of hepatic fibrosis[26-28]. In the present research, we found that increased CCN2 concentrations were present in the serum of patients with CHB and HBV-induced cirrhosis. Serum CCN2 levels were consistent with those in liver tissue and were strongly correlated with the stage of hepatic fibrosis. Taken together, our data indicate that CCN2 is a potential valuable biomarker of HBV-induced hepatic fibrosis, and further support the classification of CCN2 as class I fibrosis biomarker, defined as one that is derived from changes of the fibrogenic cell types and which reflects the activity of the fibrogenic and/or fibrolytic process[29].

Finding the best method to evaluate and diagnose the stage of liver fibrosis continues to be a challenge[30]. Although liver biopsy is a gold-standard procedure for determining the grade of liver inflammation and stage of fibrosis[30-33], there are well recognized difficulties including complications, high hospital expenses[30,34], false sample recording[35], contra-indications during the procedure, and dependence on the pathologists' skills in examining samples. Serum fibrosis tests with AUCs ranging from 0.85 to 0.90 have been proposed as good biochemical markers with high diagnostic value[36,37]. In our research, serum CCN2 was valuable not only in distinguishing normal liver controls from patients with F1 stage liver fibro-

sis but also in distinguishing between mild and significant liver fibrosis. We therefore propose that further studies are warranted to further evaluate the potential utility of serum CCN2 as a biomarker of liver fibrosis in HBV-induced CLD-B.

COMMENTS

Background
Millions of individuals around the world are infected with hepatitis B virus (HBV), resulting in chronic liver disease. In many cases, affected individuals suffer from hepatic fibrosis, a highly debilitating pathology in which the normal cellular architecture and function in the liver are severely compromised through the deposition of collagen and other insoluble extracellular matrix molecules. This process is driven by connective tissue growth factor (CCN2) which is known to be produced at high levels in fibrotic livers and which acts to drive fibrogenic pathways in hepatic stellate cells (HSCs), a principal fibrotic cell type in the liver.

Research frontiers
Currently, parameters used to assess liver fibrosis are inaccurate. There is optimism that measurement of CCN2 levels in either the livers or serum of affected patients will have useful diagnostic or prognostic value.

Innovations and breakthroughs
To date, there have been a limited number of studies regarding the value of serum CCN2 for assessment of hepatic fibrosis. In this study, the authors employed more systemic detection techniques to evaluate the relationship among serum CCN2 levels, hepatic CCN2 content and liver fibrosis severity in patients with chronic liver diseases. Furthermore, the authors described the expression characteristics of CCN2 in liver tissues and its role and mechanism in HBV-induced hepatic fibrosis.

Applications
These studies suggest that serum CCN2 concentrations are a reliable diagnostic indicator of HBV-induced liver fibrosis and that CCN2 can be used a part of the platform for evaluation of the severity of liver fibrosis.

Terminology
CCN2: a pro-fibrogenic molecule that is over-expressed in many fibrotic diseases and which stimulates collagen synthesis in HSC.

Peer review
This is an interesting and important issue in the utility of CCN2 for assessing hepatic fibrosis. Correlations of the serum levels of CCN2 in HBV infected patients with hepatic fibrosis have been well documented in literature.

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CASE REPORT

Mycophenolate mofetil for maintenance of remission in steroid-dependent autoimmune pancreatitis

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Abstract

Systemic corticosteroids represent the standard treatment for autoimmune pancreatitis with IgG4-associated cholangitis. For steroid-dependent disease, azathioprine has been used for maintenance of remission. Mycophenolate mofetil has been used for transplant immunosuppression and more recently for autoimmune hepatitis; however, there are no case reports to date on the use of mycophenolate mofetil in adult patients with autoimmune pancreatitis. A patient with IgG4-mediated autoimmune pancreatitis and IgG4-associated cholangitis refractory to steroids and intolerant of azathioprine was treated with mycophenolate mofetil, which inhibits de novo guanosine synthesis and blockade of both B and T lymphocyte production. Introduction of mycophenolate mofetil and uptitration to 1000 mg by mouth twice daily over a treatment period of 4 mo was associated with improvement in the patient's energy level and blood glucose control and was not associated with any adverse events. The patient was managed without a biliary stent. However, there was a return of symptoms, jaundice, increase in transaminases, and hyperbilirubinemia when the prednisone dose reached 11 mg per day. In the first report of mycophenolate mofetil use in an adult patient with IgG4-associated autoimmune pancreatitis and IgG4-associated cholangitis, the introduction of mycophenolate mofetil was safe and well-tolerated without adverse events, but it did not enable discontinuation of the steroids. Mycophenolate mofetil and other immunomodulatory therapies should continue to be studied for maintenance of remission in the large subset of patients with refractory or recurrent autoimmune pancreatitis.

Sodikoff JB, Keilin SA, Cai Q, Bharmal SJ, Lewis MM, Raju GS, Willingham FF. Mycophenolate mofetil for maintenance of remission in steroid-dependent autoimmune pancreatitis. *World J Gastroenterol* 2012; 18(18): 2287-2290 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i18/2287.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i18.2287

INTRODUCTION

Autoimmune pancreatitis (AIP) represents a chronic dis-

ease of the pancreas with a presumed autoimmune etiology. There are characteristic serologic, morphologic, clinical and pathologic features, specifically, lymphoplasmacytic infiltration with parenchymal fibrosis. Some patients develop comorbid involvement of the biliary tract characterized by steroid responsive stricturing, a condition that has been termed IgG4-associated cholangitis (IAC). Steroid therapy is the first-line and mainstay treatment for AIP but relapse is frequent on steroid withdrawal. Azathioprine has been used for patients who fail steroid tapering, but is not tolerated by some patients and may be contraindicated in patients with deficient thiopurine methyltransferase (TPMT) activity. The use of mycophenolate mofetil as a possible treatment alternative for autoimmune pancreatitis with IAC has been described once in the pediatric population[1] and has been mentioned in discussions of AIP[2,3]. However, there have been no case reports to date in an adult population. We describe a 65-year-old man with a history of AIP and IAC who failed multiple attempts at steroid tapering and was intolerant of azathioprine. This is the first case report of the use of mycophenolate mofetil in an adult patient with IgG4-mediated AIP and IAC.

CASE REPORT

A 65-year-old Caucasian male presented to another medical center for a routine colonoscopy. A chest radiograph obtained prior to the procedure revealed multiple nodules in the lower lung fields with pleural scarring and a small pleural effusion. A subsequent computed tomography (CT) scan of the chest revealed multiple pulmonary nodules in the right lower lobe, right lateral middle lobe, and left middle lobe, as well as intrathoracic lymphadenopathy. Because of concerns of malignancy, bronchoalveolar lavage was performed, which revealed infection with *Aspergillosis fumigatus*. The patient began treatment with micofungin.

At the time of bronchoscopy, the patient was also noted to be jaundiced. Laboratory findings revealed an elevated serum total bilirubin level of 4.8 mg/dL and an elevated conjugated bilirubin level of 4 mg/dL. Serum lipase was 16 U/L and serum amylase was 40 U/L, both within normal limits. CT scan of the abdomen revealed diffuse enlargement of the pancreas, evidence of cholelithiasis, as well as biliary ductal dilatation. An upper endoscopic ultrasound with fine-needle aspiration of the pancreas revealed ductal epithelium with fibrosis, inflammatory cells, and debris and no evidence of malignancy, suggestive of autoimmune pancreatitis. Endoscopic retrograde cholangiopancreatography (ERCP) revealed a stricture in the intra-pancreatic portion of the main bile duct, for which the patient underwent stent placement. Biliary ductal brushings revealed no malignant cells. The serum IgG4 level was elevated at 213 mg/dL. Treatment with a tapering course of prednisone for 6 weeks was initiated, with significant improvement in symptoms, reduction in the size of the pancreas on cross sectional imaging, and normalization of the liver enzymes.

After 3 mo, the patient presented to the same hospital emergency department with a relapse of jaundice, steatorrhea, and chills. The total bilirubin was 6 mg/dL, direct bilirubin was 5.2 mg/dL, aspartate aminotransferase (AST) was 122 U/L, alkaline phosphatase (ALP) was 557 U/L, and lactate dehydrogenase was 369 mg/dL. Ultrasound of the gallbladder showed development of mild intrahepatic biliary dilatation, possibly representing stent malfunction. CT of the abdomen revealed significant interval increase in the size of the pancreas. ERCP was performed with exchange of the common bile duct stent. Cholangiography revealed diffuse irregular stricturing of the common bile duct to the hilum of the liver. The patient was restarted on prednisone 40 mg daily. There was symptomatic and clinical improvement.

The patient was referred to our medical center to receive care closer to his home. He was taking 30 mg prednisone daily on presentation and reported feeling well. The biliary stent was extracted. His serum IgG4 level was elevated at 120 mg/dL. The other autoimmune work-up was negative: Anti-nuclear antibody IgG was not detected, Anti-neutrophil cytoplasmic antibody IgG was less than 1:20, f-actin antibody IgG was 15 units (negative), anti-mitochondrial M2 antibody IgG was 0.9 units (negative), and liver kidney microsomal antibody IgG was less than 1:20 (negative). Two months later, the patient presented once again with recurrent jaundice, fatigue, and discomfort. By this time, he had been tapered to a dose of 15 mg prednisone daily and had completed micafungin treatment for his pulmonary aspergillosis. Magnetic resonance imaging (MRI) of the abdomen with and without gadolinium revealed a diminished T1 signal and abnormal delayed enhancement in the pancreas. There was no pancreatic ductal dilatation, and the overall morphology was normal to mildly enlarged. The T2 signal was mildly elevated in the pancreatic parenchyma. The impression was of changes consistent with autoimmune pancreatitis. The MRI also revealed diffuse intrahepatic biliary ductal dilatation and intrahepatic ductal stenosis as well as periportal arterial blush enhancement and delayed uptake of contrast, consistent with active cholangitis. ERCP showed a single severe 18 mm stricture in the distal common bile duct with irregular stricturing of the bilateral intrahepatic ducts (Figure 1). A stent was placed in the bile duct. Biopsy of the pancreatic ampulla demonstrated chronic active inflammation with approximately 20-25 IgG4-containing plasma cells/high power field, consistent with the clinical impression of autoimmune pancreatitis (Figure 2). The patient was maintained on 15 mg prednisone and was started on 50 mg azathioprine daily in an attempt to transition to a steroid-sparing immunosuppressive regimen. However, he was unable to tolerate azathioprine due to debilitating nausea, fatigue, lethargy and diarrhea despite dose reduction, evening administration, and concurrent antiemetic therapy.

Over the next 6 mo, the patient underwent 2 additional biliary stent exchanges at 3-mo intervals. Brush specimens obtained from the common bile duct stricture were

Figure 1 Cholangiogram obtained during endoscopic retrograde cholangiopancreatography demonstrating a marked 18 mm stricture in the distal common bile duct with diffuse irregular stricturing of the intrahepatic ducts bilaterally.

negative for malignant cells by cytology and fluorescence *in situ* hybridization analysis. The patient was maintained on 15 mg prednisone daily. Mycophenolate mofetil was discussed as an alternative immunomodulatory treatment and an alternative to long-term prednisone. The patient was initiated on mycophenolate mofetil at a dose of 750 mg twice daily.

The patient tolerated the mycophenolate mofetil without side effects. At this point, he was being maintained on mycophenolate mofetil and prednisone. After 3 mo on mycophenolate mofetil and prednisone, he had no jaundice or steatorrhea. His hyperglycemia was mild and his diabetes medications were being tapered, likely a result of the lower dose of prednisone required. He required 3 half-tablets of glipizide 5 mg per month to maintain normal serum glucose values. The serum AST level was 30 U/L, ALP level was 101 U/L, total bilirubin level was 0.7 mg/dL, albumin was 3.4 mg/dL, amylase was 43 U/L and lipase was 16 U/L. At the time of his last scheduled stent exchange, the stent had passed spontaneously and no procedure was performed. However, over this 3-mo period, he had several self-limited episodes of fatigue, malaise, and pruritus lasting 24 to 48 h, associated with transient elevations in his liver enzymes. He remained without a biliary stent and felt well overall. Given this course, the mycophenolate mofetil dosage was increased to 1000 mg twice daily and a steroid taper was again resumed, decreasing the dose by 1 mg per week from an initial dose of 15 mg prednisone daily. When the patient reached 10 mg prednisone daily, he experienced a recurrence of nausea and abdominal pain, as well as darkened urine. His total bilirubin was elevated to 9.5 mg/dL, AST 146 IU/L, and ALT 266 IU/L. His prednisone dosage was increased back to 15 mg per day and his symptoms again resolved. He is presently maintained on 15 mg prednisone per day and the mycophenolate mofetil is being tapered due to an inability to stop systemic corticosteroids. He is able to continue an active lifestyle working 12-14 h days on his farm. He is being considered for alternative immunomodulatory therapy.

Figure 2 Histopathologic images demonstrating the findings from the patient's ampullary biopsy. A: A hematoxylin eosin stained biopsy at 20 × magnification revealed chronic active inflammation; B: An IgG4 stain revealed approximately 20-25 IgG4-containing plasma cells/high power field, consistent with autoimmune pancreatitis.

DISCUSSION

Systemic corticosteroid therapy is the standard treatment for AIP with IAC[3]. AIP and IAC likely represent organ-specific manifestations of a broader systemic disease described as IgG4-related sclerosing disease. The disease process involves deposition of IgG4 antibodies into various tissues, causing fibrosis and organ dysfunction. Other proposed manifestations of IgG4-associated disease include Sjögren's syndrome, primary sclerosing cholangitis, and retroperitoneal fibrosis[4]. There is a strong association between AIP and IAC, as was seen in our patient[5].

Although the response to corticosteroids is a defining feature of AIP, representing one of the 5 diagnostic criteria (histology, imaging, serology, other organ involvement and response to therapy) for the disease[6], relapse of biliary strictures after steroid withdrawal in cases of IAC is not uncommon. In one recent study of 53 patients with IAC, 54% of patients experienced relapse after steroid withdrawal[3]. In such cases, alternative treatment with immunomodulating medications such as azathioprine and 6-mercaptopurine has been proposed to avoid long-term steroid use and its associated consequences[3]. However, azathioprine and 6-mercaptopurine are not tolerated by all patients. In addition, reduced TPMT activity may be found in approximately 11% of the population, and undetectable enzyme activity in 0.3% of the population[7]. Patients with insufficient TMPT activity may develop

bone marrow toxicity and myelosuppression[8]. In addition, while azathioprine and 6-mercaptopurine may be tolerated, not all patients started on these medications will be able to taper their steroid therapy. For these reasons, alternatives are being examined. For example, a patient refractory to steroid taper and 6-mercaptopurine was successfully managed with rituximab, experiencing cholangiographic improvement, normalization of liver enzymes, and resolution of extrapancreatic manifestations of IgG4 disease[2]. However, rituximab requires infusional therapy, is not indicated for AIP, and has a high cost, which limits its use in this setting.

There have been 2 references to mycophenolate mofetil as a potential alternative treatment in refractory cases of autoimmune pancreatitis[2,3]; however, there are no case reports in the adult populations to date. Mycophenolate mofetil acts as an inhibitor of inosine monophosphate dehydrogenase, which inhibits *de novo* guanosine nucleotide synthesis. Through this mechanism, mycophenolate mofetil exhibits a cytostatic effect on T and B lymphocytes by blocking proliferation[9]. Mycophenolate mofetil has been used in transplantation medicine, inflammatory bowel disease, and rheumatoid arthritis, often as a second-line immunosuppressant. More recently, mycophenolate mofetil use has been described in cases of refractory autoimmune hepatitis[10].

In the case described herein, the steroid taper was not successful with the use of mycophenolate mofetil and the patient had recurrence of symptoms, jaundice and elevated liver enzymes; however, the medication may be efficacious in a subset of patients with refractory disease, and we believe that this merits further investigation in larger studies.

In summary, this is the first case report of mycophenolate mofetil for AIP and IAC. The addition of mycophenolate mofetil to the patient's therapy was well-tolerated but did not permit steroid tapering below 11 mg prednisone per day. The patient may have experienced an improvement in energy and in remission of mild diabetes while on mycophenolate mofetil, possibly because the treatment allowed for a lower dose of prednisone.

However, the decision was made to taper and ultimately discontinue the mycophenolate mofetil due to failure to enable cessation of systemic corticosteroids. We believe that mycophenolate mofetil should continue to be examined for use in patients with steroid-dependent AIP and/or IAC, especially in the subset for whom azathioprine and 6-mercaptopurine are ineffective or contraindicated. Additional immunomodulatory therapies are needed for maintenance of remission in the large cohort of AIP and IAC patients who fail steroid tapering.

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CASE REPORT

Hepatic artery pseudoaneurysm caused by acute idiopathic pancreatitis

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Abstract

Hepatic artery pseudoaneurysm (HAP) is a very rare disease but in cases of complication, there is a very high mortality. The most common cause of HAP is iatrogenic trauma such as liver biopsy, transhepatic biliary drainage, cholecystectomy and hepatectomy. HAP may also occur with complications such as infections or inflammation associated with septic emboli. HAP has been reported rarely in patients with acute pancreatitis. As far as we are aware, there is no report of a case caused by acute idiopathic pancreatitis, particularly. We report a case of HAP caused by acute idiopathic pancreatitis which developed in a 61-year-old woman. The woman initially presented with acute pancreatitis due to unknown cause. After conservative management, her symptoms seemed to have improved. But eight days after admission, abdominal pain abruptly became worse again. Abdominal computed tomography (CT) was rechecked and it detected a new HAP that was not seen in a previous abdominal CT. Endoscopic retrograde cholangiopancreatography (ERCP) was per-

formed because of a suspicion of hemobilia as a cause of aggravated abdominal pain. ERCP confirmed hemobilia by observing fresh blood clots at the opening of the ampulla and several filling defects in the distal common bile duct on cholangiogram. Without any particular treatment such as embolization or surgical ligation, HAP thrombosed spontaneously. Three months after discharge, abdominal CT demonstrated that HAP in the left lateral segment had disappeared.

Yu YH, Sohn JH, Kim TY, Jeong JY, Han DS, Jeon YC, Kim MY. Hepatic artery pseudoaneurysm caused by acute idiopathic pancreatitis. *World J Gastroenterol* 2012; 18(18): 2291-2294 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i18/2291.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i18.2291

INTRODUCTION

Hepatic artery pseudoaneurysm (HAP) is rare[1,2]. But rupture is common and occurs in 76% of patients with HAP. There are many causes of HAP, but it usually results from procedures such as liver biopsy, transhepatic biliary drainage, cholecystectomy, and hepatectomy[3]. HAP may also occur with complications such as infections or inflammation associated with septic emboli[4-7]. HAP has rarely been reported in patients with acute pancreatitis[8,9]. As far as we are aware, there is no report of a case caused by acute idiopathic pancreatitis. We report a case of HAP thought to be caused by acute pancreatitis due to unknown cause, in a 61-year-old Korean woman.

HAP spontaneously thrombosed without any particular treatment and finally disappeared several months later. We also review the literature concerning HAP.

CASE REPORT

A 61-year-old Korean woman was admitted to the emergency room with right upper quadrant pain and vomiting starting 8 h before admission. She was previously well-nourished, and had no past medical history. The patient denied a history of percutaneous intervention, abdominal surgery, trauma, viral hepatitis, bleeding disorders, jaundice or blood transfusion. On admission, her blood pressure was 120/80 mmHg, pulse rate was 78/min, and body temperature was 36.8 ℃. On examination, her appearance was that of acute illness and her tongue was mildly dehydrated. But she was clinically non-anemic and non-icteric. Abdominal examination revealed right upper quadrant tenderness without rebound and hepatomegaly. Rectal examination showed no melena.

Laboratory data on admission were as follows: white blood cell count 14 900/μL with 90.5% increase in neutrophils; hemoglobin 13.4 g/dL; hematocrit 39.0%; platelet count 226 000/μL; prothrombin time 15.0 [international normalized ratio (INR) 1.27]; serum total protein 7.0 g/dL; albumin 4.4 g/dL; alkaline phosphatase 103 U/L; total bilirubin 1.0 g/dL; Alanine aminotransferase (ALT) 87 U/L; aspartate aminotransferase (AST) 56 U/L; gamma-glutamyl transferase 46 U/L. Amylase (1167 U/L) and lipase (2630 U/L) were increased. Abdominal computed tomography (CT) demonstrated infiltration with swelling in an uncinate process of the pancreas head and infiltration and mural enhancement in adjacent duodenum, described as grade C according to Balthazar classification (Figure 1).

After conservative management, her symptoms were improved but eight days after admission, her abdominal pain abruptly deteriorated again. Laboratory data were as follows: hemoglobin 12.2 g/dL; hematocrit 35.7%; white blood cell count 9200/μL with 69.2% neutrophils; platelet count 118 000/μL; prothrombin time 14.1 s (INR 1.20); alkaline phosphatase 83 U/L; total bilirubin 0.6 g/dL; ALT 13 U/L; AST 22 U/L; gamma-glutamyl transferase 33 U/L. Amylase (69 U/L) and lipase (153 U/L) were within normal ranges. On a second check-up with abdominal CT, the infiltration and swelling of the pancreas head was improved and there was no stone in the biliary tract. But it showed a newly developed hepatic artery pseudoaneurysm in the left lateral segment and no evidence of acute bleeding related to it as a complication (Figure 2). We performed endoscopic retrograde cholangiopancreatography (ERCP) in order to find the cause of aggravated pain with suspicion of hemobilia. Several filling defects with labile and indeterminate shapes were observed on cholangiogram and, after sphincterotomy, these findings were confirmed to be caused by fresh blood clots without a stone, and blood clots were removed with a balloon catheter and basket (Figure 3). Emergent

Figure 1 Abdominal computed tomography scans showed diffuse swelling and infiltration in head, body (A) and tail (B) of pancreas without obvious stone, indicating acute pancreatitis.

Figure 2 Follow up of abdominal computed tomography scan (A) due to abruptly aggravated abdominal pain and reconstructed computed tomography angiogram (B) showed newly developed hepatic artery pseudoaneurysm in the left lateral segment of liver (white arrows).

Figure 3 Cholangiogram by endoscopic retrograde cholangiopancrea-tography showed several amorphous filling defects in the common bile duct (A) and lots of blood clots were seen and removed by basket after endoscopic sphincterotomy (B).

Figure 5 Follow-up of abdominal computed tomography scans 3 mo later showed that both previously noted hepatic artery pseudoaneurysm in the left lateral segment (A) and diffuse swelling and infiltration of pancreas (B) had disappeared.

Figure 4 Magnetic resonance imaging scans of the abdomen revealed that hepatic artery pseudoaneurysm was replaced by thrombus formation (white arrows) in the left lateral segment in-phase (A) and out-of-phase (B) T1-weighted images and T2-weighted image (C).

angiography was not performed due to no further acute bleeding evidence.

After ERCP, the patient's pain improved and filling defects caused by blood clots of the extrahepatic duct were not observed on endoscopic nasobiliary drainage cholangiogram. Magnetic resonance imaging showed that dilatation or filling defects of the bile duct disappeared and bleeding of proximal HAP was replaced by throm-bus formation (Figure 4). Two days later, angiography re-vealed no visible contrast leakage and definite HAP. Her symptoms resolved rapidly, and she was discharged on the 16th day after clot removal.

Three months after discharge, abdominal CT dem-onstrated that minimal bile duct dilatation was noted but HAP in the left lateral segment had disappeared and pan-creatitis improved (Figure 5).

DISCUSSION

HAP is mainly caused by acute or chronic artery injuries such as blunt or penetrating injuries and interventional radiological procedures[10]. A minority of their formation may occur as a result of bile duct damage usually associated with stone impaction or procedure-related infection[4-6]. Unlike these reports, this case suggests that HAP occurred due to no invasive procedure and infection but acute pancreatitis. In this case, acute pancreatitis due to unknown cause may have eroded the arterial wall and led to pseudoaneurysm formation and then HAP's rupture causing hemobilia. The mechanism of pseudo-aneurysm formation in pancreatitis is thought to be due to autodigestion of pancreatic enzymes. Among HAP caused by pancreatitis, most cases have been reported to be due to chronic pancreatitis, but HAP caused by acute pancreatitis has only rarely been reported[11]. Furthermore, among reports of HAP due to acute pancreatitis, acute idiopathic pancreatitis causing HAP has not been reported so far.

In this case, it can be presumed that a rupture of preexisting HAP was not detected on initial abdominal CT imaging, resulting in hemobilia and eventually, acute pancreatitis was complicated by hemobilia. However, considering the fact the patient had normal levels of hemoglobin and no bile duct dilatation or filling defects when visiting hospital and the clinical course worsened (e.g., pain) despite the improvement of acute pancreatitis during the treatment, this possibility is very low. She was hospitalized for 8 d without any evidence of this procedure, and hemobilia-induced abdominal pain developed without aggravation of acute pancreatitis. This makes the scenario of pancreatitis-induced HAP more reasonable.

HAP is a rare and potentially fatal disease if it ruptures. In most cases, conservative management is not recommended due to the high rupture rate. Treatment comprises reconstructive surgery, or ligation depending on the size of the lesion and its location in the past. Today, the treatment of choice is selective transcatheter embolization[10,12,13]. However, in very limited cases, which means, unless HAP is not at risk of immediate rupture because of progressively enlarging size or instability, it can be managed by closed medical observation and imaging follow-up with appropriate treatment of the associated infection[14]. In this case, there was only conservative management of acute pancreatitis which is thought to be a cause of HAP. But we could confirm that HAP spontaneously thrombosed without any particular treatment at the image study. Three months after discharge, abdominal CT demonstrated that HAP in the left lateral segment had disappeared.

In summary, we report a rare case of a 61-year-old Korean woman who had HAP caused by acute idiopathic pancreatitis. In this case, HAP spontaneously thrombosed and then disappeared after recovery of acute pancreatitis. This case alerts clinicians that acute pancreatitis can be considered as a potential cause of HAP.

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FRONTIER

Pathogenesis of NSAID-induced gastric damage: Importance of cyclooxygenase inhibition and gastric hypermotility

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Abstract

This article reviews the pathogenic mechanism of non-steroidal anti-inflammatory drug (NSAID)-induced gastric damage, focusing on the relation between cyclooxygenase (COX) inhibition and various functional events. NSAIDs, such as indomethacin, at a dose that inhibits prostaglandin (PG) production, enhance gastric motility, resulting in an increase in mucosal permeability, neutrophil infiltration and oxyradical production, and eventually producing gastric lesions. These lesions are prevented by pretreatment with PGE_2 and antisecretory drugs, and also *via* an atropine-sensitive mechanism, not related to antisecretory action. Although neither rofecoxib (a selective COX-2 inhibitor) nor SC-560 (a selective COX-1 inhibitor) alone damages the stomach, the combined administration of these drugs provokes gastric lesions. SC-560, but not rofecoxib, decreases prostaglandin E_2 (PGE_2) production and causes gastric hypermotility and an increase in mucosal permeability. COX-2 mRNA is expressed in the stomach after administration of indomethacin and SC-560 but not rofecoxib. The up-regulation of indomethacin-induced COX-2 expression is prevented by atropine at a dose that inhibits gastric hypermotility. In addition, selective COX-2 inhibitors have deleterious influences on the stomach when COX-2 is overexpressed under various conditions, including adrenalectomy, arthritis, and *Helicobacter pylori*-infection. In summary, gastric hypermotility plays a primary role in the pathogenesis of NSAID-induced gastric damage, and the response, causally related with PG deficiency due to COX-1 inhibition, occurs prior to other pathogenic events such as increased mucosal permeability; and the ulcerogenic properties of NSAIDs require the inhibition of both COX-1 and COX-2, the inhibition of COX-1 upregulates COX-2 expression in association with gastric hypermotility, and PGs produced by COX-2 counteract the deleterious effect of COX-1 inhibition.

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used to treat inflammatory pain. A major limitation to their use, however, is the adverse reaction they cause to the gastrointestinal (GI) tract, including the formation of gastric lesions, the potentiation of ulcerogenic responses to stress, and the impairment of gastric ulcer healing[1-4]. Concerning the mechanism of NSAID-induced gastric damage, prostaglandin (PG) deficiency is

of prime importance to the gastric ulcerogenic response to NSAIDs, yet it has proven to be more complicated than expected and involves multiple, closely interacting elements, including hypermotility, neutrophils, free radicals, and so on[1,5-13].

The PG deficiency caused by NSAIDs is due to the inhibition of cyclooxygenase (COX). COX exists in two isozymes, COX-1 and COX-2; the former is constitutively expressed in various tissues, including the stomach, while the latter appears to be expressed in most tissues in response to growth factors and cytokines[14,15]. This tissue specificity of the COX isozymes has led to the idea that COX-1 is critical for housekeeping actions in the GI mucosa, whereas COX-2 functions under pathological conditions such as inflammation. Indeed, it has been reported that the gastric ulcerogenic properties of NSAIDs are due to the inhibition of COX-1, but not COX-2[16]. However, studies using selective COX-1 and COX-2 inhibitors demonstrated that the GI ulcerogenic effects of NSAIDs are not accounted for solely by inhibition of COX-1, but require inhibition of COX-2 as well[17-21]. It has also been shown that inhibition of COX-1 upregulated COX-2 expression in the GI mucosa, and PGs produced by COX-2 may help maintain the mucosal integrity when there is a deficiency of PGs due to COX-1 inhibition[18,20,21]. This idea was supported by the finding that the selective COX-2 inhibitor by itself damaged the gastric mucosa when the expression of COX-2 was upregulated in the stomach of rats subjected to adrenalectomy (glucocorticoid deficiency) or induction of adjuvant arthritis or *Helicobacter pylori* (*H. pylori*) infection[22-24].

In this article, we reviewed the pathogenesis of NSAID-induced gastric damage, mainly based on our own publications, including the roles of functional events, particularly, gastric hypermotility, as well as the influences of arthritis and *H. pylori* infection, and discussed the relation between COX-1 or COX-2 inhibition and pathogenic elements such as gastric motility and neutrophil infiltration.

GENERAL ASPECTS OF NSAID-INDUCED GASTRIC DAMAGE

Relation to PG deficiency

There is no doubt that a deficiency of endogenous PG is a background factor in NSAID-induced gastric ulceration. Indeed, when various NSAIDs, such as indomethacin (30 mg/kg), flurbiprofen (20 mg/kg), naproxen (40 mg/kg), dicrofenac (40 mg/kg) and aspirin (200 mg/kg), were administered to rats subcutaneously, all of these agents, except aspirin, produced damage in the stomach at doses that significantly decreased the mucosal prostaglandin E2 (**PGE2**) concentration[18,19] (Figure 1). Characteristically, the damage was observed along the long axis of the stomach and consisted mostly of hemorrhagic lesions, with a few non-hemorrhagic lesions. Interestingly, aspirin given subcutaneously did not produce any damage, despite inhibiting PG production as effectively as other NSAIDs[18,25]. Notwithstanding, it is assumed that PG deficiency is causally related to the gastric ulcerogenic

Figure 1 Gastric ulcerogenic responses (A) and changes in mucosal prostaglandin E2 content (B) induced by various non-steroidal anti-inflammatory drugs in rat stomach. The animals were given indomethacin (30 mg/kg), aspirin (200 mg/kg), naproxen (40 mg/kg), flurbiprofen (20 mg/kg) and diclofenac (40 mg/kg) s.c., and killed 4 h later. Data are presented as the mean ± SE in 5 rats. [a]$P <$ 0.05 *vs* control (data from ref. 18 after modification).

action of NSAIDs, but this factor alone is not sufficient for the development of gastric lesions. The reason why parenterally administered aspirin does not cause gastric damage will be discussed in another section of this article.

Effect of various drugs

The development of gastric lesions in response to indomethacin was inhibited by prior administration of PGE2. These lesions were also prevented by antisecretory drugs such as cimetidine, omeprazole and atropine[6,26,27], confirming the importance of luminal acid in the pathogenesis of these lesions. Of interest, since atropine was effective even when 150 mmol of HCl was applied to the lumen, it is assumed that this protective action is not associated with the antisecretory effect and initiated by factors other than inhibition of acid secretion (Figure 2). Neither cimetidine nor omeprazole was effective against indomethacin-induced gastric damage in the presence of exogenous acid. In addition, anti-neutrophil antiserum also reduced the severity of these lesions, but much less effectively than other agents[28]. Pretreatment with both

Figure 2 Effects of atropine, cimetidine and omeprazole on gastric lesions produced by indomethacin in rats. The animals were given indomethacin (30 mg/kg, s.c.) and killed 4 h later. Omeprazole (30 mg/kg), cimetidine (100 mg/kg) and atropine (3 mg/kg, s.c.) were given 1 h before indomethacin. In some cases, the animals were given 1 mL of 100 mmol HCl p.o. immediately after the administration of indomethacin. Data are presented as the mean ± SE for 5-8 rats. [a]$P < 0.05$ vs vehicle (data from refs. 6, 26 and 27 after modification).

atropine and dmPGE2 significantly inhibited the development of gastric lesions at all time points during a 4 h-test period following administration of indomethacin. By contrast, the anti-neutrophil antiserum did not affect the onset but significantly reduced the severity of lesions at 4 h after indomethacin treatment. It is assumed that the gastric ulcerogenic response to indomethacin is prevented by supplementation with PGE2 and inhibition of acid secretion as well as an atropine-sensitive mechanism, not related to the antisecretory action. Neutrophils do not play a role in the onset of these lesions but may be involved in the later extension of the damage. Suematsu et al[29] recently reported that the severity of gastric lesions produced by indomethacin was worse in mice lacking heat shock factor 1 (HSF1), a transcription factor for *HSP* genes, than in control mice, while these lesions were ameliorated in transgenic mice expressing HSP70. They suggested that expression of HSP70 ameliorates indomethacin-induced gastric damage by affecting mucosal apoptosis, probably *via* the activation of Bax.

Functional alterations involved in pathogenesis
Gastric hypermotility: Mersereau et al[7] first emphasized the importance of stomach hypermotility and mucosal foldings in the genesis of gastric lesions in response to phenylbutazone. As expected, all NSAIDs, except aspirin, increased gastric motility at ulcerogenic doses, leading to the development of gastric lesions[19] (Figure 3). Gastric hypermotility causes microvascular disturbances, especially at specific sites on mucosal foldings, leading to various events including neutrophil-endothelial interaction. Garrick et al[30] reported that high-amplitude contractions during cold-restraint stress resulted in a temporal restriction of mucosal blood flow and lowered the mucosal resistance to injury. The gastric damage induced by indomethacin occurred linearly along the long axis of the

stomach, and microscopically, was seen at the top or the bottom of mucosal foldings, the sites most influenced by mucosal compression due to contraction of the stomach, where mucosal blood flow is restricted, leading to microvascular disturbances (Figure 4A and B). The inhibition of gastric motility may lead to a flattening of the mucosal foldings and a decrease in microvascular disturbances, resulting in prevention of the fold-related band-like lesions, as observed after the administration of indomethacin[6,10,26,31]. A role for muscle elements in the pathogenic mechanism of gastric ulceration has been demonstrated[6,10,31-33]. Yamaguchi et al[32] monitored gastric mucosal hemodynamics and motility simultaneously and found oscillatory changes in the hemodynamics during gastric hypermotility induced by water-immersion stress. We also found that indomethacin caused oscillatory changes in mucosal blood flow associated with hypermotility of the stomach, and such blood flow changes were prevented when the hypermotility was inhibited by atropine[10] (Figure 4C). It is assumed that indomethacin induces the sequential events in the early stage of lesion formation in the stomach during hypermotility; the microcirculatory disturbances due to abnormal compression of the gastric wall, followed by increased vascular permeability, leading to cellular damage[10,33]. Anyway, the indomethacin-induced gastric hypermotility was inhibited by both atropine and PGE2 but not by either omeprazole or the anti-neutrophil antiserum[6,10]. Since atropine prevented indomethacin-induced gastric damage, even in the presence of exogenous acid[26], the inhibitory effect on gastric hypermotility may account for the protective action of this agent. In addition, indomethacin caused oxyradical production and lipid peroxidation in the gastric mucosa, probably resulting from the ischemic-reperfusion changes due to rhythmic hypercontraction of the stomach[10]. Certainly, these changes were prevented by atropine, again confirming an importance of gastric hypermotility. At present, the exact mechanism by which NSAIDs cause gastric hypermotility remains unknown. However, it is assumed that indomethacin-induced gastric hypermotility is mediated by a vagal-cholinergic mechanism, involving a glycoprivic response[6,31].

Neutrophils: Neutrophils have been implicated in the damage associated with NSAIDs[35]. These cells are recruited to a site of injury by chemotaxins and participate in amplifying the inflammatory response. Many studies including ours have shown that indomethacin-induced gastric damage could be prevented by an anti-neutrophil antiserum or monoclonal antibody against the CD18 adhesion molecule[13,34,35]. However, there have been few studies showing the less importance of neutrophils in NSAID-induced gastric damage[36,37]. Trevethick et al[36] reported that neutrophil infiltration does not contribute to the ulcerogenic effects of indomethacin in the rat gastric mucosa. Similarly, Melange et al[37] showed that neutropenia does not prevent indomethacin-induced gastrointestinal damage in rats. Santucci et al[38] even showed that

Figure 3 Representative recordings showing the effects of various non-steroidal anti-inflammatory drug on gastric motility in rats. Indomethacin (35 g/kg), aspirin (200 mg/kg), naproxen (40 mg/kg), flurbiprofen (20 mg/kg) or diclofenac (40 mg/kg) was given s.c. after basal motility had stabilized (data from ref.18 after modification).

Figure 4 Macro- and microscopical observations of gastric lesions induced by indomethacin in rats (A and B) and simultaneous recordings of gastric motility and mucosal blood flow in the rat before and after administration of indomethacin (C). A, B: The animals were given indomethacin (25 mg/kg, s.c.), and the stomachs were excised 4 h later. Note that the lesions were located, in most cases, on the upper part of the mucosal folds (arrow) and in some cases at the base of the folds (arrows); C: Indomethacin (25 mg/kg, s.c.) was given, while atropine (1 mg/kg, s.c.) was given 1 h after indomethacin treatment. Note that during hypermotility states the mucosal blood flow repeated a decrease and an increase, respectively, corresponding to contraction and relaxation of the stomach wall (data from refs. 8 and 33 after modification). GMBF: Gastric mucosal blood flow.

Figure 5 Time-course of changes in gastric lesions following administration of indomethacin (30 mg/kg, s.c.) in rats, with or without pretreatment. Atropine (1 mg/kg) was given s.c. 30 min before indomethacin, while dmPGE₂ (10 μg/kg) or anti-neutrophil antiserum (ANS, 0.2 mL/rat) was given i.v. 10 min and 1 h, respectively, before indomethacin. Data are presented as the mean ± SE in 4-6 rats. $^aP < 0.05$ vs control group given normal serum (data from ref. 28 after modification).

Figure 6 Effects of various prostaglandin E agonists on gastric lesions generated by indomethacin in rats. The animals were given indomethacin (30 mg/kg) s.c. and killed 4 h later. Prostaglandin E₂ (PGE₂, 0.3 mg/kg), 17-phenyl PGE₂ (0.3 mg/kg; EP1 agonist), sulprostone (0.3 mg/kg; EP1/EP3 agonist), butaprost (10 mg/kg; EP2 agonist), ONO-NT-012 (10 mg/kg; EP3 agonist) and 11-deoxy PGE₁ (3 mg/kg; EP3/EP4 agonist) were given i.v. 10 min before indomethacin. Data are presented as the mean ± SE in 4-6 rats. $^aP < 0.05$ vs control (data from ref. 39 after modification).

granulocyte colony stimulating factor, though it markedly increased myeloperoxidase (MPO) activity, significantly prevented gastric lesions from forming, suggesting no relationship between MPO activity and the ulcerogenic response to indomethacin. A study by Morise et al[34] also showed that indomethacin provoked the development of gastric lesions even in CD18, intercellular adhesion molecule 1, or P-selectin-deficient mice, the degree of severity being about 70% of that in wild-type mice. We reported that the anti-neutrophil antiserum caused a significant inhibition of indomethacin- induced gastric damage, yet the degree of inhibition was much less than that shown by atropine or dmPGE₂[28] (Figure 5). Furthermore, it was shown that the anti-neutrophil antiserum did not prevent the onset of damage until 3 h after indomethacin treat-

ment and significantly reduced the severity of damage 4 h later. These results suggest that the neutralization of neutrophils itself is not sufficient to prevent the onset of damage but reduces the overall expression of gastric lesions in response to indomethacin. Anthony et al[32] examined the sequence of histological changes in the rat stomach after indomethacin treatment and identified an early phase of injury that involves mucosal contraction and vascular fibrin deposition but does not involve neutrophil infiltration. Thus, the neutrophil infiltration may be secondary to the events associated with gastric hypermotility, and not a primary event preceding the onset of gastric damage. Indeed, the increase in MPO activity as well as formation of lesions induced by indomethacin was prevented when the enhanced gastric motility was inhibited by atropine[28].

PROSTAGLANDIN E RECEPTOR SUBTYPE INVOLVED IN PGE₂-INDUCED PROTECTION

Although exogenous PGs, especially PGE₂, prevent NSAID-induced gastric damage, how they do so remains unknown. We examined the effect of various prostanoids, subtype-specific prostaglandin E (EP) agonists, on the development of gastric lesions in response to indomethacin and determined which functional alteration is most closely associated with this action[39]. Such an approach would be helpful to understanding of which event(s) may be critically important to the pathogenic mechanism of NSAID-induced gastric damage.

Gastric ulcerogenic response
PGE₂ exhibited a potent inhibitory effect on indomethacin-induced gastric damage. This effect was mimicked by other prostanoids such as 17-phenyl PGE₂ (EP1 agonist) and sulprostone (EP1/EP3 agonist)[39] (Figure 6). Neither

Figure 7 Effect of sulprostone on the increased gastric motility caused by indomethacin in rats. Indomethacin (35 mg/kg) was given s.c.. Sulprostone (Sul, 1 mg/kg) was given i.v. as a single injection 2 h after indomethacin, while ONO-AE-829 (40 mg/kg) was given s.c. 30 min before the administration of sulprostone. Data are presented as the mean ± SE of values determined every 10 min in 4-5 rats. Significant difference at $P < 0.05$; [a]from basal values in the corresponding group; [c]from saline group; [e]from indomethacin plus sulprostone (data from ref. 39 after modification).

butaprost (EP2 agonist), ONO-NT-012 (EP3 agonist), nor 11-deoxy PGE₁ (EP3/EP4 agonist), was effective in reducing the severity of these lesions, indicating that the activation of the EP2, EP3, and EP4 receptors does not provide gastric protection against indomethacin[39,40]. These results strongly suggest that the protective effect of PGE₂ against indomethacin- induced gastric damage is brought about by activation of the EP1 receptor. This idea is supported by the finding that the protective action of PGE₂ against indomethacin was totally mitigated by prior administration of ONO-AE-829, a selective EP1 receptor antagonist. In addition, indomethacin caused gastric damage similarly in both wild-type and knockout mice lacking EP1 or EP3 receptors, yet the protective action of PGE₂ was observed in wild-type and EP3-receptor knockout mice but not in mice lacking EP1 receptors. Given the above findings, it is assumed that PGE₂ prevents indomethacin-induced gastric ulceration through the activation of EP1 receptors.

Gastric functional alterations

The prostanoids exhibiting a preference for the EP1 receptors inhibited gastric hypermotility and damage in response to indomethacin (Figure 7). These effects were antagonized by ONO-AE-829, an EP1 antagonist, strongly suggesting that the antigastric motility effect of PGE is paralleled by a reduction in gross mucosal injury of the stomach with the use of indomethacin. Both butaprost and ONO-NT-012 reportedly increased gastric mucosal blood flow[41], yet these drugs did not provide any protection against indomethacin-induced gastric damage, suggesting that the protective action is not functionally associated with the increased mucosal blood flow.

Certainly, since inhibition of gastric motility may lead to an attenuation of microvascular disturbances due to contraction of the stomach, prostanoids acting through EP1 receptors may help maintain mucosal blood flow after the administration of indomethacin. It is assumed that the actions of PGE₂ to prevent indomethacin-induced gastric damage are functionally associated with the inhibition of gastric hypermotility. The mechanism by which PGE₂ inhibits gastric motility through EP1 receptors remains unknown. Milenov *et al*[42] reported that PGE₂ relaxed the circular muscle but contracted the longitudinal muscle of the canine stomach. Narumiya and his group reported the distribution of mRNA of the EP receptors along the gastrointestinal tract[43,44]. They found that strong signals for EP1 transcripts occurred in the smooth muscle cells in the muscularis mucosa throughout the tract. Since EP1 receptors are coupled to phosphatidyl inositol turnover[45], it is assumed that contraction of longitudinal smooth muscle by PGE₂ is associated with an increase of cytosolic calcium. Contraction of circular smooth muscle leads to the appearance of mucosal folds, which have been implicated in the pathogenesis of ulcers including indomethacin-generated gastric lesions[6-8,10,13]. At present, the mechanism by which PGE₂ relaxes circular smooth muscle through activation of EP1 receptors still remains unclear.

It is known that PGE₂ has an inhibitory effect on neutrophil functions, including chemotaxis[46]. We confirmed that PGE₂ exhibited an inhibitory effect on the migration of neutrophils caused by formyl-methionyl-leucyl-phenylalanine *in vitro*[39]. The same inhibitory action was shown by both butaprost and 11-deoxy PGE₁, but not by 17-phenyl PGE₂, sulprostone, or ONO-NT-012, clearly indicating that the anti-neutrophil chemotaxis action of PGE₂ is mediated by activation of EP2 and EP4 receptors (Figure 8). Thus, it is assumed that the inhibition of neutrophil migration by itself is not sufficient to reduce the overall expression of gastric lesions in response to indomethacin. Since the increase in MPO activity as well as ulceration induced by indomethacin was prevented when the enhanced gastric motility was inhibited by atropine[13,28,47], it is likely that the neutrophil infiltration is secondary to the event associated with gastric hypermotility following indomethacin treatment. As mentioned before, Melange *et al*[37] even showed that NSAID-induced gastric injury is neutrophil-independent in the neutropenic rats. These results strongly suggest that the protective effect of PGE₂ is functionally associated with the inhibition of gastric motility, but not neutrophil infiltration.

ROLE OF COX INHIBITION IN NSAID-INDUCED GASTRIC DAMAGE

Ulcerogenic properties of various COX inhibitors

COX, the enzyme responsible for PG production, exists in two isozymes, the constitutively expressed COX-1 and the inducible COX-2[14,15]. NSAIDs inhibit the activity of both COX-1 and COX-2, yet it is believed that the inhibi-

Figure 8 Effects of atropine and various prostaglandin E agonists on the neutrophil chemotaxis stimulated by formyl-methionyl-leucyl-phenylalanine. Neutrophils were pretreated for 45 min with atropine and various prostaglandin E agonists such as PGE_2, 17-phenyl PGE_2, butaprost, ONO-NT-012 and 11-deoxy PGE_1 at the indicated concentrations, and then the cells were stimulated by incubation with formyl-methionyl-leucyl-phenylalanine (fMLP, 1×10^{-7} mol) for another 45 min. Data are expressed as a percentage of the stimulated values (control) observed in the presence of fMLP and represent the mean ± SE from 4 experiments. Significant difference at $P < 0.05$; [a]from normal; [c]from control (data from ref. 39 after modification).

Figure 9 Gastric ulcerogenic responses to various cyclooxygenase inhibitors and their effects on prostaglandin E_2 content in rat stomachs. A: Gastric ulcerogenic responses induced by various cyclooxygenase (COX) inhibitors in rat stomach. The animals were given indomethacin (nonselective COX inhibitor; 10 and 30 mg/kg), SC-560 (selective COX-1 inhibitor; 10 and 30 mg/kg), or rofecoxib (selective COX-2 inhibitor; 10 and 30 mg/kg) p.o. and killed 8 h later; B: Effects of various COX inhibitors on gastric mucosal prostaglandin E_2 (PGE_2) content in rats. The animals were given indomethacin (10 mg/kg), SC-560 (10 mg/kg), or rofecoxib (10 mg/kg) p.o. and killed 2 h later. Data are presented as the mean ± SE in 5-6 rats. [a]$P < 0.05$ vs control (data from refs. 18 and 19 after modification).

tion of COX-1 is critical for their ulcerogenic properties in the stomach. However, Wallace et al[17] reported that inhibition of both COX-1 and COX-2 is required for the induction of gastric lesions. This finding was confirmed in our experiment using the selective COX-1 inhibitor SC-560 and the COX-2 inhibitor rofecoxib[19,20,40]. As shown in Figure 9, indomethacin at 30 mg/kg produced gastric lesions with a marked decrease in mucosal PGE_2 content. As expected, the selective COX-2 inhibitor rofecoxib did not induce any damage at 30 mg/kg, with no effect on mucosal PGE_2 content. Likewise, the COX-1 inhibitor SC-560 did not cause gastric damage even at 30 mg/kg, despite inhibiting PGE_2 production, as effectively as indomethacin. However, these agents given together provoked damage in the stomach. In this case, when SC-560 at 10 mg/kg was given together with various doses of rofecoxib, the severity of the damage increased depending on the dose of the selective COX-2 inhibitor (Figure 10). Similarly, when rofecoxib at 10 mg/kg was given together with SC-560, the damage increased in a manner dependent on the dose of SC-560. These results do not support the paradigm that COX-1 but not COX-2 plays a "housekeeping" role in the stomach, and strongly suggest that inhibition of both COX-1 and COX-2 is required for the occurrence of NSAID-induced gastric injury. Langenbach et al[47] reported that the indomethacin-induced gastric lesions were inhibited in animals lacking

the COX-1 enzyme, casting a doubt on the role of PG/COX-1 in the pathogenesis. However, since the inhibition of COX-1 induces the expression of COX-2[19,48], it is possible that the PGs produced by COX-2 compensate for the PG deficiency in COX-1 knockout animals.

COX inhibition and various pathogenic events
The pathogenic mechanism of NSAID-induced gastric damage involves multiple functional alterations, including gastric hypermotility, microcirculatory disturbance, neutrophil activation, and microvascular permeability[5-13]. A marked increase in gastric motility was observed after the administration of SC-560 but not rofecoxib, although the duration of the hypermotility was short as compared with that induced by nonselective COX inhibitors, suggesting that gastric hypermotility induced by NSAIDs is associated with a PG deficiency caused by COX-1 inhibition[18]. Likewise, SC-560 but not rofecoxib increased

Figure 10 Gastric ulcerogenic response induced by combined administration of SC-560 and rofecoxib in rats. The animals were administered SC-560 (3-30 mg/kg) and rofecoxib (3-30 mg/kg) p.o. either alone or in combination, and killed 8 h later. Data are presented as the means ± SE in 5-6 rats (data from ref. 18 after modification).

Figure 11 Gene expression of cyclooxygenase-1 and cyclooxygenase-2 in rat gastric mucosa after administration of various non-steroidal anti-inflammatory drugs (A) or various cyclooxygenase inhibitors (B). The animals were given indomethacin (IM, 30 mg/kg), naproxen (NAP, 40 mg/kg), flurbiprofen (FLU, 20 mg/kg), dicrofenac (DIC, 40 mg/kg), SC-560 (SC, 30 mg/kg), or rofecoxib (Rof, 30 mg/kg) p.o., and the expression of cyclooxygenase (COX)-1 and COX-2 mRNA was examined by reverse transcription polymerase chain reaction 4 h later. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; M: Marker, V: Vehicle (data from refs. 18 and 19 after modification).

microvascular permeability in the stomach, similar to indomethacin. These results for SC-560 are reasonable, because indomethacin at an ulcerogenic dose is known to cause microcirculatory disturbances resulting from abnormal mucosal compression of the stomach wall due to gastric hypermotility[10]. On the other hand, Wallace *et al*[17] reported that SC-560, but not celecoxib, decreased the gastric mucosal blood flow, suggesting a role for PGs derived from COX-1 in the maintenance of mucosal blood flow. They also showed that the selective COX-2 inhibitor celecoxib elicited neutrophil adherence in mesenteric venules, as potently as indomethacin, whereas the selective COX-1 inhibitor SC-560 did not. However, we observed that neither SC-560 nor rofecoxib alone affected MPO activity in the gastric mucosa, yet these two agents together apparently increased MPO activity to the levels comparable to those induced by indomethacin[19]. This event might be hampered by PGs derived from COX-2, probably at later stages following gastric hypermotility, since microcirculatory disturbances are known to enhance the adhesion of neutrophils to endothelial cells[13,34]. These results strongly suggest that the inhibition of both COX-1 and COX-2 is required for enhancement of neutrophil migration in the gastric mucosa and that neutrophils may be involved in the damage process later on, but do not play a role in the onset of gastric damage induced by NSAIDs.

Upregulation of COX-2 expression

The most important event is that the expression of COX-2 mRNA was induced in the gastric mucosa after administration of NSAIDs[18,19] (Figure 11A). The upregulation of COX-2 expression was similarly observed in the rat stomach after administration of SC-560 but not rofecoxib, suggesting a causal relationship between COX-1 inhibition and COX-2 expression (Figure 11B). We also reported the upregulation of COX-2 expression

in the small intestine following administration of both indomethacin and SC-560[20,48,49]. It is assumed that inhibition of COX-1 induces a PG deficiency but upregulates the expression of COX-2, which contributes to a restoration of PG production. Indeed, the mucosal PGE2 content of the stomach was markedly decreased by SC-560, yet values recovered significantly 8 h after the administration in a rofecoxib-sensitive manner[18] (Figure 12A). Thus, the upregulation of COX-2 expression following inhibition of COX-1 may represent a compensatory response to inhibition of PG biosynthesis and contribute to maintenance of the mucosal integrity of the stomach. This speculation is supported by the fact that combined treatment with SC-560 and rofecoxib did provoke gross damage in the stomach, and that such damage was prevented by administration of PGE2 4 h after the use of COX inhibitors[19]. The exact mechanism by which the expression of COX-2 is induced by inhibition of COX-1 remains unknown. Since the expression of COX-2 induced by indomethacin was attenuated by atropine at the dose that inhibited the gastric hypermotility[6,21,48], it is possible that the upregulation of COX-2 expression is due to vascular injury caused by abnormal mucosal compression of the stomach wall during gastric hypermotility (Figure 12B). Indeed, atropine significantly inhibited the recovery of PGE2 levels following administration of SC-560, similar to rofecoxib[48]. Alternatively, because NSAIDs release tumor necrosis factor α (TNF-α)[38,50],

Figure 12 Effects of various drugs on prostaglandin E₂ production and cyclooxygenase-2 expression in rat gastric mucosa. A: Effects of various drugs on prostaglandin E₂ (PGE₂) levels in the rat gastric mucosa at 8 h after administration of SC-560. The animals were administered SC-560 (10 mg/kg) p.o., and killed 2 or 8 h later. Rofecoxib (30 mg/kg) was given p.o. together with SC-560 while omeprazole (OM, 30 mg/kg) or atropine (AT, 3 mg/kg) was given s.c. 1 h before the administration of SC-560. Data are presented as the mean ± SE in 6 rats. Significant difference at $P < 0.05$; ^{a}from control; ^{c}from SC-560 (2 h); ^{e}from vehicle; B: Effect of OM and AT on cyclooxygenase (COX)-2 expression after administration of indomethacin (IM) in rat stomach. The animals were administered IM (30 mg/kg) p.o., and killed 4 h later. OM (30 mg/kg) or AT (3 mg/kg) was given s.c. 1 h before indomethacin. Some animals were given 1 mL of 100 mmol HCl immediately after administration of IM. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; M: Marker; V: Vehicle (data from ref. 48 after modification).

the upregulation of COX-2 expression observed under COX-1 inhibition is mediated by TNF-α. Omeprazole had no effect on the expression of COX-2 induced by indomethacin, suggesting no role for luminal acid in this phenomenon[48].

POTENTIATION OF NSAID-INDUCED GASTRIC DAMAGE

Adrenalectomy

Takeuchi et al[9] demonstrated that indomethacin-induced gastric damage was markedly aggravated in adrenalectomized rats and the dose required to produce lesions was decreased in these rats. Filaretova et al[22] confirmed the aggravation of NSAID-induced gastric ulceration in adrenalectomized rats (glucocorticoid-deficient conditions)

and further investigated the influence of adrenalectomy on the expression of COX-2 in the stomach as well as the ulcerogenic effect of celecoxib (a selective COX-2 inhibitor) in these rat stomachs. It was found that adrenalectomy decreased plasma corticosterone levels and markedly aggravated indomethacin-induced gastric lesions. This aggravation was significantly prevented by corticosterone replacement, suggesting that glucocorticoid deficiency is the reason for the aggravation of indomethacin-induced gastric injury in adrenalectomized rats. Moreover, in adrenalectomized rats, celecoxib provoked gross damage that was prevented by corticosterone pretreatment. Mucosal PGE₂ content was increased 3-fold after adrenalectomy, and this response was prevented by both celecoxib and corticosterone. COX-2 mRNA expression was up-regulated in the stomach of adrenalectomized rats, but suppressed by corticosterone replacement. It is assumed that adrenalectomy, probably via a glucocorticoid deficiency, increases PGE₂ production in the stomach due to COX-2 expression, and the selective COX-2 inhibitor produces gastric lesions by suppressing this additional PG production in adrenalectomized rats. These findings also support the idea that COX-2 as well as COX-1 play a role in maintaining gastric mucosal integrity under glucocorticoid-deficient conditions.

Adjuvant arthritis

Patients with rheumatoid arthritis (RA) are reportedly more susceptible to NSAID-induced gastropathy than other NSAID users[51,52]. This observation has been validated in arthritic rat models induced by injecting Freund's complete adjuvant into the planter region of a hindfoot, where the gastric ulcerogenic response to indomethacin was markedly aggravated in comparison with normal animals[23,53,54]. Since the aggravation of these lesions in arthritic rats was dependent on the degree of arthritic change, it is assumed that there is a cause-effect relationship between the systemic inflammation and the increased gastric mucosal susceptibility to indomethacin. As several studies including ours showed increased serum gastrin levels and acid secretion in arthritic rats[53,55], it is speculated that the increased gastric ulcerogenic response is partly attributable to hyperacidity in the stomach. However, because the aggravation of these lesions was similarly observed in arthritic rats, even in the presence of exogenous acid to mask endogenous hyperacidic conditions[53], it is unlikely that the increased mucosal susceptibility to indomethacin in arthritic rats is associated with the increase of acid secretion. Interestingly, the aggravation of indomethacin-induced gastric damage in arthritic rats was prevented by prior administration of N^G-nitro-L-arginine methyl ester, a nonselective nitric oxide synthase (NOS) inhibitor, and aminoguanidine, a selective inducible NOS (iNOS) inhibitor, as well as dexamethasone, an inhibitor of iNOS mRNA transcription, although they did not affect the severity of the lesions observed in normal rats[53]. Moreover, the distinct expression of iNOS mRNA was observed in the stomach of arthritic rats, accompanied with an increase in NO production. These

Figure 13 Gastric ulcerogenic effect of indomethacin, rofecoxib and SC-560 and the expression of cyclooxygenase-1 and cyclooxygenase-2 mRNA in the gastric mucosa of normal and arthritic rats. A: Arthritis was induced by injecting Freund's complete adjuvant (FCA) into the plantar region of the right hindfoot, and the experiments were performed 14 d after the injection. Indomethacin (Indo) (3 mg/kg), rofecoxib (Rof) (30 mg/kg), or SC-560 (SC) (30 mg/kg) were administered p.o., and the animals were killed 4 h later. Data are presented as the mean ± SE in 4-8 animals, [a]$P < 0.05$ vs the corresponding group in normal rats; B: COX-2 mRNA was not detected in the normal rats, but clearly observed in the arthritic rats on day 14 after the FCA injection, whereas COX-1 mRNA was observed in the stomach of both normal and arthritic rats. Lane 1, marker; lanes 2-4, normal rats; lanes 5-7, arthritic rats (data from ref. 23 after modification). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

findings suggest that the increased ulcerogenic response to NSAIDs in arthritic rats is associated at least partly with endogenous NO, mainly produced by iNOS. It is possible that the increased susceptibility of arthritic rat stomachs to NSAIDs might be explained by production of peroxynitrite, resulting from the interaction of NO/iNOS with superoxide radicals[56].

As mentioned, selective COX-2 inhibitors such as rofecoxib and celecoxib, even at a higher dose (100 mg/kg), did not damage the normal rat stomach[18]. However, they produced gross lesions in the stomach of arthritic rats[23] (Figure 13A). Moreover, PG generation in the arthritic rat stomach was significantly enhanced with a concomitant increase of COX-2 expression (Figure 13B). Certainly, the mucosal PG content was reduced by indomethacin in both normal and arthritic rat stomachs. In contrast, the COX-2 inhibitor rofecoxib did not affect PG generation in normal rats but significantly decreased PG content in

the stomach of arthritic rats, suggesting that COX-2 activity caused the increase in PG production in arthritic rat stomachs. These findings suggest that COX-2 plays an important role in maintaining the integrity of the gastric mucosa in arthritic rats. It is possible that the increased COX-2 expression level in the stomach occur in association with inflammation or stress caused by pain. Since SC-560, a selective COX-1 inhibitor, worsened stress-induced gastric lesions[57], SC-560 may produce hemorrhagic lesions in the stomach by potentiating the ulcerogenic response to arthritis-related stress. Further study is certainly required to verify this point.

H. pylori infection

Takahashi et al[24] examined the expression of COX proteins and production of PGE2 in the gastric mucosa during H. pylori infection. The level of COX-1 remained nearly constant during the infection. In contrast, the COX-2 protein was not found in normal mucosa or in H. pylori -infected mucosa at 2 wk, but was markedly elevated 4 wk after the infection, with a significant rise in PGE2 production. To investigate the role of COX-2 in H. pylori-induced gastritis, they also examined the effects of NSAIDs on PGE2 production and gastric pathology caused by H. pylori. NS-398 (a COX-2-selective inhibitor) at 10 mg/kg or indomethacin at 2 mg/kg was administered for 4 wk to normal and H. pylori-infected animals. NS-398 failed to inhibit PGE2 production in normal mucosa but significantly reduced the H. pylori-increased PGE2 production. In contrast, indomethacin potently inhibited PGE2 production in both normal and H. pylori-infected mucosa. Hemorrhagic erosions, neutrophil infiltration, lymphoid follicles, and epithelium damage were induced by H. pylori infection. NS-398 and indomethacin aggravated these pathological changes, but did not increase viable H. pylori numbers. Overall, these results indicate that both COX-2 and COX-1 might play anti-inflammatory roles in H. pylori-induced gastritis. Similar findings were also obtained by Tanigawa et al[58] who showed that PGE2 derived from either COX-1 or COX-2 is involved in the regulation of gastric mucosal inflammation and contributes to the maintenance of mucosal integrity during H. pylori infection via inhibition of TNF-α expression.

BIPHASIC EFFECT OF ASPIRIN

Conventional NSAIDs cause gastric damage with a concomitant decrease in mucosal PGE2 production, irrespective of the route of administration[18,19]. However, since aspirin is not ulcerogenic in the stomach, despite that it reduces mucosal PGE2 production as effectively as other NSAIDs, it is likely that a depletion of endogenous PGs by itself is not sufficient for gastric lesions to form and other factors are required for the onset of gastric damage. However, when administered orally, aspirin damages the stomach, similar to other NSAIDs. Several studies have proposed a role for neutrophils or TNF-α in the

Figure 14 Effects of aspirin and salicylic acid on gastric lesions (A) and gastric hypermotility (B) caused by indomethacin in rats. A: The animals were given indomethacin (35 mg/kg) s.c., and killed 24 h later. Aspirin (20-200 mg/kg) or salicylic acid (17.8-178 mg/kg) was given s.c. 30 min before indomethacin. Data are presented as the mean ± SE in 4-6 rats. [a]$P < 0.05$ *vs* control; B: Animals were given indomethacin (30 mg/kg) s.c. and subsequently aspirin (200 mg/kg) or salicylic acid (178 mg/kg) s.c. 2 h later. Note that both aspirin and salicylic acid markedly inhibited the intestinal hypermotility induced by indomethacin, with the effect of salicylic acid appearing much earlier than that of aspirin (data from ref. 25 after modification).

pathogenesis of NSAID-induced gastric damage[34,35,50]. These events are considered to occur in relation to a decrease in PG biosynthesis in the gastric mucosa due to suppression of COX activity. However, aspirin given parenterally inhibited PGE2 production in the stomach, yet did not cause any damage in the mucosa[19]. Furthermore, salicylate reportedly inhibited TNF-α production by suppressing nuclear factor kappa B[59]. Considering all these points, we assumed that the topical irritant action of oral aspirin is most crucial in causing gastric mucosal damage.

As mentioned earlier, aspirin does not damage the stomach but shows a dose-dependent inhibition of indomethacin-induced gastric injury[24] (Figure 14A). This result is consistent with the finding by Robert *et al*[60], who showed for the first time that aspirin provided protection against gastric damage in response to various noxious

agents including indomethacin. Following the subcutaneous administration of aspirin (200 mg/kg) in rats, plasma levels of salicylate increased with time, reaching almost a plateau within 30 min, and remained elevated for more than 4 h. A small amount of aspirin was detected in the blood for the first 15 min, but it had disappeared almost totally 30 min later. As expected, since salicylate, the major metabolite of aspirin, also prevented indomethacin-induced gastric damage, it is possible that the protective action of aspirin is mediated by salicylate. Interestingly, aspirin and salicylate did not increase basal gastric motility but suppressed the enhanced gastric motility following indomethacin treatment, suggesting again a relationship between the inhibition of gastric hypermotility and prevention of gastric damage (Figure 14B)[25]. At present, the exact mechanism by which salicylate (aspirin) suppresses the gastric hypermotility induced by indomethacin remains unknown.

Unlike other NSAIDs, COX-2's acetylation by aspirin switches eicosanoid biosynthesis from PGE2 to lipoxin A4, which exerts protective effects in the stomach. Co-administration of aspirin and a selective COX-2 inhibitor, such as celecoxib or rofecoxib, resulted in substantially more severe gastric injury than that produced with either agent alone[61,62]. We also observed that the gastric ulcerogenic response to aspirin was significantly worsened by co-administration of rofecoxib but not SC-560[63]. These results confirmed the importance of COX-2's inhibition in this phenomenon related to the suppression of lipoxin A4's production.

SUMMARY AND FUTURE PROSPECTS

The gastric ulcerogenic properties of NSAIDs are not accounted for solely by the inhibition of COX-1 and require the inhibition of both COX-1 and COX-2[17-19]. This idea is supported by the finding that neither the selective COX-1 nor COX-2 inhibitor alone caused gross damage in the stomach, but the combined administration of these two inhibitors provoked the development of gastric lesions. Indomethacin caused an increase of gastric motility, microvascular permeability and MPO activity following administration of indomethacin[6,10,26,28,34,64,65] and showed that the former two events were due to COX-1 inhibition, but the increase of MPO activity occurred only when both COX-1 and COX-2 were inhibited[19]. On the other hand, NSAIDs up-regulate the expression of COX-2, and the PGs produced by COX-2 may suppress the neutrophil-endothelial interaction caused by the vascular disturbances due to COX-1 inhibition. These sequential events related to COX-1 and/or COX-2 inhibition explain why gastric damage occurs only when both COX-1 and COX-2 are inhibited (Figure 15). It should also be noted that selective COX-2 inhibitors by themselves damage the gastric mucosa when an overexpression of COX-2 occurs in the stomach under conditions of adrenalectomy, arthritis, or *H. pylori* infection[22-24]. Independent of the type of NSAIDs, the users of NSAIDs should be aware of these side effects if they are

Figure 15 Working hypothesis on the roles of COX-1 and COX-2 in the pathogenic mechanism of non-steroidal anti-inflammatory drug-induced gastric damage. Non-steroidal anti-inflammatory drugs (NSAIDs) cause gastric hypermotility, followed by microvascular disturbances and neutrophil activation, leading to gastric damage. Gastric hypermotility and subsequent vascular disturbances are associated with a prostaglandin (PG) deficiency caused by COX-1 inhibition. The inhibition of COX-1 up-regulates COX-2 expression, and PGs produced by COX-2 may suppress the neutrophil-endothelial interaction caused by microvascular disturbances due to COX-1 inhibition.

infected with *H. pylori* or have a glucocorticoid deficiency or arthritic condition. Interestingly, aspirin acts to protect against indomethacin-induced gastric damage, although this agent given p.o. damages the stomach due to its direct irritative action. The failure of aspirin to induce gastric injury may be explained, at least partly, by a protective action of salicylic acid, the metabolite of aspirin, and this action is also functionally associated with inhibition of gastric hypermotility in response to indomethacin.

There is no doubt that gastric hypermotility plays a primary role in the pathogenesis of NSAID-induced damage in the stomach[3-7]. This response, causally related with PG depletion due to COX-1 inhibition, occurs prior to other pathogenic events involved in NSAID-induced gastric damage, such as microvascular disturbances and neutrophil infiltration as well as COX-2 expression[6,10,19,28,63]. However, the mechanism underlying NSAID-induced gastric hypermotility remains unknown. Since the gastric hypermotility induced by indomethacin was inhibited by atropine and vagotomy as well as intravenous glucose infusion[6,7,66], it is assumed that the response occurs in association with PG deficiency caused by COX-1 inhibition and is mediated by the vagal-cholinergic pathway through central glucose receptors. The upregulation of NSAID-induced COX-2 expression is functionally associated with gastric hypermotility[47]. Because atropine prevented both gastric hypermotility and COX-2 expression in response to indomethacin[21,47] and because gastric microvascular permeability increased in association with gastric hypermotility[8,63], the upregulation of COX-2 expression may result from

mild mucosal injury and/or vascular injury caused by gastric hypermotility. However, the cells responsible for COX-2 expression induced by COX-1 inhibition also remain to be identified. In addition, other possible actions, such as inhibition of phosphorylative oxidation, injury of mitochondrial membrane and cell apoptotic change, have been demonstrated as the cellular mechanisms of NSAID-induced gastropathy[66-70], although these effects are shared by NSAIDs, including aspirin that does not cause gastric damage through parenteral administration. Further study is certainly needed to clarify these points, and these approaches should contribute to the development of gastric-sparing NSAIDs that are devoid of ulcerogenic properties.

BIOGRAPHY

Professor Koji Takeuchi received his PhD degree from the University of Tokyo, Tokyo, Japan. He had an extensive 4-year postdoctoral training at Department of Physiology and Cell Biology, University of Texas, Houston and Department of Surgery, Harvard Medical School, Boston, United States. He is presently Professor and Chairman of the Department of Pharmacology and Experimental Therapeutics, Dean of the Graduate School and Vice President of the Kyoto Pharmaceutical University, Kyoto, Japan. His research interest covers numerous areas of GI pharmacology and physiology, and one of his most notable contributions is the understanding of mucosal defense, focusing on the regulation of acid/bicarbonate secretion, the influences of non-steroidal anti-inflammatory drugs and prostaglandins; their mode of action, cyclooxygenase isoforms, receptors that drive physiological responses, and their role in mucosal injury, protection and healing. He has had 448 papers published on peer-reviewed journals, including 45 book chapters, and gave numerous presentations at national and international meetings. Professor Koji Takeuchi has enjoyed quite a few prestigious academic awards and honors.

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Clopidogrel and proton pump inhibitors - where do we stand in 2012?

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Abstract

Clopidogrel in association with aspirine is considered state of the art of medical treatment for acute coronary syndrome by reducing the risk of new ischemic events. Concomitant treatment with proton pump inhibitors in order to prevent gastrointestinal side effects is recommended by clinical guidelines. Clopidogrel needs metabolic activation predominantly by the hepatic cytochrome P450 isoenzyme Cytochrome 2C19 (CYP2C19) and proton pump inhibitors (PPIs) are extensively metabolized by the CYP2C19 isoenzyme as well. Several pharmacodynamic studies investigating a potential clopidogrel-PPI interaction found a significant decrease of the clopidogrel platelet antiaggregation effect for omeprazole, but not for pantoprazole. Initial clinical cohort studies in 2009 reported an increased risk for adverse cardiovascular events, when under clopidogrel and PPI treatment at the same time. These observations led the United States Food and Drug Administration and the European Medecines Agency to discourage the combination of clopidogrel and PPI (especially omeprazole) in the same year. In contrast, more recent retrospective cohort studies including propensity score matching and the only existing randomized trial have not shown any difference concerning adverse cardiovascular events when concomitantly on clopidogrel and PPI or only on clopidogrel. Three meta-analyses report an inverse correlation between clopidogrel-PPI interaction and study quality, with high and moderate quality studies not reporting any association, rising concern about unmeasured confounders biasing the low quality studies. Thus, no definite evidence exists for an effect on mortality. Because PPI induced risk reduction clearly overweighs the possible adverse cardiovascular risk in patients with high risk of gastrointestinal bleeding, combination of clopidogrel with the less CYP2C19 inhibiting pantoprazole should be recommended.

CLOPIDOGREL AND PROTON PUMP INHIBITORS - WHERE DO WE STAND IN 2011?

Clopidogrel in association with Aspirine has become

Figure 1 Potential intrahepatic mechanism of Proton pump inhibitor-clopidogrel interaction by the example of omeprazole (adapted from Tantry et al[50]). CYP2C19: Cytochrome 2C19; CYP2C19*2: Poor metabolizing cytochrome 2C19 isoenzyme.

the basis of pharmaceutical treatment in patients treated either medically or with percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS), by significantly reducing the risk of new ischemic cardiovascular events[1].

To prevent gastrointestinal bleeding as a drug-induced side effect, proton pump inhibitors (PPI) are often associated with clopidogrel use. This strategy is recommended by consensus guidelines[2] and endorsed by a recent meta-analysis, especially for patients taking dual antiplatelet therapy but in a lesser extent for those on clopidogrel alone due to sparse data[3-5]. Gilard et al[6,7] first reported in 2006 and 2008 a significant decrease of the clopidogrel effect in association with omeprazole in vitro. In opposite to that, no decrease was found in further pharmacodynamic studies for pantoprazole or esomeprazole[8-13]. Several retrospective observational studies showed an increased risk of new cardiovascular events in patients on clopidogrel-PPI association[14-22], thus leading the United States Food and Drug Administration and the European Medicines Agency to recommend to avoid the clopidogrel-PPI combination, especially with omeprazole[23,24]. More recently, one randomized double-blind trial[25], one post-hoc analysis of a randomized double-blind trial comparing prasugrel with clopidogrel[26] and several predominantly propensity matched cohort studies[27-35] have not shown clinically relevant adverse cardiovascular interaction between clopidogrel and PPI. Moreover, three recent meta-analyses, one by Kwok et al[36] reviewing 23 studies with the majority in abstract form, one by Siller-Matula et al[37] including 25 studies and the most recent by Lima et al[38] reviewing 18 studies pointed out that an elevated risk of bias was present in these studies indicating a possible interaction between clopidogrel and PPI. Furthermore, there was no significance for a drug interaction by analysing propensity matched and randomized trials.

The aim of this review is to focus on these recent studies, in order to reevaluate the present recommendations.

CLOPIDOGREL

Clopidogrel is a thienopyridine, inhibiting adenosine diphosphate (ADP) induced platelet activation by blocking the $P2Y_{12}$ receptor on the platelet surface. It is a prodrug that needs to be metabolized in an intrahepatic two-step oxidative process. First, the cytochrome P450 isoenzymes CYP1A2, CYP2B6 and CYP2C19 form 2-oxo-clopidogrel, which is then oxidized by CYP2B6, CYP2C19 and CYP3A4 to the clopidogrel active metabolite. The further formation of a disulfide bond with the $P2Y_{12}$ receptor unables the binding of ADP and finally platelet activation[12,39]. This is associated with dephosphorylation of intraplatelet vasodilator-stimulated phosphoprotein (VASP), providing an index of platelet reactivity to clopidogrel: the higher the platelet reactivity index (PRI), the less important the antithrombotic effect of clopidogrel[7]. Cytochrome P450 CYP2C19 seems to be of major importance in the metabolisation and activation of clopidogrel (Figure 1). Recent studies investigating the genetic polymorphism of the CYP2C19 allele have found a decreased platelet inhibition and increased cardiovascular risk in patients treated by clopidogrel, when carriers of even one reduced function CYP2C19 allele[40-42]. The CYP2C19*2 mutation was the most frequent variant found in the poor metabolizer (decreased platelet inhibition) group[43-45]. The prevalence of reduced function alleles differs among various populations, while an increase effect is observed from West to East: In the Caucasian population, 30%-40% of the normal function *1/*2 genotype and 2%-5% of the reduced function *2/*2 genotype are reported, whereas in East Asian and Chinese populations up to 24% of the poor metabolizing genotypes *2/*2, *2/*3 and *3/*3 are present[46-48].

PROTON PUMP INHIBITORS

PPI are benzimidazole derivates consisting of two heterocyclic moieties linked via a methylsulfinyl group. Being weak bases, they reach the parietal cell membrane as prodrugs and can thereby cross cell membrane to accumulate in the canalicular space, where the environment is highly acid. After a two step pronation, the drug reacts with cysteine sulfhydryls on the gastric H+/K+-ATPase by forming covalent disulfide bonds and inhibiting its activity[49-53]. So far, we dispose of five different PPIs on the market: omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole. Each among them is mainly metabolized by the intrahepatic P450 cytochrome system, especially CYP2C19 and CYP3A4, inhibiting them competitively. Interestingly, in vitro studies showed important differences in the inhibition of CYP2C19, with lansoprazole

and omeprazole being the most powerful inhibitors while pantoprazole and rabeprazole are the less potent inhibitors[49,54,55]. Of note, only pantoprazole showed significant acid inhibition after a single dose in the fast metabolizing genotype CYP2C19*1[46].

PHARMACODYNAMIC STUDIES ON CLOPIDOGREL- PPI INTERACTION

Gilard *et al*[6] demonstrated in 2006 an *in vitro* reduction of the antiaggregatory activity of clopidogrel in patients after coronary revascularisation under PPI treatment. The same group ran out the randomized double-blind OCLA (Omeprazole Clopidogrel Aspirine) trial in 2008: 124 patients undergoing elective coronary artery stent implantation receiving 75 mg of aspirine and clopidogrel daily, were randomized to receive either omeprazole 20 mg/d or placebo. The clopidogrel effect was assessed by measuring the phosphorylated VASP expressed in the PRI on day 1 and 7. On day 7, the mean PRI was significantly higher in the omeprazole-associated group (51.4% *vs* 39.8%, *P* > 0.0001), indicating less effective platelet antiaggregation. To investigate whether this potential interaction was due to a class effect, Cuisset *et al*[11] compared in the PACA (PPI And Clopidogrel Association) study 104 patients undergoing coronary stent implantation for non-ST-elevation ACS by randomizing them to a 20 mg omeprazole or pantoprazole treatment in association with 75 mg of aspirine and 150 mg of clopidogrel. After 1 mo, the VASP PRI was significantly lower in the pantoprazole group (36% ± 20% *vs* 48% ± 17%, *P* = 0.007), suggesting that pantoprazole, being a less potent CYP2C19 inhibitor, leads to a lower decrease of the clopidogrel antithrombotic effect. These results were confirmed in a prospective observational study, including a multivariable logistic regression analysis on 300 patients with coronary artery disease undergoing PCI and being already under aspirine 100 mg/d and clopidogrel 75 mg/d for at least 5 d. No difference was found for the VASP-PRI and the ADP induced platelet aggregation (ADP Ag) either between the PPI and no-PPI-group (51% *vs* 49%, *P* = 0.724) or between the different PPIs (pantoprazole and esomeprazole)[8]. In the same line, a prospective observational study including 336 patients undergoing coronary stent implantation showed no difference in ADP induced platelet aggregation between patients treated concomitantly by clopidogrel (600 mg loading and 75 mg maintenance dose) and pantoprazole *vs* clopidogrel only (OR 0.59, 95% CI: 0.31-1.13). For omeprazole and esomeprazole, a non significant increase in platelet aggregation persisted even after multiple adjustment (OR 1.84, 95% CI: 0.64-5.31), but due to the relatively small number of patients (26 *vs* 122 pantoprazole users), definite conclusions couldn't be drawn[13]. The authors of the post-hoc analysis of the PRINCIPLE (Prasugrel In Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation)-TIMI 44 study evaluated the impact of concomitant PPI use in 201 patients undergoing planned PCI and random-

ly assigned to either prasugrel (a new third generation thienopyridine) or high dose clopidogrel (600 mg loading dose and 150 mg/d maintenance dose) treatment. Fifty-six patients (26.4 %) were recorded to take a PPI at the time of randomization and the mean inhibition of platelet aggregation measured by ADP induced platelet aggregation was significantly lower at 2, 6 and 24 h after the loading dose, with a non-significant trend still persisting after 15 d. For prasugrel, no significant lowering of the mean inhibition of platelet aggregation was observed in the first 24 h, becoming only significant after 15 d in patient treated by PPI[26]. Recently, Angiolillo *et al*[12] conducted four randomized placebo-controlled crossover comparison studies among 282 healthy subjects, addressing the questions whether the PPI-clopidogrel interaction should be considered as a class effect or is rather due to more or less potent CYP2C19 inhibition and if a time interval between clopidogrel and PPI administration might diminish the inhibitory effect as evoked by the rapid metabolization of clopidogrel and omeprazole. After randomization in either interventional or placebo groups, the interventional arm entered a two period (clopidogrel only and clopidogrel with PPI) crossover study with four interventions during the clopidogrel-PPI period: The first study investigated an interaction between clopidogrel (300 mg loading and 75 mg maintenance dose) and omeprazole 80 mg/d when administered simultaneously. Study 2 investigated the administration of clopidogrel and omeprazole staggered by 12 h and study 3 an increased clopidogrel dose (600 mg loading and 150 mg maintenance dose) with omeprazole 80 mg/d. Finally, study 4 used a standard clopidogrel dose with pantoprazole 80 mg/d. Dosages of the active metabolite of clopidogrel (clopi H4) were significantly decreased in study 1, 2 and 3 while ADP induced platelet aggregation as well as VASP-PRI were significantly increased, indicating a less effective platelet antiaggregation in patients treated concomitantly with clopidogrel and omeprazole. Of note, these results were irrespective of the administration time or the clopidogrel dose. In contrast, the decrease of clopi H4 (40%, *P* < 0.001 for omeprazole and 14%, *P* < 0.002 for pantoprazole) was smaller in study 4 as well as the increase of ADP induced platelet aggregation, both differences remaining statistically significant. The increase of VASP-PRI was not significant when treated with pantoprazole, leading the authors to conclude that the clopidogrel-PPI interaction was not a class effect, whereas the combination with pantoprazole was a more optimal treatment option[12]. However, omeprazole was given at 80 mg per day, which represents 2 to 4 times the dose commonly prescribed, leaving unclear the hypothesis of a possible interaction when using standard doses. Furthermore, other molecules like rabeprazole, which does not inhibit the CYP2C19 isoenzyme, haven't been tested. In the same line, Ferreiro *et al*[56] conducted two supplementary randomized crossover studies in healthy subjects: In the first study, 20 volunteers received a 600 mg loading dose followed by 75 mg of clopidogrel combined with 40 mg of omeprazole concomitantly or staggered by 8-12 h with

Table 1 Overview of important pharmacodynamic studies on the clopidogrel-proton pump inhibitor interaction

Study	PPIs used	Population	Primary outcome	Author's conclusions
Gilard *et al*[7] OCLA study (double-blind, placebo-controled, randomized)	Omeprazole	124 patients undergoing elective coronary stent implantation	VASP-PRI on 7 d	Omeprazole significantly decreases clopidogrel inhibitory effect
Cuisset *et al*[11] PACA study (prospective, randomized)	Omeprazole *vs* Pantoprazole	104 NSTE-ACS patients undergoing coronary stenting	VASP-PRI/ADP-Ag after 1 mo	Significantly better platelet response under pantoprazole (VASP-PRI), no difference for ADP-Ag
O'Donoghue *et al*[26] PRINCIPLE-TIMI 44 (post hoc analysis of a RCT)	Not specified	201 patients undergoing planned PCI	ADP Ag	Mean inhibition of platelet aggregation significantly lower for patients on PPI
Siller-Matula *et al*[8] (prospective observational)	Pantoprazole esomeprazole	300 patients with CAD undergoing PCI	VASP-PRI/ADP-Ag in the catheter laboratory	No association of PPIs with impaired response to clopidogrel
Neubauer *et al*[13] (prospective observational)	Pantoprazole omeprazole esomeprazole	336 patients undergoing coronary stent implantation	ADP Ag	Pantoprazole does not diminish the antiplatelet effectiveness of clopidogrel
Angiolillo *et al*[12] (placebo controlled, randomized, cross-over)	Omeprazole pantoprazole	282 healthy subjects	Clopi H4 ADP Ag VASP-PRI after 5 d	Presence of a metabolic drug-drug interaction between clopidogrel and omeprazole but not for pantoprazole

VASP-PRI: Vasodilator-stimulated phosphoprotein platelet reactivity index; NSTE-ACS: Non-ST-elevation acute coronary syndrome; ADP Ag: Adenosine DiPhospate induced platelet aggregation; CAD: Coronary artery disease; PCI: Percutaneous coronary intervention; PPI: Proton pump inhibitor; OCLA: Omeprazole Clopidogrel Aspirine; PACA: PPI and clopidogrel association; **PRINCIPLE-TIMI: Prasugrel in comparison to clopidogrel for inhibition of platelet activation and aggregation-TIMI.**

a crossover washout period after a 2-4 wk followed by 1 wk of clopidogrel alone after a new washout period: No difference was observed in VASP-PRI after 1 wk between the concomitant and the staggered omeprazole administration, but PRI was significantly lower in the clopidogrel alone period compared with the omeprazole period, irrespective of the timing of administration (comcomitant omeprazole: $P = 0.02$; staggered omeprazole: $P = 0.001$). In the second study, 80 mg of pantoprazole were administered with the same regimen, but no differences in VASP-PRI were found between a concomitant or staggered administration of pantoprazole. Moreover, no difference was noted between clopidogrel alone and clopidogrel plus pantoprazole after 1 wk of treatment. The authors concluded that a time interval between the administration of clopidogrel and PPI doesn't afford any benefit and that pantoprazole seems to be a safer choice when combined with clopidogrel[57] (Table 1).

CLINICAL TRIALS ON CLOPIDOGREL- PPI INTERACTION

In 2009, Ho *et al*[15] published a retrospective cohort study including 8205 patients hospitalized for ACS in Veterans Affairs Hospitals. Analysis of prescription records identified 63.9% of patients being concomitantly under clopidogrel and PPI with a mean follow-up of 521 d. Concomitant use of clopidogrel and PPI (predominantly omeprazole and rabeprazole) was associated with an elevated risk of death or rehospitalisation for ACS after multivariable analysis (OR 1.25, 95% CI: 1.11-1.41). Of note, 98% were men and no information on the patient's race was available. In the same line, a Canadian popula-

tion-based nested case-control study based on discharge abstracts and prescription records of 13636 patients being hospitalized for ACS, found an increased risk of reinfarction when under concomitant clopidogrel and PPI use (OR 1.27, 95% CI: 1.03-1.57). An analysis according to the PPI molecule used found no association with increased myocardial reinfarction for pantoprazole users in contrast to a 40% risk increase when using other PPIs (OR 1.40, 95% CI: 1.10-1.77). This result should be interpreted carefully, due to the small number of pantoprazole users (46 of 734 reinfarction patients)[16]. Moreover, only patients aged 66 years or older were included, introducing potential age bias. Another retrospective observational study based on diagnosis and prescription records of two Dutch health insurances, included 18139 new clopidogrel users, of whom 5734 (32%) were on concomitant PPI treatment. In this particular study, patients under PPI cotherapy had a significantly higher risk for the composite endpoint of myocardial infarction, unstable angina, stroke and all-cause mortality (HR 1.75, 95% CI: 1.58-1.94). In the subanalysis of secondary endpoints, PPI use was associated with a higher risk of myocardial infarction (ST-elevation and non-ST-elevation), unstable angina and all-cause mortality, but not with stroke[14]. Selection bias may be present in these two insurance databases, covering only 25% of the Dutch population. All three studies evidence significant differences in the baseline characteristics between the clopidogrel and the clopidogrel-PPI groups with significantly older patients with several comorbidities (e.g., heart failure, diabetes mellitus and renal failure) in the latter group, raising concern about unmeasured confounders in patients whith cardiovascular risk treated by PPI. In addition to that, no data about the efficacy of antihypertensive and statin treatment as well as on smok-

ing status were available. Finally as the medication exposure was based on prescription records, drug compliance data were not available.

More recently, several studies have been designed to include propensity scores in their analysis to improve confounding adjustment. Especially confounding by indication, an important bias in pharmacoepidemiologic studies, is diminished by using propensity score matching by calculating the probability to be exposed to a treatment or not. Moreover, adjustment for unmeasured or mismeasured covariates is improved by including hundreds of items in the propensity score calibration[58]. However, by the fact that many unexposed subjects of the initial study population aren't matched to exposed subjects and unmatched exposed subjects are excluded from the propensity matched analysis, precision of the estimated drug interaction could be decreased[59-62]. Rassen *et al*[31] analysed 18 565 patients aged over 65 years having been hospitalized for ACS and consecutive PCI in a retrospective cohort study based on Canadian and United states insurance records. Patients under clopidogrel and PPI had a slightly increased risk for rehospitalization for myocardial infarction or death of any cause (RR 1.26, 95% CI: 0.97- 1.63) leading the authors to conclude to no evidence of a substantial interaction. Major efforts for bias reduction have been made in this study by including only clopidogrel naïve patients, using a 7 d run-in period and a high-dimensional propensity score, permitting further adjustment for 400 additional variables empirically identified in their databases[63]. However, Aspirine use was unfortunately not measured in this coronary disease population[31]. A similar analysis was conducted on 20 596 patients of the Tennessee Medicaid program after hospitalization for ACS and PCI. Concomitant clopidogrel and PPI use was not associated with serious cardiovascular disease (HR 0.99, 95% CI: 0.82-1.19). Subanalysis concerning the different types of PPI has not found any increased risk of serious cardiovascular disease either, but confidence bounds were wide except for pantoprazole[27]. Another retrospective cohort study using the national Danish patient and prescription registry, included 56 406 patients older than 30 years and hospitalized for acute myocardial infarction. Concomitant clopidogrel and PPI users had a significant increased risk for cardiovascular death or rehospitalization for myocardial infarction and stroke compared to non-PPI users (HR 1.35, 95% CI: 1.22-1.50). In the same time, PPI users not receiving clopidogrel presented a similar increased risk (HR 1.43, 95% CI: 1.34-1.53), indicating no interaction between clopidogrel and PPI. The authors suspected that the increased cardiovascular risk in PPI users might be due to imperfectly measured differences in the baseline characteristics (lack of data on smoking status, lipid levels and body mass index)[28]. The strength of this study lies in the unselected nationwide population (patients older than 30 years hopitalized for myocardial infarction allover in Denmark) and the probably high concordance between the measured drug dispension (from data of the Danish national prescription registry) and real drug consumption

due to only partial reimboursement of drug expenses and the fact that PPIs weren't available over the counter during the study period. However, the study is based on data from 2000 to 2006 and the low antiplatelet drug exposure (only 50%-70% of patients were under aspirine and 27% under clopidogrel on follow-up) dramatically contrasts with the current practice and questions the validity of the final conclusions.

In 2011, two analyses of PCI registries, including large data on cardiovascular risk factors and comorbities, were not able to show any difference on cardiovascular events: The American Guthrie Health Off-Lable Stent (GHOST) investigators studied 2651 patients discharged after coronary stenting and found no increase of Major Adverse Cardiovascular Events (MACE: death, myocardial infarction, target vessel revascularisation or stent thrombosis) for PPI users after propensity adjusted analysis (HR 0.89, 95% CI: 0.63-1.27) and in the propensity matched subgroup including 685 pairs of patients [42 (6.1%) without PPI against 40 (5.8%) with PPI; adjusted *P* = 0.60], the latter indicating even a trend to a protective effect of PPI treatment when under clopidogrel, perhaps due to less discontinuation of the antiaggregation as shown at the 6 mo follow-up (78% under clopidogrel in the PPI group against 70% without PPI, *P* = 0.0085). Furthermore, no difference according to the PPI used (omeprazole and esomeprazole) was observed[29]. Similar results came from the French Registry of Acute ST-Elevation and Non-ST Elevation Myocardial Infarction (FAST-MI), including 3670 post myocardial infarction patients. No increase in death, reinfarction or stroke was observed for concomitant PPI and clopidogrel use after one year (HR 0.98, 95% CI: 0.90-1.08). Furthermore, no difference existed regarding the PPI used (predominantly omeprazole and esomeprazole) and the presence of no or 1 to 2 CYP2C19 loss-of-function alleles. Of note, only a low number of 2 CYP2C19 loss-of-function alleles patients has been integrated (44 of 1579), leaving a higher risk of adverse cardiovascular outcome in this group still possible[30]. Both studies are based on PCI registries with detailed data assessment on baseline until hospital discharge. In contrast, follow-up was restricted on recording the patient's hospital readmission or death, without reliable information on medication exposure after hospitalization. The analysis restricted to the clopidogrel naïve population (2651 of 4421 respectively 2744 of 3670 patients) in order to avoid bias du to the occurrence of the index episode, limited the number of patients included, and may have underpowered the individual subgroups to detect a significant difference. Pointing out that the majority of the previous observational studies relied on discharge prescription records, Banerjee *et al*[32]. conducted a study on 23 200 post-PCI patients, including postdischarge drug exposure patterns using data from the Veteran Affairs Pharmacy Benefits Management database to assess drug exposure during the follow-up throughout a 6 years period. After propensity score adjustment, no difference in MACE (composite of all-cause death, nonfatal myocardial infarction or repeated revasularisation)

was observed between PPI and no PPI use in the group of continuous clopidogrel users (HR 0.97, 95% CI: 0.65-1.44). A rigorous control according to the consistency and duration of the clopidogrel and PPI exposure has been done, by revising daily exposure derived from prescription release dates and days of supply-a method considered superior to patient self-reported medication use[64]. In a subanalysis, rescue nitroglycerin and/or PPI use in patients < 30 d before MACE was significantly greater in patients taking clopidogrel and PPI ($P < 0.001$), suggesting a potential indication bias for PPI use due to misdiagnosed angina, a fact that may have contributed to a confounding bias in previous observational studies[32].

Conducting a post hoc analysis of the randomized Clopidogrel for Reduction of Events During Observation (CREDO) trial, Dunn *et al*[65] reported an increased risk of death, myocardial reinfarction or urgent target vessel revascularization at 28 d for patients using PPIs, independent on the underlying treatment [clopidogrel (OR 1.63, 95% CI: 1.02-2.63) or placebo (OR 1.55, 95% CI: 1.03-2.34)]. Baseline characteristics of the PPI group are not available, but as already discussed by Charlot *et al*[28], patients under PPI might be sicker than those who are not, explaining the higher rate of adverse cardiovascular events. Another post hoc analysis of a double-blind randomized trial, the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel (TRITON)-TIMI 38 trial included 13 608 patients with an ACS undergoing PCI and being randomly assigned to prasugrel or clopidogrel. Thirty three percent (4529 patients) were on PPIs at randomisation and exposure during the follow-up was identified by landmark analyses at 3 d, 3 and 6 mo and at the end of follow-up. Baseline characteristics showed that patients treated with PPIs were once again significantly older and had more often preexisting cardiovascular disease. After multivariable adjustment and propensity score matching, PPI use was not associated with the composite endpoint of cardiovascular death, myocardial infarction or stroke when prescribed with either clopidogrel (HR 0.94, 95% CI: 0.80-1.11) or prasugrel (HR 1.00, 95% CI: 8.84-1.20). Sensitivity analysis of patients being on PPI during the whole follow-up and patients never taking PPIs has not found any increase in adverse cardiovascular events either. Finally, no difference regarding the PPI subtype prescribed was found[26]. Both analyses have the advantage, that each end-point was strictly defined and controlled according to the initial randomized design. However, the analyses weren't designed to assess PPI use and therefore didn't randomize PPI treatment, leaving a potential risk of residual confounding even after multivariable adjustment and propensity score analysis. Furthermore, PPI compliance has not been memorized during follow-up- a fact attempt to be adjusted by landmark analyses in the second study.

So far, the only existing randomized controlled double-blind multicenter trial is the Clopidogrel and the Optimization of Gastrointestinal Events (COGENT) trial, including 3878 patients presenting with an ACS or undergoing PCI. Patients were randomized to receive CGT-2168, a fixed combination of 75 mg of clopidogrel and 20 mg of omeprazole *vs* 75 mg of clopidogrel alone. After a median follow-up of 106 d, a significant reduction in the primary endpoint, a composite of upper gastrointestinal bleeding, was observed in the CGT-2168 group (1.1% *vs* 2.9%, HR 0.34, 95% CI: 0.18-0.63, $P < 0.001$). Moreover, analysis of the primary cardiovascular safety end-point, (a composite of death from cardiovascular causes, myocardial infarction, coronary revascularisation and ischemic stroke) have not shown any difference between the placebo and omeprazole group (HR with omeprazole 0.99, 95% CI: 0.68-1.44, $P = 0.96$). Unfortunately, the study was interrupted prematurely due to the bankruptcy of the sponsor, after having included only 3873 of the 5000 initially planned patients. Moreover, wide confidence intervals around the hazard ratio of cardiovascular events and the fact that 94% of the study population was white, do not permit to rule out any significant clinical interaction between clopidogrel and omeprazole[25].

The first of three recent meta-analyses was conducted by Kwok *et al*[36] selecting 23 studies with 93 278 patients. Of note, more than half of the included studies have been available only as abstracts (12 of 23). Studies have been divided into three groups: nonrandomized studies with unadjusted risk ratios, nonrandomized studies with adjusted RR and randomized trials or studies including propensity score matching. Overall analysis of 19 studies reporting the incidence of MACE showed a significantly increased risk in the PPI group (RR 1.43, 95% CI: 1.15-1.77), but data were substantially heterogeneous ($I^2 = 77\%$), partially due to considerable variation of the definition of MACE within the studies. Of interest, subanalysis of the propensity matched and randomized trails didn't show any increased risk (RR 1.15, 95% CI: 0.89-1.48) and data were much less heterogeneous ($I^2 = 53\%$). Identical results were found when analysing the risk of myocardial infarction or ACS, leading the authors to conclude that unmeasured confounders may contribute to the results of the lower quality studies[36]. Siller-Matula *et al*[37] re-analysed 25 studies with 159 138 patients, finding a 29% increase of MACE (RR 1.29, 95% CI: 1.15-1.44) and myocardial infarction (RR 1.31, 95% CI: 1.12-1.53) for concomitant PPI and clopidogrel use. Again heterogeneity in the overall analysis was very important ($I^2 = 72\%$ *vs* 77%, respectively) and sensitivity analysis assessing the study quality showed a decreased risk of MACE in high quality studies (RR 1.23, 95% CI: 1.09-1.39) *vs* low quality studies (RR 1.65, 95% CI: 1.43-1.90), rising again the question of unmeasured confounders and differences in baseline characteristics[37]. Lima *et al*[38] reviewed 18 studies according to the PRISMA guidelines[66] by classifying them into high (well-performed randomized clinical trials), moderate (post hoc analysis of RCTs and propensity matched studies) and low (observational studies without propensity matching) quality studies. Due to important study heterogeneity, data pooling was a priori not effected. A stratified analysis comparing studies of low (13) with those of moderate quality (5) demonstrated an inverse correlation

Table 2 Overview of important clinical studies on the proton pump inhibitor-clopidogrel interaction

Study	PPIs used	Procedures to minimize bias	Population	Primary outcome	Results
Bhatt *et al*[25] (randomized, controlled, double-blind trial)	Omeprazole		3873 patients with ACS or undergoing PCI	Mean 133 d- composite safety endpoint of cardiovascular death, MI, coronary revascularisation	No difference between PPI and placebo group (HR with omeprazole 0.99, 95% CI: 0.68-1.44)
O'Donaghue *et al*[26] (post-hoc analysis of a RCT)	Pantoprazole omeprazole esomeprazole lansoprazole rabeprazole	Propensity score matching; multivariable and sensitivity analysis	13 608 patients undergoing planned PCI for ACS	Composite endpoint of cardiovascular death, MI or stroke after 6-15 mo	No difference between PPI and clopidogrel alone group (HR 0.94, 95% CI: 0.80-1.11)
Dunn *et al*[65] (post-hoc analysis of a RCT)	Not specified	Multivariable analysis	2116 patients undergoing PCI	28 d death, MI, urgent target vessel revascularisation 1 yr death, MI or stroke	Increased risk for adverse cardiovascular outcome regardless of clopidogrel use (clopidogrel/PPI: OR 1.63, 95% CI: 1.02-2.63 *vs* placebo/PPI: OR 1.55, 95% CI: 1.03-2.34)
Charlot *et al*[28] (retrospective cohort study)	Esomeprazole pantoprazole lansoprazole omeprazole rabeprazole	Propensity score matching; multivariable and sensitivity analysis	56 406 patients discharged with first-time myocardial infarction	1 yr composite end point of MI, stroke or cardiovascular death	Increased risk for adverse cardiovascular outcomes in PPI users regardless of clopidogrel use (HR for PPI/clopidogrel: 1.35, 95% CI: 1.22-1.50 *vs* HR for PPI alone: 1.43, 95% CI: 1.34-1.53)
Banerjee *et al*[32] (retrospective cohort study)	Predominantly omeprazole (88,9%)	Propensity score matching; multivariable and sensitivity analysis	23 200 post PCI patients	6-yr MACE	No increased risk for MACE in PPI users (HR 0,97, 95% CI: 0.65-1.44)
Ray *et al*[27] (retrospective cohort study)	Pantoprazole lansoprazole esomeprazole omeprazole rabeprazole	Propensity score matching; multivariable and sensitivity analysis	20 596 patients discharged after PCI or ACS	1 yr composite end point of ACS, stroke or cardiovascular death	No increased risk for serious cardiovascular disease in PPI users (HR 0.99, 95% CI: 0.82-1.19)
Rassen *et al*[31] (retrospective cohort study)	Pantoprazole omeprazole rabeprazole lansoprazole esomeprazole	Propensity score matching;	18 565 patients discharged after PCI or ACS (age > 65 yr)	180 d composite end point of hospitalization for MI and PCI or death of any cause	Trend towards a higher risk of composite end point in PPI users (RR 1.26, 95% CI: 0.97-1.63)
Simon *et al*[30] (retrospective cohort study)	Omeprazole esomeprazole pantoprazole lansoprazole	Propensity score matching; multivariable and sensitivity analysis	2744 clopidogrel and PPI-naive patients with definite MI	In hospital and 1-yr death, reinfarction or stroke	No increased risk of cardiovascular events and mortality in PPI users (HR 0.98, 95% CI: 0.90-1.08)
Harjai *et al*[29] (retrospective cohort study)	Omeprazole esomeprazole	Propensity score matching; multivariable and sensitivity analysis	2651 patients discharged after PCI for stable and unstable CAD	6-mo MACE	No increased risk for MACE in PPI users (HR 0.89, 95% CI: 0.63-1.27)
van Boxel *et al*[14] (retrospective cohort study)	Pantoprazole omeprazole rabeprazole lansoprazole	Multivariable analysis	18 139 clopidogrel users	2 yr composite endpoint of ACS, stroke and any cause death	Increased risk of composite endpoint (HR 1.75, 95% CI: 1.58-1.94), myocardial infarction (HR 1.93, 95% CI: 1.40-2.65) and unstable angina pectoris (HR 1.79, 95% CI: 1.60-2.03)
Juurlink *et al*[16] (population-based nested case-control study)	Omeprazole rabeprazole lansoprazole pantoprazole	Nested case –control; multivariable and sensitivity analysis	13 636 patients discharged after ACS (age > 65 yr)	90-d readmission for acute MI	Increased risk of reinfarction (OR 1.27, 95% CI: 1.03-1.57) in PPI users except pantoprazole
Ho *et al*[15] (retrospective cohort study)	Omeprazole rabeprazole lansoprazole pantoprazole	Multivariable and sensitivity analysis	8205 patients discharged after ACS	3 yr death or rehospitalization for ACS	Increased risk for death or rehospitalization in PPI users (OR 1.25, 95% CI: 1.11-1.41)

ACS: Acute coronary syndrome; PPI: Proton pump inhibitor; OR: Odds ratio; CI: Confidence interval; MI: Myocardial infaction; PCI: Percutaneous coronary intervention; RR: Relative risk; HR: Hazard ratio; MACE: Major adverse cardiovascular event; RCT: Randomized controlled trial.

between clopidogrel-PPI interaction and study quality (P = 0.007), as none of the moderate quality studies reported an association *vs* 10 in the low quality group[38]. The

authors pointed out that according to the large CURE (Clopidogrel in Unstable Angina to Prevent Recurent Events) trial[67] no or very little advantage in reduction of

Table 3 Summary of studies reporting on adverse bleeding events

Study	Observed adverse event	Ascertainment	Results
Bhatt *et al*[25]	Composite of upper gastrointestinal bleeding (of known and unknown origin): overt bleeding, ulcers, symptomatic erosions, obstruction, perforation or decrease in hemoglobin of 2 g/dL	Endoscopic and radiologic confirmation (in known origin subgroup)	Significative reduction of upper gastrointestinal bleeding in the omeprazole treated group (1.1% against 2.9% under placebo; HR 0.34, 95% CI: 0.18-0.63)
Ray *et al*[27]	Hospitalization for bleeding at a gastroduodenal site (excluding angiodysplasia) or other gastrointestinal and non-gastrointestinal sites	Validated diagnostic codes with PPV of 91%	Adjusted 50% reduction of hospitalization in the PPI treated group (HR 0.50, 95% CI: 0.39-0.65), no significant difference concerning bleeding at other sites
van Boxel *et al*[14]	Occurence of complicated or non complicated peptic ulcer disease	ICD-9 diagnostic codes	Low incidence (0.7% with PPI against 0.2%) but significant increase of peptic ulcer disease in the PPI treated group even after multivariable adjusting (HR 4.76, 95% CI: 1.18-19.17)
Charlot *et al*[28]	Hospitalization for gastrointestinal bleeding	ICD-9 diagnostic codes	No reduction between the clopidogrel with PPI and clopidogrel alone group
Harjai *et al*[29]	TIMI major bleeding: intracranial hemorrage or a ≥ 5 g/dL decrease in hemoglobine TIMI minor bleeding: observed blood loss with decrease ≥ 3 g/dL in hemoglobine	Guthrie Health System database	No significant difference between the clopidogrel with PPI and clopidogrel alone group
Simon *et al*[30]	In-hospital major bleeding (not specified) or need for blood transfusion	FAST-MI registry	No significant difference between the clopidogrel with PPI and clopidogrel alone group

HR: Hazard ratio; CI: Confidence interval; PPV: Positive predictive value; PPI: Proton pump inhibitor; ICD-9: International classification of disease-9th revision; FAST-MI: French registry of acute-ST-elevation and non-ST-elevation myocardial infarction.

adverse cardiovascular events when treated with clopidogrel was observed later than 3 mo after an ACS. In contrast to that, in the study of Ho *et al*[15] the increased risk of adverse cardiovascular events for concomitant PPI and clopidogrel use appears in the long term (not before 180 d), a period when clopidogrel has not been shown to be therapeutically useful any more[38]. Theses results might be explained by unmeasured residual confounders rather than by the existence of a clopidogrel-PPI interaction, a hypothesis endorsed by the three studies having found an elevated risk for adverse cardiovascular events in PPI-users, regardless whether on clopidogrel or not[28,65,68]. Characteristics and results of the cited studies are overviewed in Table 2 by classifying them according to their scientific weight, while Table 3 summarizes the studies reporting on adverse bleeding events.

To summarize, pharmacodynamic studies suggest an existing interaction between clopidogrel and omeprazole but not with pantoprazole, a phenomenon that may be explained by the higher inhibitory potency of omeprazole for the cytochrome P450 CYP2C19[54], a key enzyme in the metabolic activation of clopidogrel[39].

Nevertheless, the clinical impact of this biochemical interaction still remains unclear, as several cohort studies report an interaction and consecutive increase in adverse cardiovascular events for omeprazole[14-22]. In contrast to that, recent retrospective studies including propensity score matching in order to minimise underlying bias have not show any clopidogrel-PPI interaction[27-35] (except one recent study using a larger endpoint including overall death, myocardial infarction, stroke and critical limb ischemia[69]). In addition to that, post-hoc analyses of randomized trials[26,65] and the only randomized double blinded trial available so far have not found any increase in adverse cardiovascular events for the PPI treated group[25]. Finally, several meta-analyses pointed out that

there was an inverse correlation between study quality and a reported statistically positive interaction[36-38]. Despite of that, the United states Food and Drug Administration and the European Medecines Agency still discourage the use of PPI (especially omeprazole) concomitantly with clopidogrel[23,24].

Three recommendations to health care providers could therefore be made for the moment: (1) A gastrointestinal risk evaluation (e.g., history of gastrointestinal bleeding, dyspepsia, therapeutic anticoagulation, concomitant NSAIDS use especially in elderly persons and in the presence of helicobacter pylori[70-73]) has to be performed in each patient, as clopidogrel treatment and dual antiplatelet therapy rise the risk of adverse gastrointestinal events and mortality[3,4,25,74,75]. Patients at high risk of gastrointestinal bleeding should have prescribed concomitant PPIs when under clopidogrel, due to the high mortality rate in case of bleeding[2]; (2) Favoring pantoprazole over omeprazole pharmacologically leads to less inhibition of the CYP2C19 isoenzyme, but the clinical impact of this pharmacologic difference has not been proved so far. Nevertheless, to the best of our knowledge, no clinical trial (regardless of its quality) has ever demonstrated a clear interaction for pantoprazole, making it a rather safe choice, especially regarding recent moderate and high quality publications. Furthermore the standard daily dose of 40 mg doesn't seem to induce any significant pharmacodynamic interaction with clopidogrel, as none was found for a 80 mg/d dose[12,57]; and (3) Widening the delay between clopidogrel and PPI intake by a minimum of 12 h (a concept based on the rapid metabolization of clopidogrel[49]), doesn't seem to avoid the possible drug interaction between clopidogrel and PPIs[12,56,57].

In conclusion, rising evidence accumulates to infirm an interaction between PPIs and clopidogrel. This point

suggests that the bleeding reduction benefit overweighs the possible adverse cardiovascular risk in patients with an indication for PPI treatment taking dual antiplatelet treatment. Of course, adequate powered randomized controlled trials with pharmacodynamic assessment are still needed to infirm the persisting doubt upon the PPI-clopidogrel interaction.

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REVIEW

Enhanced apoptosis in post-liver transplant hepatitis C: Effects of virus and immunosuppressants

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Abstract

Hepatitis C (HCV)-infected patients have a poorer survival post-liver transplantation compared to patients transplanted for other indications, since HCV recurrence post-transplant is universal and commonly follows an aggressive course. There is increasing evidence that in the non-transplant setting, induction of hepatocyte apoptosis is one of the main mechanisms by which HCV drives liver inflammation and fibrosis, and that HCV proteins directly promote apoptosis. Recent studies have shown that post-liver transplant, there is a link between high levels of HCV replication, enhanced hepatocyte apoptosis and the subsequent development of rapidly progressive liver fibrosis. Although the responsible mechanisms remain unclear, it is likely that immunosuppressive drugs play an important role. It is well known that immunosuppressants impair immune control of HCV, thereby allowing increased viral replication. However there is also evidence that immunosuppressants may directly induce apoptosis and this may be facilitated by the presence of high levels of HCV replication. Thus HCV and immunosuppressants may synergistically interact to further enhance apoptosis and drive more rapid fibrosis. These findings suggest that modulation of apoptosis within the liver either by changing immunosuppressive therapy or the use of apoptosis inhibitors may help prevent fibrosis progression in patients with post-transplant HCV disease.

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INTRODUCTION

Hepatitis C (HCV)-related liver failure is now the commonest indication for liver transplantation in the United States, Australia and Europe[1]. HCV-infected patients have a poorer survival post-transplantation compared to patients transplanted for other indications[2]. This is because HCV recurrence occurs in virtually all patients and commonly follows an aggressive course, with 20%

or more of patients developing cirrhosis within 5 years of transplantation[3]. The cause of this accelerated disease has not been fully elucidated, but risk factors include advanced donor age, early high HCV viral load post-transplant[4], acute graft rejection and treatment thereof, and the degree of immunosuppression[5].

In the non-transplant setting, induction of hepatocyte apoptosis is one of the main mechanisms via which HCV drives liver inflammation and fibrosis[6]. Recent evidence suggests a link between high levels of HCV replication, high rates of apoptosis and the subsequent development of rapidly progressive graft injury and fibrosis after liver transplantation[7]. The mechanisms responsible for this high levels of apoptosis found in aggressive post-liver transplant HCV disease remain unclear. It is well known that immunosuppressants impair immune control of HCV, thereby allowing increased viral replication. There is also recent evidence that some commonly used immunosuppressants may directly induce apoptosis and this may be facilitated by the presence of high levels of HCV replication. This suggests that HCV and immunosuppressants may synergistically interact to enhance apoptosis and drive rapid fibrosis.

OVERVIEW OF APOPTOSIS

Apoptosis is a highly regulated physiological process that plays an important role in organogenesis and the maintenance of tissue homeostasis[8]. Cells posing a threat to the integrity of an organ, such as virus-infected cells, may be eliminated by apoptosis, which occurs by two major pathways - extrinsic and intrinsic. The extrinsic pathway is activated when death ligands [tumor necrosis factor (TNF), FasL/CD95L and TRAIL] secreted by cells of the immune system in response to foreign (for example, viral) antigens bind to their respective cell surface receptors, to trigger signaling pathways that result in the activation of caspases[9]. The caspases are a class of enzymes responsible for the execution of apoptosis within the cell. In the intrinsic pathway, intracellular apoptotic stimuli, such as viral antigens, cause disruption of mitochondrial membrane integrity, releasing cytochrome c that activates the caspase pathway[10]. The integrity of the outer mitochondrial membrane is predominantly maintained by anti-apoptotic members of the Bcl-2 family (e.g., Bcl-2 and Bcl-xL), which antagonize pro-apoptotic members (for example, Bax and Bak).

LINK BETWEEN HEPATOCYTE APOPTOSIS AND LIVER FIBROSIS

There are increasing amounts of experimental data implicating apoptosis as a driving force for fibrogenesis in a range of different liver diseases, including alcohol-related and cholestatic liver diseases and viral hepatitis[11]. Apoptotic hepatocytes are engulfed and cleared by both Kupffer cells and hepatic stellate cells (HSCs). Activated HSCs are the primary cell type responsible for promoting

fibrogenesis within the damaged liver, and the uptake of apoptotic bodies by HSCs result in their activation and secretion of the key pro-fibrogenic cytokine transforming growth factor-β (TGF-β)[12]. In activated HSCs, TGF-β induces a marked upregulation of genes encoding fibrillar collagens and other extracellular matrix components, resulting in the abnormal deposition of collagen within the liver[13]. Kupffer cells, which are the resident liver macrophages, upon ingestion of apoptotic hepatocytes, also secrete TGF-β, thereby promoting a pro-fibrogenic response in activated HSCs[14]. Furthermore, TGF-β itself induces hepatocyte apoptosis via two independent pathways, SMAD and DAXX[15], thus providing a positive feedback loop that could further potentiate apoptosis-induced fibrosis. In support of these *in vitro* observations, inhibition of apoptosis reduces hepatic inflammation and fibrosis in experimental models of fibrotic liver disease[16].

HEPATITIS C AND HEPATOCYTE APOPTOSIS

In HCV infection, hepatocyte apoptosis is an important part of the host anti-viral defense mechanism since it interrupts viral replication and assists in the elimination of virus-infected cells. However, in keeping with the observed effects of apoptosis in laboratory studies, there is now evidence to suggest that the severity of liver damage in chronic HCV is associated with the degree of hepatocyte apoptosis[6]. Furthermore, the degree of apoptosis correlates with the level of viraemia[17]. Bantel and colleagues have studied a serum apoptosis biomarker, the proteolytic neoepitope of the caspase substrate cytokeratin-18, as a means of determining caspase activity to monitor liver injury and predict the progression of hepatic fibrosis in HCV-infected patients[18]. This biomarker was markedly elevated in the sera of HCV-infected patients compared to healthy controls, and in patients with normal transaminase levels, raised serum caspase activity was associated with advanced fibrosis on liver biopsy.

Hepatocyte apoptotic rates on liver biopsy are significantly greater in HCV-positive patients post-liver transplant compared to the non-transplant setting, with the severity of liver inflammation correlating with the level of hepatocyte apoptosis[7], and HCV viral load is known to be higher post-liver transplantation[19]. Thus one potential explanation for accelerated fibrosis post-transplantation is that the high levels of HCV replication that occurs due to impaired immune control of HCV replication may drive increased hepatocyte apoptosis.

How then does HCV affect apoptosis? One likely mechanism is that virus-specific cytotoxic T-cells may induce apoptosis of HCV-infected hepatocytes by upregulating death receptor ligands (TNF, FasL/CD95L and TRAIL), by producing antiviral cytokines (for example, interferon-γ), and by direct cell killing with perforins and granzymes[20]. HCV infection is also associated with an upregulation of death receptors on hepatocytes, and the levels of Fas/CD95 and FasL/CD95L have been shown

Figure 1 Where the hepatitis C proteins and immunosuppressants are thought to interact with the apoptotic pathways within the hepatocyte. CyA: Cyclosporine; FK506: Tacrolimus; MMF: Mycophenolate mofetil; Sirol: Sirolimus; TNF-α: Tumor necrosis factor-alpha.

to increase in parallel with the severity of inflammation and disease progression[21].

There is also considerable experimental evidence that HCV structural proteins can directly influence hepatocyte apoptosis. HCV core protein has been reported to sensitize hepatocytes to TNF-α-[22] and FasL/CD95L-[23] mediated apoptosis, by interacting with the cytoplasmic domains of TNFR1 and Fas/CD95 to enhance downstream signaling events. It also induces oxidative stress, enhances mitochondrial-mediated hepatocyte apoptosis[24] and upregulates *TGF-β1* gene expression, thereby promoting apoptosis and fibrogenesis. However, the expression of core protein has also been shown to have a number of possible anti-apoptotic effects. These include inhibition of TNF-α- and Fas/CD95-mediated apoptosis through the upregulation NF-κB[25], and interaction with cFLIP, an endogenous caspase-8 inhibitor[26]. Core protein has also been reported to promote the anti-apoptotic Bcl-xL expression, inhibit interferon-α-mediated STAT1 signaling and activate STAT3, thereby protecting infected hepatocytes from T-cell-mediated apoptosis[27]. Both the E1 and E2 glycoproteins of HCV have been shown to induce hepatocyte apoptosis[28], with the E2 protein noted to activate the mitochondrial caspase pathway. However,

E2 protein has also been shown to inhibit interferon-α-mediated STAT1 signaling and TRAIL-induced apoptosis, as well as enhance the proliferation of transfected Huh7 human hepatoma cells[29]. The data on the effect of HCV on caspase-independent apoptosis are lacking. One study showed that core protein expression promoted apoptosis-like caspase-independent cell death in osteosarcoma-derived cells[30], but the effect in liver cells is unknown.

The non-structural proteins of HCV have also been shown to affect hepatocyte apoptosis. By using a NS3-5B subgenomic replicon of HCV, Huh7.5 human hepatoma cells were shown to be sensitized to TRAIL-induced apoptosis[31]. Accumulation of NS4A on mitochondria has been found to promote mitochondrial-mediated apoptosis[32]. Similarly, the HCV protease NS3, can induce apoptosis in a caspase 8-dependent manner. On the other hand, NS2 has been found to inhibit the mitochondrial release of cytochrome c, thereby inhibiting mitochondrial-mediated apoptosis[33]. NS5A inhibits interferon-α-mediated STAT1 signaling[34] and protects hepatocytes against interferon-α- and TNF-α-mediated apoptosis. NS5A also prevents apoptosis by activating NF-κB, inhibiting TGF-β, and upregulating STAT3 expression to

promote hepatocyte proliferation[35].

Thus HCV proteins have been shown to have a number of both pro- and anti-apoptotic effects in cultured hepatocytes but the net of contribution of these changes to hepatocyte apoptotic rates and liver fibrosis *in vivo* remains unclear. The discrepancies in these effects may be partly explained by differences in experimental conditions, cell types, apoptotic stimuli and HCV genotype-specific proteins expressed in various *in vitro* systems that may not mimic the true *in vivo* situation. Our current understanding of how the HCV proteins interact with apoptotic pathways within the hepatocyte is summarized in Figure 1.

HEPATITIS C AND APOPTOSIS OF OTHER LIVER CELL TYPES

Activated HSCs are the key cell type promoting fibrogenesis in the liver. HSC activation is increased in patients with chronic HCV infection and the degree of activation correlates with necroinflammatory grade and fibrosis stage[36]. Interestingly, patients with chronic HCV infection have elevated plasma levels of TGF-β1 and increased expression of TGF-β1 in the liver, while the clearance of HCV infection with anti-viral treatment is associated with normalization of plasma TGF-β1 levels[37]. This argues for an important role of TGF-β in HCV-mediated HSC activation and liver fibrogenesis.

Normally, hepatocytes do not express TGF-β, but hepatocytes exposed to HCV non-structural proteins upregulate TGF-β expression, resulting in the activation of HSCs[38]. HSCs express CD81 and LDL receptor, the putative receptors for HCV, and may perhaps be infected by HCV *in vivo*[39]. Expression of HCV core and non-structural proteins in HSCs was found to activate HSCs, resulting in upregulation of TGF-β and procollagen 1 expression[39]. The interaction of HCV E2 glycoprotein with HSCs is noted to upregulate HSC expression of matrix metalloproteinase 2, thus facilitating hepatic fibrogenesis.

Activated HSCs are primarily cleared by apoptosis, a process that would normally restrict the fibrogenic response within an inflamed liver. However, in patients with chronic HCV and advanced fibrosis, HSC apoptosis is reduced compared to patients with mild fibrosis[40]. This suggests that the inhibition of HSC apoptosis by HCV may contribute to the progression of liver fibrosis in this disease. Also, HCV-infected patients who are noted to have a high number of activated HSCs in liver biopsies done several months after liver transplantation developed advanced fibrosis within 2 years of transplantation, indicating that the degree of HSC activation may be an early predictor of post-transplant rapid fibrosis[41].

Kupffer cells have an integral role in the development of chronic liver inflammation in response to hepatocyte injury. Activated Kupffer cells contribute to HSC activation and thereby promote liver fibrosis. The interaction between HCV core protein and toll-like receptor (TLR) 2 on human Kupffer cells has been shown to upregulate cell surface programmed death-ligand 1 (PD-L1). The binding of Kupffer cell PD-L1 to PD-1 receptors on T-cells promotes T-cell apoptosis, thereby impairing the host adaptive anti-viral response[42]. HCV core protein has also been shown to inhibit TLR3-mediated induction of interferon-α, interferon-β and TRAIL, and this may impair the anti-viral activity of Kupffer cells[42]. HCV has not been shown to affect Kupffer cell apoptosis.

IMMUNOSUPPRESSIVE DRUGS AND APOPTOSIS

The aim of post-liver transplant immunosuppression is to dampen the adaptive immune response and prevent graft rejection. However, robust CD4+ and cytotoxic CD8+ T-cell responses play a central role in controlling HCV replication. The experimental evidence that the increased HCV viraemia that occurs post-transplant may directly drive higher rates of apoptosis suggests a likely link between immunosuppressive drug therapy, the resultant loss of immune control of HCV replication, and apoptosis-induced liver injury and fibrosis.

It is has been suggested that the overall level of immunosuppression, rather than the individual agent, is associated with the level of HCV viraemia and the degree of hepatic injury on liver biopsy in patients with post-transplant HCV recurrence[43]. Thus the use of pulse methylprednisolone for the treatment of acute graft rejection has been shown to dramatically elevate HCV viral load[43], while OKT3, another highly potent immunosuppressant used to treat steroid-refractory acute rejection, has been shown to accelerate HCV-associated liver fibrosis.

However, there is emerging evidence that individual immunosuppressive drugs used in long-term maintenance therapy may also have individual specific effects on both HCV replication and HCV-mediated liver injury. Some groups have shown that cyclosporine therapy is associated with less severe histological recurrence and improved graft survival post-liver transplantation compared to tacrolimus[44]. One possible explanation for this effect is that cyclosporine is known to inhibit HCV replication *in vitro* by the inhibition of NS2 and NS5A[45]. Tacrolimus, on the other hand exhibits no anti-viral effect *in vitro* and in fact impairs interferon-α activity by interfering with STAT-1 phosphorylation, and thus, may promote viral replication and persistence[46]. Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), inhibits HCV replication in Huh7 human hepatoma cells without inhibiting cell proliferation or inducing apoptosis[47]. A synergistic inhibition of viral replication has also been shown when MPA was combined with cyclosporine or interferon-α[48].

In addition to their possible effects on viral replication, there is increasing evidence that some of the immunosuppressive agents may also directly contribute to apoptosis. Figure 1 summarizes our current understand-

ing of where individual immunosuppressants interact with intracellular apoptotic pathways.

Cyclosporine has been shown to prevent hepatocyte necrosis in mice exposed to concanavalin A[49], but data on its effect on hepatocyte apoptosis are lacking. Cyclosporine is noted to cause apoptosis of renal vascular endothelial cells via endoplasmic reticulum stress, as well as fibrosis of the renal tubulointerstitium by upregulating TGF-β expression[50]. These findings raise concerns that similar effects may occur within the liver. Indeed, cyclosporine has been found to promote hepatocyte expression of pro-apoptotic Bak in a rat model of liver injury[51]. On the other hand, cyclosporine has also been shown to prevent apoptosis of human gingival fibroblasts by inhibiting Bax and upregulating anti-apoptotic Bcl-2[52], as well as reducing mitochondrial permeability and inhibiting cytochrome c release in human platelets and rat vascular endothelial cells *in vitro*[53]. In an animal model of colitis, cyclosporine was found to have a protective role against epithelial apoptosis through the upregulation of anti-apoptotic cFLIP and inhibition of caspase-8 activity[54].

Tacrolimus has also been shown to have both pro-apoptotic and anti-apoptotic effects in various cell lines in culture. Treatment with tacrolimus promotes Jurkat T-cell G0/G1 phase cell cycle arrest and the generation of reactive oxygen species, mitochondrial dysfunction and thereby apoptosis[55]. In contrast, in human islet cells exposed to pro-inflammatory cytokines such as IL-1 and interferon-γ, tacrolimus has an anti-apoptotic effect, causing a reduction in TNF-α and down-regulation of caspase-3, -8 and -9[56]. Tacrolimus has also been shown to promote hepatic expression of anti-apoptotic Bcl-2 in a rat model of liver injury[51]. However the effect of tacrolimus on apoptosis in human liver is unknown.

After solid organ transplantation, treatment with MMF has been associated with increased mucosal apoptosis in the upper gastrointestinal tract and colon, producing an appearance similar to graft-*vs*-host disease[57]. While MMF has been shown to induce apoptosis via promoting endoplasmic reticulum stress and increasing caspase-3 activity in human pancreatic islet cells[58], the opposite effect has been observed in renal transplant recipients, where reduced apoptosis of renal tubular epithelial, glomerular and interstitial cells was noted[59]. MMF has also been shown to reduce pancreatic β-cell apoptosis in a rodent model of diabetes, and reduce hepatocyte oxidative stress and apoptosis in a rat model of ischaemia/reperfusion injury[60]. The effect of MMF on human hepatocyte apoptosis is currently unknown.

Sirolimus has been found to induce apoptosis in acute lymphoblastic leukemia cells by inhibiting the PI3K/Akt pathway[61]. It also induces apoptosis in vascular smooth muscle cells by activating caspase-3 and inhibiting NF-κB nuclear translocation[62]. However, sirolimus is known to inhibit HSC proliferation *in vitro*, reduce TGF-β expression and inhibit collagen deposition, thereby reducing hepatic fibrosis in a rat model of liver injury[63]. Indeed, sirolimus has also been shown to reduce liver fibrogen-

esis, improve liver function and enhance survival in rats with established cirrhosis[64]. Huh7 hepatoma cells transfected with the HCV-1b genome have upregulated PI3K-Akt-mTOR signaling[65], possibly rendering HCV-infected cells more resistant to apoptosis. Sirolimus, by inhibiting the mTOR pathway, has been shown to inhibit NS5A phosphorylation, thereby inhibiting HCV replication[66]. Sirolimus-based maintenance immunosuppression has been associated with lower HCV RNA levels at 12 mo following liver transplantation and improved patient survival at 6 years compared to calcineurin inhibitors[67].

THERAPEUTIC IMPLICATIONS

Understanding the role of hepatocyte apoptosis in the pathogenesis of post-transplant HCV-mediated liver injury and the likely contributing role of the immunosuppressive agents has a number of important therapeutic implications. It is hoped that increased knowledge of the pro- or anti-apoptotic effects of different immunosuppressive agents and whether they exacerbate HCV-induced apoptosis may allow the development of immunosuppressive regimes that minimize this aspect of HCV-mediated liver injury. In this regard, sirolimus is of particular interest given its possible anti-apoptotic and anti-fibrotic effects both *in vitro* and in animal models.

These findings also suggest a possible therapeutic role for apoptosis inhibitors in post-transplant HCV. There is increasing experimental and clinical experience with the use of this class of compounds in liver disease. The pan-caspase inhibitor IDN-6556 was found to reduce hepatocyte apoptosis and liver fibrosis in bile duct-ligated mice[16], and improve liver function tests in patients with hepatic dysfunction[68]. VX-166, another pan-caspase inhibitor, has been shown to reduce hepatocyte caspase-3 expression and apoptosis, thereby decreasing hepatic fibrosis in a murine model of non-alcoholic steatohepatitis[69]. Given the evidence linking HCV-induced hepatocyte apoptosis with liver fibrosis, 2 randomized, double-blind, placebo-controlled studies have been conducted using pan-caspase inhibitors in patients with chronic HCV, one using PF-03491390[70] and the other using IDN-6556[71]. In both studies, the orally administered pan-caspase inhibitors were well tolerated with minimal adverse effects and showed significant reductions in serum transaminases. Besides directly targeting caspases, compounds that inhibit other components of the apoptotic pathway upstream to caspases are currently in development. There are currently no drugs that inhibit the caspase-independent apoptotic pathway in the literature.

Conversely, the promotion of HSC apoptosis may also act to reduce hepatic fibrosis. Cortex Dictamni extract was noted to induce apoptosis of activated HSCs, resulting in decreased hepatic collagen deposition and attenuated fibrosis in a murine model of liver injury[72]. Another compound, 2',4',6'-tris(methoxymethoxy) chalcone, is noted to induce apoptosis of activated HSCs by enhancing FasL/CD95L expression without affecting

hepatocyte apoptosis[73]. The tyrosine kinase inhibitor sorafenib has also been found to increase HSC expression of caspase-3 and induce HSC apoptosis resulting in reduced hepatic collagen deposition and fibrosis in bile duct-ligated rats[74]. These compounds raise the possibility of treatment to reduce the population of activated HSCs within the transplanted liver in HCV-recurrence.

In conclusion, the management of post-liver transplant HCV disease remains one of the major challenges in transplant medicine. Enhanced hepatocyte apoptosis appears to contribute to much of the liver injury that drives rapid liver fibrosis in this disease, and in the near future clinically useful serum biomarkers of apoptosis may be available to monitor for this. The precise mechanisms that drive this accelerated hepatocyte apoptosis post-transplant require further study, but it appears that both HCV itself and immunosuppressants play contributory and possibly synergistic roles. In the future as the effects of various immunosuppressive agents on HCV-induced liver cell apoptosis are clarified, a combination of fine-tuning immunosuppressive regimens as well as the manipulation of apoptosis within the liver represents novel therapeutic possibilities for the management of this complex disease.

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ORIGINAL ARTICLE

Side-stream smoking reduces intestinal inflammation and increases expression of tight junction proteins

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Abstract

AIM: To investigate the effect of side-stream smoking on gut microflora composition, intestinal inflammation and expression of tight junction proteins.

METHODS: C57BL/6 mice were exposed to side-stream cigarette smoking for one hour daily over eight weeks. Cecal contents were collected for microbial composition analysis. Large intestine was collected for immunoblotting and quantitative reverse transcriptase polymerase chain reaction analyses of the inflammatory pathway and tight junction proteins.

RESULTS: Side-stream smoking induced significant changes in the gut microbiota with increased mouse intestinal bacteria, *Clostridium* but decreased *Fermicutes* (*Lactoccoci* and *Ruminococcus*), *Enterobacteriaceae* family and *Segmented filamentous baceteria* compared to the control mice. Meanwhile, side-stream smoking inhibited the nuclear factor-κB pathway with reduced phosphorylation of p65 and IκBα, accompanied with unchanged mRNA expression of tumor necrosis factor-α or interleukin-6. The contents of tight junction proteins, claudin3 and ZO2 were up-regulated in the large intestine of mice exposed side-stream smoking. In addition, side-stream smoking increased c-Jun N-terminal kinase and p38 MAPK kinase signaling, while inhibiting AMP-activated protein kinase in the large intestine.

CONCLUSION: Side-stream smoking altered gut microflora composition and reduced the inflammatory response, which was associated with increased expression of tight junction proteins.

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INTRODUCTION

Cigarette smoking is a remarkable etiological factor in the pathogenesis of cardiovascular diseases, hypertension, pulmonary diseases and gastroenterological diseases[1-4]. Meanwhile, passive smoking (second-hand smoking) is also a contributing factor for the development of coronary artery disease[5-7], lung cancer[7] and Crohn's disease[8], which pose a substantial health risk to non-smoking adults and young children worldwide[9]. It was estimated in 2004 that more than 600 thousand deaths were due to

second-hand smoke, which accounted for about 1% of worldwide mortality[9]. On the other hand, it was reported that smoking had a protective effect in reducing ulcerative colitis mostly based on the epidemiologic studies[8,10,11].

Chronic inflammatory bowel diseases, mainly Crohn's disease and ulcerative colitis, are characterized by chronic inflammation of the intestines[8]. Recent studies clearly show that gut epithelial integrity and barrier function are the central predisposing factors in inflammatory bowel diseases, autoimmune and related allergic diseases[12-16]. The intestinal epithelium is composed of tightly assembled intestinal epithelial cells which form a protective barrier against pathogenic and commensal bacteria, preventing their penetration from the lumen to initiate inflammatory responses in the mucosal system[17]. Impairment of the tight junction barrier is associated with chronic diseases such as inflammatory bowel diseases, obesity and type 1 diabetes[18-21]. Epithelial cells form an integrated web through interaction of tight junction proteins including intracellular proteins, zona occludens (ZO)-1, (ZO)-2 and (ZO)-3, cingulin, 7H6 and ZA-1, and membrane proteins, occludin, claudin and junctional adhesion molecules[22,23]. The tight junction functions are affected by extracellular stimuli such as the microbial components, pro-inflammatory cytokines and stress[24,25].

Inflammation disrupts tight junctions. Inflammatory cytokines such as interleukin (IL)-13, and IL-6, increase tight junction permeability through increasing claudin 2 expression[26,27]. The activation of the inflammatory pathway nuclear factor (NF)-κB by TNF-α, down-regulates *ZO-1* gene expression and induces its relocation in Caco-2 cells[28]. Therefore, local inflammation impairs the barrier function of gut epithelium.

The "microflora hypothesis" suggests that gut microflora composition plays an important role in the immunological response of the gut[29]. Lactic acid bacteria are known to have an anti-inflammatory effect[30-34], and alteration of microflora composition is linked to the incidence of inflammatory bowel diseases[35,36]. Up to now, there is no published studies assessed gut microflora changes due to smoking.

We hypothesized that side stream smoking may possess a potent anti-inflammatory effect on the gut mucosal immune system which promotes the expression of tight junction proteins in the intestine, exerting beneficial effects on the prevention of ulcerative colitis.

MATERIALS AND METHODS

Animal care and experiment design
C57BL/6 female mice at 6 mo of age were housed in a temperature-controlled room with a 12 h light and 12 h darkness cycle and were given food and water *ad libitum*. Mice were placed in an exposure box and exposed to side-stream smoke for 1 h daily for 40 d. Commercial cigarettes (golden monkey, tar: 13 mg; nicotine: 1.1 mg; CO: 15 mg) were used at a dose equivalent to one commercial cigarette's smoke per day[37]. The animal care procedures described in this study was approved by the University of Wyoming Institutional Animal Use and Care Committee.

Tissue collection
On the day of necropsy, mice were anesthetized intraperitoneally with tribromoethanol (250 mg/kg body wt). Blood samples were collected from the orbital sinus while mice were under general anesthesia. Mice were then sacrificed by cervical dislocation. Large intestines were dissected, flushed with phosphate-buffer saline and then frozen in liquid nitrogen for immunoblotting and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses. Cecal contents from each mouse were collected and frozen for microflora analyses.

Reagents and antibodies
Antibodies against ZO1, ZO2, Claudin3 and Occludin were purchased from Invitrogen (Camarillo, CA). Antibodies against phospho- c-Jun N-terminal kinase (SAPK/JNK) (Thr183/Tyr185), SAPK/JNK, phospho-NF-κB p65 (ser536), NF-κB p65, phospho-IκB kinase (IKK) α/β (Ser176/180), IKKβ, phospho-IκBα, IκBα, phosho-p38 MAP kinase and p38 MAP kinase, phospho-AMP-activated protein kinase (AMPK) α and AMPKα were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against xanthine oxidase (XO), heat shock protein (HSP) 60 and superoxide dismutase (SOD) 1 were purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Affinity BioReagents (Golden, CO).

Quantitative reverse transcription PCR
Total RNA was extracted from powdered large intestine using Trizol®Reagent (Sigma, St. Louis, MO), treated with DNase I (Qiagen, Valencia, CA) and purified with RNeasy Mini kit (Qiagen). cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). qRT-PCR was conducted on a Bio-Rad CFX96 machine and SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) was used for all qRT-PCR reactions. Mouse GAPDH was used as the housekeeping gene. Primer sequences are listed in Table 1. The final primer concentration was 200 nmol for each gene. The amplification efficiency was 0.90-0.99. The qRT-PCR conditions were 95 ℃, 3 min, and 35 cycles of 95 ℃ for 10 s, 58 ℃ for 20 s and elongation step at 72 ℃ for 20 s. At the end of each run, dissociation melting curve was obtained to confirm the purity of PCR products[38].

Microflora analyses
The frozen caecal contents (0.1 g) were homogenized and bacterial genomic DNA was extracted using a QIAamp DNA stool mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The abundance of specific intestinal bacterial groups was measured by qPCR using Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules, CA) as described above. Group specific

Table 1 Primer sets used for quantitative reverse transcriptase polymerase chain reaction of mouse large intestine tissue

Gene name	Accession no.	Product size	Direction	Sequence (5'→3')	Source
IL-6	NM_031168.1	107 bp	Forward	GCTGGTGACAACCACGGCCT	This study
			Reverse	AGCCTCCGACTTGTGAAGTGGT	
TNF-α	NM_013693.2	67 bp	Forward	TGGGACAGTGACCTGGACTGT	[58]
			Reverse	TTCGGAAAGCCCATTTGAGT	
Claudin 3	NM_009902.4	132 bp	Forward	CAGGGGCAGTCTCTGTGCGAG	This study
			Reverse	GCCGCTGGACCTGGGAATCAAC	
Occludin	NM_008756.2	308 bp	Forward	ATGTCCGGCCGATGCTCTC	[58]
			Reverse	TTTGGCTGCTCTTGGGTCTGTAT	
ZO-1	NM_009386.2	403 bp	Forward	ACCCGAAACTGATGCTGTGGATAG	[58]
			Reverse	AAATGGCCGGGCAGAACTTGTGTA	
ZO-2	AF113005.1	106 bp	Forward	CCCAGCACCAAGCCACCTTTTCA	This study
			Reverse	TCGGTTAGGGCAGACACACTCCC	
GAPDH	NM_008084.2	132 bp	Forward	AACTTTGGCATTGTGGAAGG	This study
			Reverse	GGATGCAGGGATGATGTTCT	

IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α; ZO: Zona occludens; GPADH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Primer sets used for quantitative polymerase chain reaction of 16S rRNA of specific bacterial species or genus

Target organism	Primer set	Sequence (5' to 3')	Product size	Annealing temp (℃)	Reference
Bacteroides	BactF285	GGTTCTGAGAGGAGGTCCC	53	61	[59]
	UniR338	GCTGCCTCCCGTAGGAGT		61	
Clostridium butyricum	Cbut825F	GTGCCGCCGCTAACGCATTAAGTAT	213	72	[60]
	Cbut1038R	ACCATGCACCACCTGTCTTCCTGCC		72	
Clostridium clostridiiforme	Cclos99F	AATCTTGATTGACTGAGTGGCGGAC	148	62	[60]
	Cclos247R	CCATCTCACACTACCGGAGTTTTTC		62	
Clostridium perfringens	Cperf165F	CGCATAACGTTGAAAGATGG	104	61	[59]
	Cperf269R	CCTTGGTAGGCCGTTACCC		61	
Enterobacteriaceae	Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	195	63	[60]
	Eco1652R	CTCTACGAGACTCAAGCTTGC		63	
Enterococcus	Ec-ssu1F	GGATAACACTTGGAAACAGG	115	60	[61]
	Ec-ssu1R	TCCTTGTTCTTCTCTAACAA		60	
Eubacteria	UniF340	ACTCCTACGGGAGGCAGCAGT	210	63	[62]
	UniR514	ATTACCGCGGCTGCTGGC		63	
Faecalibacterium prausnitzii	Fprau223F	GATGGCCTCGCGTCCGATTAG	199	58	[60]
	Fprau420R	CCGAAGACCTTCTTCCTCC		58	
Lactoccoci	LabF362	AGCAGTAGGGAATCTTCCA	315	56	[59]
	LabR677	CACCGCTACACATGGAG		56	
Mouse intestinal Bacteria	Uni516F	CCAGCAGCCGCGGTAATA	161	58	[59]
	MIBR677	CGCATTCCGCATACTTCTC		58	
Segmented filamentous bacteria	SFB736F	GACGCTGAGGCATGAGAGCAT	108	58	[59]
	SFB844R	GACGGCACGGATTGTTATTCA		58	
Ruminococcus albus	Ralb561F	CAGGTGTGAAATTTAGGGGC	246	63	[60]
	Ralb807R	GTCAGTCCCCCCACACCTAG		63	

or kingdom specific 16S rRNA gene primers were listed in Table 2. Eubacteria 16S rRNA was used as the housekeeping gene.

Immunoblotting analyses

Immunoblotting analyses were conducted as previously described[39,40]. Briefly, protein extracts from the mouse large intestine were separated by 5%-15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels and transferred to nitrocellulose membranes for immunoblotting analyses. Band density was normalized according to the GAPDH content[39,40].

Statistical analysis

Statistical analyses were conducted as previously described[41-43]. Data were analyzed as a complete randomized design using General Linear Model of Statistical Analysis System (2000). Mean ± SEM are reported. Mean difference was separated by a least significant difference multiple comparison test. Statistical significance is considered as *P* < 0.05.

RESULTS

Effect of side-stream cigarette smoking on the gut microflora composition

Quantitative PCR analysis of 16S rRNA showed that exposure of C57BL6 mice to side-stream cigarette smoking increased the amount of *Clostridium clostridiforme* and mouse intestinal bacteria (MIB) in the cecal microflora, while decreasing the content of *Lactoccoci, Ruminococcus albus, Enterobacteriaceae* and segmented filamentous bacteria

Figure 1 Cecal microflora composition of Con and side-stream smoking mice. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group (mean ± SEM; *n* = 6 per group).

Figure 2 NF-κB signaling pathway in large intestine of Con and side-stream smoking mice. A: Phos-p65 and p65; B: Phos-IκBa and IκBa; C: Phos-IKKα/β and IKKβ. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group (mean ± SEM; *n* = 6 per group).

(SFB) compared with those of control mice (Figure 1).

Intestinal inflammatory responses of gut to side stream smoking

Side-stream smoking decreased phosphorylation of NF-κB p65, a key mediator of the NF-κB inflammatory signaling pathway. Consistently, phosphorylation of IκBα and IKKα/β were also down-regulated in mice exposed to side-stream smoking, indicating that smoking is capable of reducing inflammation in the gut (Figure 2). qRT-PCR analysis indicated that mRNA expression of the two main inflammatory cytokines, TNFα and IL-6, were not changed (data not shown).

Side-stream smoking induced oxidative stress in large intestine

There was an enhanced oxidative stress in side-stream

smoking mice compared to that of control mice, as indicated by increased XO ($P = 0.06$) and decreased SOD1 ($P < 0.01$) protein content in the side-stream smoking mice (Figure 3). Meanwhile, the heat shock protein 60 (HSP60) decreased in the side-stream smoking mouse large intestine when compared to that of control mice (Figure 3). Consistently, the phosphorylation of stress signaling mediators, JNK and p38 MAP kinase, were increased in the large intestine of side-stream smoking mice (Figure 4). However, the phosphorylation of another kinase related to stress, AMPK, was reduced in response to side-stream smoking (Figure 5).

Tight junction protein expression

Both mRNA expression and protein content of selected tight junction proteins were further analyzed. Protein content of claudin3 ($P < 0.01$) and ZO2 ($P < 0.05$) were

Figure 3 Xanthine oxidase, superoxide dismutase 1 and heat shock protein 60 content in large intestine of Con and side-stream smoking mice. [b]P < 0.01 *vs* control group (mean ± SEM; n = 6 per group). XO: Xanthine oxidase; SOD1: Superoxide dismutase 1; HSP60: Heat shock protein 60.

increased in the large intestine of side-stream smoking mice (Figure 6B), while there is no difference in their mRNA expression (Figure 6A).

DISCUSSION

Epidemiology studies have shown that smoking, including passive smoke inhalation, reduces the incidence of ulcerative colitis, which may be due to the reduction of epithelial permeability[44]. Intestinal permeability was reduced in healthy smokers compared to the non-smokers[45,46].

Mechanisms by which side-stream smoking improves intestinal tight junctions are not well understood. Previous studies suggest that activation of NF-κB signaling increases intestinal permeability[47]. In this study, we observed that the NF-κB signaling was down-regulated in mice exposed to side-stream smoking. This indicates that side-stream smoking negatively regulates NF-κB signaling which might be a contributing factor to the reduction of intestinal permeability. We also observed that side-stream smoking increased Claudin3 and ZO-2 content without affecting Occludin and ZO-1. In summary, our data revealed that side-stream smoking up-regulated the expression of tight junction proteins and inhibited NF-κB signaling, which may be responsible for the preventive effect of smoking on ulcerative colitis.

Smoking generates reactive oxygen species and nitrogen species in blood, resulting in oxidative stress[48-50]. In this study, we also observed that oxidative stress related enzymes such as xanthine oxidase and superoxide dismutase 1 were altered in the large intestine due to side-stream smoking. Consistent with altered oxidative stress, two pivotal stress signaling mediators, the activation of JNK and p38 signaling were enhanced in the large intes-

Figure 4 MAP kinase signaling pathways in large intestine of Con and side-stream smoking mice. A: JNK; B: MAP kinase p38. [a]P < 0.05, [b]P < 0.01 *vs* control group (mean ± SEM; n = 6 per group).

tine of mice exposed to side stream smoking. Previously, it was reported that oxidative stress related signaling promotes tight junction protein claudin1 expression in hepatocytes and Sertoli cells[51,52].

A recent published study in gut epithelial cells shows that AMPK is related to the impairment of tight junction and barrier properties of gut induced by inflammation[53]. Our data showed that AMPK activity was dramatically inhibited in the gut tissue of side-stream smoking mice, which may provide an additional mechanism for the association between passive smoking and gut epithelial barrier function.

Furthermore, we found that microflora were altered due to the side-stream smoking. The "microflora hypothesis" suggests that gut microflora composition plays an important role in the immunological response of the gut[29]. Up to now, there have been no published studies assessing changes in gut microflora due to smoking. Our data showed that exposure to side-stream smoking altered the composition of cecal microflora, reducing *Fermicutes*

Figure 5 Total AMP-activated protein kinase α **subunit content and its phosphorylation at Thr 172 in large intestine of Con and side-stream smoking mice.** [b]$P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group). AMPK: Total AMP-activated protein kinase.

Figure 6 Tight junction protein content in large intestine of Con and side-stream smoking mice. A: mRNA expression; B: Protein content. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group (mean ± SEM; $n = 6$ per group).

and *Enterobacteriaceae*. Both *Fermicutes* and *Enterobacteriaceae* belong to a group of bacteria contributing to fermentation and nutrient intake. *Lactoccoci* and other lactic acid bacteria are known to have anti-inflammatory effects[30-34]. The dramatic reduction of *Lactoccoci* in side-stream smoking mice indicates that *Lactoccoci* might not be responsible for the reduced inflammation in the gut of side-stream smoking mice. The reason for the reduction of *Lactoccoci* in cecal microflora due to smoking is unclear, but might be related to oxidative stress. Many *Lactoccoci* lack catalase and are sensitive to oxidative stress[54], which may render them less competitive in the oxidative environment induced by smoking. We also observed that MIB was increased while SFB was decreased in smoking mice. Because SFB is known to have important roles in maturation of the gut immune system, its reduction in smoking mice could be associated with the adverse effect of smoking on Crohn's disease[55]. MIB refers to a group of bacteria called *Cytophaga-Flavobacter-Bacteroides* phylum[56], and their abundance in the gut is known to be altered by environmental factors[57]. The biological effect of MIB alteration due to smoking is unclear.

In conclusion, data from our present study demonstrated that exposure to side-stream smoking inhibited mucosal inflammation and enhanced the expression of tight junction proteins in the large intestine. Further, side-stream smoking increased oxidative stress and altered gut microflora composition.

COMMENTS

Background

Despite its apparent harmful effects, side-stream smoking reduces the risk of inflammatory gastrointestinal diseases. Gut epithelial integrity and barrier function is a central predisposing factor to inflammatory bowel diseases. Local inflammation impairs the barrier function of gut epithelium. We hypothesized that side stream smoking may possess potent anti-inflammatory effects, which promote the expression of tight junction proteins in the intestine, exerting ben-

eficial effects on the prevention of ulcerative colitis.

Research frontiers

Epidemiologic studies indicate that smoking had a protective effect on ulcerative colitis though the underlying mechanisms remain elusive. In this study, we demonstrated that exposure to side-stream smoking inhibited mucosal inflammation, improved gut tight junction protein expression, and altered gut microflora composition in mice, which could partially explain the preventive effects of smoking on ulcerative colitis.

Innovations and breakthroughs

Recent epidemiologic studies have highlighted the preventive effect of smoking on ulcerative colitis. This is the first study to report that side-stream smoking has anti-inflammatory effect on gut mucosal, improving gut tight junction protein expression and altering gut microflora composition.

Applications

By understanding how side-stream smoking affects gut mucosal immune response and tight junction protein expression, the authors can develop alternative strategies to reduce the risk of ulcerative colitis and possibly other inflammatory bowel diseases without the harmful effects of smoking.

Terminology

Inflammatory bowel diseases are characterized by chronic inflammation in the intestine. Side-stream smoking, mimicking secondhand smoking, has anti-inflammatory effect, which may be responsible for its beneficial effects against ulcerative colitis.

Peer review

The authors address the observation that passive smoking decreases inflammatory response in large intestine. The authors are to be commended for excel-

lent work in performing a very important and informative study. The experimental methods are well summarized and explained. The statistics are appropriate for this study.

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ORIGINAL ARTICLE

Agmatine induces gastric protection against ischemic injury by reducing vascular permeability in rats

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Abstract

AIM: To investigate the effect of administration of agmatine (AGM) on gastric protection against ischemia reperfusion (I/R) injury.

METHODS: Three groups of rats (6/group); sham, gastric I/R injury, and gastric I/R + AGM (100 mg/kg, i.p. given 15 min prior to gastric ischemia) were recruited. Gastric injury was conducted by ligating celiac artery for 30 min and reperfusion for another 30 min. Gastric tissues were histologically studied and immunostained with angiopoietin 1 (Ang-1) and Ang-2. Vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) were measured in gastric tissue homogenate. To assess whether AKt/phosphatidyl inositol-3-kinase (PI3K) mediated the effect of AGM, an additional group was pretreated with Wortmannin (WM) (inhibitor of Akt/PI3K, 15 μg/kg, i.p.), prior to ischemic injury and AGM treatment, and examined histologically and immunostained. Another set of experiments was run to study vascular permeability of the stomach using Evan's blue dye.

RESULTS: AGM markedly reduced Evan's blue dye extravasation (3.58 ± 0.975 μg/stomach *vs* 1.175 ± 0.374 μg/stomach, $P < 0.05$), and VEGF (36.87 ± 2.71 pg/100 mg protein *vs* 48.4 ± 6.53 pg/100 mg protein, $P < 0.05$) and MCP-1 tissue level (29.5 ± 7 pg/100 mg protein *vs* 41.17 ± 10.4 pg/100 mg protein, $P < 0.01$). It preserved gastric histology and reduced congestion. Ang-1 and Ang-2 immunostaining were reduced in stomach sections of AGM-treated animals. The administration of WM abolished the protective effects of AGM and extensive hemorrhage and ulcerations were seen.

CONCLUSION: AGM protects the stomach against I/R injury by reducing vascular permeability and inflammation. This protection is possibly mediated by Akt/PI3K.

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INTRODUCTION

Agmatine (AGM), 4-(aminobutyl) guanidine, is a natural biogenic endogenous dicationic amine metabolite mainly present in the deprotonated form at physiological pH and produced by decarboxylation of L-arginine *via* arginine decarboxylase in bacteria, plants, invertebrates, and mammals[1-5]. It is not supplied by nutritional com-

ponents or bacterial colonization. AGM is metabolized by two distinct pathways depending on the tissue where it is contained: by "agmatinase" (AGM uryl hydrolase) to putrescine with cleavage of urea, mainly in the brain, and by "diamineoxidase" (DAO), in peripheral tissues, to 4-guanidinobutyraldehyde, then dehydrogenated and hydrolyzed by specific enzymes and excreted out of the body. The heterogeneous location of DAO suggests that certain tissues or organs may have the capacity to regulate local AGM levels[6,7]. AGM is transported to organs by an energy-dependent mechanism which is inhibited by dose-dependent administration of putrescine, suggesting a correspondence between the transport mechanism of polyamines and AGM, probably using a carrier[8,9].

After its discovery in the brain, AGM was demonstrated in nearly all organs of rats, with organ-specific distribution. Its highest concentrations were found in the stomach (71 ng/g wet weight), followed by the aorta, small and large intestine, and spleen[10,11]. AGM was also shown in vascular smooth muscle and endothelial cells[12], and in plasma of rats at a concentration of 0.45 ng/mL, which is similar to that of catecholamines[10]. The source of circulating AGM remains undefined. In humans, higher plasma concentrations (47 ng/mL) were determined in comparison to rats[13]. The reasons underlying this large difference remain to be clarified.

It is becoming clear that AGM has multiple physiological functions in the body. It acts as a potential neurotransmitter in the brain[14,15], and a regulator of polyamine concentration[16] by acting on different enzymes involved in the polyamine pathway. It inhibits all isoforms of nitric oxide synthase (NOS), providing evidence of its important role in modulating NO production as an endogenous regulator[17]. In particular, AGM irreversibly inhibits the endothelial NOS and downregulates the inducible form (iNOS), and exhibiting a neuroprotective role since NO contributes to ischemic brain injury[18].

It has been reported that AGM is protective against ischemia reperfusion (I/R) injury in different organs including the brain, retina, kidney and heart[19-22]. However, no previous reports on its protective effect in gastric reperfusion injury have been investigated. Despite the fact that AGM is a strong base[23] and is found in mucous-secreting cells and in parietal cells where it localizes in the canaliculi, it was reported to be deleterious in ethanol-induced gastric lesions[5] as well as in gastric stress-induced lesions[24,25]. Therefore, the aim of the present study was to investigate whether or not the administration of AGM is protective to rat stomach subjected to I/R injury, and the mechanisms involved.

MATERIALS AND METHODS

Animals
Male Wistar rats weighing 170-210 g were obtained from the College of Medicine Animal House at King Saud University. Rats were maintained on standard rat chow and tap water *ad libitum*. Rats were kept in an air-condi-

tioned room with a 12 h day/light cycle. Animals were fasted 12 h prior to the experimental procedure. All studies were approved by the Ethics Committee of King Saud University.

Experimental design
Rats were divided into 3 experimental groups (6 rats/group): (1) control sham-operated group; (2) a gastric I/R group; and (3) I/R + AGM (100 mg/kg) group, administered AGM 15 min prior to I/R injury induction. Rats were anesthetized by i.p. injection of urethane at a dose of 125 mg/100 g body weight (BW). To investigate whether or not the protection induced by AGM is mediated by the Akt/IPK3 pathway, wortmannin (WM), an inhibitor of this pathway, was given at a dose of 15 μg/kg, i.p.[26], 15 min prior to AGM treatment in an additional I/R injury group. The stomach was observed macroscopically for hemorrhages and ulceration. The dose of AGM, selected for the current study was based on the previously published doses used in models of brain[19] and myocardial I/R injury[22]. AGM was dissolved in normal saline and controls received saline in an equivalent volume. WM was dissolved in dimethyl sulfoxide (10%).

Experimental model
Gastric IR lesions were produced as described by Yoshikawa *et al*[27]. Briefly, the stomach was exposed and the esophagus and the pylorus were occluded using bulldog clamps. The celiac artery was clamped and 100 mmol hydrochloric acid (HCL, 1 mL/100 g BW) was placed in the stomach to maintain acid levels during ischemia. The acid was then removed 25 min after ischemia and clamps were removed 30 min after ischemia. The tissues were allowed to re-perfuse for 30 min and then the stomach was removed and examined. AGM 100 mg/kg was found to be effective depending on histological examination and Evans blue (EB) dye extravasation.

At the end of the experiment gastric tissues were collected. Gastric samples were snap-frozen in liquid nitrogen and stored at -80 ℃ for subsequent assays of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1). A piece of each stomach was fixed in 4% phosphate-buffered formalin, embedded in paraffin, and cut. Paraffin sections were hydrated and stained with hematoxylin and eosin (HE) for assessment of mucosal damage or stained with sera specific for angiopoietin-1 (Ang-1) and Ang-2, (R and D Systems, United States).

Determination of vascular permeability
EB dye, an azo dye, is widely used as an indicator of increased capillary permeability[28-30]. Systemic administration of EB leads to the formation of a dissociable complex with serum albumin, and when there is microvascular tissue damage, EB extravasates. In another three experimental groups, 1 mL of EB (0.5% v/w) was injected i.v. after reperfusion or sham operation. The amount of EB that accumulated in the stomach within

Figure 1 Evan's blue extravasation as a measure of vascular permeability in the studied groups. Gastric ischemia reperfusion (I/R) injury induced extravasation of Evan's blue dye due to increased vascular permeability. Agmatine (AGM) pre-treatment (100 mg/kg, i.p.) significantly attenuated the vascular leakage (gastric I/R *vs* I/R + agmatine, [b]*P* < 0.001). Results are expressed as mean ± SD.

the reperfusion period was measured. Briefly, the animals were killed and the stomach was removed. After collecting the gastric content carefully by lavage with 5 mL cold distilled water, the stomach was opened along the greater curvature and the corpus mucosa was scraped off and put into a tube containing 5 mL distilled water. The EB was extracted by a modified method of Lange *et al*[29] and its concentration was spectrophotometrically quantified. The EB present in the gastric contents and mucosa was extracted by adding 5 mL formamide to each tube and kept in a shaking water bath at a temperature of 50 ℃ for 24 h. This was followed by centrifugation at 3000 *g* for 10 min and the absorbance of supernatant was measured at 612 nm (Lambada 5, Perkin-Elmer, Pomona, CA, United States). The amount of EB was calculated from a previously prepared standard curve and expressed as μg per stomach.

Histological study
Gastric tissues from the studied groups were fixed in 10% phosphate-buffered formalin, embedded in paraffin and 4 μm sections were made, followed by staining with HE and were examined histologically for mucosal damage.

Enzyme-linked immunosorbent assay
VEGF and MCP-1 were assayed in a supernatant of gastric tissue homogenate and calculated according to protein concentration in each sample. Protein was determined in each sample using Bradford Reagent (Biorad, United States). Concentrations of VEGF and MCP-1 were measured using an ELISA kit according to the manufacturer's instructions (R and D Systems, United States).

Immunohistochemistry
Immunostaining was performed using formalin fixed, paraffin-embedded sections (4 μm) after dewaxing and rehydration. Endogenous peroxidase was quenched with 3% H$_2$O$_2$ for 30 min and sections were blocked with 10% normal goat serum (Sigma). Sections were incubated with

Ang-1 and Ang-2 (Santa Cruz, Biotech., United States) at a concentration of 1:200 and were kept at room temperature for 2 h. Sections were then washed and incubated with secondary antibody, and immunoperoxidase staining was carried out using the Vectastain ABC Elite reagent kit (Vector Laboratories, CA, United States). Di-amino-benzidine was used as a chromogen. All slides were counterstained with HE.

Chemicals and reagents
All chemicals were purchased from Sigma (St, Louis, MO, United States) unless otherwise specified.

Statistical analysis
All values are expressed as the mean ± SD. Statistical significance of differences was determined using one-way analysis of variance. Further statistical analysis for *post hoc* comparisons was carried out using the Tukey test. A level of *P* < 0.05 was considered statistically significant.

RESULTS

Effect of AGM on gastric tissue vascular permeability
Gastric perfusion for 30 min following 30 min of ischemia induced a marked leakage of EB dye in the stomach lumen (3.58 ± 0.975 μg/stomach). The administration of AGM 100 mg/kg prior to induction of ischemia attenuated the leakage by about 60% when compared with no treatment (1.173 ± 0.374 μg/stomach, *P* < 0.05) (Figure 1).

Histological assessment
The histological features of gastric I/R injury included hemorrhage, and ulceration of the mucosa with inflammatory cell infiltration (Figure 2B). Gastric mucosa of normal rats showed intact mucosa and sub-mucosa (Figure 2A). The administration of AGM at a dose of 100 mg/kg attenuated the mucosal damage by some of 80% of the surface exposed (Figure 2C) with reduction in hemorrhage, ulceration and cellular infiltration. WM prevented the protective effect resulting in extensive ulceration, edema, and hemorrhage (Figure 2D).

Effect of AGM on VEGF gastric tissue level
Following ischemic injury to the stomach, VEGF demonstrated an increase in gastric tissue homogenate (48.4 ± 6.53 pg/100 mg protein) compared with normal gastric tissue (32.725 ± 37.7 pg/100 mg protein). AGM treatment significantly reduced this increase (36.87 ± 2.71 pg/100 mg protein *vs* 48.4 ± 6.53 pg/100 mg protein, *P* < 0.05) (Figure 3).

Effect of AGM on MCP-1 gastric tissue level
As shown in Figure 4, the I/R injury of the gastric mucosa induced an increase in the level of MCP-1 compared with the mucosa in control sham rats (41.17 ± 10.3 pg/100 mg protein *vs* 18.3 ± 7.4 pg/100 mg protein, *P* < 0.01). AGM pretreatment markedly reduced the MCP-1

Figure 2 Histological appearance of the gastric mucosa of HE stained sections of the studied groups. A: Sham-operated showing intact mucosa; B: Gastric ischemia reperfusion (I/R) showing hemorrhages, edema and ulceration; C: Agmatine (AGM) treated group (100 mg/kg, i.p. prior to I/R) with marked preservation of gastric mucosa and disappearance of ulceration and hemorrhages; D: Effect of inhibition of Akt/phosphatidyl inositol-3-kinase (PI3K) (wortmannin 15 μg/kg, i.p.) prior to AGM treatment, showing extensive lesions and salvage of gastric mucosa into the lumen (× 20 magnification).

Figure 3 Effect of administration of agmatine on gastric vascular endothelial growth factor tissue level. Gastric vascular endothelial growth factor (VEGF) protein significantly increased after ischemia reperfusion (I/R) injury, [b]*P* < 0.01 *vs* sham group. Administration of agmatine administration (100 mg/kg, i.p.) 15 min prior to gastric I/R reduced the VEGF level in gastric tissue homogenate, [d]*P* < 0.01 *vs* I/R group. Results are expressed as mean ± SD.

Figure 4 Effect of AGM administration on gastric monocyte chemoattractant protein-1 tissue level. Gastric monocyte chemoattractant protein-1 (MCP-1) protein significantly increased after ischemia reperfusion (I/R) injury, [b]*P* < 0.01 *vs* sham group. Administration of agmatine (100 mg/kg, i.p.) 15 min prior to gastric I/R reduced the MCP-1 level in gastric tissue homogenate, [d]*P* < 0.01 *vs* I/R group. Results are expressed as mean ± SD.

levels relative to I/R injury (29.5 ± 7 pg/100 mg protein *vs* 41.17 ± 10.4 pg/100 mg protein; *P* < 0.01).

Ang-1 and Ang-2 immunostaining

A very faint staining for Ang-1 was seen in normal rat stomach (Figure 5A), while it was extensively expressed in areas where congestion and damage occurred in ischemia reperfused stomach (Figure 5B). However, AGM pretreatment (100 mg/kg) markedly attenuated the expression of Ang-1 (Figure 5C). Ang-2 expression of normal rat stomach (Figure 6A), was almost undetected. Exten-

sive expression of Ang-2 was seen mostly in the mucosa of ischemia reperfused stomach (Figure 6B). AGM markedly attenuated Ang-2 expression and protected the mucosa from injury (Figure 6C).

Effect of blocking the phosphatidyl inositol-3-kinase pathway on protection induced by AGM

AKt/phosphatidyl inositol-3-kinase (PI3K) is described as an important component of cell survival pathways in many cell types[26]. Pretreatment of rats with WM (15 μg/kg per i.p.), an inhibitor of AKt/PI3K, 15 min prior to

Figure 5 Photomicrographs representative of angiopoietin-1 immunostaining of gastric sections of the studied groups. A: Sham-operated showing lack of expression of angiopoietin-1 (Ang-1); B: Gastric ischemia reperfusion (I/R) group showing extensive cytoplasmic expression of Ang-1 particularly in areas of congestion and damage; C: Agmatine (AGM) treated group (100 mg/kg, i.p.), with marked attenuation of Ang-1 expression in intact mucosa; D: Wortmannin (15 μg/kg, i.p.) treated group prior to AGM administration abolished the effect of AGM and the expression of Ang-1 is marked (× 20 magnification).

Figure 6 Photomicrographs representative of angiopoietin-2 immunostaining of gastric sections of the studied groups. A: Sham-operated showing faint expression of angiopoietin-2 (Ang-2); B: Gastric ischemia reperfusion (I/R) group showing extensive cytoplasmic expression of Ang-2 in areas of congestion and damage, and in the submucosa; C: Agmatine (AGM) treated group (100 mg/kg, i.p.), with marked attenuation of Ang-2 expression in intact mucosa; D: Wortmannin (15 μg/kg, i.p.) treated group prior to AGM administration abolished the effect of AGM and the expression of Ang-2 is marked in mucosa and sloughed tissues (× 20 magnification).

AGM administration, markedly prevented its protective effect and extensive hemorrhages and ulceration were demonstrated microscopically (Figure 2D), suggesting that AGM probably acted on this signaling pathway to protect the stomach from I/R injury. The administration of WM prior to AGM also induced marked expression of both Ang-1 and Ang-2 in the mucosa and even in the submucosa (Figures 5D and 6D).

DISCUSSION

As mentioned earlier, the present study examined whether or not the administration of AGM prior to gastric I/R injury has a protective effect. Our results revealed a gastroprotective effect of AGM by reducing vascular permeability of the gastric mucosa as evidenced by reduction of EB dye extravasation, Ang-1 and Ang-2 protein expression in gastric sections, as well as VEGF and MCP-1 concentrations in gastric tissue homogenate. This effect is probably mediated by the Akt/PI3K pathway.

The gastric injury model we used in the present study is a gastric ischemia reperfusion model. In this model we injected 100 mmol HCL (1 mL/100 g BW) to maintain the gastric acid level during the procedure. The acid was then removed 25 min after ischemia and clamps were removed 30 min after ischemia. I/R injury was demonstrated macroscopically and microscopically in the form of congestion, hemorrhages, and mucosal ulceration. With prior administration of AGM, hemorrhages, blood clots and ulceration were attenuated. This protective effect, while supported by some studies, was opposed by others.

Circumstantial evidence was provided in a recent study that AGM is protective to the gastric mucosa[23]. That study, utilizing immunohistochemistry, showed that AGM is found in the mucus-secreting cells of the stomach and in the parietal cells where it is localized in the lumen of the canaliculi. Biochemically, basic amino acids are ideally suited to act as a source of carbon dioxide in the stomach, and the most basic amino acid is arginine (isoelectric point 11.15). A significant source of arginine is found in the stomach[23]. CO_2 can be produced from the decarboxylation of arginine. The activation segment of pepsinogen in the parietal cell canaliculi can provide a source of arginine[31]. AGM is the decarboxylation product of arginine. Interestingly, the highest concentration of AGM is found in the stomach[10]. The fact that AGM is such a strong base and its cellular localization is in the gastric mucosa makes AGM a strong candidate for a protective role against HCL formed in the stomach[23]. In addition, *Helicobacter pylori* (*H. pylori*) infection is associated with a decrease in the amount of mucous-secreting cells in the stomach. This change is associated with a decrease in the amount of AGM in these mucous-secreting cells[32]. It was speculated that such a decrease in the amount of AGM in the epithelium of the *H. pylori*-infected stomach would make this epithelium more vulnerable to damage by gastric acid[23]. On the other hand, a group of inves-

tigators reported higher AGM concentrations in gastric juice from *H. pylori*-positive patients than from *H. pylori*-negative patients, and concluded that AGM is deleterious to the stomach and may be involved in the pathogenesis of gastroduodenal lesions[33]. However, these results suggest an association rather than a causal link. Furthermore, these results might implicate AGM as a counter-regulatory molecule to *H. pylori*. Supporting our speculation, a recent report demonstrated that bacteria such as *Escherichia coli* and *H. pylori* utilize AGM to survive the highly acidic medium of the stomach and even prevent AGM being taken up by stomach cells[34]. This explains the high levels of AGM previously reported in gastric juice of *H. pylori* patients, suggesting a compensatory mechanism.

The pattern of gastric response to AGM in our study is opposite to what has been reported by some other previous studies[24,25], which showed that AGM augments gastric acid and pepsin secretion, decreases gastric adherent mucus and worsens experimental gastric mucosal injury in rats in a pylorus-ligated ischemic stress model. Similar results demonstrating exacerbation of gastric mucosal injury by AGM pretreatment in ethanol-induced stress model in rats have been reported[5]. The inconsistency of these results with ours could be attributed to the models used and the duration of gastric stress. In our model, the rats were exposed to 30 min of ischemia and 30 min of reperfusion, while these investigators exposed the animals to 4 h of stress. In addition, these studies administered AGM at a dose of 10 mg/kg[24] or 20 mg/kg[5], which is much lower than that used in the present study or other studies. For example in a rat model of brain I/R injury AGM was given at a dose of 100 mg/kg[19].

The mechanisms by which AGM induced gastric protection were the focus of the present study. Increased vascular permeability occurs after insult to the gut[35] and hence, reduction of hyper-permeability can induce tissue protection. The current work provides evidence that AGM works by reducing vascular permeability of the stomach in response to I/R injury. This was investigated using EB dye extravasation as a measure of vascular permeability. Also, we measured VEGF concentration in gastric tissues and Ang-1 and Ang-2 distribution in gastric sections. The present study showed an elevation of VEGF content in the stomach following 30 min of ischemia and 30 min of reperfusion. This increase is most probably due to increased blood to the tissue upon reperfusion, but the possibility of increased expression of VEGF protein could be a factor. The prevention of this increase by AGM could be explained by the capability of AGM to reduce vascular permeability as was seen in the present study by the EB dye experiment. Interestingly, previous studies showed that transgenic mice overexpressing VEGF induced hyper-permeable vessels[36], providing evidence that VEGF increases vascular permeability. VEGF has been implicated in the pathophysiology of liver I/R injury, and its increase during reperfusion injury was seen to mediate leukocyte trafficking and ischemic injury response early after reperfusion, whereby

VEGF acts *via* MCP-1[37]. Interestingly, the administration of VEGF antibodies was reported to block reperfusion injury[37]. The proinflammatory functions of VEGF have been demonstrated by previous studies[37-39]. Therefore, agents attenuating VEGF during the early period of reperfusion could possibly indirectly reduce the inflammatory response resulting from reperfusion injury.

Other vascular molecules studied in the current work were Ang-1 and Ang-2. We demonstrated the attenuation of both Ang-1 and Ang-2 expression in rats receiving AGM prior to induction of gastric I/R, compared with untreated rats. Ang-2, in contrast to Ang-1, was shown to increase vascular permeability, and to promote vascular leakage from blood vessels *in vivo*[40]. AGM seems to reduce vascular permeability at least partly by reducing VEGF and Ang-2 in gastric tissues.

MCP-1 is an inflammatory mediator molecule[41] whose upregulation is responsible for recruitment of inflammatory cells after I/R injury[42]. Importantly, the interaction of MCP-1 with its receptor CCR2 has been attributed a central role in experimental cardiac, renal and cerebral I/R models[43]. Therefore, decreased MCP-1 production will attenuate attraction and recruitment of monocytes and subsequently ameliorate the post-ischemic inflammatory responses. The present study demonstrated an increase in gastric tissue content of MCP-1 after I/R which was suppressed by AGM pre-treatment. Indeed, we provided evidence for the protective anti-inflammatory effect of AGM, whereby it reduced gastric MCP-1 in the ischemic group. In support of our observation, previous reports also showed an anti-inflammatory effect of AGM[44,45], a mechanism by which AGM can attenuate I/R injury. Furthermore, AGM was shown to inhibit the production of NO in macrophages, thus providing a molecular basis for the anti-inflammatory actions of AGM[42]. We, previously demonstrated that iNOS is upregulated in the gastric mucosa in response to I/R injury[46]. Interestingly, Mu *et al*[18] reported that iNOS was also upregulated in brain in response to I/R injury and was significantly downregulated by AGM. With these data in mind we may speculate that AGM could contribute to gastric protection against I/R injury by reducing NO production, possibly by inhibiting iNOS. Other suggested mechanisms that might be involved in the protection offered by AGM could be *via* preservation of endothelial dysfunction[47] and inhibition of matrix metalloproteinase-9, which is known to be upregulated in ischemic injury and degrades the basement membrane of blood vessels[48].

The signaling pathway by which AGM induced gastric protection is possibly mediated *via* Akt/PI3K. The present study provided indirect evidence, as inhibition of this pathway by WN, prior to AGM administration, prevented protection offered by AGM. A limitation to this study is that we did not measure Akt/PI3K activity in gastric tissues.

There are many reasons making AGM a good candidate for gastric protection. First, it is a strong base[22]; second, the highest concentration of AGM is found in the stomach[10]; third, AGM is localized in the mucus-secret-

ing cells[22]. In the parietal cells, it is localized to the lumen of the canaliculi. Finally, a decrease in AGM concentration in mucus-secreting cells in *H. pylori* infection of the stomach is also associated with a decrease in the amount of mucus in the mucus-secreting cells of the stomach[32]. Recently, it has been reported that bacteria such as *E-coli* and *H. pylori* utilize AGM to survive the highly acidic medium of the stomach and even prevent AGM being taken up by stomach cells[34].

In conclusion, the present study revealed that AGM protects the rat stomach exposed to I/R injury at least for brief periods by reducing vascular permeability possibly mediated *via* Akt/PI3K pathway.

COMMENTS

Background

Gastric ischemia reperfusion (I/R) injury which can occur after major surgeries, may lead to extensive gastric ulceration and even perforation if it is not prevented/treated. Although there are therapies that treat gastric lesions, the search for novel molecules is needed. Agmatine (AGM) an endogenously produced amine has its highest concentration in the stomach with alkaline pH. Many studies reported that AGM protected rat kidneys and brains against ischemic injury. Its effectiveness in a model of gastric ischemia reperfusion injury needs investigation.

Research frontiers

AGM is a biological amine found in most cells with the highest concentration in the stomach. The study focused on the effectiveness of AGM in protecting the stomach against I/R injury.

Innovations and breakthroughs

Previous application of AGM to rats in which gastric injury was performed by ethanol administration was found to be unprotective. It was given at a dose of 20 mg/kg. The present study used a model of gastric I/R injury by clamping the gastric vessels for 30 min followed by declamping. AGM was administered at 100 mg/kg before reperfusion. I/R injury induced gastric ulceration and hemorrhages and increased gastric permeability. AGM treatment was accompanied by preservation of gastric histology and vascularity. Also this protection was produced by an anti-inflammatory effect. AGM was demonstrated to be effective in preventing gastric injury induced by I/R.

Applications

The study results suggest that the AGM can be a potential therapeutic option that could be used in preventing gastric injury induced by I/R injury.

Terminology

Ischemia reperfusion (I/R) injury: Injury induced in tissue when blood supply to a tissue/organ stops for a while and then resumes. During reperfusion some injurious substances are released from the tissues including inflammatory, vasodilator and oxidative stress molecules. Therefore it is important to protect tissues against reperfusion injury. Agmatine (AGM): A naturally occurring amine found in our body organs, with the highest concentration in the stomach. It is also found in bacteria and animals. It has diverse functions that deserve future investigations.

Peer review

The authors demonstrated the protection against gastric ischemia-reperfusion injury in rats by agmatine. This study has an interesting finding.

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BRIEF ARTICLE

Protective effects of 5-methoxypsoralen against acetaminophen-induced hepatotoxicity in mice

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Abstract

AIM: To investigate the hepatic protective effects of 5-methoxypsoralen (5-MOP) and to learn if 5-MOP causes hepatotoxicity at protective doses.

METHODS: C57BL/6J mice were administrated orally with 5-MOP at doses of 12.5, 25 and 50 mg/kg body weight respectively every morning for 4 d before given acetaminophen (APAP) subcutaneously at a dose of 500 mg/kg. The 5-MOP alone group was treated with 5-MOP orally at a dose of 50 mg/kg body weight for 4 d without APAP. Twenty-four hours after APAP administration, blood samples of mice were analyzed for serum enzyme alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) levels, and malondialdehyde (MDA), reduced glutathione (GSH) and oxidized glutathione (GSSG) of liver tissues were measured and histopathologic changes of the liver were observed.

RESULTS: Compared with the vehicle control group, the serum levels (IU/L) of ALT, AST and LDH were all increased significantly in APAP group (8355 ± 3940 *vs* 30 ± 21, $P < 0.05$; 6482 ± 4018 *vs* 146 ± 58, $P <$ 0.05; 24627 ± 10975 *vs* 1504 ± 410, $P < 0.05$). Compared with APAP group, the serum ALT levels (IU/L) (1674 ± 1810 *vs* 8355 ± 3940, $P < 0.05$; 54 ± 39 *vs* 8355 ± 3940, $P < 0.05$; 19 ± 9 *vs* 8355 ± 3940, $P < 0.05$), AST levels (IU/L) (729 ± 685 *vs* 6482 ± 4108, $P < 0.05$; 187 ± 149 *vs* 6482 ± 4108, $P < 0.05$; 141 ± 12 *vs* 6482 ± 4108, $P < 0.05$) and LDH levels (IU/L) (7220 ± 6317 *vs* 24 627 ± 10 975, $P < 0.05$; 1618 ± 719 *vs* 24 627 ± 10 975, $P < 0.05$; 1394 ± 469 *vs* 24 627 ± 10 975, $P < 0.05$) were all decreased drastically in the three-dosage 5-MOP pretreatment groups. Pretreatment of 5-MOP could attenuate histopathologic changes induced by APAP, including hepatocellular necrosis and infiltration of inflammatory cells, and the effect was dose-dependent. MDA levels (nmol/mg) were decreased by 5-MOP in a dose-dependent manner (0.98 ± 0.45 *vs* 2.15 ± 1.07, $P > 0.05$; 0.59 ± 0.07 *vs* 2.15 ± 1.07, $P < 0.05$; 0.47 ± 0.06 *vs* 2.15 ± 1.07, $P < 0.05$). The pretreatment of 5-MOP could also increase the GSH/GSSG ratio (3.834 ± 0.340 *vs* 3.306 ± 0.282, $P > 0.05$; 5.330 ± 0.421 *vs* 3.306 ± 0.282, $P < 0.05$; 6.180 ± 0.212 *vs* 3.306 ± 0.282, $P < 0.05$). In the group treated with 5-MOP but without APAP, the serum enzyme levels, the liver histopathologic manifestation, and the values of MDA and GSH/GSSG ratio were all normal.

CONCLUSION: 5-MOP can effectively protect C57BL/6J mice from APAP-induced hepatotoxicity and possesses an antioxidative activity, and does not cause liver injury at the protective doses.

Key words: 5-Methoxypsoralen; Protection; Acetaminophen; Hepatotoxicity; Antioxidation

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INTRODUCTION

Acetaminophen (APAP), a widely used antipyretic and analgesic drug, could induce hepatotoxicity and even acute liver failure (ALF) when taken at overdose[1]. APAP overdose is a common cause of adult and children ALF in the United States and other countries[2-5]. APAP can be metabolized by cytochrome P450 enzymes (CYPs) to N-acetyl-P-benzoquinoneimine (NAPQI)[6]. At overdoses of APAP, a large number of NAPQIs is generated, which can deplete reduced glutathione (GSH) and then bind to mitochondrial proteins to cause mitochondrial dysfunction and oxidant stress[7,8], leading to hepatocellular damage and centrilobular hepatic necrosis. In this process, APAP can increase the level of malondialdehyde (MDA) both in the liver and plasma[9] and NAPQI is capable of lowering GSH/oxidized glutathione (GSSG) ratio by oxidizing the thiol group of GSH[10]. Oxidant stress plays a central role in the hepatic damage induced by APAP[11].

5-methoxypsoralen (5-MOP), a furocoumarin found in many medicinal plants, possesses slight antioxidative activity evidenced from researches *in vitro*[12,13]. 5-MOP has been used in combination with UV radiation in skin photochemotherapy for decades[14], and some studies also found that it has anticancer[15-19], antidepressant[20-24], anticonvulsion[25] and anti-inflammatory effects[26,27], but none of previous studies have shown that 5-MOP could prevent hepatotoxicity.

In addition, some patients suffered from toxic hepatitis induced by 5-MOP when it was used as photochemotherapeutic agent[28,29], and one animal experiment demonstrated that high doses of 5-MOP can induce hepatotoxicity in mice[30]. So it is essential to examine if 5-MOP can cause liver injury at therapeutic doses.

This study was designed to determine the protective effects of 5-MOP in APAP-induced hepatotoxicity using mouse hepatotoxic models, and to investigate if 5-MOP can cause hepatotoxicity in mice at effective doses.

MATERIALS AND METHODS

Chemicals

5-MOP was purchased from Tokyo Chemical Industry (Tokyo, Japan), and APAP from Jiaozuo Xin'An Science and Technology Company (Henan, China). Tween 80, which was used to prepare 5-MOP suspension, was bought from Biodee Biotechnology Company (Beijing, China). APAP was dissolved in normal saline before use. GSH and N-ethylmathione gained from Lizhudongfeng Biotechnology Company (Shanghai, China), GSSG from Hongxing Biotechnology Company (Beijing, China), o-phthalaldehyde (OPT) from Jinlong Chemical Company (Beijing, China), and thiobarbituric acid (TBA) from Acros (United States).

Animals and treatment

Male C57BL/6J mice, 18-22 g in weight, were purchased from Peking University Laboratory Animal Department, Beijing, China. They were housed in a well-ventilated room and the room temperature was controlled at 21 ℃ -23 ℃ and humidity at 65%-70% with a 12 h light-12 h dark cycle. All the mice were fed adaptively for three d before experiment, and they had free access to water and were fed with forage supplied by Laboratory Animal Center of Military Medical Science Academy.

5-MOP was suspended in 1% Tween 80 at different concentrations of 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL, and all of these suspensions were administrated to mice at 10 mL/kg body weight; that is, mice were administrated with 5-MOP at doses of 12.5 mg/kg, 25 mg/kg and 50 mg/kg, respectively. A 5-d experiment was performed with 36 mice which were randomly divided into 6 groups by weight. Group 1 was the vehicle control group and group 2 was APAP alone group, both groups were orally treated with 1% Tween 80 (10 mL/kg body weight) every morning for 4 d. Groups 3, 4 and 5 were 5-MOP multiple-dose groups administered with oral 5-MOP at doses of 12.5, 25 and 50 mg/kg body weight respectively every morning for 4 d. Group 6 was 5-MOP alone group treated with oral 5-MOP at a dose of 50 mg/kg body weight also for 4 d. Thirty minutes after the administrations, all mice except those in the vehicle control group and 5-MOP alone group were subcutaneously administrated with APAP (500 mg/kg body weight). Twenty hours after APAP administration, blood samples were collected from orbital venous plexus of the mice. After the mice were sacrificed, their livers were dissected out immediately and washed with normal saline, dried on a filter paper and weighted. Then the livers were prepared immediately for further examinations.

The animal care and surgical procedures were performed in compliance with the Guidelines for Animal Care and Use of Peking University.

Biochemical test

The blood samples were collected to determine serum enzyme [alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH)] levels by HI-TACHI-7170A automatic analyzer. The liver tissues were homogenized with potassium chloride (KCl) solution (0.15 mol/L) on ice to yield a 5% (w/v) homogenates for MDA test. The hepatic MDA levels were determined as thiobarbituric acid reactive substances levels using a published colorimetric method[31].

The liver tissues were homogenized with phosphate buffered solution on ice to yield a 5% (w/v) homogenates for glutathione test. The GSH and GSSG levels in liver tissues were measured by the improved Hission method[32], a fluorometric method that uses OPT as a fluorescent reagent. Then GSH/GSSG ratio was calculated.

Table 1 Effects of acetaminophen alone, 5-methoxypsoralen multi-dose and 5-methoxypsoralen alone on alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase activities, hepatic malondialdehyde and reduced glutathione/oxidized glutathione ratio in mice (mean ± SE)

Treatments groups	ALT (IU/L)	AST (IU/L)	LDH (IU/L)	MDA (nmol/mg)	GSH/GSSH
Vehicle control	30 ± 21	146 ± 58	1504 ± 410	0.18 ± 0.11	6.045 ± 0.629
APAP alone at 500 mg/kg	8355 ± 3940[a]	6482 ± 4018[a]	24 627 ± 10975[a]	2.15 ± 1.07[a]	3.306 ± 0.282[a]
5-MOP at 12.5 mg/kg	1674 ± 1810[c]	729 ± 685[c]	7220 ± 6317[c]	0.98 ± 0.45	3.834 ± 0.340
5-MOP at 25 mg/kg	54 ± 39[c]	187 ± 149[c]	1618 ± 719[c]	0.59 ± 0.07[c]	5.330 ± 0.421[c]
5-MOP at 50 mg/kg	19 ± 9[c]	141 ± 12[c]	1394 ± 469[c]	0.47 ± 0.06[c]	6.180 ± 0.212[c]
5-MOP alone at 50 mg/kg	37 ± 20	138 ± 22	1471 ± 191	0.15 ± 0.09	6.858 ± 0.678

[a]$P < 0.05$ *vs* vehicle control group; [c]$P < 0.05$ *vs* acetaminophen (APAP) alone group. 5-MOP: 5-methoxypsoralen; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; GSH: Reduced glutathione; GSSG: Oxidized glutathione.

Histopathologic examination

The left liver lobes were scissored out and fixed in 10% formalin solution for 48 h. The liver samples were then cut into thin transverse sections with the help of microtome and permanent slides were prepared with HE staining. Liver histopathologic changes were examined under an optical microscope with the original magnification × 200.

Data treatment and statistical analysis

The experimental results were expressed as mean ± SE (standard error). Statistical comparison between groups was performed by one-way analysis of variance with SPSS 13.0 statistical software. A P value < 0.05 was indicated as a statistically significant difference.

RESULTS

Effects of APAP alone, 5-MOP multiple-dose and 5-MOP alone on serum enzyme levels

The hepatocellular damage induced by a toxic dose (500 mg/kg) of APAP and the effects of pretreatment with 5-MOP were investigated by measuring the serum levels of ALT, AST and LDH. As shown in Table 1, APAP significantly increased the serum ALT, AST and LDH levels compared with the control group, and the multiple-dose 5-MOP pretreatment significantly prevented the increases of serum enzyme levels. The effect of 5-MOP was dose-dependent, and in the highest dose group, serum levels of ALT, AST and LDH were close to the normal levels as compared with the vehicle control group ($P > 0.05$).

The influences of 5-MOP alone on serum enzyme levels were also observed. There were no statistically significant differences in the serum levels of ALT, AST and LDH between 5-MOP alone group (50 mg/kg) and the vehicle control group ($P > 0.05$).

Effects of APAP alone, 5-MOP multiple-dose and 5-MOP alone on liver tissue MDA and GSH/GSSG ratio

As seen in Table 1, compared with the vehicle control group, a toxic dose of APAP elevated liver MDA and lowered the hepatic GSH/GSSG ratio. With the escalating dose of 5-MOP (12.5, 25 and 50 mg/kg), the content of MDA decreased and ratio of GSH/GSSG increased.

In the 5-MOP alone group, the MDA level in liver was as low as that in the vehicle control group (0.15 ± 0.09 *vs* 0.18 ± 0.11, $P > 0.05$). In addition, the hepatic GSH/GSSG ratio in the 5-MOP alone group was not significantly changed as compared with that in the vehicle control group (6.858 ± 0.678 *vs* 6.045 ± 0.629, $P > 0.05$).

Effects of APAP alone, 5-MOP multi-dose and 5-MOP alone on histopathologic changes

The liver histopathologic changes of mice in the six groups are shown in Figure 1. The liver sections displayed the representative hepatocellular morphological changes of each group.

In the vehicle control group, hepatocytes, presenting normal morphology, arranged around the central vein in a radical pattern, and liver lobule structures were clear and regular (Figure 1A). Normal liver lobule structures were damaged and collapsed in the APAP alone group. Large areas of hepatocellular necrosis and infiltration of inflammatory cells were also observed (Figure 1B). 5-MOP administration could alleviate the pathological injury induced by APAP in a dose-dependent manner. 5-MOP at a dose of 12.5 mg/kg could slightly relieve the pathological injury. In this group, no hepatocellular necrosis and infiltration of inflammatory cells were observed, but hepatocellular hydropic degeneration and sinusoidal dilation occurred (Figure 1C). There was no necrosis and hydropic degeneration of hepatocytes, no sinusoidal dilation and infiltration of inflammatory cells in the 25 mg/kg 5-MOP dose group. However, liver lobule structures were still not clear in this group (Figure 1D). 5-MOP at a dose of 50 mg/kg could significantly prevent APAP-induced hepatotoxicity with an almost normal lobular structure comparable to the vehicle control group (Figure 1E). There were no significant liver histopathologic changes in the 5-MOP alone group (Figure 1F).

DISCUSSION

The protective effect of 5-MOP against hepatocellular injury and oxidative stress, and the potential toxic effect of 5-MOP on the liver were investigated in this study. C57BL/6J mice were used because our previous research found that C57BL/6J mice were more suscep-

Figure 1 Representative pathological changes of liver section of the six groups (original magnification × 200). A: Section of liver from the vehicle control group showing a normal lobular structure; B: Section of liver from acetaminophen alone group showing large areas of centrilobular necrosis with inflammatory cell infiltration; C: Section of liver from the 12.5 mg/kg 5-methoxypsoralen (5-MOP) dose group showing absence of hepatocellular necrosis and infiltration of inflammatory cells but presence of hepatocellular hydropic degeneration and sinusoidal dilation; D: Section of liver from the 25 mg/kg 5-MOP dose group showing normal hepatocellular morphology but liver lobule structure damage; E: Section of liver from the 50 mg/kg 5-MOP dose group showing a significant alleviation of liver pathological injury with an almost normal lobular structure; F: Section of liver from 5-MOP alone group (50 mg/kg) showing presence of normal lobular structure.

tible to APAP[33]. The serum levels of ALT, AST and LDH are main indices of liver injury[34] and the levels of MDA, GSH and GSSG can be used as indices of oxidative stress[9]. We evaluated the hepatic protective effect of 5-MOP based on these indices. It is well known that any chemical can be toxic if its dose is high enough, so a 5-MOP alone group was designed to see if the highest therapeutic dose of 5-MOP could cause hepatotoxicity.

APAP used alone can significantly increase the serum levels of ALT, AST and LDH and cause pathological changes as compared with the vehicle control group. Oxidative stress also took place as shown by the increase of MDA level and decrease of GSH/GSSG ratio. The model of APAP-induced hepatotoxicity was successfully established in this experiment.

5-MOP can protect mice from APAP-induced acute liver injury based on the fact that it can decrease the serum ALT, AST and LDH levels in a dose-dependent manner and alleviate the liver histopathologic alterations. Moreover, 5-MOP decreased the MDA level and increased the GSH/GSSG ratio in a dose-related manner, which reflected that 5-MOP could significantly attenuate the oxidative stress induced by APAP and suggested that the hepatoprotective effect of 5-MOP may be associated with its antioxidative activity.

However, besides antioxidant activity, 5-MOP also possesses biological activities to inhibit the mouse and human CYPs both *in vivo* and *in vitro*[14]. And CYPs-catalyzed formation of NAPQI is the key mechanism in APAP-induced hepatotoxicity[35]. So we presume that

inhibition of CYPs of 5-MOP may also account for the protective mechanism against APAP-induced hepatotoxicity, which should be further investigated.

In the 5-MOP alone group, the serum enzyme (ALT, AST and LDH) levels and histopathologic changes were as normal as in the vehicle control group, which indicated that 5-MOP could not cause liver injury at a dose of 50 mg/kg (the highest therapeutic dose used in this study). The MDA level and the GSH/GSSG ratio were not significantly changed as compared with the vehicle control group, which showed that 5-MOP did not influence the normal oxido-reduction levels.

In conclusion, 5-MOP could protect against APAP-induced hepatotoxicity in mice and had an antioxidative activity, and caused no hepatotoxicity at protective doses.

COMMENTS

Background
Overdose of acetaminophen (APAP) can induce hepatotoxicity and oxidative stress plays a central role in the hepatic damage. Though 5-methoxypsoralen (5-MOP) possesses antioxidative activity suggested by researches *in vitro*, none of previous studies has found that 5-MOP could prevent APAP-induced hepatotoxicity.

Research frontiers
It is important to search for effective methods to protect human from APAP-induced hepatotoxicity. Despite the various applications of 5-MOP, no research has been conducted to determine if 5-MOP could prevent APAP-induced hepatotoxicity. In addition, although the antioxidative activity of 5-MOP has been evidenced from researches *in vitro*, this activity was not manifested *in vivo*. Besides, 5-MOP may also cause hepatotoxicity when taken at high doses.

Innovations and breakthroughs
This study manifested that 5-MOP could protect mice from APAP-induced hepatotoxicity *in vivo*, and this hepatoprotective effect was associated with its antioxidation activities. In addition, 5-MOP caused no hepatotoxicity at protective doses.

Applications
This study has suggested that 5-MOP can be used at appropriate doses as a drug against APAP-induced hepatotoxicity in human. However, before clinical use, more researches are needed to confirm the safety of APAP administration at protective doses.

Peer review
The authors investigated the protective effects of 5-MOP against APAP-induced hepatotoxicity and whether 5-MOP could cause hepatotoxicity in mice. The results suggested that 5-MOP resisted APAP-induced hepatotoxicity, reduced APAP-induced oxidative stress, and did not cause liver injury at protective doses.

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BRIEF ARTICLE

Effect of soy protein supplementation in patients with chronic hepatitis C: A randomized clinical trial

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Abstract

AIM: To evaluate the effects of soy supplementation on insulin resistance, fatty liver and alanine aminotransferase (ALT) levels in non-diabetic patients with chronic hepatitis C (CHC).

METHODS: In a prospective, randomized and single-blinded clinical trial, we compared patients with CHC who had casein as a supplement (n = 80) (control group), with patients who consumed a soy supplement diet (n = 80) [intervention group (IG)]. Both groups received 32 g/d of protein for 12 wk.

RESULTS: Patients' baseline features showed that 48.1% were overweight, 43.7% had abdominal fat accumulation, 34.7% had hepatic steatosis and 36.3% had an homeostasis model assessment index of insulin resistance (HOMA-IR) $\geqslant$ 3.0. Descriptive analysis showed that protein supplementation diet reduced hepatic steatosis in both groups; however, significant reductions in ALT levels occurred in the soy group. Multiple regression modeling indicated that in the presence of severe fibrosis (F3/F4), γ glutamyl transferase elevation and high density lipoprotein (HDL) reduction, the intervention group had 75% less chance of developing hepatic steatosis (OR= 0.25; 95% CI: 0.06-0.82) and 55% less chance of presenting with an ALT level $\geqslant$ 1.5 × the upper limit of normal (ULN) (OR = 0.45, 95% CI: 0.22-0.89). Soy treatment did not have any effect on insulin resistance (OR = 1.92; 95% CI: 0.80-4.83), which might be attributed to the fact that the HOMA-IR values at baseline in most of our patients were in the normal range. Advanced hepatic fibrosis, an ALT level > 1.5 × ULN and visceral fat were predictors of an HOMA-IR $\geqslant$ 3. The IG group had a reduced risk of an ALT level > 1.5 × ULN. An HOMA-IR $\geqslant$ 3.0 and HDL < 35 mg/dL were also risk factors for increased ALT.

CONCLUSION: Soy supplementation decreased ALT

levels and thus may improve liver inflammation in hepatitis C virus (HCV) patients; it also reduced hepatic steatosis in a subgroup of patients but did not change insulin resistance. It should be considered in the nutritional care of HCV patients.

Key words: Chronic hepatitis C; Soy supplementation; Insulin resistance; Hepatic steatosis; Hepatitis C virus

INTRODUCTION

Hepatitis C virus (HCV) infection is considered an important public health problem[1] and is the leading cause of liver transplantation in the Western world. Chronic HCV infection increases the risk for hepatic steatosis, insulin resistance, glucose intolerance and type 2 diabetes[2-4].

The pathophysiology of nonalcoholic fatty liver disease (NAFLD) involves histology ranging from fat alone (hepatic steatosis) to fat plus inflammation (nonalcoholic steatohepatitis, NASH) to fat plus hepatocyte injury (ballooning degeneration) with or without fibrosis or Mallory's bodies which can lead to liver failure[5]. NAFLD and NASH have been associated with insulin resistance resulting in glucose intolerance and hyperglycemia[6]. Insulin resistance also contributes to increased lipolysis, which reduces fat uptake and oxidation by peripheral tissues. Both mechanisms lead to fat influx and accumulation in hepatic tissue[7].

Insulin resistance induced by HCV may involve several mechanisms, such as an immune response mediated by Th1 lymphocytes, the action of pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-6], the degradation of intracellular components that participate in the insulin signaling system, including insulin receptor substrates (IRS-1 and IRS-2), and the reduced activation of phosphatidyl inositol 3-kinase (PI3-K) and protein kinase B (AKT)[8-12].

Considering these factors, appropriate nutrition becomes an essential tool to minimize HCV's comorbidities such as fatty liver, inflammation and insulin resistance. Recently, functional foods have been considered essential for promoting and maintaining health. According to some reports, soy protein and its derivatives might be able to lower insulin resistance in patients with chronic liver diseases due its constituents, such as fiber, isoflavones and high biological value protein[13], and modulation of hepatic lipid metabolism[14,15]. The aim of this study was to evaluate the influence of soy protein supplementation on insulin resistance, liver fat content and alanine transaminase (ALT) levels in non-diabetic patients with chronic hepatitis C.

MATERIALS AND METHODS

Subjects

Non-diabetic patients with chronic hepatitis C were recruited from a reference ambulatory unit of the Federal University of Bahia's Hospital between June 2008 and December 2009. The diagnosis of HCV infection was made by the presence of serum anti-HCV, which was confirmed by qualitative determination of HCV RNA. Inclusion criteria were the following: patients aged over 18 years, with or without liver cirrhosis; patients with ethanol consumption below 20 g/d; patients with normal liver function (Child-Pugh A); and patients who were not under antiviral therapy or who had discontinued antiviral therapy for at least three months.

Patients co-infected with HIV and/or HBV with renal failure as well as those with heart disease, decompensated cirrhosis, pregnancy, any malignancy, diabetes mellitus or obesity (BMI > 30 kg/m^2) were excluded. The subjects gave written informed consent before participating in the study. The Ethics Committee of the Federal University of Bahia approved the study.

Study design

The study was a prospective, randomized and single-blinded clinical trial. Patients who were regularly followed in the Hepatology outpatient clinic were informed about the protocol and referred to the Nutrition clinic. Subjects who met the inclusion criteria of the study were randomly allocated into one of two study groups. The study was single-blinded (only blinded for patients). The estimated sample size was 160 patients.

The 160 patients were equally divided into two groups ($n = 80$), and each group received isonitrogenous protein supplementation with 32 g of protein per day for twelve weeks. The control group (CG) was supplemented with animal protein (casein), and the intervention group (IG) was supplemented with vegetable protein (soy). The nutritional composition of the supplements used in this study is reported in Table 1.

Patients were instructed to dissolve the protein supplement in water, juice, soup, porridge or to consume it with fruits. Additionally, considering their nutritional status and dietary habits, patients received dietary guidelines to promote healthy eating and weight control. Diet counseling aimed to promote the ingestion of a normocaloric, normoglycidic and high protein (1.5 g/kg per day) diet by both groups. Patients returned monthly to receive their supplements.

Table 1 Nutritional content per 100 g of supplement

	Whole soy powder[1]	Calcium caseinate
Energy (Kcal)	392	371
Carbohydrate (g)	14	0.2
Protein (g)	40	97.4
Total fat (g)	18.8	2.4
Alpha linolenic acid (mg)	1252	0
Linoleic acid (mg)	9332	0
Dietary fiber (g)	18	0
Isoflavones (mg)	53.82	0

[1]Soyos® ingredients.

Clinical parameters

Clinical survey data such as clinical diagnosis, viral genotype, necroinflammatory activity index and fibrosis (METAVIR classification) were either collected from medical records or from patient examinations.

Patients underwent ultrasonography of the upper abdomen with a team of three examiners using a single piece of equipment at the University Hospital's radiology service. Hepatic steatosis was graded as mild, moderate or severe according to the classification of Saverumuttu *et al*[16].

Measurement of waist circumference was performed according to the World Health Organization recommendations using an inelastic tape measure (TBW Import Ltd.) that was 0.5 cm wide and 200 cm in length. Waist circumference was measured at a level midway between the superior aspect of the iliac crests and the lower lateral margins of the ribs. The cutoff points adopted for classifying central obesity and increased risk of metabolic complications were above 80 cm for women and 94 cm for men[17]. Socio-demographic and lifestyle information was also collected using a structured questionnaire during the first appointment of follow-up (baseline).

Patients underwent follow-up visits once a month with registered dietitians to elucidate the adherence to the diet prescription and protein supplementation. Schedule monitoring also included weekly telephone calls in the first month and biweekly thereafter. After 12 wk of supplementation, physical, biochemical and anthropometric tests as well as a questionnaire were applied to evaluate possible changes during the intervention program. Ultrasound was also performed in patients with a diagnosis of hepatic steatosis at baseline. All procedures were performed within a maximum interval of ten days after the nutrition counseling.

Laboratory measurement

After a 12-h fast, a blood sample was collected for the determination of aspartate aminotransferase (AST), ALT, gamma glutamyl transferase (γGT), alkaline phosphatase, plasma glucose, insulin, total cholesterol and cholesterol fractions. Analyses were performed on a Beckman Coulter LX-20 PRO and CX-9 equipment. Serum insulin was measured using an electrochemiluminescence method with an Elecsys 2010 device.

The insulin resistance index was predicted according to the homeostasis model assessment index of insulin resistance (HOMA-IR). The formula was as follows: insulin resistance (HOMA-IR) = fasting insulinemia (microU/mL) × fasting glycemia (mmol/L)/22.5[18]. We considered $\geqslant 3.0$ as the cutoff point to define insulin resistance.

Virological tests

An enzyme linked immunosorbent assay was performed on all serum samples to detect the presence of anti-HCV using third generation commercial kits (anti-HCV Hepatitis C® Wiener Lab.) following the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on all samples for qualitative determination of HCV RNA. The method used was the nested PCR (HCV-RNA detectable using the COBAS® AMPLICOR HCV Test, v2.0, Roche). HCV genotyping was performed using the technique of restriction fragment polymorphism (RFLP-PCR).

Histological analysis

The stages of fibrosis and inflammation were determined according to the METAVIR scoring system: F0 = no fibrosis, F1 = expansion of fibrosis in portal areas without septa, F2 = fibrous portal expansion with septa, F3 = numerous septa or fibers with nodular transformation, and F4 = cirrhosis[19].

Statistical analysis

Descriptive analysis was performed to characterize the population. Mann-Whitney *U* and Wilcoxon's rank sum tests were used to compare the biochemical values in the intervention and control groups and to evaluate the differences obtained at baseline and after intervention. Logistic regression analysis was used to evaluate risk predictor factors for hepatic steatosis, insulin resistance (HOMA-IR $\geqslant 3.0$) and changes in ALT levels (1.5 above the upper limit of normal). Confounding and interaction analyses were performed to select the final models. Multiple regression analysis was performed after the intervention considering the groups had similar demographic, clinical and laboratory features before protein supplementation.

The sample size was calculated using an estimated 20% loss to follow-up, a confidence level of 95% and 80% power. A statistical significance was inferred at $P < 0.05$. In some biochemical analyses, the Bonferroni method was applied to adjust the multiple comparisons *P*-value between the groups at the significance level of 0.007. Statistical analysis was performed with the statistical package R version 2.12[20].

RESULTS

Characteristics of patients

The characterization of the study population is presented in Table 2. Males (63.8%) were predominant. The prevalence of HCV genotype 1 infected patients was 83.5%.

Table 2 Characterization of the study population *n* **(%)**

	CG-casein	IG-soy	Total	*P* value
Demographic characteristics				
Gender				
Male	49 (61.2)	53 (66.2)	102 (63.8)	0.62[1]
Female	31 (38.8)	27 (33.8)	58 (36.2)	
Marital Status				
Married	56 (70.0)	55 (68.7)	111 (69.4)	1.00[1]
Single, widowed or divorced	24 (30.0)	25 (31.3)	49 (30.6)	
Anthropometric data				
Body mass index				
< 25 kg/m^2	40 (50.0)	43(53.8)	83 (51.9)	0.751[1]
⩾ 25 kg/m^2	40 (50.0)	37(46.2)	77 (48.1)	
Waist circumference				
Adequate	43 (53.7)	46 (59.0)	89 (56.3)	0.525[1]
Inadequate	37 (46.3)	32 (41.0)	69 (43.7)	
Clinical data				
Genotype				
1	66 (88.0)	61 (79.2)	127 (83.5)	0.189[1]
2/3	9 (12.0)	16 (20.8)	25 (16.5)	
Necroinflammatory activity				
A0	26 (41.3)	29 (46.8)	55 (44.0)	0.792[1]
A1	26 (41.3)	22 (35.5)	48 (38.4)	
A2 e A3	11 (17.4)	11 (17.7)	22 (17.6)	
Stage of fibrosis				
F0/F1/F2	35 (55.6)	40 (63.5)	75 (59.5)	0.468[1]
F3/F4	28 (44.4)	23 (36.5)	51 (40.5)	
Hepatic steatosis				
Yes	24 (40.0)	19 (29.7)	43 (34.7)	0.260[1]
No	36 (60.0)	45 (70.3)	81 (65.3)	
HOMA-IR				
< 3.0	55 (53.9)	47 (46.1)	102 (63.7)	0.25[1]
⩾ 3.0	25 (43.1)	33 (56.9)	58 (36.3)	
Biochemical data	Median (iq r)	Median (iq r)		
Glucose (mg/dL)	91.0 (14.2)	91.5 (14.2)		0.70[2]
HOMA-IR	2.35 (2.24)	2.26 (2.59)		0.99[2]
Triglycerides (mg/dL)	96.5 (60.2)	96.0 (67.2)		0.57[2]
Total cholesterol (mg/dL)	149.0 (49.7)	160.0 (44.0)		0.42[2]
HDL (mg/dL)	44.0 (14.2)	44.0 (15.5)		0.30[2]
LDL (mg/dL)	90.5 (40.7)	90.0 (43.0)		0.94[2]
AST (U/L)	59.5 (40.7)	52.0 (44.5)		0.14[2]
ALT (U/L)	76.5 (54.5)	73.5 (53.0)		0.38[2]
γGT (U/L)	102.0 (118.0)	87.0 (114.0)		0.71[2]
Alkaline phosphatase (U/L)	79.0 (33.5)	80.0 (53.2)		0.49[2]

[1]Fisher's exact test; [2]Mann-Whitney; iq r: Interquartile range. *n* = 160. CG-casein: Control group-casein; IG-soy: Intervention group-soy; Adequate waist circumference: ⩽ 80 cm for women and ⩽ 94 cm for men. HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; LDL: Low density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transferase.

Advanced fibrosis (Metavir F3/F4) was detected in 40.5% of patients. The average age was 52.2 (± 10.5) years, 48.1% were overweight and 51.9% had a BMI < 25 kg/m^2; 43.7% individuals had abdominal fat accumulation. It was also observed that 34.7% of the patients had hepatic steatosis, and 36.3% had an HOMA-IR ⩾ 3.0 at baseline.

Biochemical data presented in Table 2 show that glucose and lipid profiles were not altered; however, high transaminase levels were observed in both groups. It is also observed that the median value for γGT is greater

Table 3 Comparison of the distribution of hepatic steatosis before and after intervention between groups of patients with hepatitis C virus

Hepatic Steatosis	*n*	Yes	No	*P* value
Before				0.2604[1]
IG-soy	64	19 (29.7)	45 (70.3)	
CG-casein	60	24 (40.0)	36 (60.0)	
After				0.1850[1]
IG-soy	64	10 (15.6)	54 (84.4)	
CG-casein	60	16 (26.7)	44 (73.3)	
IG-soy: before *vs* after				0.0076[2]
With hepatic steatosis	19	10 (52.6)	9 (47.4)	
Without hepatic steatosis	45	0 (0.0)	45 (100.0)	
GC-casein: before *vs* after				0.0133[2]
With hepatic steatosis	24	16 (66.7)	8 (33.3)	
Without hepatic steatosis	36	0 (0.0)	36 (100.0)	

[1]Fisher's exact test: Comparison between groups before and after intervention; [2]Paired analysis: McNemar's test: Comparison in each group. CG-casein: Control group-casein; IG-soy: Intervention group-soy.

Figure 1 Hepatic esteatosis before and after intervention between groups of patients with hepatitis C virus.

in the CG than in the IG (102.0 U/L *vs* 87.0 U/L); however, no significant difference was detected between the groups. Median alkaline phosphatase was in the normal range for both groups. Baseline demographic, anthropometric, clinical and laboratory data show that the study population had a homogeneous distribution between the groups.

Prevalence of steatosis at baseline and after intervention

Table 3 shows the comparison of the prevalence of hepatic steatosis before and after the intervention in the IG and CG. The prevalence of hepatic steatosis was different between the groups at baseline (29.7% *vs* 40.0%) and remained different after the interventions (15.6% *vs* 26.7%), but without statistical significance in both instances (Figure 1).

Paired analysis, within groups (before and after protein supplementation), showed the reduction in hepatic steatosis was significant for both groups. The IG showed a reduction from 19 to 10 cases (47.4% reduction, *P* =

Figure 2 Prevalence of hepatic steatosis condition after intervention for those who begin with hepatic steatosis.

Table 4 Median values of biochemical tests in patients with hepatitis C virus after protein supplementation

| Exams | CG-casein | | IG-soy | | Treatment difference |
	n	median (iq r)	*n*	median (iq r)	*P* value[1]
Total cholesterol (mg/dL)	80	152.0 (61.5)	80	153.0 (44.7)	0.54
HDL (mg/dL)	80	46.0 (17.0)	80	43.5 (15.2)	0.21
LDL (mg/dL)	80	89.5 (45.0)	80	84.5 (41.5)	0.39
AST (U/L)	78	61.5 (40.5)	80	49.5 (44.2)	0.02
ALT (U/L)	78	73.0 (65.7)	79	64.0 (50.0)	0.007
γGT (U/L)	75	108.0 (120.5)	73	84.0 (107.0)	0.09
HOMA-IR	79	2.4 (2.7)	80	2.6 (2.2)	0.91

[1]Mann-Whitney test ($P < 0.05$): comparison between groups; Bonferroni correction ($P < 0.007$); CG-casein: Control group-casein; IG-soy: Intervention group-soy; iq r: Interquartile range; $n < 80$: When an examination was not performed; HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; LDL: Low density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transferase.

0.0076) and the CG decreased from 24 to 16 cases (33.3% reduction, $P = 0.0133$) (Figure 2 and Table 3).

Biochemical data before and after intervention
After protein supplementation, a significant reduction was observed in the transaminase levels of the IG *vs* the CG (AST: 49.5 U/L *vs* 61.5 U/L, $P = 0.02$; ALT: 64.0 U/L *vs* 73.0 U/L, $P = 0.007$). A reduction in the levels of γGT was observed in the IG compared to the CG (84.0 U/L *vs* 108.0 U/L, $P = 0.09$) but without statistical significance (Table 4).

The IG had a significant reduction in ALT levels (73.5 U/L *vs* 64.0 U/L; 12.92% change, $P = 0.006$) (Figure 3) and γGT levels (87.0 U/L *vs* 84.0 U/L; 3.45% change, $P = 0.007$) after 12 wk of intervention. Reductions were also observed in total cholesterol (160.0 mg/dL *vs* 153.0 mg/dL; 4.37% change), but without statistical significance (Table 5). It is noteworthy that the CG did not present improvements in these biochemical tests.

Figure 3 Distribution of alanine aminotransferase and homeostasis model assessment according to intervention groups and time.

Predictors of hepatic steatosis after intervention
Table 6 presents the logistic regression analysis for predictive factors of hepatic steatosis. Multiple regression modeling indicates that, in the presence of severe fibrosis (F3/F4), γGT elevation and HDL reduction, the IG had a 75% less chance of developing hepatic steatosis (OR = 0.25; 95% CI: 0.06-0.82). Nevertheless, in the IG, those with an HOMA-IR ≥ 3 were three times more likely to develop hepatic steatosis (OR = 3.49; 95% CI: 1.10-11.90) (Table 6). The bivariate analysis also revealed that an age ≥ 60 years (crude OR = 3.81; 95% CI: 1.50-9.70), abdominal fat accumulation (crude OR = 3.87; 95% CI: 1.57-10.29) and BMI ≥ 25.0 kg/m² (crude OR = 1.32; 95% CI: 1.12-1.61) were independent risk factors for hepatic steatosis (data not shown).

Predictors of insulin resistance (HOMA-IR ≥ 3.0) after intervention
Soy treatment did not have any effect on insulin resistance (OR = 1.92; 95% CI: 0.80-4.83). In patients with advanced fibrosis, an ALT level ≥ 1.5 times upper limit of normal (ULN) and increased abdominal fat accumulation were independent risk factors for insulin resistance.

Table 5 Median values of biochemical tests in patients with hepatitis C virus at the beginning of monitoring and after protein supplementation

| | | IG-soy | | | | | | CG-casein | | | | |
| | | Baseline | 12 wk | | | | | Baseline | 12 wk | | | |
Exams	*n*	Median (iq r)	Median (iq r)	change	% change	*P* value[1]	*n*	Median (iq r)	Median (iq r)	change	% change	*P* value[1]
Total cholesterol (mg/dL)	80	160.0 (44.0)	153.0 (44.7)	-7.0	-7.37	0.05	80	149.0 (49.7)	152.0 (61.5)	+3.0	+2.01	0.63
HDL (mg/dL)	79	44.0 (15.5)	43.5 (15.2)	-0.5	-1.14	0.69	80	44.0 (14.2)	46.0 (17.0)	+2.0	+5.54	0.13
LDL (mg/dL)	78	90.0 (43.0)	84.5 (41.5)	-5.5	-6.11	0.03	80	90.5 (40.7)	89.5 (45.0)	-1.0	-1.10	0.66
AST (U/L)	80	52.0 (44.5)	49.5 (44.2)	-2.5	4.81	0.32	78	59.5 (40.7)	61.5 (40.5)	+2.0	+3.36	0.04
ALT (U/L)	76	73.5 (53.0)	64.0 (50.0)	-9.5	-12.92	0.006	78	76.5 (54.5)	73.0 (65.7)	-3.5	-4.57	0.48
γGT (U/L)	73	87.0 (114.0)	84.0 (107.0)	-3.0	-3.45	0.007	75	102.0 (118.0)	108.0 (120.5)	+6.0	+5.88	0.19
HOMA-IR	80	2.3 (2.6)	2.6 (2.2)	+0.3	+13.04	0.23	79	2.3 (2.2)	2.4 (2.7)	+0.1	+4.35	0.09

[1]Paired analysis: Wilcoxon test ($P < 0.05$): Change in the group, the Bonferroni correction ($P < 0.007$). HDL: High density lipoprotein; LDL: Low density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transferase; CG-casein: Control group-casein; HOMA-IR: Homeostasis model assessment index of insulin resistance; IG-soy: Intervention group-soy; iq r: Interquartile range; $n < 80$: When an examination was not performed.

Table 6 Logistic regression model for factors predictive of hepatic steatosis in patients with hepatitis C virus after 12 wk of protein supplementation

| | Bivariate analysis | | Multivariate analysis | |
	Crude OR (95% IC)	*P* value	Adjusted OR[1] (95% IC)	*P* value
Groups				
Control-casein	1		1	
IG-soy	0.51 (0.20-1.22)	0.135	0.25 (0.06-0.82)	0.032
HOMA-IR				
< 3.0	1		1	
≥ 3.0	1.97 (0.82-4.77)	0.129	3.49 (1.10-11.90)	0.037
Stage of fibrosis				
F0/F1/F2	1		1	
F3/F4	1.78 (0.68-4.69)	0.239	1.59 (0.50-5.40)	0.436
γGT				
< 85 U/L	1		1	
≥ 85 U/L	2.07 (0.81-5.64)	0.135	1.52 (0.47-5.18)	0.487
HDL-C				
≥ 35 mg/dL	1		1	
< 35 mg/dL	1.25 (0.41-3.40)	0.677	2.18 (0.56-8.21)	0.247

[1]Adjusted for the other variables shown in the table; γGT: 85 U/L upper limit of normal. γGT: Gamma glutamyl transferase; HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; IG-soy: Intervention-soy; OR: Odd ratio; 95% IC: 95% confidence interval.

Table 7 Logistic regression model for factors predictive of homeostasis model assessment of insulin resistance in patients with hepatitis C virus after 12 wk of protein supplementation

| | Bivariate analysis | | Multivariate analysis | |
	Crude OR (95% IC)	*P* value	Adjusted OR[1] (95% IC)	*P* value
Groups				
Control-casein	1		1	
Intervention-soy	0.90 (0.47-1.70)	0.745	1.92 (0.80-4.83)	0.15
Stage of fibrosis				
F0/F1/F2	1		1	
F3/F4	4.21 (1.99-9.19)	0.0002	5.25 (2.17-13.67)	0.0004
Alanine aminotransferase				
1.5 times below ULN	1		1	
1.5 times above ULN	2.22 (1.16-4.30)	0.017	3.26 (1.30-8.71)	0.014
Marital status				
Married	1		1	
Single, widowed or divorced	0.52 (0.24-1.07)	0.081	0.24 (0.08-0.65)	0.007
Waist circumference[2]				
Adequate	1		1	
Inadequate	2.62 (1.37-5.16)	0.004	5.57 (2.23-15.27)	0.0004

[1]Adjusted for the other variables shown in the table; [2]Adequate waist circumference: ≤ 80 cm for women and ≤ 94 cm for men; ULN: Upper limit of normal; OR: Odd ratio; 95% IC: 95% confidence interval.

Logistic regression analysis showed that the presence of severe fibrosis promoted a five-fold increase in the chance (OR = 5.25; 95% CI: 2.17-13.67) for an HOMA-IR ≥ 3.0 as well as accumulation of abdominal fat (OR = 5.57; 95% CI: 2.23-15.27). Those with an ALT level ≥ 1.5 × ULN had a three times greater chance of developing insulin resistance. Being single, widowed or divorced was a protective factor (OR: 0.24; 95% CI: 0.08-0.65) for an HOMA-IR ≥ 3.0 (Table 7).

Predictors of elevated an ALT level after intervention

The independent predictive factors for changes in ALT levels (≥ 1.5 × ULN) were an HOMA-IR ≥ 3.0, HDL < 35 mg/dL and being a male subject (Table 8). Severe fibrosis and alterations in AST and γGT levels were also

independent predictors of an increased ALT level (≥ 1.5 × ULN). Multivariate analysis showed that supplementation with soy protein *per se* represents a protective factor; the IG had a 55% less chance of presenting with an ALT level ≥ 1.5 × ULN (OR = 0.45, 95% CI: 0.22-0.89), and subjects with an HOMA-IR ≥ 3.0 were three times more likely to have an increased ALT level (OR = 3.16, 95% CI: 1.51-6.93). However, females had 72% less chance to have an increased ALT level (OR = 0.28, 95% CI: 0.12-0.60) (Table 8).

DISCUSSION

Our population was predominantly male, infected with HCV genotype 1, overweight and presented abdominal

Table 8 Logistic regression model for factors predictive of alanine aminotransferase levels in patients with hepatitis C virus after 12 wk of protein supplementation

	Bivariate analysis		Multivariate analysis	
	Crude OR (95% IC)	*P* value	Adjusted OR[1] (95% IC)	*P* value
Groups				
Control-casein	1		1	
IG-soy	0.55 (0.29-1.04)	0.068	0.45 (0.22-0.89)	0.024
HOMA-IR				
< 3.0	1		1	
≥ 3.0	2.22 (1.16-4.30)	0.017	3.16 (1.51-6.93)	0.001
Gender				
Male	1		1	
Female	0.39 (0.20-0.77)	0.007	0.28 (0.12-0.60)	0.003
HDL-C				
≥ 35 mg/dL	1		1	
< 35 mg/dL	3.15 (1.37-7.76)	0.008	2.85 (1.18-7.40)	0.024

[1]Adjusted for the other variables shown in the table. HOMA-IR: Homeostasis model assessment index of insulin resistance; HDL: High density lipoprotein; IG-soy: Intervention-soy; OR: Odd ratio; 95% IC: 95% confidence interval.

fat accumulation. Both studied groups had similar characteristics. These clinical conditions increase the chances of developing insulin resistance and hepatic steatosis, which have a negative impact in patients with chronic hepatitis C[21]. In patients infected with HCV genotype 1, steatosis is frequently associated with metabolic syndrome and insulin resistance and is also called "metabolic steatosis"[4,8,21].

A large proportion of our patients had increased liver enzymes (i.e., ALT, AST, γGT) and had not yet been subjected to antiviral treatment. In our population, at baseline, there was a 34.7% prevalence of hepatic steatosis and a 36.3% prevalence of an HOMA-IR ≥ 3.0. The prevalence of hepatic steatosis associated with HCV varies widely in the literature[22,23] and may differ depending on the population profiles[24].

In this study, protein supplementation caused a significant reduction of hepatic steatosis in both groups; however, this reduction was not significant between the groups. The probable mechanism is not associated with the quality of protein supplementation (animal or vegetable) but likely the nutritional care offered to both groups, which promoted changes in eating habits and consequently improved the overall quality of the diet. Of note, there was no change in body mass index (BMI) or in the pattern of physical activity of these patients.

It is controversial whether insulin resistance is a cause or consequence of steatosis, however the literature suggests that it seems to work more like a cause than a consequence of steatosis in patients infected with HCV genotype 1[2]. In the present study, the regression model showed that an HOMA-IR ≥ 3 increased the chances of hepatic steatosis more than three-fold. The regression model also revealed that advanced age (≥ 60 years) and a higher waist circumference and BMI were independent predictor factors for hepatic steatosis. These results are in agreement with other studies which have observed a

direct correlation between BMI, visceral obesity and liver steatosis[25-27].

Clinical and experimental studies suggest that soy protein and isoflavones can synergistically act to promote a greater benefit in controlling hypercholesterolemia, hypertriglyceridemia, insulin resistance and steatosis[13,28,29]. We observed that consumption of soy protein had a protective effect and was associated with 75% less chance of having hepatic steatosis.

In an experimental study with obese Zucker rats, a diet with isolated soy protein favored reduced triglycerides in the liver. The proportions of AST/ALT, alkaline phosphatase, bile acids in plasma and pro-inflammatory cytokines (TNF-α and IL1) were also reduced. The authors suggested that soy protein enriched with isoflavones has a favorable effect on the inflammatory status of obese mice, which may promote a favorable outcome in NAFLD patients[28]. It is known that oxidative stress is a decisive factor in the progression of steatosis[30,31]; thus, if isoflavones can act as an antioxidant, then they may minimize the negative progression of steatosis[32,33]. The morbidity of hepatic steatosis is increasing and has been recognized as a liver component of the metabolic syndrome, which also has a negative effect on HCV treatment[8,26,34-36].

Our data revealed a significant decrease in ALT values after supplementation with soy protein compared to the control patients who consumed casein. These findings agree with experimental studies, which have found that soy protein enriched with isoflavones reduces plasma aminotransferase levels[32] and the proportion of AST/ALT[28]. However, a reduction in HOMA-IR levels after supplementation with soy was not observed in our study, which can be attributed to the fact that the HOMA-IR values at baseline in most of our patients were in the normal range. In contrast, Jayagopal *et al*[13] in a study conducted with diabetic women in which the mean value of the HOMA-IR was 5.54 in the intervention group and 5.14 in the control group, supplementation with soy protein enriched with isoflavones significantly reduced serum insulin and the HOMA-IR.

We found that insulin resistance (HOMA-IR ≥ 3.0) and lower HDL values were predictors for increased ALT. Soy protein intervention in female subjects presented *per se* as a protective factor for increased ALT levels. Our data are in agreement with a recent study that showed higher levels of ALT were significantly associated with gender, a low HDL level and a high HOMA-IR[25]. When evaluating patients with HCV with and without changes of ALT levels and healthy controls, Addel-Azziz *et al*[37] found a higher value of HOMA-IR in patients with abnormal ALT levels compared with those with no change in ALT levels and healthy controls (3.98 *vs* 2.69 *vs* 1.92, respectively), with a significant difference between those with abnormal ALT levels and controls (3.98 *vs* 1.92).

In our study, abdominal fat concentration, an ALT level ≥ 1.5 the upper limit of normal and the presence of advanced fibrosis were independent predictors of insulin resistance, and even in a multivariate model, they

remained significant. Addel-Azziz *et al*[37] also detected a positive correlation between the HOMA-IR and fibrosis. We detected that marital status was a predictor for insulin resistance as well; single, widowed or divorced patients were less susceptible to inadequacy of the HOMA-IR. This could be associated with the fact that a higher prevalence of overweight and abdominal fat accumulation has been described among married subjects.

In patients with hepatitis C, the presence of a high BMI, insulin resistance and high cholesterol are important predictors for mortality. Multivariate analysis has shown increased mortality associated with metabolic disorders such as diabetes, hypertension and a higher BMI[36,38]. Mehta *et al*[39] found that individuals with HCV and an age above 40 years had a three-fold higher chance of presenting with type 2 diabetes. Therefore, it is recommended that all patients with chronic hepatitis C avoid excess weight and maintain blood glucose, cholesterol levels and blood pressure within normal ranges[40].

Soy supplementation decreased ALT levels and thus may improve liver inflammation in HCV patients. It also reduced hepatic steatosis in a subgroup of individuals with advanced fibrosis, insulin resistance, increased γGT levels and low HDL. On the other hand, soy supplementation did not change insulin resistance, which might be attributed to the fact that the HOMA-IR values at baseline in most of our patients were in the normal range. To our knowledge, this is the first study to show that soy protein supplementation reduces hepatic steatosis and decreases ALT levels in chronic hepatitis C patients. Control of insulin resistance, hepatic steatosis, abdominal obesity and body weight seems to play an essential role in nonpharmacological therapies for chronic hepatitis C treatment. These practices should therefore be encouraged by a multidisciplinary team. Supplementation with soy protein should be considered as an important choice of nutritional management of patients with chronic hepatitis C.

COMMENTS

Background
Hepatitis C virus (HCV) infection is an important public health problem and is the leading cause of liver transplantation in the Western world. Chronic HCV infection increases the risk for hepatic steatosis, insulin resistance, glucose intolerance and type 2 diabetes. The improvement of these comorbidities may benefit the clinical course of the patients.

Research frontiers
Several studies have shown that soy protein may stimulate peroxisome proliferator-activated receptors-α and thus might increase liver fatty oxidation and decrease hepatic steatosis. It also may inhibit sterol regulatory element-binding transcription factor 1 and decrease hepatic lipogenesis. Clinical studies have previously demonstrated that soy consumption may reduce plasma lipid levels, promote insulin resistance reduction and maintain normal glucose levels. HCV infection may be associated with hepatic steatosis and increased insulin resistance. The morbidity of hepatic steatosis is increasing and it has been recognized as a liver component of the metabolic syndrome, which also has a negative effect on HCV treatment. The role of soy supplementation in the improvement of liver diseases is still a matter of debate and there are no studies that have evaluated its effect on insulin resistance, liver fat content and alanine transaminase (ALT) levels in non-diabetic patients with chronic hepatitis C.

Innovations and breakthroughs
Our work is characterized by its originality since it evaluated the impact of soy nutritional intervention in a population of patients infected with hepatitis C. To our knowledge, this is the first study to show that soy protein supplementation decreases ALT levels and reduces hepatic steatosis in a subgroup of individuals with advanced fibrosis, insulin resistance, increased γGT levels and low HDL in chronic hepatitis C. In an experimental study with obese Zucker rats, a diet with isolated soy protein favored reduced triglycerides in the liver. The AST/ALT ratio, alkaline phosphatase, bile acids in plasma and pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1) were also reduced. However, there are no clinical studies that have evaluated the role of soy supplementation on liver enzymes levels, insulin resistance and hepatic steatosis of patients with chronic HCV infection. The authors emphasize the need for further clinical trials to confirm the soy effects on ALT levels and hepatic steatosis of patients with chronic HCV infection.

Applications
This study showed that soy supplementation may improve liver inflammation (decrease ALT level), and may improve steatosis in a sub-group of patients with HCV. Therefore, supplementation with soy protein should be considered in the nutritional management of patients with chronic hepatitis C. However, further clinical trials are necessary to confirm our results.

Terminology
HOMA-IR: Homeostasis model assessment index of insulin resistance; PPARs: Peroxisome proliferator-activated receptors. These are nuclear receptors that function as transcription factors regulating the expression of genes. PPARs play essential roles in the regulation of cellular differentiation and metabolism (carbohydrate, lipid, protein); SREBP-1: Sterol regulatory element-binding transcription factor 1 is a transcription factor involved in sterol biosynthesis.

Peer review
The topic of the study is interesting and based on rational logic. The design of the study is appropriate, but to be sure of that we need to know some more information about the recruitment, randomization and blinding method. In a clinical trial design the conclusions are based only in the analysis performed for the main objectives of the study, otherwise the conclusion from sub-analysis must be biased.

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BRIEF ARTICLE

Satisfaction with patient-doctor relationships in inflammatory bowel diseases: Examining patient-initiated change of specialist

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Abstract

AIM: To assess the reasons for, and factors associated with, patient-initiated changes in treating specialist in inflammatory bowel diseases (IBD).

METHODS: Prospectively identified IBD patients (n = 256) with $\geq$ 1 encounter at a metropolitan hospital were surveyed, including whether they had changed treating specialist and why. Negative reasons included loss of confidence, disagreement, and/or personality clash with the specialist.

RESULTS: Of 162 respondents, 70 (43%) had ever changed specialists; 30/70 (43%) for negative reasons, 52/70 (74%) in the preceding year. Patients with negative reasons for changing (n = 30) were younger (median, 35.2 years *vs* 45.3 years) ,had higher IBD knowledge (median, 5.0 years *vs* 4.0 years), yet had lower medication adherence and satisfaction scores (median, 19.0 years *vs* 22.0 years, 14.0 years *vs* 16.0 years respectively, Mann-Whitney tests, all P < 0.05), compared to all other responders (n = 132). Patients

with a recent change (for any reason) were more likely to have Crohn's disease, currently active disease, previous bowel resection and recent hospitalization [OR 2.6, 95% CI (1.3-5.4), 2.2 (1.0-4.7), 5.56 (1.92-16.67), 2.0 (1.3-3.0), each P < 0.05].

CONCLUSION: Changing specialist appears associated with patient- related (age, nonadherence) and contemporaneous disease-related factors (recent relapse) which, where modifiable, may enhance patient-doctor relationships and therefore quality of care.

INTRODUCTION

In chronic inflammatory bowel diseases (IBD), a positive patient-doctor relationship (PDR) appears integral to optimizing quality of care (QoC). Although specific IBD data are lacking, a positive PDR is associated with improved patient satisfaction and health outcomes in other chronic diseases like diabetes and hypertension[1]. In a consumer-driven society, patient-centered care appears essential for a positive PDR[2], and thus assessing patients' satisfaction with their specialist is important[3]. A positive PDR should facilitate the development of trust essential

to the therapeutic process and ensure continuity of care, thus consolidating high-quality care[4,5].

Conversely therefore, PDR discord may compromise QoC, leading to adverse outcomes[6]. Discord may result from personality clashes, lack of rapport, misunderstandings, patient disenfranchisement over management decisions, or adverse disease outcomes. Yet, quantifying PDRs is difficult. Patient self-report instruments to rate PDRs are emerging, but understanding of their relevance to QoC is limited[7,8].

In Australia, as in many countries worldwide, there is significant variation in care models available to IBD patients; ranging from those managed solely by their primary care practitioner; those seen by a colorectal or general surgeon, general physician/internist, or by a specialist gastroenterologist either in private rooms or within a local hospital clinic setting; to those seen in the secondary/tertiary care setting within a dedicated hospital-based "IBD service" with multiple gastroenterologists and clinicians co-located with specific interests in IBD[9]. Each model has inherent strengths and weaknesses and may be attractive to different patients for various reasons. Moreover, with the multiple options potentially available, patients are theoretically able to select and change treating specialist (either in the government-funded public or private sectors), pending appropriate referral from their primary care practitioner and subject to regional availability. Thus one may theoretically assume the patient, as the consumer, may change their treating IBD specialist if their perceived PDR discord "threshold" was overcome and a suitable alternative existed (i.e., "voting with their feet").

Hence, we aimed to explore PDRs in an IBD cohort with the simple, tangible measure of whether patients changed treating specialists and whether this had occurred recently and/or for negative reasons, as a marker of patient satisfaction with the PDR and their medical care. We also explored potential factors associated with patients switching specialist for their care. Although patients logically may report changing specialists for innocuous reasons (e.g., relocation), instances where change occurs, especially for negative reasons from the patients' perspective, represent vulnerable moments in QoC delivery, but also provide an insight into the importance of the PDR, and its continuity[10].

MATERIALS AND METHODS

Subjects and recruitment

All patients with confirmed IBD diagnoses who had an inpatient or outpatient encounter for any reason at the Royal Adelaide hospital (RAH), in a 6-mo period from November 1 2007 to April 30 2008, were prospectively identified as previously described[9].

Subsequently, medical record review was performed to verify diagnoses, and extract further data including demographics, psychological comorbidity, previous surgery and healthcare utilization data. A contemporaneous (± median 14 d), physician global assessment[11] of disease activity (0 = inactive through to 4 = severe) was

Patient satisfaction with their medical care' questionnaire
(four questions used in study)

In response to each of the statements below, please mark on the line exactly where you think most accurately describes your own feelings:

1. I have a good relationship with my inflammatory bowel diseases (IBD) doctor and look forward to my appointments with him/her.

| Never | Rarely | Sometimes | Often | Very often |

2. The hassle of taking medications for IBD makes me wonder if it is worthwhile

| Never | Rarely | Sometimes | Often | Very often |

3. I wonder if there was another doctor available who could manage my IBD better.

| Never | Rarely | Sometimes | Often | Very often |

4. Overall I am satisfied with the treatment I am taking for IBD

| Never | Rarely | Sometimes | Often | Very often |

Figure 1 Scoring system. Maximum 5 points per question, maximum total of 20 indicates complete, 100% satisfaction with medical care. Score less than 16 indicates "suboptimal" satisfaction. Before scoring, responses to questions 2 and 3 must be inverted (i.e., never = 5, rarely = 4, *etc.*) prior to calculating absolute scores.

performed based on all information available, including clinical data (Harvey-Bradshaw criteria)[12], pathology and histological/endoscopic activity grading.

Patient survey

Surveys were distributed to each patient comprising multiple components; IBD patient knowledge was assessed using two validated questionnaires[13,14], health-related quality of life was assessed using the shortened inflammatory bowel disease questionnaire (sIBDQ10)[15], the medication adherence report scale (MARS-5) assessed medication adherence[16], and the hospital anxiety depression scale screened for anxiety and depression[17]. Patient satisfaction with medical care was measured using a novel instrument (Figure 1), yet to be validated but the use of which has previously been reported[9]. This comprises four questions with a total score of 20 indicating 100% patient satisfaction. A score < 16 arbitrarily indicates suboptimal satisfaction.

Endpoints relating to change in treating specialist

IBD patients were asked "Have you ever changed your treating specialist," and "If yes, why?" Reasons for changing were deemed negative if the patients' response included at least one of: (1) loss of confidence; (2) disagreement/dissatisfaction regarding management; (3) personality clash with specialist; or (4) other (including "specialist too busy", "waiting time too long"). Alternative reasons including "doctor retired", "doctor/patient moved" or "financial reasons" were not deemed negative responses. The endpoint "Change within 12 mo of survey completion, for any reason" was used to determine contemporaneous disease- and patient-related associations with chang-

Table 1 Clinical and disease characteristics of inflammatory bowel diseases patients who responded to survey (*n* = 162)

Patient variable	*n* (%)
Female sex	85 (52.5)
Crohn's disease	95 (58.6)
Ulcerative colitis	65 (40.1)
Previous bowel resection surgery (ever)	50 (30.9)
Recent inpatient admission[1]	80 (49.4)
Active disease[2]	69 (42.6)
Current stoma	19 (11.7)
Current perianal disease	29 (18.0)
Current cigarette smoker	31 (19.1)
Documented history of psychological comorbidity	41 (25.3)
Currently unable to work due to illness	39 (24.1)
Proportion of lowest socioeconomic group[3]	16 (9.9)

[1]Inpatient admission in period between January 1, 2007-April 31, 2008; [2]as determined by physician global assessment at time of survey completion; [3]according to Social Health atlas, Central Northern Adelaide Health Service, Department of Health, SA 2004.

ing specialist. At the time of survey, 108 (66.6%) of the cohort saw their current treating specialist at an outpatient clinic at RAH, whereas 54 (33.3%) had a current treating specialist based externally to the hospital (either in public or private sector).

Analysis
Statistical analyses were performed using SPSS 15.0.1.1 (Chicago, IL, United States). Bivariate correlations were conducted between variables and changing endpoints. Subsequently, exploratory logistic regression analyses assessed variables for inclusion in the final multivariable model. Those of definite clinical relevance were retained in the model regardless of statistical significance or fit; continuous variables remained unchanged wherever possible.

Ethics
The study was approved by the RAH Research Ethics Committee. Return of a completed survey was accepted as implied patient consent.

RESULTS
Patient characteristics
Two hundred and fifty-six confirmed IBD patients were prospectively identified over 6 mo, and 162 (63.3%) returned a completed survey. Responders had a median age of 43 years (range, 18-90), median IBD duration of 7 years (range, 0-47) (Table 1). As reported elsewhere, survey responders and non-responders did not differ significantly[9].

Changing specialist for any reason
Overall, 70/162 (43.2%) respondents had ≥ 1 change in specialist for any reason since IBD diagnosis. Of these, the median number of changes per patient since diagnosis was 2.0 (range: 1-6, Figure 2). Thirty of 70 patients

who changed specialists (42.9%) gave a negative reason, and 52/70 patients changing specialist (74.3%) had done so within the prior 12 mo (for any reason). The total number of changes per patient correlated weakly positively with IBD duration (r_s = 0.19), and when controlling for disease duration, positively with IBD knowledge (r_s = 0.20) and negatively with age (r_s = -0.22) (Spearman's partial correlations, all *P* < 0.02).

IBD patients with four or more changes in specialists had lower median quality of life scores (sIBDQ10, median score 37 *vs* 48 respectively, *P* = 0.01) and higher disease activity scores (median score 2.75 *vs* 2.28 respectively, *P* = 0.04) than those with a lower number of changes over their total duration of IBD.

Recent change in specialist
In order to identify temporal associations with changing specialist, bivariable (Table 2) then multivariable logistic regression analyses were conducted with the endpoint of specialist change within 12 mo prior to survey completion. The multivariable model (incorporating associations from bivariable analyses where *P* ≤ 0.05 plus age) showed those with a recent change were more likely to have Crohn's disease, had recent hospitalization, had a past bowel resection, and trended towards having currently active disease (Table 3).

Patients reporting negative reasons for changing specialist
Thirty patients gave 34 negative reasons for changing specialist, including dissatisfaction with management (*n* = 23), lost confidence (*n* = 10) and personality clash (*n* = 1) (Figure 3). These 30 patients were generally younger (median, 35.2 years *vs* 45.3 years), had higher IBD knowledge (median, 5.0 score *vs* 4.0 score respectively), yet had lower medication adherence and satisfaction scores (median, 19.0 score *vs* 22.0, respectively, 14.0 score *vs* 16.0, respectively, Mann-Whitney, all *P* < 0.05) compared to all other responders (*n* = 132). There were no other statistically significant differences including no difference in disease duration, IBD-related characteristics, hospitalization outcomes, or QoL scores between patients changing for negative reasons and other responders (data not shown). However, the frequency of changing specialist [i.e. the duration (years) since IBD diagnosis divided by the total number of changes in specialist over the same period] trended towards being higher in those with one or more negative reasons for changing specialist, compared to other responders (median, 2.8 *vs* 4.0 years between each change, *P* = 0.06, Mann-Whitney).

Finally, in order to identify factors associated with changing specialist for a negative reason, bivariable (Table 2) then multivariable logistic regression analyses were conducted where a negative reason for change in specialist was the dependent variable. The multivariable model (incorporating associations from bivariable analyses with *P* ≤ 0.05, plus sex) showed those with a negative reason had poorer medication adherence and trended towards being of male sex (Table 4).

Table 2 Bivariable logistic regression analyses of relevant clinical and demographic factors potentially associated with a change in treating specialist

Variable	Associated with change in treating specialist within 12 mo of survey completion		Associated with change in specialist for a "negative" reason at any time	
	OR [95% CI]	*P* value	OR [95% CI]	*P* value
Age under 30	1.13 [0.52, 2.47][5]	0.01[5]	0.60 [0.25, 1.46]	0.33
Female gender	1.15 [0.59, 2.24]	0.73	1.57 [0.71, 3.49]	0.31
Crohn's as inflammatory bowel diseases diagnosis	2.03 [1.03, 4.03][5]	0.04[5]	0.79 [0.35, 1.78]	0.68
Recent inpatient admission[3]	1.70 [0.86, 3.35][5]	0.13[5]	0.82 [0.37, 1.82]	0.69
Previous bowel resection surgery[1]	3.23 [7.81, 1.41][5]	0.005[5]	0.61 [0.27, 1.38]	0.28
Moderate/severe disease activity[4]	2.09 [1.06, 4.12][5]	0.04[5]	0.96 [0.43, 2.15]	0.93
Polypharmacy[2]	1.09 [0.51, 2.36]	0.85	1.50 [0.57, 3.97]	0.49
Living alone	1.06 [0.54, 2.07]	1.0	1.59 [0.71, 3.57]	0.31
Low socioeconomic status	1.62 [0.75, 3.48]	0.26	1.33 [0.53, 3.36]	0.66
Limited employment status	1.09 [0.52, 2.28]	0.85	0.65 [0.29, 1.49]	0.37
Poor inflammatory bowel diseases knowledge	0.39 [0.18, 0.88][5]	0.03[5]	2.04 [0.90, 4.55][5]	0.12[5]
Poor medication adherence	1.22 [0.62, 2.40]	0.60	2.22 [0.99, 4.95][5]	0.06[5]
Poor HRQoL	1.20 [0.57, 2.49]	0.71	0.91 [0.37, 2.24]	1.0
Possible psychological disorder (HADS > 7)	1.13 [0.58, 2.20]	0.74	0.75 [0.34, 1.68]	0.55
Dissatisfaction with medical care	0.95 [0.47, 1.90]	1.0	2.39 [1.05, 5.42][5]	0.04[5]

[1]At any time in the past; [2]taking ⩾ 6 prescription medications currently; [3]in observation period between January 1, 2007-April 31, 2008; [4]as determined by physician global assessment at time of survey; [5]items included in multivariate model (see Tables 3 and 4). OR: Odd ratio; 95% CI: 95% confidence interval; HADS: Hospital anxiety and depression scale; HRQoL: Health-related quality of life.

Table 3 Factors associated with change of treating specialist within 12 mo of survey completion - multivariable logistic regression analysis results

Variable	OR [95% CI]	*P* value
Crohn's disease as IBD diagnosis	2.60 [1.25, 5.41]	0.01
Age[1]	0.98 [0.97, 0.99]	0.01
Previous bowel resection surgery[2]	5.56 [1.92, 16.67]	0.002
Recent inpatient admission[3]	1.97 [1.29, 3.01]	0.002
Moderate/severe disease activity[4]	2.16 [0.99, 4.71]	0.05

[1]For every one year increase in age; [2]at any time in the past; [3]within observation period between 2007-April 31, 2008; [4]as determined by physician global assessment at time of survey. OR: Odd ratio; 95% CI: 95% confidence interval; IBD: Inflammatory bowel diseases.

Table 4 Factors associated with change in specialist for a negative reason - multivariable logistic regression analysis results

Variable	OR [95% CI]	*P* value
Female gender[1]	0.36 [0.13, 1.01]	0.053
Suboptimal satisfaction with medical care	1.22 [0.40, 3.75]	0.73
Poor disease knowledge	0.54 [0.20, 1.50]	0.24
Poor medication adherence[1]	3.49 [1.12, 10.89]	0.03

[1]Variables indicate those statistically, significantly associated with a negative reason for change in specialist. OR: Odd ratio; 95% CI: 95% confidence interval.

DISCUSSION

Accepted IBD dogma dictates that expert specialist care is vital in optimizing outcomes, as per recent IBD consensus guidelines[18,19]. However, these guidelines do not specifically address continuity of care or patient satisfaction with their PDR. Moreover, the United Kingdom IBD Standards Group emphasizes the importance of maintaining

Figure 2 Number of changes in specialist ever by each patient. N.A.: Not available.

Figure 3 Reasons stated for patient change of treating specialist (72 reasons from 70 patients).

patient-centered care (Standard C), "offering personalized and responsive healthcare so that any patient can migrate between models of care according to activity and

complexity of disease, local facilities and personal preference[20]. In this prospectively encountered IBD cohort, we showed that patients frequently changed treating specialist (43.2% of responders) and of these, many changed (42.9%) for negative reasons, which appears to represent a high prevalence of discordant/poor PDR, which has not previously been documented. Suboptimal PDRs and/or discontinuity of care appear to impair QoC[18], therefore, clinicians (for both the sake of the patient and retaining their own practice) should be cognizant of and, wherever possible, endeavor to address when and why this discord exists[21].

Quantifying PDRs is inherently difficult, however assessing past and hence, risk of future changes in treating specialist *via* patient self-report is a novel, tangible method of surrogately measuring patient satisfaction with the PDR and their medical care. Increasingly, health providers are utilizing patient feedback *via* satisfaction questionnaires as a means to establish a patient-centered approach and for evaluating and improving quality performance[22,23]. In IBD, this has led to development of a limited number of disease-specific satisfaction questionnaires such as the comprehensive QUOTE-IBD survey[24] and others such as the Treatment Satisfaction Questionnaire for Crohn's disease[25]. Given the lack of brief but validated surveys focusing on specialist care in IBD, an unvalidated four-item survey was used in this study[9]. Further refinement and validation of this and similar surveys are needed, whereupon specialists may utilize these in routine practice as a means to identify and address problems with patient dissatisfaction and thus potentially enhance the PDR, and hence quality of care[26].

We thus examined the data to identify characteristics of patients likely to opt for change. Patient factors temporally associated with a recent change, accepting these associations may not be causal, although nevertheless potentially modifiable, include higher disease activity, diagnosis of Crohn's disease, and previous surgery. We believe that these adverse disease factors may jeopardize patient satisfaction and the PDR. In this context, subsequent hospitalization may be the "final straw" in an already vulnerable PDR, prompting a patient-initiated change[10]. Additionally, given all patients were identified *via* a recent hospital encounter at the study centre (RAH), this encounter may have resulted in a sample biased towards those provided with an opportunity to change IBD specialist, given that their inpatient care may have been conducted by a different treating specialist than their usual doctor. However as mentioned, one-third of the cohort continued seeing a treating specialist external/unaffiliated to the study hospital at the time of survey.

Indeed, the data may also reflect societal changes. First, patient care is increasingly often disjointedly administered across multiple primary care, hyper-specialized and provider-specific boundaries[5]. This frequently tests the ability of health systems to ensure a seamless flow of clinical information and correspondence responsive to patient transitions between health providers, especially where larger institutions are involved[27,28]. For instance, a gastroenterologist with a solely community-based practice caring for a patient who suffers an IBD flare may remain unaware when their patient is admitted to a tertiary hospital, and conversely the hospital staff may not be aware of the treating specialist's long-term relationship with the patient. Post-discharge follow-up may then be routinely arranged in the hospital-based IBD clinic instead of the private gastroenterologist's rooms. Hence, depending on the patient's own initiative, a "system-induced change" in specialist may occur, resulting in a loss of continuity of care and potentially, reduced patient satisfaction and jeopardized QoC. Second, consumer expectations of doctors and health outcomes, congruent with medical technological advances, continue to escalate, thus conceivably, patients do (and will) change specialists more readily than ever before[5,29]. Finally, there is a consumer perception that larger entities (e.g., large department stores analogous to large hospitals) offer better products, more conveniently at a lower cost which may also drive patient-initiated changes[30].

Interestingly, we also found that patients with superior IBD knowledge appeared more likely to change specialists. Potentially, knowledgeable IBD patients who expect to participate in management decisions, desire a patient-centered emphasis within the PDR, otherwise this unmet need may drive patients to change specialist[10,31]. Moreover, given those with negative reasons generally exhibited lower adherence again underlines potential disadvantages of a discordant PDR, although these data cannot ascertain whether this discord elicits nonadherence or vice versa. Hence, these patients may be deemed at risk of future adverse disease outcomes in the context of nonadherence, which is unlikely to be salved by changing specialist[21,32].

Regardless in many ways, the patient's self-reported reasons for changing specialist may over-simplify the complex interplay of patient beliefs and expectations, their underlying illness and the PDR, resulting in the change. Hence the fact that the change occurred, rather than the stated reason may be more relevant to consider. For instance, we showed that patients with four or more changes for any reason had lower QoL scores, yet there was no statistically significant difference between patients stating a negative reason for changing specialist and other responders. Thus, the prevalence and frequency of changes (regardless of reason or timing) warrant attention so as to determine whether these lead to increased risk of adverse outcomes *via* disjointed care and underlying dissatisfaction, and whether these outcomes are potentially preventable. Possible avenues begin with the specialist, including engendering patient involvement in clinical decision making and self-empowerment[33], patient-friendly doctor-patient communication, and regular opportunities for patients to provide feedback on their care received[34,35]. Furthermore, patients suspected of medication nonadherence must be sensitively confronted and efforts made to rectify this as previously documented elsewhere[36,37]. Also, fail-safe systems of timely referral and correspondence between health providers must be instituted in order to prevent

loss to treating specialist follow-up and discontinuity of care, which often may occur during times of disease deterioration where continuity and high QoC may in fact be most needed[20,38]. Indeed, in occasional scenarios where a discordant PDR is irreconcilable, and upon mutual agreement between doctor and patient, patients may ultimately benefit from referral on to alternative colleagues or services for ongoing care[39].

In conclusion, in this novel study, we demonstrated that a patient-initiated change in treating specialist in IBD occurs frequently and appears temporally associated with adverse disease traits. Continuity of care, within a positive PDR, is an important element of high-quality care, thus, we recommend that treating specialists should monitor their patients for history and future risk of changing specialists. In view of the recent genesis of national standards in the United Kingdom and United States[20,40], we recommend that continuity of care and institution of efficient, fail-safe referral mechanisms are included as markers of quality in IBD. Adopting a patient-centered approach to IBD management, regularly surveying patient satisfaction and maintaining best practice therapeutic strategies, may result in durable benefits to both patients and doctors alike, although proven formulae to minimize avoidable change in specialist and maintain positive PDRs require further evaluation.

COMMENTS

Background
Inflammatory bowel diseases (IBD) is typically an unpredictable, relapsing- remitting condition and thus patient satisfaction and a robust patient-doctor relationship (PDR) are fundamental to high quality care. Conversely however, relational discord between patient and doctor may compromise quality of care (QoC), leading to adverse outcomes. Thus, in this study, we aimed to explore whether patients changed treating specialists as a tangible marker of patient satisfaction with the PDR and their medical care.

Research frontiers
In the 21st century, patient-centered care is integral in chronic disease management. Measuring patient satisfaction is an important component of ensuring a high standard of care delivery. A compromised PDR, and therefore, QoC, may result in inferior disease outcomes.

Innovations and breakthroughs
A patient-initiated change in treating specialist represents a vulnerable moment in delivery of care but also is a surrogate, tangible measure of patient satisfaction. By establishing factors associated with a change in specialist, one may better understand to what extent these are preventable, and/or how these are best identified so as to minimize disruptions to QoC, thus avoiding adverse outcomes.

Applications
Monitoring patient satisfaction is important to maintain continuity of care and therefore quality. Ensuring failsafe referral and follow-up mechanisms, especially in times of IBD relapse may reduce contemporaneous changes in specialists, thus ultimately improving outcomes for patients.

Peer review
This is an interesting study that sheds some light on the nature and complexity of the PDR in IBD patients. Given the importance of a positive PDR in IBD, such a study is necessary to understand the full dynamics of that relationship. The manuscript should be a good addition to the existing literature on the subject.

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World Journal of Gastroenterology

MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
United States

April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

World Journal of Gastroenterology

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid

evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

Instructions to authors

Editor-in-chief

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SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing

Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (n). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

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Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

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Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

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Title: Title should be less than 12 words.

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Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Abstract

There are unstructured abstracts (no less than 256 words) and structured abstracts (no less than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no less than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections.

AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, *P* < 0.001; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

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Data that are not statistically significant should not be noted. [a]P < 0.05, [b]P < 0.01 should be noted (P > 0.05 should not be noted). If there are other series of P values, [c]P < 0.05 and [d]P < 0.01 are used. A third series of P values can be expressed as [e]P < 0.05 and [f]P < 0.01. Other notes in tables or under illustrations should be expressed as [1]F, [2]F, [3]F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be la-

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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1   **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224   DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2   **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3   **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4   **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462   PMCID:2516377   DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5   **Vallancien G**, Emberton M, Harving N, van Moorse-laar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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]

Volume with supplement

7   **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8   **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10   **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11   **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12   **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13   **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14   **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15   Morse SS. Factors in the emergence of infectious dis-eases. 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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

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Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
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World Journal of *Gastroenterology*

Contents

Weekly Volume 18 Number 19 May 21, 2012

Contents

Contents

ABOUT COVER Zhou FX, Chen L, Liu XW, Ouyang CH, Wu XP, Wang XH, Wang CL, Lu FG.
Lactobacillus crispatus M206119 exacerbates murine DSS-colitis by interfering with inflammatory responses.
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EDITORIAL

Hepatitis C virus infection and health-related quality of life

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Abstract

Hepatitis C virus (HCV) hepatitis and other diseases related to HCV, such as cryoglobulinemia, lymphoma and renal failure, impair health-related quality of life (HRQoL). In addition, HCV *per se* might directly influence HRQoL *via* colonization of microglia in the brain or, indirectly, *via* the effect of systemic inflammatory cytokines which, in turn, can trigger brain interleukin production. The treatment of HCV-related disorders with interferon (IFN) has an effect on HRQoL. Initially, IFN causes a transient deterioration of HRQoL, due to the induction of depression and other side effects of treatment. Subsequently, the subjects who obtain a sustained virologic response experience an improvement in HRQoL. Only rarely does interferon treatment causes permanent detrimental effects on HRQoL, due to residual psychiatric or neurologic side effects. Liver transplantation is the only treatment for end-stage HCV-related liver disease. HRQoL generally improves massively a few months after transplantation, except in the case of serious complications of the transplant procedure. Furthermore, high levels of anxiety and neuroticism pre-transplant are associated with lower HRQoL one year after transplant. Additionally, six months after transplant, patients with HCV who experience virologic recurrence show significantly greater depression, anxiety, phobic anxiety, and paranoid ideation than anti-HCV-negative patients. In conclusion, optimal care for the overall well-being of patients with HCV infection requires adequate knowledge of their neurological and psychological status.

INTRODUCTION

The concept of quality of life (QoL) is an attempt to define, in analyzable terms, the effect of functional outcome of a disease and its treatment on the patient. What emerges should be a functional definition that is measurable over time. The questions that are included in the evaluation of QoL are drawn from various domains of the experience of the patient and should, therefore, represent in the most complete way his/her self-perception concerning physical, psychological, relational and working experience. This conceptualization is based on the World Health Organization definition of health as "A state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity". This definition is so broad that it includes elements which are beyond the traditional domain of medicine and health

caring systems. Opportunity, education, spiritual attitudes, social security, working satisfaction, social relationships, goods availability are elements of QoL that are independent of medicine. What we deal with, by using the concept of health-related quality of life (HRQoL), is the functional effect of an illness and of its therapy on an individual.

Four broad domains contribute to HRQoL: (1) somatic sensations; (2) psychological state; (3) social interactions; and (4) physical and occupational functions.

Various questionnaires, if possible self-administered, have been developed to assess HRQoL. They are subdivided into generic tools, which encompass a global overview of the above-mentioned domains, and specific tools which are oriented to the peculiar consequences of a disease or a class of diseases.

The interest of HRQoL assessment in relation to hepatitis C virus (HCV) infection depends firstly on the acknowledgment that HCV infection is a major public health problem. It has been estimated that about 3% of humans in the word are infected by the HCV[1]. In Europe, it is estimated that there are approximately 5 million HCV carriers; the peak prevalence among individuals is in the fifth decade of age[2]. Therefore, HCV infection mainly involves adults in the period of their active life.

About 10%-20% of the individuals who are chronically infected by HCV are likely to develop liver cirrhosis over a median period of about 30 years[3] and, out of the HCV-infected patients with liver cirrhosis, 2%-6% per year develop hepatocellular carcinoma[4]. In addition, a minority of subjects will develop immunological disorders, such as cryoglobulinemia and lymphoma[5]. Obviously all these individuals will suffer from symptoms due to the diseases caused by HCV infection (but that can also have other causes), rather than from the virus infection *per se*.

Decompensated liver cirrhosis, the final stage of cirrhosis, has an ominous consequence both on survival[6,7] and on HRQoL[8]. This group of subjects display obvious limitations to HRQoL that are mainly related to the consequences of liver insufficiency and portal hypertension (*i.e.*, hepatic encephalopathy, ascites, upper intestinal tract bleeding, sexual dysfunction, lower leg cramps and itch)[8]. However, the number of subjects with these complications of end-stage liver disease is relatively small, compared with the vast majority of patients having HCV in the absence of clinically significant liver disease.

Despite the initial idea that the majority of patients with HCV infection have merely asymptomatic seropositivity, some data suggest that HCV itself may diminish HRQoL even in the absence of advanced liver disease, perhaps due to HCV brain colonization or to systemic activation of cytokines which, in turn, influence brain cytokine production and neurotransmission.

Other important issues concerning HRQoL in HCV-infected patients are those related to the side effects of interferon (IFN) treatment and to liver transplantation, which is the treatment of choice of end-stage liver disease (Figure 1).

Figure 1 Pathways producing a reduction of health-related quality of life in hepatitis C virus-infected patients: both direct and indirect mechanisms play a role. HCV: Hepatitis C virus; HRQoL: Health-related quality of life.

HCV INFECTION *PER SE*

The issue of whether HCV infection *per se* (i.e., independent of the development of liver disease or other severe organic diseases related to HCV infection) may have an effect of HRQoL is a focus of debate. Several studies reported that patients with chronic HCV have a significant reduction in their HRQoL that is not related to the severity of the liver disease[9].

Depression can account for a reduction of HRQoL, since HRQoL depends on a patient's self-perception and evaluation. Therefore, any change in mood can obviously influence HRQoL. The prevalence of depression in HCV-infected individuals was reported to be higher than that in the general population (59% *vs* 21%)[10]. It should be noted, however, that depressive symptoms in patients with HCV infection might be due to pre-existing psychiatric disorders, to a reaction to the awareness of being infected, to adverse biological effects of HCV and, finally, to the consequences of liver disease; at least in the individuals who develop severe chronic hepatitis. In addition, coping styles and sources of information are of paramount importance in perceiving disease severity and disability, since they influence patients' stress levels and/or a pessimistic/optimistic view about the future[11].

An association between HCV infection and depression is therefore likely. However, the role of confounders-such as intravenous substance abuse or promiscuous sexual behaviour-related both to HCV infection and possibly to mood and psychological dimensions may bias statistical assessment. Therefore, further studies are required to determine with certainty the existence of a true association between depression and chronic HCV infection, together with its extent and the mechanisms implicated.

Moreover, strong clues for the existence of a true pathophysiological link between HCV infection and depression derive from the observation that depression has a higher prevalence in drug addicts who are infected with

HCV compared to those who are not infected[12]. In addition, HCV-infected patients who report no history of intravenous drug abuse complain of lower HRQoL compared with healthy normal subjects[13].

As mentioned above, the most difficult confounder to be controlled is the effect of the patient's awareness of his/her infection. In fact, it is reasonable to assume that the awareness of being infected with a virus which potentially is both life-threatening and transmittable by sexual intercourse may have an adverse emotional affect *per se*, regardless of any biological effect of the virus. However, the observation that patients with chronic HCV infection have reduced HRQoL scores compared to those with hepatitis B virus infection, suggests that HCV infection *per se* reduces HRQoL, rather than the non-specific patient awareness of a viral infection[13]. Also, HRQoL was found to be reduced even in patients who were not aware of being HCV carriers[14].

Some pathophysiological insight into the possible mechanisms involved in the reduction of HRQoL due to HCV reduction was provided by Weissenborn *et al*[15] who reported alterations of mood (increased anxiety and depression) and cognition, together with changes in both the midbrain serotoninergic and striatal dopaminergic systems, irrespective of viraemia and normal liver function, in patients with HCV infection. The existence of brain alteration directly caused by HCV is provided by the evidence of deficits of attention, executive function and verbal learning, of electroencephalogram slowing in the absence of liver cirrhosis and/or substance use disorder, and of peculiar alterations on magnetic resonance spectroscopy in HCV-infected patients[16-18]. It has been suggested that monocytes infected by HCV can cross the blood-brain barrier producing a secondary infection of microglia[19-22]. Such an infection is thought to be facilitated by immunosuppression caused by human immunodeficiency virus or by immunosuppressive treatment in transplanted patients[17,21-23].

Microglia infection by HCV may disturb neuronal function due to local cytokine production[16,20,23]. Another hypothesis is that the noxious effect on the brain is due to systemic induction of inflammatory cytokines [interleukin (IL)-1, IL-6 and tumor necrosis factor-α] by HCV which, *via* the blood-brain barrier, cause microglial activation and in-brain cytokine production[24]. This is the mechanism of the "sickness behaviour" syndrome, which includes non-specific depressive symptoms: fatigue, anhedonia, apathy, emotional lability, irritability, agitation, anorexia, psychomotor retardation, sleep disturbance, social withdrawal, hyperalgesia, decreased libido, and cognitive impairment[25-27].

HCV INFECTION AND IFN TREATMENT

Another approach to solve the problem of the existence, if any, of a direct effect of HCV infection on mood and HRQoL, is to examine the results of trials on HCV treatment. Such studies concern patients with chronic viral

hepatitis; therefore, the dimension of liver disease cannot be dissociated from that of HCV infection *per se*. At any rate, large studies based on the SF36 health questionnaire have shown a significant improvement in quality of life scores in those patients with chronic hepatitis who obtain a sustained viral response[28,29]. However, these kinds of studies cannot remove the bias caused by patient satisfaction due to the awareness of being healed of hepatitis C.

In addition, the interpretation of the change of HRQoL in HCV-infected patients who are treated with IFN is biased by the fact that IFN itself is a proinflammatory cytokine that induces neuropsychiatric symptoms. In fact, 12%-41% of subjects with HCV hepatitis who are treated with IFN develop neuropsychiatric symptoms, even if they did not have a history of previous mental disorder[10]. An even higher percentage of individuals, ranging from 17%-58%, develop neuropsychiatric symptoms, mainly depression, if they had had a history of previous mental disorder[10]. The depressive syndrome induced by IFN has two expressions: (1) a depression-specific syndrome characterized by depressed mood, anhedonia, anxiety, subjective cognitive disturbance and sometimes suicidal ideation; and (2) a neurovegetative syndrome, characterized by fatigue, insomnia, anorexia, pain, psychomotor slowness. Generally, symptoms remit within 2-3 wk of discontinuation of IFN treatment[30]. Nevertheless, some patients may continue to complain of cognitive difficulties 12-24 mo after discontinuation of IFN treatment[31]. Obviously, these neuropsychiatric IFN side effects influence HRQoL, which depends on subjective perception and self-report of personal well-being. Therefore, the cessation of IFN treatment and, thus, of the discomfort caused by treatment itself, might explain a rebound perception of increased HRQoL.

LIVER TRANSPLANTATION

Liver transplantation (LT) is a life-saving intervention for many patients with end-stage liver disease: survival after this intervention ranges between 90%-70% at one and five years, respectively. Many studies have reported significant improvements in HRQoL and satisfactory psychological outcome after LT[32,33]. The topic was recently reviewed by Tome *et al*[34] who observed that general HRQoL improves dramatically after LT; however, when compared with the general population, the vast majority of LT patients have significant deficits in most HRQoL domains. In addition, while some domains, such as physical functioning and psychosocial adaptation, significantly improve after transplantation, psychological health shows a lower improvement than physical functioning. On the whole, sexual life does not improve significantly; however, results in the literature so far are extremely heterogeneous, suggesting that this effect is essentially unpredictable in individual patients. A relationship between the aetiology of liver disease and HRQoL has been reported: HCV infections impinge on HRQoL in the patients with recur-

rence of liver disease[35]. The impairment is mainly related to physical functioning and fatigue[35,36]. An intriguing issue is the interplay amongst HRQoL, psychological status and brain function. Interestingly, elevated levels of anxiety and neuroticism on pre-transplantation assessment were associated with poor psychological outcome one year after transplantation. These findings suggest that personality and affective status may be important determinants of long-term psychological health after LT. Accordingly, patients with HCV who experienced virologic recurrence within 6 mo of LT showed significantly greater depression, anxiety, phobic anxiety, and paranoid ideation than anti-HCV-negative patients[33].

In conclusion, independent of the underlying pathophysiologic mechanisms, HCV infection is a signal of vulnerability for poor HRQoL and should be considered in patient assessment.

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Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

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Histopathology

Abstract

Nonalcoholic fatty liver disease (NAFLD) is a condition in which excess fat accumulates in the liver of a patient without a history of alcohol abuse. Nonalcoholic steatohepatitis (NASH), a severe form of NAFLD, can progress to liver cirrhosis and hepatocellular carcinoma. NAFLD is regarded as a hepatic manifestation of metabolic syndrome and incidence has been increasing worldwide in line with the increased prevalence of obesity, type 2 diabetes, and hyperlipemia. Animal models of NAFLD/NASH give crucial information, not only in elucidating pathogenesis of NAFLD/NASH but also in examining therapeutic effects of various agents. An ideal model of NAFLD/NASH should correctly reflect both hepatic histopathology and pathophysiology of human NAFLD/NASH. Animal models of NAFLD/NASH are divided into genetic, dietary, and combination models. In this paper, we review commonly used animal models of NAFLD/NASH referring to their advantages and disadvantages.

Key words: Animal model; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Metabolic syndrome;

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a condition in which excess fat accumulates in the liver of a patient without a history of alcohol abuse[1]. NAFLD is classified into simple steatosis and nonalcoholic steatohepatitis (NASH). In NASH, not only steatosis but also intralobular inflammation and hepatocellular ballooning are present, often accompanied by progressive fibrosis[2]. Long-standing NASH may progress to liver cirrhosis, and hepatocellular carcinoma (HCC) may be an outcome[3-5].

NAFLD is regarded as a hepatic manifestation of metabolic syndrome[6,7]. NAFLD has been increasing worldwide over recent decades in line with the increased prevalence of obesity, type 2 diabetes, and hyperlipemia. NAFLD/NASH is currently regarded as the most common chronic liver disease worldwide. It is estimated that about 20% of all adults have NAFLD and 2%-3% of adults have NASH[8].

The pathogenesis of NASH has not been completely elucidated, and treatments for NASH other than lifestyle modification by diet and exercise have not been fully established[5]. Studies of NAFLD/NASH using human materials have limitations, because the occurrence and progression of NAFLD/NASH require a long period of several decades, and ethical limitations exist in admin-

istering drugs to patients or collecting liver tissues from patients. Animal models of NAFLD/NASH give crucial information, not only in elucidating the pathogenesis of NAFLD/NASH but also in examining therapeutic effects of various agents. These animal models need to reflect correctly both the histopathology and pathophysiology of human NAFLD/NASH. Recently, several review articles on animal models of NAFLD/NASH have been published[9,10]. In this paper, we firstly review the histopathology and pathogenesis of NAFLD/NASH, and thereafter, we review current animal models of NAFLD/NASH referring to their advantages and disadvantages, with emphasis on a fructose-enriched diet model that was established by us.

HISTOPATHOLOGY OF NAFLD

The most important histological characteristic of NAFLD is steatosis of hepatocytes. Steatosis of hepatocytes is classified into macrovesicular and microvesicular. Macrovesicular steatosis is characterized by large vacuoles occupying almost the entire cytoplasm and pushing the nucleus to the periphery of the cell. Microvesicular steatosis is characterized by multiple small lipid vacuoles, and the nucleus is located at the center of the cell. Typically, steatosis in NAFLD is centrilobular and macrovesicular. However, steatosis may be present throughout the lobule, and microvesicular steatosis may also be present. Steatosis in more than 5% of hepatocytes is necessary for a diagnosis of NAFLD[11].

In NASH, not only steatosis but also intralobular inflammation and hepatocellular ballooning are present, and this is usually accompanied by fibrosis. Mallory's hyaline (MH) may also be present. Intralobular inflammation in NASH is typically mild and of a mixed type and includes a small number of lymphocytes, macrophages, and neutrophils. Neutrophils tend to infiltrate in the area of marked steatosis and around MH. Portal inflammation is usually mild; however, relatively intense chronic inflammation may be present in the portal area.

Hepatocellular ballooning is a form of liver cell injury recognized as a swollen hepatocyte with a rarefied cytoplasm. It is most apparent near steatotic liver cells, typically in zone 3.

MH is an eosinophilic and amorphous structure in the cytoplasm of hepatocytes and is observed in alcoholic hepatitis, NASH, chronic cholestasis, and HCC[2]. MH in NASH is most frequently found in the areas of perisinusoidal fibrosis in zone 3 and may be accompanied by neutrophilic infiltration. MH in NASH is usually less distinct than that in alcoholic hepatitis. Immunohistochemically, MH is positive for cytokeratin (CK) 8, CK 18, p62, and ubiquitin[2].

Other pathological features that are often observed in NASH include fat cysts and lipogranulomas, and glycogenated nuclei frequently occur in hepatocytes in zone 1. Furthermore, megamitochondria and iron deposition may be observed in NASH.

Fibrosis usually originates in the perisinusoidal regions of zone 3 (perisinusoidal fibrosis) and may also be present in the periportal area. As the disease progresses, bridging fibrosis and liver cirrhosis may develop. In the process, steatosis and lesion activity may resolve, resulting in a diagnosis of "cryptogenic cirrhosis" (burn-out NASH).

Brunt *et al*[12] proposed the grading and staging system of NASH, and the NASH Clinical Research Network designed and validated the NAFLD activity score[13]. These systems are frequently used in both clinical studies and animal experiments. Intralobular changes are milder and portal inflammation is more severe in pediatric NAFLD than in adult NAFLD[14,15]. Furthermore, steatosis in pediatric NAFLD is not necessarily predominant in zone 3[15,16].

PATHOGENESIS OF NAFLD/NASH

The "two-hit" hypothesis proposed by Day *et al*[17] is widely accepted as the pathogenesis of NAFLD/NASH; the first hit causes fat accumulation in hepatocytes, and the second hit causes inflammation and fibrosis. Fat accumulation in the liver is closely associated with metabolic derangements that are related to central obesity and insulin resistance[18]. The most reproducible risk factors for NAFLD/NASH are central obesity, insulin resistance, fasting hyperglycemia, and hypertriglyceridemia[19], and NAFLD/NASH is regarded as a hepatic manifestation of metabolic syndrome[6,7].

Hepatic steatosis occurs when the rate of import or synthesis of fatty acids by hepatocytes exceeds the rate of export or catabolism[20,21]. Accordingly, the following 4 mechanisms are possible causes of lipid accumulation within the liver: (1) increased delivery and uptake into hepatocytes of long-chain fatty acids (LCFA) due to excess dietary intake or release from adipose tissue; (2) increased *de novo* hepatic LCFA and triglyceride synthesis; (3) failure of very low-density lipoprotein (VLDL) synthesis and triglyceride export; and (4) failure of LCFA elimination due to impaired hepatic mitochondrial β-oxidation[22].

Once steatosis has developed, the liver is sensitized; thus, an inflammatory response may be precipitated by a variety of stimuli[22]. Oxidative stress, pro-inflammatory cytokine [e.g., tumor necrosis factor (TNF)-α]-mediated hepatocyte injury, altered lipid partitioning and hepatotoxicity mediated by free fatty acids, abnormal intrahepatic cholesterol loading, hyperinsulinemia, hyperleptinemia, hypoadiponectinemia, and apoptosis are all thought to be important second hits causing NASH[22-24].

ANIMAL MODELS OF NAFLD/NASH

An ideal animal model of NAFLD/NASH should reflect hepatic histopathology and pathophysiology of human NAFLD/NASH. Accordingly, the liver of the animal model of NASH should show steatosis, intralobular inflammation, hepatocellular ballooning, and, ideally, perisinusoidal fibrosis in zone 3 and susceptibility to liver

Table 1 Biochemical and pathological characteristics of animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

Model	Obesity	Insulin resistance	Steatosis	Steatohepatitis	Fibrosis
SREBP-1c transgenic mice	No (decreased adiposity)	Yes	Yes	Yes	Yes
Ob/ob mice	Yes	Yes	Yes	No (does not develop spontaneously)	No (resistant to fibrosis)
Db/db mice	Yes	Yes	Yes	No (does not develop spontaneously)	No (does not develop spontaneously)
KK-A^y mice	Yes	Yes	Yes	No (does not develop spontaneously)	No (does not develop spontaneously)
PTEN null mice	No	No	Yes	Yes	Yes
PPAR-α knockout mice	No	No	No (steatosis occurs in the starved state)	No	No
AOX null mice	No	No	Yes	Yes	No
MAT1A null mice	No	No	Yes	Yes	Yes
Methionine and choline deficiency	No (decreased weight and adiposity)	Hepatic insulin resistance	Yes	Yes (severe)	Yes
High fat	Yes	Yes	Yes	Yes (mild)	Yes
Cholesterol and cholate (atherogenic diet)	No (decreased weight)	Hepatic insulin resistance	Yes	Yes	Yes
Fructose	No	Yes	Yes	No/Yes	No

SREBP: Sterol regulatory element binding protein; PTEN: Phosphatase and tensin homologue deleted on chromosome 10; AOX: Acyl-coenzyme A oxidase; MAT1A: Methionine adenosyltransferase-1A; PPAR: Peroxisome proliferator-activated receptor.

tumors. Furthermore, the animal should show metabolic abnormalities such as obesity, insulin resistance, fasting hyperglycemia, dyslipidemia, and altered adipokine profile. It is questionable whether the results of a study using an animal model that does not completely fulfill these conditions can be extrapolated to human disease. Animal models of NAFLD/NASH are classified into genetic models, nutritional models, and combination models of genetic and nutritional factors. Numerous animal models of NAFLD/NASH have been reported to date; however, no animal model completely reflects hepatic histopathology and pathophysiology of human NAFLD/NASH. It is, therefore, important to select the animal model that best conforms to the aim of the study. Biochemical and pathological characteristics of animal models described in this paper are summarized in Table 1. As described below, in many of the genetic models of NASH [e.g., sterol regulatory element binding protein (SREBP)-1c transgenic mice and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) null mice], hepatic steatosis occurs first, and steatohepatitis develops later. Ob/ob, db/db, and KK-A^y mice do not progress to steatohepatitis spontaneously. On the other hand, in a dietary model induced by methionine and choline deficiency, steatohepatitis occurs very quickly.

GENETIC MODELS

SREBP-1c transgenic mice
In the fat tissue of these mice, SREBP-1c, a lipogenic transcription factor, is overexpressed. This creates a model of congenital lipodystrophy in which severe insulin resistance and diabetes develop secondary to impaired adipose differentiation[25]. In these mice, decreased fat tissue with lipid accumulation in the liver is observed,

and marked hepatic steatosis occurs by 8 d of age. When fed a standard diet, steatosis, lobular inflammation, and perivenular and pericellular fibrosis develop at 20 wk[26]; ballooned hepatocytes and MH are also observed. These mice appear to be appropriate for the lipodystrophy-associated steatohepatitis model. However, it is questionable whether they can be used as a model for typical NAFLD/NASH, because visceral fat characteristically increases in human NAFLD/NASH.

Ob/ob mice
Ob/ob mice possess a spontaneous mutation in the leptin gene (leptin-deficient). Leptin is an adipokine produced by white adipose tissue and operates on the hypothalamic ventral median nucleus exerting a marked anorexic effect[22]. Ob/ob mice are hyperphagic, inactive, and extremely obese, and show hyperglycemia, insulin resistance, and hyperinsulinemia[27]. Ob/ob mice develop spontaneous hepatic steatosis[28]; this does not, however, progress to steatohepatitis spontaneously. Secondary insults such as a methionine- and choline-deficient (MCD) diet, a high fat (HF) diet, or low-dose lipopolysaccharide (endotoxin) are needed to trigger steatohepatitis in ob/ob mice[29,30]. Another feature of ob/ob mice is that they are protected against fibrosis[31], a phenomenon which led to the characterization of leptin as an essential mediator of hepatic fibrogenesis[31,32]. Mutations in the *ob* gene are not prevalent in obese subjects or NASH patients, and leptin levels correlate poorly with the development of NASH[33].

Db/db mice
Db/db mice possess a natural mutation in the leptin receptor (*Ob-Rb*) gene[34] and, therefore, show normal or elevated levels of leptin but are resistant to the effects of leptin. These mice are obese, insulin resistant, and dia-

betic, and develop macrovesicular hepatic steatosis. They develop NASH when a second hit such as an MCD diet is added[35]. When db/db mice are fed an MCD diet, significant liver fibrosis is observed as compared to that in ob/ob mice[36]. The advantage of ob/ob and db/db mice is that the phenotype of these mice simulates the human condition of metabolic syndrome in many aspects. However, these mice have the disadvantage that they do not spontaneously develop steatohepatitis or liver fibrosis.

KK-A^y mice

A heterozygous mutation of the agouti gene (*KK-Ay/a*) results in a loss of melanocortin and an obese phenotype due to hyperphagia from impaired hypothalamic appetite suppression[9]. Although these mice develop hepatic steatosis in conjunction with obesity and insulin resistance, significant steatohepatitis does not occur spontaneously. It was reported that KK-A^y mice exhibited increased susceptibility to MCD diet-induced steatohepatitis, where hypoadiponectinemia most likely played a key role in exacerbation of both inflammatory and profibrogenic responses[37].

PTEN 10 null mice

PTEN is a tumor suppressor gene encoding a lipid phosphatase whose major substrate is phosphatidylinositol-3,4,5-triphosphate (PIP3). PTEN is a negative regulator of several signaling pathways such as phosphatidylinositol 3-kinase and serine-threonine protein kinase B (PKB, or Akt)[38]. These pathways regulate apoptosis, cell proliferation, and tumor formation[38]. Liver-specific *Pten* knockout mice (*AlbCrePTEN flox/flox* mice) show extensive hepatomegaly and steatohepatitis, and the histopathology is similar to that in human NASH[39]. Steatosis is observed at 10 wk of age, and steatohepatitis with fibrosis is observed at 40 wk. Hepatocellular adenomas occur with an incidence of 47% by 44 wk, and by 74-78 wk, all of the livers show adenomas, whereas 66% develop HCCs[40]. *Pten* knockout mice are hypersensitive to insulin, and *Pten* null hepatocytes have high proliferative activity *in vitro*[40]. The advantage of this model is that the histological phenotype resembles that of human NASH. The disadvantage of this model is that it is hypersensitive to insulin.

Peroxisome proliferator-activated receptor-α knockout mice

Peroxisome proliferator-activated receptor-α (PPAR-α) is a key regulator of genes involved in peroxisomal, mitochondrial, and microsomal fatty acid oxidation systems in the liver, and a significant decrease in PPAR-α is observed in the HF diet model[41]. Fat does not accumulate in the liver of mice with a homozygous mutation of the *PPAR*-α gene under conditions of normal feeding, but in the starved state, hepatic steatosis occurs because fatty acid oxidation is inhibited[42].

Acyl-coenzyme A oxidase null mice

Acyl-coenzyme A oxidase (AOX) is the rate-limiting enzyme of peroxisomal β-oxidation of LCFA. *AOX* null (*AOX* -/-) mice have defective peroxisomal β-oxidation of LCFA, which accumulate in the liver and lead to steatohepatitis. *AOX* -/- mice begin to exhibit microvesicular fatty change of hepatocytes in zones 2 and 3 of liver lobules at 7 d of age[43]. By 30 d of age, the *AOX* -/- mouse liver shows increased severity of steatosis of liver parenchymal cells and concomitant occurrence of focal inflammatory cell infiltrate[43]. At 2 mo of age, clusters of hepatocytes with peroxisome-rich eosinophilic granular cytoplasm are observed in periportal areas[43]. By 4-5 mo of age, increased expressions of PPAR-α, cytochrome P450 (Cyp) 4a10 and Cyp 4a14, and increased levels of H_2O_2 are observed[44]. However, compensative increase of fatty acid oxidation is observed by 6-7 mo of age, and hepatic steatosis recovers by regeneration of hepatocytes. *AOX* -/- mice develop hepatocellular adenomas and HCCs by 15 mo of age[44].

Methionine adenosyltransferase-1A null mice

Methionine adenosyltransferase-1A (MAT1A) is a liver-specific rate-limiting enzyme of methionine metabolism and catalyzes the formation of S-adenosylmethionine. *MAT1A* null mice have decreased levels of antioxidants, including glutathione, and decreased expressions of genes involved in lipid oxidation such as Cyp 4a10 and Cyp 4a14[44]. *MAT1A* null mice spontaneously develop steatohepatitis after 8 mo of age, and proliferation of hepatocytes increases, resulting in tumor development[45,46]. The mice are susceptible to choline-deficient diet-induced fatty liver at 3 mo of age[45]. Although *MAT1A* null mice are hyperglycemic, their insulin levels are normal and they do not appear to develop other features of metabolic syndrome[46].

DIETARY MODELS OF NASH

Methionine and choline deficiency

The MCD diet contains high sucrose and fat (40% sucrose, 10% fat) but lacks methionine and choline, which are essential for hepatic β-oxidation and production of VLDL[22]. In addition, choline deficiency impairs hepatic VLDL secretion[47]. As a result, lipid is deposited in the liver. Furthermore, oxidative stress[48,49] and changes in cytokines and adipocytokines[50] occur, contributing to the liver injury. Antagonizing oxidative stress by increasing antioxidant capacities attenuates the degree of steatohepatitis and stresses the importance of reactive oxygen species in this model[51].

Serum alanine aminotransferase (ALT) level is consistently increased after MCD-diet feeding in mice[52]. Steatohepatitis occurs at day 10[52], and perisinusoidal fibrosis is observed by 8-10 wk in mice[48,53]. After 10 wk of MCD feeding, extensive macrovesicular steatosis is observed in

all areas except for the periportal region, and many necro-inflammatory foci containing lymphocytes and neutrophils are observed in mice[54]. Although the MCD model causes more severe inflammation, oxidative stress, mitochondrial damage, apoptosis, and fibrogenesis than other animal nutritional models of NASH[55], the severity of NASH in rodents fed the MCD diet may depend on the species, gender, and strain of the animal[56]. Kirsch *et al*[57] compared the effects of MCD diet using male and female Wistar, Long-Evans, and Sprague-Dawley rats, and C57BL/6 mice. As a result, the Wistar strain and the male sex were associated with the greatest degree of steatosis in rats. Of the groups studied, male C57BL/6 mice developed the most inflammation and necrosis, and best approximated the histological features of NASH.

The main disadvantage of the MCD model is that the metabolic profile of the model is opposite to that of typical human NASH. Namely, mice fed the MCD diet show significant weight loss (often, more than 20% weight loss after 3 wk), low fasting blood sugar, peripheral insulin sensitivity, low serum insulin and leptin levels, and unchanged or increased serum adiponectin levels[50,58-61]. To improve these problems, genetically obese mice, such as ob/ob and db/db mice, are occasionally used as the MCD-fed animal. The main advantages of the MCD diet are that it is easy to obtain and use.

HF diet

Lieber *et al*[62] reported a diet model of NASH by using an HF diet (71% of energy from fat, 11% from carbohydrates, and 18% from proteins). Rats fed this diet *ad libitum* for 3 wk showed elevated plasma insulin levels reflecting insulin resistance. Rats fed the HF diet developed marked panlobular steatosis, and the hepatic lipid concentrations of these rats were approximately twice those of control rats fed the standard Lieber-DeCarli diet (35% fat, 47% carbohydrates, and 18% protein). Like human NASH, the rats fed the HF diet developed oxidative damage in the liver. When dietary consumption was restricted, steatosis and inflammation in the liver, oxidative stress, and plasma insulin levels were decreased. Feeding of an HF emulsion to Sprague-Dawley rats also induced changes closely resembling human NASH[63]. In the longitudinal study by Ito *et al*[64], chronic administration of an HF diet (60% of calories from fat) caused steatohepatitis in male C57BL/6J mice.

Intragastric overfeeding of mice with an HF diet up to 85% in excess of standard intake for 9 wk has been reported to replicate the histopathological and pathogenic features of NASH[65]. Overfed C57BL/6 mice became obese (71% higher body weight), had increased visceral fat (white adipose tissue: WAT), and showed hyperglycemia, hyperinsulinemia, hyperleptinemia, glucose intolerance, and insulin resistance. Almost half (46%) of the animals developed NASH, and their plasma ALT levels showed 9- to 10-fold increases. Neutrophilic infiltration and perisinusoidal fibrosis reminiscent of human NASH were observed. The WAT exhibited increased TNF-α and leptin

expressions and reduced adiponectin expression.

An HF diet is widely used to cause hepatic steatosis and NASH in experimental animals. However, it seems that the HF diet model produces variable results with regard to the degree of steatosis, inflammation, and fibrosis, and the results depend on rodent species and strain, the fat content in the diet, the composition of dietary fat, and the duration of treatment. For example, Sprague-Dawley rats appear susceptible to steatohepatitis development when fed an HF diet, and this is likely associated with their susceptibility to diet-induced obesity[54]. On the other hand, it was reported that long term high saturated fat feeding did not induce hepatic steatosis and NASH in Wistar rats[66]. In mice, it was reported that BALB/c male mice accumulated more hepatic lipid than C57BL/6J male mice when fed an HF diet[67]. In general, compared with the MCD model, the degree of liver injury in the HF model is less severe[10]. Among the HF models, the histopathology and pathophysiology of the intragastric overfeeding method most resemble those of human NASH. However, this method is difficult to implement, because it requires specific equipment and expertise. The optimization of the composition of the HF diet to reliably cause NASH in animals by *ad libitum* administration warrants future investigation. Recently, Ogasawara *et al*[68] reported a combined model of HF diet and gold thioglucose (GTG) administration. GTG is known to induce lesions in the ventromedial hypothalamus, leading to hyperphagia and obesity. They administered GTG intraperitoneally to C57BL/6 mice, and thereafter, fed an HF diet to the mice for 12 wk. As a result, obesity with increased abdominal adiposity, glucose intolerance, insulin resistance, and steatohepatitis with hepatocyte ballooning, MH, and pericellular fibrosis were induced.

Cholesterol and cholate

Matsuzawa *et al*[69] fed mice an atherogenic diet containing 1.25% cholesterol and 0.5% cholate and observed the progressive formation of steatosis, inflammation, and fibrosis in a time-dependent manner over 6-24 wk. In the model, hepatocellular ballooning, characteristic of human NASH, was observed at 24 wk. When 60% fat (cocoa butter) was added to the diet, development of these histopathological features was accelerated, and hepatocellular ballooning was observed at 12 wk. Furthermore, the atherogenic diet induced oxidative stress. Thus, it is conceivable that a combination diet of HF, cholesterol, and cholate in animals would cause histological features reminiscent of human NASH. However, the mice fed this diet were systematically insulin sensitive, albeit they showed hepatic insulin resistance. In fact, the mice lost 9% body weight during the experiment and had small epididymal fat pads and low plasma triglyceride levels compared with those in control mice. Therefore, this model appears to differ from human NASH in metabolic status.

Fructose

NAFLD/NASH is regarded as a hepatic manifestation of

Figure 1 Liver histology of rats fed a high-fructose diet for 5 wk. A: Hepatic steatosis, mainly distributed in zone 1, is observed (azan stain, × 40); B: Both macrovesicular and microvesicular steatosis are evident as well as scattered necroinflammatory foci (arrows) (hematoxylin and eosin stain, × 200).

Figure 2 Liver histology of rats fed a high-fructose diet + plant leaf extract. Hepatic fatty change is completely suppressed by the plant leaf extract (hematoxylin and eosin stain, × 40).

metabolic syndrome. Experimental animals fed a fructose-enriched diet are recognized as good models of metabolic syndrome. We examined livers of Wistar rats fed a high-fructose (70%) diet for 5 wk and found significantly higher macrovesicular steatosis (Figure 1A) and intralobular inflammation (Figure 1B) grades, liver:body weight ratios, and hepatic triglyceride concentrations than those in control rats[70]. In this study, the distribution of steatosis in the rats fed a high-fructose diet was characteristically predominant in zone 1. This pattern differs from that of human adult NAFLD, in which steatosis is usually predominant in zone 3. Rats fed a high-fructose diet showed significantly higher expressions of interleukin (IL)-6 protein and TNF-α protein in the liver compared with those in control rats (unpublished data). By adding plant leaf extract to the high-fructose diet, hepatic fatty change (Figure 2) and expression of IL-6 protein in the liver were completely suppressed (unpublished data).

Other groups also reported induction of NAFLD/NASH by fructose in experimental animals. Ackerman et al[71] found that male Sprague-Dawley rats fed a fructose-enriched (60%) diet developed macrovesicular and microvesicular steatosis in the liver. Armutcu et al[72] reported that male Wistar albino rats provided with drinking water containing 10% fructose for 10 d developed macrovesicular and microvesicular steatosis but did not develop inflammation in the liver. In mice, by adding 30% fructose to the drinking water, 3- to 4-fold increases in

hepatic triglyceride levels and marked increases in hepatic steatosis and weight were observed at 8 wk[73]. Recently, it was reported that C57BL/6 mice fed a high-fat high-carbohydrate diet and provided with drinking water containing 55% fructose for 16 wk developed a NASH-like phenotype with significant fibrosis as well as obesity[74]. Inflammation caused by endogenous toxins of fructose metabolites is suggested as one of the mechanisms of the second hit in the pathogenesis of NASH[75].

COMBINED MODEL OF GENETIC MODIFICATION AND NUTRITIONAL/ DIETARY CHALLENGES

Many animal models combine naturally occurring genetic mutations or targeted gene modifications with dietary or chemical challenges so that the histopathology and pathophysiology of the models more closely resemble those of human NAFLD. Sahai et al[36] fed an MCD diet to ob/ob and db/db mice and observed that db/db mice had significantly higher serum ALT levels and more severe hepatic inflammation and fibrosis than those in ob/ob and wild-type mice. PPAR-α null mice fed an MCD diet show more severe steatohepatitis than that in wild-type mice fed the same diet[76,77]. Many other models combining genetic abnormalities with nutritional challenges have been reported[78-80].

CONCLUSION

As reviewed in this paper, many animal models of NASH have been developed to date. These animal models do not replicate the full spectrum of the disease in humans; however, they can be used in verifying hypotheses on the pathogenesis of NASH and in performing interventional studies. We hope that animal models which more closely reflect the histopathology and pathophysiology of human NASH will be developed in the future, and information on pathogenesis and treatment of NASH will increase by using these models.

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REVIEW

Gastric stimulation for weight loss

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Abstract

The prevalence of obesity is growing to epidemic proportions, and there is clearly a need for minimally invasive therapies with few adverse effects that allow for sustained weight loss. Behavior and lifestyle therapy are safe treatments for obesity in the short term, but the durability of the weight loss is limited. Although promising obesity drugs are in development, the currently available drugs lack efficacy or have unacceptable side effects. Surgery leads to long-term weight loss, but it is associated with morbidity and mortality. Gastric electrical stimulation (GES) has received increasing attention as a potential tool for treating obesity and gastrointestinal dysmotility disorders. GES is a promising, minimally invasive, safe, and effective method for treating obesity. External gastric pacing is aimed at alteration of the motility of the gastrointestinal tract in a way that will alter absorption due to alteration of transit time. In addition, data from animal models and preliminary data from human trials suggest a role for the gut-brain axis in the mechanism of GES. This may involve alteration of secretion of hormones associated with hunger or satiety. Patient selection for gastric stimulation therapy seems to be an important determinant of the treatment's outcome. Here, we review the current status, potential mechanisms of action, and possible future applications of gastric stimulation for obesity.

INTRODUCTION

Obesity is a major public health challenge. The evolving concepts of how nutrient excess and inflammation modulate metabolism provide new opportunities for strategies to correct the damaging health consequences of obesity. The traditional approaches of caloric restriction, exercise, and behavioral therapies can each produce substantial weight loss, but it is not sustained in the majority of patients. The pharmacological agents that are currently licensed for weight loss produce only modest results and may have unwanted side effects. Clearly, a safe, effective, and durable treatment for obesity is needed.

The role of the gastrointestinal tract in regulating energy balance is now well recognized, and the expanding understanding of gut endocrinology and electrical signaling has produced a number of potentially fruitful avenues for developing obesity therapies.

In the last few years, gastric stimulation has gained attention as a possible new approach for treating obesity. Gastric electrical stimulation (GES) is a new method for invoking gastric contractions using a microprocessor con-

troller. The effect of this long-term gastric stimulation on food intake and body weight was first studied in pigs[1,2]. Pigs treated for eight months with electrical antral stimulation showed a net decrease of food intake[1]. In other studies, a significant decrease in food intake has been noted with gastric stimulation in dogs, and a significant decrease in fat absorption has been reported in rats[3-6].

Because GES has shown promising results in several animal models, the technique has been further refined for obesity treatment in humans. In 1995, the technique of an implantable gastric pacing device was studied in 24 morbidly obese patients. The results demonstrated the safety of the method and revealed changes in eating habits that resulted in reduced food intake and weight loss[7]. In a subsequent human study, there was a reported increase in meal-related satiety and enhanced inter-meal satiety during the treatment[8]. From this information, gastric pacing was hypothesized to facilitate weight loss by enhancing neuroendocrine satiety mechanisms.

Here, we review some of the techniques and the potential mechanisms associated with using gastric stimulation for treating obesity.

ROLE OF THE GUT-BRAIN AXIS IN REGULATING APPETITE

Food intake is influenced by emotional factors, social cues, and learned behavior. These influences overlay highly conserved systems within the brain that sense and integrate signals reflecting overall energy stores, recent energy intake and the presence of specific classes of nutrients.

Signals from the gut are important for controlling appetite and for regulating energy balance and glucose homeostasis. The active brain-gut axis sets the basis for options to stimulate different brain areas or neural connections between the brain and the gut to induce sensations of satiety.

There are several potential means by which the gut and the brain are associated. Some involve connections from the gut to hormones and from hormones to the nervous system. The gut continuously sends information to the brain regarding the quality and quantity of ingested food. These signals are not only important for satiation and meal termination but also for the appetitive phase of eating behavior[9]. By acting on the brainstem and hypothalamus, this flow of sensory information from the gut to the brain generates the feeling of satisfaction that is observed after a satiating meal[9].

The vagus nerve (VN) contributes to the bidirectional communications between the gastrointestinal tract and central nervous system (CNS)[10]. Afferent neurons of the VN are important targets of gut hormones, particularly the hormones involved in controlling food intake. Vago-vagal reflexes are involved in feeding homeostasis, making neuromodulation an attractive method for managing obesity[10].

Ziomber *et al*[10] have described the parameters of vagal neuromodulation required to decrease food intake in rats and cause a resulting reduction in body mass. Rats with solenoid electrodes placed on the left VN significantly decreased their food intake, weight gain and serum leptin concentrations. Another study has shown the suppressive effects of vagal nerve stimulation (VNS) on long-term feeding regulation in rats[11]. VNS has been shown to increase vagal afferent satiety signals, leading to reduced food intake and decreased weight gain[11]. Chronic microchip vagal stimulation significantly decreases epididymal fat pad weight and meal size in VNS rats, which causes decreased weight gain.

In addition to the VN, several other areas in the brain are involved in regulating gastrointestinal motility and satiety. The central nervous system regulates the homeostasis between nutrient intake and body reserves by sensing nutrient levels, integrating the information, and regulating energy intake and/or energy expenditure[12]. Grill *et al*[13] have summarized several potential mechanisms for connections between the gut and the brain. The nucleus tractus solitarius (NTS) and circuits within the hindbrain mediate the intake-inhibition effects of gastrointestinal (GI) signals. Short-term eating behavior is also controlled by the hindbrain. The NTS receives inputs from VN afferent neurons, while the area postrema is a target for circulating factors, such as amylin and glucagon-like peptide 1 (GLP-1)[14]. Classical studies have shown that when the higher inputs are surgically interrupted, the hindbrain can continue to regulate food intake in response to peripheral signals[15].

GASTRIC HORMONES ASSOCIATED WITH HUNGER AND SATIETY

The mechanisms that modulate gut-related satiety signals are now being recognized. The apparent importance of alterations in the gut hormonal milieu caused by surgical interventions in the GI tract has led to new surgical approaches and devices. Interventions (such as gastric pacing) that modulate gut-related satiety signals may offer approaches for curbing the threat of obesity to human health. The enteroendocrine cells of the GI tract act as a luminal surveillance system, responding to either the presence or absence of food in the gut lumen[16]. Their secretion products regulate the course of digestion and determine the delivery of nutrients to the gut by controlling food intake[16]. The gut is the source of numerous peptides, many of which can alter appetite. These peptides have numerous targets, including gastrointestinal exocrine glands, smooth muscles, afferent nerve terminals, and the brain[17].

GLP-1

This incretin hormone is secreted from the lower gut in response to food intake stimulates insulin secretion and

inhibits gastric emptying[18,19]. Following an oral glucose load, insulin secretion is enhanced when compared with an intravenous glucose infusion in the presence of similar plasma glucose concentrations[20]. This phenomenon is referred to as the incretin effect. In patients with type 2 diabetes mellitus (T2DM), however, the incretin effect is significantly impaired and postprandial GLP-1 secretion is diminished[20]. Because both the incretin effect and postprandial GLP-1 concentrations are markedly reduced in T2DM patients[21], the first clinical studies addressed the question of whether raising GLP-1 levels by exogenous administration of the incretin hormone could help to restore normal glucose regulation. However, GLP-1 has a plasma half-life of just a few minutes because it is readily degraded by the ubiquitous enzyme dipeptidyl peptidase-IV. Although the beneficial clinical effects of GLP-1 are evident, the need to continuously infuse it have hampered its broad clinical applications. Therefore, incretin mimetic agents, such as exenatide and dipeptidyl peptidase-IV inhibitors, are used to treat patients with T2DM[22]. Some studies have indicated that this treatment also results in weight loss[23,24]. Because of its short half-life, it has been shown that peripheral administration of GLP-1 lowers postprandial glucose dysregulation by inhibiting glucagon secretion. Beta-cell function is also improved, along with increased satiety and decreased food intake, which then results in weight loss. GLP-1 stimulates insulin secretion, which also delays gastric emptying[25-27]. Obese subjects have less GLP-1 release after a meal, which may lead to less inter-meal satiety when compared with lean subjects[28]. Low postprandial levels of GLP-1 in obese children are responsible for excessive ingestion of food and decreased inhibition of gastric emptying, both of which result in obesity[29]. The ability of Roux-en-Y gastric bypass surgery to increase GLP-1 levels has been reported[30]. In gastric pacing, up to 40% of excess body weight can be lost after 2 years of treatment[31]. A limited study in morbidly obese patients has shown that gastric pacing is associated with decreased peripheral levels of GLP-1. This effect correlates with decreased food intake and the resulting weight loss. From these results, a mechanistic role for the stimulation of vagal afferents has also been propounded[8].

Leptin

The nervous system regulates energy balance at the whole-body level by constantly adjusting energy intake, expenditure, and storage[32,33]. The *ob* gene encodes the protein leptin, which is an adipose tissue-derived circulating hormone. Since the identification of *ob* as the first example of human monogenic obesity, it has become clear that leptin plays a key role in controlling mammalian food intake and body fat stores[34,35]. A leptin deficiency in mice homozygous for a mutant *ob* gene (*ob*/*ob* mice) causes morbid obesity and diabetes. Leptin replacement leads to decreased food intake, normalized glucose homeostasis and increased energy expenditure[36-39]. Leptin induces

transcriptional changes for several genes *via* the JAK/STAT3 pathway, and rapid changes in cellular activity and membrane potential may underlie the acute actions of leptin[40]. Leptin resistance limits its utility in ordinary obesity, as reflected by the increased levels of leptin in obese subjects relative to their increased body fat[41]. Therefore, while the actions of leptin in peripheral tissues have been identified, studies in genetically modified mice have demonstrated that leptin action in the CNS is sufficient to regulate body weight, feeding, energy expenditure, and glucose metabolism[42-44]. The effect of leptin on metabolism is supported by neurons distributed in the hypothalamus, midbrain, and brainstem. It has been suggested that morphological changes progressively develop in the brain during obesity[45-47], and further inquiry into the cellular mechanisms linking obesity, neural plasticity, and food cravings are needed. Certain gut peptides (e.g., ghrelin) act in an additive manner with leptin to regulate energy balance[48], which has led to the development of combination therapies to enhance leptin sensitivity in obese states. Roux-en-Y gastric bypass in obese patients is associated with a decrease in leptin levels after only minimal changes in BMI have occurred[49]. Gastric pacing in morbidly obese patients results in significant weight loss that correlates with a decrease in leptin levels[8].

Peptide YY

The gut hormone peptide YY (PYY) belongs to a family of peptides that includes pancreatic polypeptide and neuropeptide Y (NPY). It is secreted by the L cells of the lower intestine after ingesting a meal and is released into the circulation[50], where it exists in two endogenous forms: PYY $_{1-36}$ and PYY $_{3-36}$[51]. PYY $_{1-36}$ is the major form of PYY in the fasting state[51]. The latter form is produced by the action of the dipeptidyl peptidase-IV enzyme in response to food intake[52]. Between 1% and 10% of PYY is also found in the esophagus, stomach, duodenum and jejunum[53]. PYY exerts its action through NPY receptors. PYY inhibits gastric motility and increases water and electrolyte absorption in the colon[54].

Endogenous PYY may be involved in the long-term regulation of body weight. PYY enhances a reduction in hunger and food intake[55]. Peripheral administration of PYY $_{1-36}$ decreases food intake in rodents. PYY $_{3-36}$ also markedly inhibits food intake in rodents[56,57] In humans, an intravenous infusion of physiological levels of PYY$_{3-36}$ reduces caloric intake in both normal weight[56] and obese subjects[58]. This effect is not achieved exclusively by affecting energy intake; there is evidence that PYY may have some effects on energy expenditure and lipid metabolism[59]. PYY levels are higher in rats treated with solenoid electrodes placed on the left VN than in untreated controls[10]. PYY may also be associated with the rapid improvement in carbohydrate homoeostasis observed after bypass surgery. This improvement is secondary to an increase in insulin sensitivity rather than an increase in insulin secretion, which occurs later[60]. Increases in

the secretion of GLP-1 and PYY are involved in the disappearance of hypertriglyceridemia and decreases in the levels of circulating fatty acids[60]. Some studies have reported that Roux-en-Y gastric bypass surgery increases PYY levels[30].

Cholecystokinin

Cholecystokinin (CCK) is the major hormone responsible for gallbladder contraction and pancreatic enzyme secretion. Like other gastrointestinal hormones, CCK is produced in discrete endocrine cells that line the mucosa of the small intestine. CCK stimulates vagal afferent neuron discharge and controls the expression of G-protein coupled receptors and peptide neurotransmitters in these neurons[16,61]. A gatekeeper function is attributed to CCK because its presence or absence influences the capacity of vagal afferent neurons to respond to other neurohormonal signals[16]. CCK reduces food intake by exerting an effect on the CCK-1 receptors residing in the VN[62].

During fasting, plasma CCK concentrations are low and vagal afferent neurons express cannabinoid CB1 and melanin concentrating hormone (MCH)-1 receptors, which stimulate food intake[16]. Post-prandial release of CCK down-regulates the expression of both receptors and stimulates the expression of Y2 receptors in neurons projecting to the stomach. In fasting, there is increased expression in the neurons of the appetite-stimulating neuropeptide transmitter MCH, along with decreased expression of the satiety-peptide cocaine and amphetamine-regulated transcript (CART). The secretion of CCK decreases the expression of MCH and increases the expression of CART. At low plasma concentrations of CCK, vagal afferent neurons exhibit increased capacity for appetite-stimulation, while post-prandial concentrations of CCK lead to enhanced capacity for satiety signaling[16].

CCK antagonists induce hunger, leading to larger meals sizes. CCK also delays the rate at which food empties from the stomach[62]. CCK expression can be altered by prolonged gastric stimulation. Following 14 d of GES, the number of CCK-immunoreactive neurons in the hippocampus increased compared with a control group[63]. In another study performed on morbidly obese patients treated with gastric pacing, the gastric pacing resulted in decreased CCK levels[8].

Ghrelin

Ghrelin was the first identified circulating hunger hormone[64]. In contrast to the GI peptide hormones (such as GLP-1, PYY and others) that increase satiety through CNS-mediated pathways[65,66], the gastric hormone ghrelin stimulates hunger through a different CNS-mediated pathway[65]. Ghrelin is produced primarily by cells in the oxyntic glands of the stomach and intestines[67] and is secreted into the bloodstream. Ghrelin is a potent stimulator of growth hormone (GH) secretion and is the only circulatory hormone known to potently enhance feeding and weight gain and to regulate energy homeostasis fol-

lowing central and systemic administration. When administered either peripherally or centrally to rodents, ghrelin increases food intake and body weight[68-70] and stimulates gastric motility and acid secretion[71]. In humans, there is a pre-prandial rise in plasma ghrelin levels[72,73]. When administered to rodents at supra-physiological doses, ghrelin is able to activate hypothalamic neuropeptide Y agouti-related protein neurons and increase both food intake and body weight. Ghrelin participates in meal initiation. When administered either peripherally or centrally to rodents, ghrelin rapidly increases food intake and body weight[48]. Therapeutic intervention with ghrelin in catabolic situations enhances food intake and increases gastric emptying and nutrient storage. These effects, coupled with an increase in GH, link nutrient partitioning with growth and repair processes. Ghrelin-based compounds may have therapeutic utility for treating the malnutrition and wasting that are induced by various sub-acute and chronic disorders. Conversely, compounds that inhibit ghrelin action may be useful for preventing or treating components of the metabolic syndrome, such as obesity, impaired lipid metabolism and insulin resistance.

Peripheral blood ghrelin levels were studied one month prior to gastric pacing, one month following implantation, and six months after activation of the electrical stimulation. Ghrelin levels were decreased significantly in response to food intake at all these evaluations. After activating the pacemaker, ghrelin levels were significantly increased above their levels prior to activation. The weight loss was significantly correlated with the increased ghrelin levels[74]. A decrease in the number of ghrelin-immunoreactive neurons in the hypothalamic paraventricular nucleus and in the supraoptic nucleus has been reported after a short period of gastric stimulation in rats[63]. In Roux-en-Y gastric bypass surgery, the total fasting plasma ghrelin levels were nearly identical between the subjects and the matched controls[30].

METHODS OF GASTRIC STIMULATION FOR THE TREATMENT OF OBESITY

Many aspects of cell functioning are controlled by intrinsic or extrinsic electrical activity. In the normal physiological state, an action potential is generated and then causes a cellular response. When electrical signals are applied externally, they increase the rate at which this response occurs, which is called electrical pacing. Nevertheless, if the electrical signal is applied during the refractory period of the normal action potential, it does not increase the response rate. However, it has been shown in several studies that application of the electrical signal is still able to change the biochemistry of the cell, which leads to greatly increased responses to the next normal action potential[75]. Stimulating the stomach, the sub-diaphragmatic sympathetics, the vagal nerve (with or without unilateral vagotomy), and the intestines are some of the pacing approaches that have been used for treating obesity[76].

Gastric pacing with short pulse width and high frequency for the treatment of obesity

Dilatation of the stomach by food ingestion sends afferent signals through the VN to the CNS and increases satiety, thus regulating food intake[77,78-80]. Because of the many neuro-hormonal functions of the stomach and the modification of these hormones by food ingestion, modifying the function of the stomach has been hypothesized to be a potential mechanism for treating obesity.

Several methods have been used to electrically stimulate the stomachs of obese patients[76]. Changes in the intensity of the stimulus, the duration of the pacing, and the anatomical site of stimulation determine the effect of the treatment. The responses to gastric pacing are dependent on the stimulus intensity, with shorter duration stimuli being sufficient when the impulse strength is increased. The antrum can be paced to significantly higher frequencies than the more proximal gastric regions.

GES is a method for invoking gastric contractions. The main effect of the technique was originally thought to be longer retention of food in the stomach that induces early satiety and diminishes food intake. The first clinical use of gastric pacing in the early 1990s was preceded by an exploration of gastric electrical physiology in the 1980s[81] Human GES for obesity began in 1995. There have now been more than 500 subjects treated for obesity with gastric pacing. With proper screening, the weight loss can be in the range of 40% of the excess body weight[76,82].

GES uses an electrical device called a gastric pacemaker to provide mild electrical stimulation to the lower abdominal nerves. Utilizing minimally-invasive surgical techniques, the gastric pacemaker is placed subcutaneously in the abdomen. It is intended to induce early satiety through electrical stimulation of the gastric wall. Several gastric stimulation protocols have demonstrated their weight-reduction efficacy in animal obesity models and in patients with morbid obesity. The implantable gastric stimulator (IGS) induces stomach expansion *via* electrical stimulation of the VN. This type of stimulation induces neurobiological responses by using brain circuits that lead to decreased food intake[83].

IGS has been used for the treatment of obesity[84]. IGS reduces appetite and increases satiety. Its efficacy is attributed to its inhibitory effects on gastric motility and its direct effects on the central nervous system and hormones related to satiety and/or appetite[84]. Chronic gastric stimulation impairs intrinsic gastric myoelectrical activity in the fed state, induces gastric distention in the fasting state and inhibits postprandial antral contractions[84]. The impairment of gastric myoelectrical activity and contractions are associated with impaired digestion and emptying of the stomach, which leads to early satiety and reduced food intake. The induction of gastric distention in the fasting state results in activation of stretch receptors, causing satiety[84]. Modulation of neuronal activities and the release of certain hormones in response to an IGS may also explain the reduction in appetite and increase in satiety.

The first studies of GES as a treatment for obesity employed the Transcend™ Implantable Gastric Stimulator. In 2002, Favretti and colleagues showed that the Transcend device was safe and effective at inducing and maintaining weight loss in 20 morbidly obese patients[85]. The pacemaker was implanted laparoscopically below the pes anserinus, 3 cm from the edge of the lesser gastric curvature and 6 cm from the pylorus. The lead was fixed within a tunnel in the gastric muscular wall by a suture, and gastroscopy was performed to ensure that the lead did not perforate the stomach wall. The operative time was less than an hour, and the stimulator was activated 30 d after its implantation. The stimulus parameters were an amplitude of 10 mA, a pulse width of 208 ms, and a frequency of 40 Hz with 2 s on and 3 s off[85]. The Transcend device blocks vagal efferents and delays gastric emptying, leading to a 40% loss of excess body weight[76]. The pacemaker delivers 2 s pulse trains with a 40-100 Hz frequency, 3-10 mA of current, and a short pulse duration of 0.18-0.4 ms, with intervening 3 s periods of no stimulation[7,86]. In another study, two electrode positions along the lesser curvature of the stomach were used, with the low insertions 6 cm proximal to the pylorus and the high insertions just distal to the esophageal-gastric junction. Short-term weight loss has been achieved in a subset of these patients[86]. The clinical response is most often defined by the excess weight lost (EWL) in these studies; however, improvements in symptoms, quality of life and nutritional status have also been reported by most open-labeled studies[87].

Gastric pacing with long pulse width and low frequency for obesity

Other types of stimuli have been tested in animal models and in humans. In dogs, retrograde stimulation through two electrode pairs positioned proximal to the pylorus for 8 s at a frequency of 50 Hz and a voltage of 16 V with intervening periods of no stimulation elicits retrograde contractions, reduces food intake and promotes weight reduction[88]. In another animal study, electrical stimulation has been shown to reduce food intake and increase gastric volume[89]. Studies in dogs using a pulse width in milliseconds at a frequency of six cycles per minute induced gastric relaxation when the pacing occurred in the proximal stomach but not when it occurred in the distal stomach[90]. These studies demonstrated the ability to inhibit gastric contractions and delay gastric emptying[90,91]. Temporary retrograde pacing with a mucosal electrode placed endoscopically on the greater curvature of the stomach, 5 cm above the pylorus, was performed in 12 normal volunteers. The gastric slow waves were entrained at nine cycles per minute, and the symptoms of satiety, bloating, discomfort, and nausea were linearly correlated with the energy stimulation in milliamps[92]. Later, 12 normal volunteers were studied for 3 d using temporary electrodes endoscopically placed

on the greater curvature stomach, 5 cm above the pylorus. Retrograde gastric pacing at nine cycles per minute resulted in retrograde propagation of electrical waves from the antrum. These waves disrupted the normal electrical waves that propagate distally and caused gastric hypomotility. Food intake decreased by 16% and gastric retention of solids increased by 15% during this retrograde pacing. These changes were accompanied by tolerable dyspeptic symptoms[92]. Another study in 12 normal volunteers using a stimulus intensity below the threshold that induces dyspepsia was able to delay gastric emptying, decrease food intake, and decrease water intake compared with sham controls. These results suggested the possibility of performing longer stimulations to treat obesity[93].

VNS for weight loss

The VN has a role in regulating hippocampal activity, and the hippocampus has a role in modulating eating behaviors[83]. Studies with the Transcend gastric pacemaker have suggested that blocking the efferent vagal impulses can reduce gastric tone, which is accompanied by slower gastric emptying, decreased food intake, and weight loss. A laparoscopically implanted electrical device that intermittently blocked both VNs near the esophageal-gastric junction led to EWL in obese patients[94]. A vagal blocking algorithm with a duration of 90-150 s was associated with greater EWL than were either shorter- or longer-duration algorithms[94]. An association was found between the number of 90-150 s algorithms delivered daily and greater EWL. In a study conducted in rabbits, the animals had a microchip implanted on the posterior vagus by laparotomy. Over a 4-wk period, body weight decreased by 12%, food intake decreased by 40%, and pulse rate decreased. Heart rate changes suggested stimulation of the afferent vagus in addition to blocking of the efferent vagus[95]. In another study, pigs were implanted with microchips on both vagal nerves by laparotomy. At an amplitude of 170 mV, a frequency of 1 Hz, and a 170-ms impulse duration, their food intake was decreased and their body weight gain was reduced with no observable side effects. Normogastria was reduced and tachygastria was increased, which is consistent with reduced efferent vagal transmission[96]. The human data on weight loss through VN stimulation comes from trials using VN stimulators for treating epilepsy. These patients had cervical vagal stimulation, which caused hoarseness, cough, throat pain, and dyspnea. Thirty-two patients were evaluated and 17 lost weight[97].

Stimulation during the electrical refractory period to treat obesity

Stimulating the gastric antrum in rats during the absolute refractory period increases the strength of gastric contractions and increases vagal afferent firing, similar to its effects on gastric distension[98]. Pyloric stimulation in dogs decreases food intake, decreases antral contractions, and delays gastric emptying[99]. The Tantalus system (MetaCure Ltd.) is implanted laparoscopically for gastric stimulation

that does not exhibit malabsorptive or restrictive characteristics[100]. It was developed to electrically stimulate the gastric antral muscle immediately following the entrance of food into the stomach. It increases antral muscular contractions and delays gastric emptying by delivering stimulation during the absolute refractory period[76]. The Tantalus system has a pulse generator and three bipolar leads. Two pairs of electrodes are implanted in the gastric antrum and two pairs in the gastric fundus. The electrodes in the gastric fundus sense the beginning of a meal and signal the pulse generator to stimulate the antral electrodes during the absolute antral refractory period, which enhances spontaneous gastric contractions and sends a signal through the afferent vagus that the stomach is distended. The device applies gastric contractility modulation signals to the gastric antrum. The system is designed to automatically detect when eating begins and to only then deliver GES sessions using electrical pulses that are synchronized to the intrinsic antral slow waves[100,101]. This method involves surgical placement of three electrode pairs; one pair in the fundus detects food ingestion, and two pairs in the antrum detect the intrinsic slow waves and deliver stimuli in synchrony with these signals[102]. The gastric stimulation begins when food enters the stomach, so it is only delivered postprandially. The device is believed to work by increasing antral contractions. This stimulated phased motor activity enhances the satiety that is normally elicited by postprandial gastric distention[98]. The stimulation parameters that enhance the phased antral contractions include a frequency of 80 Hz, a pulse width of 1-2 s, and a current of 0.5-1 mA[98]. Because of its energy requirements, the device must be recharged weekly by an external charger.

A trial in 12 obese subjects demonstrated that the fundal electrodes were able to sense the start of a meal > 75% of the time. The subjects lost 10 kg over 20 wk, and 9 subjects who continued till week 52 lost an additional 7 kg, for a total of 30.5% of their excess body weight lost[102]. In a European multicenter, open-label study, thirteen T2DM obese patients were laparoscopically implanted with the Tantalus device. The thirteen subjects that completed 3 mo of treatment showed a significant reduction in weight that was accompanied by glycemic improvement[100]. In the eleven patients that completed 6 mo of therapy, HbA1c was significantly reduced. However, the improvement in glucose control did not correlate with weight loss[101]. The data support the hypothesis that GES can improve glucose metabolism and induce weight loss in obese diabetic patients[103].

Intestinal electrical pacing for obesity

Intestinal electrical stimulation (IES) may have promising applications for treating motility disorders associated with altered intestinal contractile activity. However, recent studies have also revealed possible applications of intestinal electrical stimulation in obesity treatment[104]. IES applied to the duodenum reduces postprandial blood

glucose levels by modulating gastric emptying and the intestinal flow rate[105]. Both vagal and extra-vagal pathways are involved in the modulatory effects of IES on the central neurons of the satiety center[106]. Electrical stimulation of the stomach, intestine, or colon with long pulses has an inhibitory effect on gastric tone[107]. IES has been reported to alter intestinal slow waves, contractions and transit, effects that are mediated by both vagal and adrenergic pathways[108]. Duodenal stimulation in 12 healthy human volunteers did not induce dyspepsia, but it did reduce water intake and slowed gastric emptying[43]. Intestinal stimulation in dogs using seven sequential electrodes with a frequency of 24 cycles per minute, a pulse duration of 50 ms, and a pulse amplitude of 1-3 mA entrains the intestinal pacesetter. This treatment is effective at stimulating intestinal transit, even in the face of fat in the distal small intestine[109]. Intestinal stimulation in rats accelerates intestinal transit and reduces fat absorption[110]. Clinical trials of intestinal pacing in humans have yet to be reported.

Some potential mechanisms for the effect of gastric pacing in obesity

Obesity is the result of an imbalance between nutrient consumption, absorption, and energy expenditure[111]. GI motility regulates the rates at which nutrients are processed and absorbed and participates in controlling appetite and satiety *via* mechanical and neuro-hormonal pathways. The relatively extensive information on gastric pacing for motility disorders has shed light on the possible mechanisms of this treatment's beneficial effects on obesity. The effects of pacing may depend on the stimulus parameters and stimulation sites. Both the entrainment of intrinsic gastric electrical activity, eliciting propagating contractions and reducing symptomatology in patients with gastroparesis and reducing appetite and food intake in morbid obesity were suggested[112]. Additionally, gastric stimulation parameters have extra-gastrointestinal effects, including altering systemic hormonal and autonomic neural activity and modulating afferent nerve pathways projecting to the central nervous system[112].

Obesity that is induced by hypothalamic damage is associated with increased vagal tone, increased insulin secretion, increased food intake, and weight gain[113]. Performing a vagotomy below the diaphragm reverses the obesity caused by hypothalamic damage in rodents. Because trials have shown that placing a gastric pacemaker is the safest and simplest surgical treatment for morbid obesity, there has been considerable interest in defining the mechanisms by which it works[31]. Obesity seems to be associated with efferent vagal stimulation, which is inhibited by the Transcend pacemaker that is currently used. Short pulse widths and high-frequency stimulation induces gastric distention, inhibits postprandial antral contractions, and slows gastric emptying, which then leads to early satiety, reduced food intake and weight loss[114]. Cholinergic vagal efferents increase gastric tone,

while nitric oxide pathways decrease it[115]. Gastric stimulation blocks the efferent vagal pathway and releases nitric oxide pathways from inhibition, resulting in gastric dilatation[84,116,117]. The gastric slow waves are inhibited postprandially, which may also contribute to the delayed gastric emptying and promotion of satiety[118].

The decreased levels of GI hormones may be more compatible with depressed vagal tone than with vagal electrical stimulation of the pes anserinus area (the spreading zone of the vagal branches in the lesser curvature of the stomach). However, it is also possible that gastric pacing leads to parasympathetic hyperstimulation and the depletion of stored peptides, which may explain the reduced plasma levels of certain gastrointestinal hormones[8]. GES may also affect several brain areas associated with satiety and hunger, and may exert an effect on the hormonal gut-brain axis. Information on the current metabolic state is transmitted to the appetite control centers of the brain by a diverse array of signals, such as VN activity and metabolic "feedback" factors that are derived from the pituitary gland, adipose tissue, stomach, intestines, pancreas and muscle[12]. These signals act directly on the neurons located in the arcuate nucleus of the medio-basal hypothalamus, a key integration and hunger (orexigenic) and satiety (anorexigenic) control center of the brain[12].

CONCLUSION

Obesity is an epidemic disease that is increasing in prevalence and is associated with a rising incidence of diabetes. Behavior and lifestyle therapy are safe and effective treatments for obesity in the short term, but the durability of the weight loss is limited. Although promising obesity drugs are in development, the currently available drugs lack efficacy or have unacceptable side effects. Surgery leads to long-term weight loss, but it is associated with morbidity and mortality. GES is a promising, minimally invasive, safe, and effective method for treating obesity. Patient selection for gastric stimulation therapy seems to be an important determinant of the treatment's outcome[86,119-121]. Several approaches with different physiologic mechanisms are in various stages of development. The use of GES as an obesity treatment should produce exciting new developments in the foreseeable future.

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ORIGINAL ARTICLE

Matrix metalloproteinase-9 contributes to parenchymal hemorrhage and necrosis in the remnant liver after extended hepatectomy in mice

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Abstract

AIM: To investigate the effect of matrix metalloproteinase-9 (MMP-9) on the remnant liver after massive hepatectomy in the mouse.

METHODS: Age-matched, C57BL/6 wild-type (WT), MMP-9(-/-), and tissue inhibitors of metalloproteinases (TIMP)-1(-/-) mice were used. The mice received 80%-partial hepatectomy (PH). Samples were obtained at 6 h after 80%-PH, and we used histology, immunohistochemical staining, western blotting analysis and zymography to investigate the effect of PH on MMP-9. The role of MMP-9 after PH was investigated using a monoclonal antibody and MMP inhibitor.

RESULTS: We examined the remnant liver 6 h after 80%-PH and found that MMP-9 deficiency attenuated the formation of hemorrhage and necrosis. There were significantly fewer and smaller hemorrhagic and necrotic lesions in MMP-9(-/-) remnant livers compared with WT and TIMP-1(-/-) livers ($P < 0.01$), with no difference between WT and TIMP-1(-/-) mice. Serum alanine aminotransaminase levels were significantly lower in MMP-9(-/-) mice compared with those in TIMP-1(-/-) mice (WT: 476 ± 83 IU/L, MMP-9(-/-): 392 ± 30 IU/L, TIMP-1(-/-): 673 ± 73 IU/L, $P < 0.01$). Western blotting and gelatin zymography demonstrated a lack of MMP-9 expression and activity in MMP-9(-/-) mice, which was in contrast to WT and TIMP-1(-/-) mice. No change in MMP-2 expression was observed in any of the study groups. Similar to MMP-9(-/-) mice, when WT mice were treated with MMP-9 monoclonal antibody or the synthetic inhibitor GM6001, hemorrhagic and necrotic lesions were significantly smaller and fewer than in control mice ($P < 0.05$). These results suggest that MMP-9 plays an important role in the development of parenchymal hemorrhage and necrosis in the small remnant liver.

CONCLUSION: Successful MMP-9 inhibition attenuates the formation of hemorrhage and necrosis and might

be a potential therapy to ameliorate liver injury after massive hepatectomy.

INTRODUCTION

In the field of hepatobiliary surgery, liver resection is considered the standard treatment for primary liver tumor and colorectal liver metastases[1-3]. Major liver resections have been associated with an increased morbidity and mortality compared with more limited resections. In particular, the outcomes are worsened when a chronic underlying liver disease is present[4]. Recent studies have shown that the volume of the remnant liver is correlated with perioperative morbidity and mortality[5-7], and an insufficient hepatic remnant (IHR) after extended hepatectomy is a critical issue.

On the other hand, liver transplantation (LT) is the treatment of choice for end-stage liver diseases. However, in the LT field, donor shortage and small-for-size grafts (SFSGs) are critical issues. Although split-liver transplantation (SOLT) is promising, failure of SFSGs in adult LT recipients[8,9] and after extensive hepatectomy for metastatic cancer hinders its application[10]. SFSG failure results in postoperative coagulopathy, cholestasis, ascites, encephalopathy, and death[11,12]. The mechanisms of SFSG failure remain incompletely understood.

One mechanism that has been implicated in the early development of SFSG failure is the formation of parenchymal hemorrhage and necrosis[13,14]. Previous investigators used electron microscopic studies to demonstrate sinusoidal breakdown leading to hemorrhage and necrosis after chemically or endotoxin-induced liver failure[15-17], hepatic ischemia-reperfusion injury[18-19], extensive hepatectomy[18,20,21], and LT with SFSG or SOLT[22-25]. These findings suggested that although the inciting factors might have different causes, the pathogenesis of sinusoidal breakdown leading to hemorrhage and necrosis might be similar.

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent neutral proteases that can degrade the extracellular matrix and basement membrane. MMPs play significant roles in cellular regulation, cell-cell communication, and tumor progression[26,27]. MMP-2 and MMP-9 have been implicated in liver injury and remodeling. Knocking out MMP-9 and other MMPs attenuate liver injury associated with interferon treatment[28]. MMP-9 contributes to the pathogenesis of experimental acute liver failure[29]. Specifically, MMP-9 has been implicated in sinusoidal breakdown leading to extravasation of circulating cells and hemorrhage[14,30]. MMP-9 plays a pivotal role in ischemia-reperfusion injury[31-33]. When MMP-9 is blocked with a synthetic inhibitor or by MMP-9 gene deletion, it successfully reverses ischemia-reperfusion injury[34,35] and cold preservation-warm reperfusion injury after LT[35]. Collectively, these data show that MMP-9 is involved in sinusoidal injury in liver failure. However, the role of MMP-9 in the pathogenesis of failure in IHR or SFSGs is not well known.

We hypothesized that MMP-9 plays an important role in the development of liver parenchymal hemorrhage and necrosis in IHR/SFSG failure. Tissue inhibitors of metalloproteinases (TIMP)-1 are physiological tissue inhibitors of MMP-9, which increase MMP-9 activity when deleted[36,37]. We hypothesized that a deletion or decrease in MMP-9 has a beneficial effect after hepatectomy. In the current study, we examined this hypothesis in IHR after 80%-partial hepatectomy (PH) in wild-type (WT), MMP-9(-/-), and TIMP-1(-/-) mice. We also examined the efficacy of MMP-9 blockade with a monoclonal antibody and synthetic MMP-9 inhibitor.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (10-14 wk old) were obtained from Jackson Laboratory (Bar Harbor, ME), housed with a 12-h light/dark cycle, and given food and water *ad libitum*. MMP-9(-/-) mice, obtained from Dr. Robert Senior (Washington University, St. Louis, MO), and TIMP-1(-/-) mice, purchased from Jackson Laboratory, both with a C57BL/6 background, were bred in our facility. The study was institutionally approved in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals".

PH in mice

Our institutional procedures for PH and postoperative care have been described elsewhere in detail[38]. Left and right posterior and left and right anterior segments were resected for an 80%-PH, similar to that described in a previous report[39]. In brief, under general anesthesia, liver lobes were mobilized. A hemostatic clip (Teleflex Medical, Triangle Park, NC) was applied across the pedicle, and the liver lobes were removed, without any obstruction of portal flow and hepatic venous drainage[38]. Laparotomy alone was performed for sham operations. Before closure, 2 mL of warm saline was administered

intraperitoneally. Cephalexin (30 mg/kg) and buprenorphine (0.1 mg/kg) were given subcutaneously. Postoperatively, the mice were kept in a temperature-controlled environment with free access to food and water. All mice were sacrificed 6 h after 80%-PH. The remnant livers were harvested, and blood samples were collected. We chose the extended hepatectomy model in mice because we hypothesized that MMP-9 plays an important role in the development of liver parenchymal hemorrhage and necrosis in IHR/SFSG failure.

MMP-9 inhibition by anti-MMP-9 monoclonal antibody and the MMP inhibitor GM6001

WT mice received 3 mg/kg of anti-MMP-9 neutralizing monoclonal antibody intravenously (clone 6-6B; EMD Chemicals, Gibbstown, NJ) 1 h before 80%-PH (MMP-9 mAb, $n = 6$). Control mice received normal IgG (EMD, Gibbstown, NJ) (control IgG, $n = 6$). The broad-spectrum MMP-inhibitor GM6001 (Millipore, Billerica, MA) at a concentration of 100 mg/kg in 10% dimethyl sulfoxide (DMSO) was administrated intraperitoneally 2 h before 80%-PH ($n = 10$), and controls received DMSO only ($n = 10$). An inhibitor of MMP-9 itself may affect liver regeneration after PH. Therefore, for the inhibition of MMP-9, we employed two inhibitory methods (*i.e.*, a monoclonal antibody and inhibitor) and used MMP-9(-/-) mice in this study.

Biochemical analysis

Serum levels of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) were determined by using a kinetic detection kit (Pointe Scientific, Inc, Canton, MI), and total bilirubin was determined by using the QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems, Hayward, CA).

Western blotting analysis

Liver samples were homogenized in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylene diamine tetra-acetic acid (EDTA), 1 mmol/L ethylene glycol tetra-acetic acid, 1 mmol/L phenyl-methyl-sulfonyl fluoride, and protease and phosphatase inhibitors. Homogenates were centrifuged at 105 000 × *g* for 1 h at 4 ℃. Supernatants were collected, and protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL). Forty micrograms of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 [20 mmol/L Tris-buffered saline (pH 7.4), 500 mmol/L NaCl, and 0.05% Tween 20] and probed using the antibody for MMP-9 (R and D Systems, Minneapolis, MN), and were then incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) followed by enhanced chemi-luminescence (ECL) or ECL Plus reagent (Am-

ersham Biosciences, Piscataway, NJ). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control (Imgenex Corporation, San Diego, CA). Signals were quantified by using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Gelatin zymography

Liver homogenates were analyzed by gelatin zymography with affinity chromatography[40]. In brief, 400 µg of liver extract samples were incubated with 100 µL of gelatin-Sepharose 4B (GE Healthcare, Piscataway, NJ) and equilibrated buffer containing 50 mmol/L Tris-HCL pH 7.5, 150 mmol/L NaCl, 5 mmol/L CaCl₂, 0.02% Tween 20, and 10 mmol/L EDTA for 2 h at 4 ℃. After being washed three times, gelatin-Sepharose beads were resuspended in the same volume of 2 × zymography sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA) and loaded onto a 10% SDS-PAGE gel containing 1 mg/mL of gelatin (Bio-Rad Laboratories, Inc.). After electrophoresis, the gel was washed twice for 30 min with 2.5% Triton X-100 for renaturing and then incubated in development buffer (Bio-Rad Laboratories, Inc.) for 20 h at 37 ℃. The gel was then fixed and stained with 0.5% Coomassie Blue R-250 (Bio-Rad Laboratories, Inc.) for 1 h and destained with 10% acetic acid in 40% methanol solution. Gelatinase zymography standards (Millipore, Billerica, MA) were used as a positive control.

Histology and immunohistochemical staining

Formalin-fixed, paraffin-embedded liver specimens in 5-µm sections were stained with hematoxylin and eosin. Immunohistochemical staining for CD11b (MAC-1) and CD68 was performed on frozen sections, and staining for desmin, myeloperoxidase, and MMP-9 was performed on paraffin-embedded sections after heat-induced antigen retrieval. We used 0.3% H₂O₂ to quench the endogenous peroxidase activity. An ABC kit (Vector Laboratories, Inc, Burlingame, CA) was used according to the manufacturer's instructions for HRP-based staining with 3,3' diaminobenzidine (DAB) tetrahydrochloride (Dako, Carpinteria, CA). Antibodies for MMP-9 [antigen affinity-purified, lyophilized from a 0.2 µm filtrated solution in phosphate buffered saline (PBS) with trehalose; AF909; R and D Systems, Minneapolis, MN], CD11b (clone M1/70, R and D Systems), CD68 (clone FA-11, Abcam Inc., Cambridge, MA), desmin (Abcam Inc.), and myeloperoxidase (Ab-1) (Thermo Scientific, Fremont, CA) were incubated for the primary reaction. Alexa Fluor 568 donkey anti-goat IgG (H + L), Alexa Fluor 488 donkey anti-rabbit IgG, and Alexa Fluor 488 donkey anti-rat antibody (Invitrogen, Carlsbad, CA) were used for secondary reactions.

Histological analysis

The HE-stained sections were scanned with a Scanscope XT system and analyzed with Aperio Imagescope software (Aperio Technologies, Inc., Vista, CA). Histologically positive areas were counted and measured by investigators blinded to the study group in three randomly

selected 3-mm^2 fields. DAB-positive staining of MMP-9 was calculated using the positive-pixel-count function of Imagescope software in 10 randomly selected fields. The value was quantified as the percentage of positive pixels per total pixels. The number of infiltrated neutrophils in the remnant liver was evaluated in five randomly captured 40 × high-power fields (hpf) with an Olympus BX50 fluorescence microscope (Olympus Optical, Tokyo, Japan).

In situ zymography

In situ gelatinolytic activity was determined using 20-μm cryostat sections for the EnzChek Gelatinase assay kit (Molecular Probes, Eugene, OR)[40]. Sections were incubated with 20 μg/mL of fluorescence-conjugated gelatin in reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L CaCl$_2$, and 0.2 mmol/L sodium azide) for 2 h at 37 ℃. After three washes with PBS, they were fixed in 4% paraformaldehyde. The sections were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA). Gelatinase activity was visualized using fluorescence microscopy (Olympus BX50; Olympus Optical, Tokyo, Japan).

Preliminary study

In our institution, 90%-PH (10% of the liver remnant with an omental segment), 80%-PH (20% of the liver remnant with right middle and omental segments) and 75%-PH (25% of the liver remnant with right middle and posterior segments) are available[38]. Initially, we performed 90%-PH in WT, MMP-9(-/-) and TIMP-1(-/-) mice, as a preliminary study. A survival study after 90%-PH was performed in each murine strain. Moreover, liver damage scores in HE staining[41], serum AST and ALT levels, and the ratios of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive nuclei/all nuclei per mm^2 and caspase 3-positive nuclei/all nuclei per mm^2 in immunohistological staining were assessed in serum or liver samples 6 h after 90%-PH in each murine strain. The results for each factor did not reach significant differences between WT and MMP-9(-/-) mice and between WT and TIMP-1(-/-) mice. Even in WT mice (control group), all mice received 90%-PH without any surgical issues but showed considerable damage and subsequently died in the early postoperative period. We therefore considered that 90%-PH is not suitable to investigate the effect of MMP-9 and TIMP-1 on the hepatic remnant after hepatectomy. To obtain relevant data according to clinical background[1-10], we finally employed 80%-PH in each strain for further experiments in this study. We decided to evaluate 80%-PH because mice could survive for a long time after surgery.

Statistical analysis

Data are presented as the mean ± SE. Statistical comparisons were performed using analysis of variance followed by a two-sample *t*-test with Bonferroni adjustment. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Parenchymal hepatic hemorrhage and necrosis occurs soon after 80%-PH

First, 80%-PH was performed in WT mice. Six hours after 80%-PH, the animals were killed and divided into two groups according to the status of parenchymal hemorrhage and necrosis in the remnant livers. Murine behavior in hepatic failure has been well described[42,43]. The animals with hemorrhage and necrosis were observed to be sick and inactive, whereas the mice without such injury were asymptomatic and active. As shown in Figure 1A and B, the groups consisted of mice with minimal or no hemorrhage and necrosis (group Ⅰ, *n* = 6) or those with multifocal hemorrhage and necrosis (group Ⅱ, *n* = 5). Group Ⅰ had a significantly greater area of necrosis (2.97% ± 0.92% *vs* 0.11% ± 0.08%, *P* < 0.05) (Figure 1C) and number of necrotic foci compared with group Ⅱ (3.60 ± 0.87 *vs* 0.26 ± 0.17, *P* < 0.01) (Figure 1D). Group Ⅱ had significantly higher AST (893 ± 72 IU/L *vs* 28 ± 12 IU/L, *P* < 0.05) (Figure 1E), ALT (744 ± 35 IU/L *vs* 32 ± 77 IU/L, *P* < 0.05) (Figure 1F) and total bilirubin levels than those in group Ⅰ (4.45 ± 0.63 mg/dL *vs* 1.41 ± 0.19 mg/dL, *P* < 0.01) (Figure 1G). These results suggested that group Ⅱ mice were in the process of liver failure, and these results are consistent with previous reports[13,44-46].

Focal and/or patchy necrosis is an important finding after hepatectomy[13,44]. Progressive necrosis is found from the early postoperative period[13,44-46]. We observed that parenchymal hemorrhage and necrosis occurred as early as 1 h after 80%-PH, and increased in number and size at 12 h and 24 h (data not shown), consistent with recent reports[13,44-46].

MMP-9 is upregulated in the remnant liver with hemorrhage and necrosis

MMP-9 has been implicated in acute liver failure[14,29]. We investigated MMP-9 expression in the remnant livers of both groups Ⅰ and Ⅱ 6 h after 80%-PH. Western blotting analysis demonstrated that MMP-9 was upregulated in group Ⅱ compared with group Ⅰ (*P* < 0.01) (Figure 2A and B). When we examined immunohistochemical staining for MMP-9, hepatocytes showed no expression of MMP-9. However, MMP-9 was mainly observed in round-shaped cells and in stellate-shaped cells (Figure 3A and B). To evaluate the association between necrosis and MMP-9, we compared the expression of MMP-9 in 10 randomly selected fields of 3-mm^2 in area with and without hemorrhage and necrosis. MMP-9 expression in the foci of hemorrhage and necrosis was higher than that in normal liver parenchyma (*P* < 0.05) (Figure 3C). These results suggest that MMP-9 expression is associated with the development of hemorrhage and necrosis in the remnant liver after 80%-PH, which is consistent with previous reports[14,29].

CD11b-positive neutrophils are the main source of MMP-9 in the remnant liver

To determine the source of upregulated MMP-9 in the

Figure 1 Histological and hematological differences between groups Ⅰ and Ⅱ at 6 h after 80%-partial hepatectomy. A: Histological findings in group Ⅰ mice; B: Representative images of remnant liver histology demonstrating significant multiple foci of hemorrhage and necrosis in group Ⅱ mice; C: Quantitation of the size (area percentage) of hemorrhage and necrosis: Significantly larger areas and more hemorrhagic and necrotic foci were observed in group Ⅱ mice than in group Ⅰ mice ([a]P < 0.05); D: Quantitation of the number of foci of hemorrhage and necrosis per mm^2: Significantly larger areas and more hemorrhagic and necrotic foci were observed in group Ⅱ mice than in group Ⅰ mice ([b]P < 0.01); E: Aspartate aminotransferase (AST) levels were significantly higher in group Ⅱ mice than those in group Ⅰ mice ([a]P < 0.05); F: Alanine aminotransaminase (ALT) levels were significantly higher in group Ⅱ mice than those in group Ⅰ mice ([a]P < 0.05); G: Total bilirubin levels were significantly higher in group Ⅱ mice than those in group Ⅰ mice ([b]P < 0.01).

remnant liver, we performed double immunofluorescent analysis with MMP-9 and cell marker antibodies. CD68 and desmin were used for Kupffer cells and hepatic stellate cells, respectively. MMP-9 was not observed in CD68-positive Kupffer cells (Figure 4A-C). Weak immunoreactivity to MMP-9 was observed in desmin-positive hepatic stellate cells (Figure 4D-F). In contrast, we consistently found MMP-9 expression in CD11b-positive cells (Figure 4G-I). These results suggest that infiltrating

neutrophils are the source of MMP-9 found in the foci of hemorrhage and necrosis in the remnant liver after 80%-PH, which is consistent with previous reports[28,35].

MMP-9 deletion reduces parenchymal hemorrhage and necrosis

To further confirm the role of MMP-9 in the development of liver hemorrhage and necrosis after 80%-PH, we performed liver resections in WT, MMP-9(-/-), and

Figure 2 Western blotting analysis for matrix metalloproteinase-9 in remnant livers with (group Ⅱ) and without (group Ⅰ) foci of hemorrhage and necrosis after 80%-partial hepatectomy. Representative immunoblot (A) and histogram (B) show enhanced expression of matrix metalloproteinase (MMP)-9 protein in group Ⅱ mice compared with that in group Ⅰ mice and sham controls ([b]$P < 0.01$).

TIMP-1(-/-) mice. TIMP-1 is a physiological tissue inhibitor of MMP-9, which increases MMP-9 activity when deleted[36,37]. We compared the size (area percentage) and the number of hemorrhagic and necrotic foci in the remnant livers. There were significantly fewer and smaller hemorrhagic and necrotic lesions in MMP-9(-/-) mice compared with WT and TIMP-1(-/-) mice (Figure 5A-C), (necrotic area: 0.49% ± 0.14% *vs* 1.74% ± 0.27% *vs* 1.82% ± 0.40%, $P < 0.01$, Figure 5D) (number of necrotic foci: 0.64 ± 0.18 *vs* 1.50 ± 0.21 *vs* 1.76 ± 0.39, $P < 0.05$, Figure 5E). There were no differences in the size and number of necrotic foci in WT and TIMP-1(-/-) animals.

Western blotting and gelatin zymography showed that MMP-9 expression and activity were present in WT and TIMP-1(-/-) mice but were absent in MMP-9(-/-) mice (Figure 6A and B). There was no difference in MMP-2 expression, a gelatinase, which is closely related to MMP-9, in the three groups of mice. In situ gelatin zymography showed significantly less MMP-9 activity in the remnant liver of MMP-9(-/-) mice compared with that in WT and TIMP-1(-/-) mice (Figure 6C-E). These results support our other findings that MMP-9 contributes to the development of parenchymal hemorrhage and necrosis in the remnant liver after 80%-PH.

MMP-9 deletion inhibits hepatic infiltration of neutrophils
To evaluate the effect of MMP-9 deficiency on hepatic neutrophil infiltration, we performed double immunofluorescent analysis of MMP-9 and myeloperoxidase,

Figure 3 Immunohistochemical staining for matrix metalloproteinase-9 in the remnant liver. A: Representative images of matrix metalloproteinase (MMP)-9 staining in a non-necrotic area in group Ⅱ; B: Representative images of MMP-9 staining in a necrotic area in group Ⅱ; C: Histogram of MMP-9 expression as described in the material and methods section: Enhanced MMP-9 expression was observed in areas close to the focus of necrosis ([a]$P < 0.05$).

as previously described[47]. As shown in Figure 7A-C, we identified myeloperoxidase-positive neutrophils by their characteristic staining pattern in cytoplasmic azurophilic granules[48]. There were significantly fewer infiltrated neutrophils in MMP-9(-/-) mice than in WT and TIMP-1(-/-) mice (3.3 ± 0.4 *vs* 7.0 ± 1.1 *vs* 6.0 ± 0.8 per hpf, $P < 0.05$) (Figure 7D). These results suggest that MMP-9 deletion inhibits neutrophil infiltration of the remnant liver after massive hepatectomy.

Inhibition of MMP-9 with MMP-9 mAb or GM6001 ameliorates liver hemorrhage and necrosis
We performed an MMP-9 inhibition experiment with a

Figure 4 Co-localization analysis by dual immunofluorescence in the remnant liver after 80%-partial hepatectomy. Co-localization analysis by dual immu-nofluorescence in the remnant liver for (A) matrix metalloproteinase (MMP)-9 labeled in red (Alexa Fluor 568), (B) CD68 labeled in green (Alexa Fluor 488), and (C) both MMP-9 and CD68; for (D) MMP-9 labeled in red (Alexa Fluor 568), (E) desmin labeled in green (Alexa Fluor 488), and (F) both MMP-9 and desmin; and for (G) MMP-9 labeled in red (Alexa Fluor 568), (H) CD11b labeled in green (Alexa Fluor 488), and (I) both MMP-9 and CD11b. These co-localization results demonstrate that MMP-9 protein expression is mainly localized in CD11b-positive cells, and to a lesser extent in desmin-positive cells.

specific MMP-9 monoclonal antibody (MMP-9 mAb) and the broad-spectrum MMP inhibitor GM6001, as previously described[40]. There were significantly smaller and fewer necrotic areas in the remnant liver in mice treated with MMP-9 mAb than in control IgG mice (necrotic area: $0.17\% \pm 0.15\%$ *vs* $1.81\% \pm 0.66\%$, $P < 0.05$; number of necrotic foci: 0.23 ± 0.16 *vs* 1.23 ± 0.44, $P < 0.05$) (Figure 8A and B). We showed inhibition of gelatinolytic activity with *in situ* gelatin zymography[40], which demonstrated significantly reduced MMP-9 activity in the MMP-9 mAb-treated group compared with that in the IgG-treated group (Figure 8C and D). Similarly, the number of infiltrated neutrophils was significantly lower in the group treated with MMP-9 mAb than that in the group treated with IgG (4.3 ± 1.1 *vs* 9.1 ± 1.9 per hpf, $P < 0.05$) (Figure 8E-G).

We observed similar results with GM6001. Treatment with GM6001 showed significant suppression of liver necrosis compared with the DMSO vehicle (necrotic area: $0.19\% \pm 0.13\%$ *vs* $1.04\% \pm 0.36\%$, $P < 0.05$; number

of necrotic foci: 0.35 ± 0.19 *vs* 1.12 ± 0.31, $P < 0.05$). Neutrophil infiltration was significantly suppressed in the GM6001 group compared with that in the DMSO group (5.5 ± 1.0 *vs* 9.2 ± 1.3 per hpf, $P < 0.05$). In situ zymographic analysis showed a significant reduction in gelatinolytic activity in the GM6001 group compared with that in the DMSO group (data not shown). Collectively, these results demonstrate that MMP-9 inhibition plays an important role in protecting mice from developing parenchymal hemorrhage and necrosis after 80%-PH.

DISCUSSION

IHR after extended liver resection or a SFSG after SOLT is prone to develop parenchymal hemorrhage and necrosis. Uncontrolled progression of the foci of hemorrhage and necrosis leads to liver failure. The role of MMP-9 in liver injury in the hepatic remnant has not been investigated. In this study, we investigated the role of MMP-9 in the development of hemorrhage and necrosis in IHR

Figure 5 Histological analysis in each strain. Histological analysis between wild-type (WT, *n* = 15) (A), matrix metalloproteinase (MMP)-9(-/-) (*n* = 15) (B), and tissue inhibitors of metalloproteinases (TIMP)-1 (-/-) mice (*n* = 14) (C) at 6 h after 80%-partial hepatectomy (PH). Representative images of remnant liver histology at 6 h after 80%-PH in WT, MMP-9(-/-), and TIMP-1(-/-) mice. Foci of hemorrhage and necrosis are denoted by white arrows. The percentage of the necrotic area (D) and the number of necrotic foci per mm^2 (E) in remnant livers of study mice are shown. Significantly smaller and fewer necrotic foci in the remnant liver were observed in MMP-9(-/-) mice compared with WT and TIMP-1(-/-) mice ($^a P < 0.05$, $^b P < 0.01$).

Figure 6 Expression and activity of matrix metalloproteinase-9. Expression and activity of matrix metalloproteinase (MMP)-9 were determined using (A) western blotting analysis for MMP-9 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control, (B) polyacrylamide gel electrophoresis gelatin zymography with human MMP-9 and -2 standards (S), and *in situ* fluorescence gelatin zymography in the remnant livers of wild-type (WT), MMP-9(-/-), and tissue inhibitors of metalloproteinases (TIMP)-1(-/-) mice at 6 h after 80%-partial hepatectomy. We observed MMP-9 protein expression and activity in WT mice (C), but they were absent in MMP-9(-/-) mice (D). Enhancement of MMP-9 protein expression and activity in TIMP-1(-/-) mice was observed (E).

Figure 7 Immunohistochemical analysis of accumulated neutrophils with myeloperoxidase staining in remnant livers of wild-type, matrix metalloproteinase-9(-/-), and tissue inhibitors of metalloproteinases-1(-/-) mice at 6 h after 80%-partial hepatectomy. Representative images of myeloperoxidase staining, labeled in green (Alexa Fluor 488), of cytoplasmic azurophilic granules in neutrophils (arrows) in wild-type (WT) mice in remnant liver sections in (A) WT, (B) matrix metalloproteinase (MMP)-9(-/-), and (C) tissue inhibitors of metalloproteinases (TIMP)-1(-/-) mice. A histogram shows the number of accumulated neutrophils in the remnant liver (D). Significantly fewer neutrophils were observed in the remnant liver in MMP-9(-/-) mice than in WT and TIMP-1(-/-) mice ($^aP < 0.05$, $^bP < 0.01$).

after 80%-PH in mice. We demonstrated that in IHR 6 h postoperatively, MMP-9 was upregulated in infiltrating neutrophils associated with the foci of hemorrhage and necrosis. Blocking MMP-9 expression by using a synthetic inhibitor, a specific monoclonal antibody, or gene deletion significantly ameliorated the formation of parenchymal hemorrhage and necrosis.

In the current study, we found that the number and percentage of necrotic foci were decreased in MMP-9(-/-) mice compared with WT mice. While TIMP-1(-/-) mice appeared to show slightly increased necrosis, there were no statistical differences between WT and TIMP-1(-/-) mice. TIMP-1 is a physiological tissue inhibitor of MMP-9[36,37], and therefore, MMP-9 activity is increased under the absence of TIMP-1[36,37]. Our results demonstrated that a decrease or absence of MMP-9 prevented liver injury, and that a physiological inhibitor of MMP-9, i.e., TIMP-1, caused similar liver damage in WT mice with IHR after 80%-PH. One possible explanation for the similar damage between WT and TIMP-1(-/-) mice is that liver injury after 80%-PH is very severe in these mice.

In our study of IHR after 80%-PH in mice, we found that infiltrating neutrophils carry MMP-9, which appears to be responsible for the development of hemorrhage and necrosis. This is consistent with previous reports[14,28,35]. Wielockx and colleagues showed that MMPs, including MMP-9, play a central role in the destruction of sinusoidal integrity, resulting in hemorrhage and necrosis[28]. They speculated that MMPs could be derived from infiltrating neutrophils, resident hepatic stellate cells, and Kupffer cells. Recently, hepatic stellate cells have been shown to play a critical role in acute liver failure induced by lipopolysaccharide and beta-galactosamine or carbon tetrachloride by releasing MMP-9, which disrupts sinusoid integrity leading to sinusoidal collapse and liver failure[29]. Similarly, in hepatic warm ischemia-reperfusion injury, hepatic MMP-9 and MMP-2 induce sinusoidal injury in a manner that is independent of neutrophil cytotoxicity[35]. Together, these data suggest that decreasing MMP-9 expression is useful to reduce early sinusoidal injury in IHR and SFSGs.

Inhibition of MMP-9 appears to be a potential therapeutic option in ischemic liver injury. In a previous study in rats, 70% of the liver was rendered ischemic for 90 minutes followed by reperfusion; a phosphinic MMP inhibitor attenuated the liver injury and necrosis[49]. Gene deletion of MMP-9 mitigates liver injury after 90 minutes of warm ischemia and reperfusion in mice[34,35]. Inhibition of MMP-9 has been shown to protect rat livers from cold preservation-warm reperfusion injury[34,35]. Interestingly, after cold ischemic preservation and warm reperfusion, it was found that MMP-9 and MMP-2 were not of hepatocellular origin but from an extrahepatic source[34,35]. Therefore, collectively, these data support MMP-9 reduction as a potential pathway to improve parenchymal integrity and function in IHR after extended liver resection or a SFSG after SOLT.

Figure 8 Effect of matrix metalloproteinase-9 monoclonal antibody on 80%-partial hepatectomy. A, B: Treatment with matrix metalloproteinase-9 monoclonal antibody (MMP-9 mAb) in mice with 80%-partial hepatectomy (PH) resulted in a significant reduction in the size (percent area) and number of hemorrhagic and necrotic foci 6 h after 80%-PH compared with treatment using control IgG ([a]$P < 0.05$); C, D: Suppression of MMP-9 activity as determined by *in situ* fluorescence gelatin zymography compared with control IgG-treated mice; E, F: There was significantly less myeloperoxidase staining (arrows) in remnant liver sections in mice treated with MMP-9 mAb compared with that in control IgG-treated mice; G: A histogram shows the number of accumulated neutrophils in the remnant liver 6 h after 80%-PH. Significantly fewer neutrophils were observed in MMP-9 mAb-treated mice than in control IgG-treated animals ([a]$P < 0.05$).

Although MMP-9 can cause liver injury in IHR after extended liver resection or SFSGs after SOLT, MMP-9 is also essential for liver regeneration[50]. It has been shown that MMP-9 releases hepatic growth factor from the hepatic extracellular matrix during the priming phase of liver regeneration[51,52]. Together with its physiological tissue inhibitor, TIMP-1, MMP-9 participates in regulating the hepatocellular cell cycle and proliferation[51]. MMP-9 is temporally correlated to angiogenesis and regeneration in the liver. Therefore, prolonged inhibition of MMP-9 might be clinically undesirable. Recent studies have suggested that inhibition of MMP-9 to ameliorate the early formation of parenchymal hemorrhage and necrosis should probably be started within and be limited to the first 2 d after extended liver resection or SOLT with SFSGs[50,52]. However, the use of MMP-9 inhibition in the clinical management of IHR or SFSGs requires further investigation and understanding to optimize its therapeutic effectiveness.

After extended liver resection or SOLT with SFSGs, portal hyperperfusion is likely to occur. Although increased portal hypertension is important in liver regeneration, uncontrolled acute portal hypertension with portal hyperperfusion can be detrimental[21,25,53]. In SOLT with SFSGs, portal hypertension and hyperperfusion are known to contribute to SFSL failure[25,54,55]. In experimental models[25,53,56] and in clinical LT with SFSGs[8,9,25,54,55], decompression of portal hyperperfusion has been shown to significantly attenuate hepatic hemorrhage and necrosis[57,58]. Evidence suggests that surgical and anatomical reduction of portal hyperperfusion is important in the management of IHR and SFSGs.

The molecular mechanisms by which portal hyperperfusion affects the outcome of IHR after extended hepatectomy and SFSGs after SOLT are poorly understood. Shear stress has been shown to induce nitric oxide production in microvascular endothelial cells[59], which might upregulate MMP-9 synthesis and production[60]. Reduction of portal hyperperfusion could thus indirectly mitigate the effect of MMP-9 production. However, it is not known how portal hyperperfusion relates to MMP-9 with respect to sinusoidal injury leading to parenchymal hemorrhage and necrosis. Other potential important mediators in the injury of IHR or SFSGs include oxidative stress[61] and tumor necrosis factor[62]. Understanding the role of MMP-9 and other signaling pathways will increase our ability to develop therapeutic options to heal and prevent liver injury associated with IHR and SFSGs.

Neutrophils have been consistently implicated in ischemia-reperfusion injury[19,35]. Neutrophils might produce and release MMPs that contribute to initiating sinusoidal injury[14]. Conversely, hepatic MMP-9 might be derived from hepatic stellate cells or sinusoidal cells, including endothelial cells and Kupffer cells[29]. Hepatic-derived MMP-9 might then attract neutrophils[30] into the liver after an injury. Neutrophils could then initiate and/or propagate sinusoidal hemorrhage and progressive necrosis[35]. Therefore, targeting neutrophils for treatment could be important in some diseases and conditions.

The limitations of this study include the following. Although MMP-9 is implicated in the formation of parenchymal hemorrhage and necrosis in IHR after 80%-PH, our study did not address how and where MMP-9 acts on the sinusoidal elements. The cascade of nitric oxide synthetase, which is important in upregulating MMP-9, was not examined[59,60]. Even though we did not observe constitutive MMP-9 in association with parenchymal hemorrhage and necrosis, we could not rule out the possibility that hepatic cells, including sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells[30], produced MMP-9 at a minute level that was adequate to initiate liver injury with subsequent recruitment of circulating neutrophils. The potential role of T cells was not evaluated[63]. The possibility of myeloperoxidase-associated oxidative inactivation of TIMP-1, thus activating MMP-9, was not considered in this study[64]. We consider that the effects of upregulation of MMP-9 on sinusoidal and endothelial cells should be investigated in detail.

Initially, we hypothesized that MMP-9 plays an important role in parenchymal hemorrhage and necrosis in the small remnant liver. We found significantly less hemorrhagic and necrotic lesions in MMP-9(-/-) remnant livers than in WT and TIMP-1(-/-) remnant livers. Similar results were observed with MMP-9 monoclonal antibody or the synthetic inhibitor GM6001. In conclusion, we demonstrated that MMP-9 plays an important role in the development of hepatic hemorrhage and necrosis in the small liver remnant 6 h after extensive partial hepatectomy. Moreover, we showed that infiltrating neutrophils are critical to the process of liver injury. Targeting MMP-9 could provide a clinically useful tool to optimize the function of a small liver volume, i.e., IHR after extended hepatectomy or SFSGs after SOLT.

ACKNOWLEDGMENTS

COMMENTS

Background

In the field of hepatobiliary surgery, liver resection is considered the standard treatment for primary liver tumors and colorectal liver metastases. Major liver resections have been associated with an increased morbidity and mortality compared with more limited resections. In particular, the outcomes are worsened when a chronic underlying liver disease is present. Recent studies have shown that the volume of the remnant liver is correlated with perioperative morbidity and mortality, and an insufficient hepatic remnant (IHR) after extended hepatectomy is a critical issue.

Research frontiers

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent neutral proteases that can degrade the extracellular matrix and basement membrane.

MMPs play significant roles in cellular regulation, cell-cell communication, and tumor progression. MMP-2 and MMP-9 have been implicated in liver injury and remodeling. Knocking out MMP-9 and other MMPs attenuates liver injury associated with interferon treatments. MMP-9 contributes to the pathogenesis of experimental acute liver failure. Specifically, MMP-9 has been implicated in sinusoidal breakdown leading to extravasation of circulating cells and hemorrhage. MMP-9 plays a pivotal role in ischemia-reperfusion injury. When MMP-9 is blocked with a synthetic inhibitor or by MMP-9 gene deletion, it successfully reverses ischemia-reperfusion injury. Collectively, these data show that MMP-9 is involved in sinusoidal injury in liver failure. However, the role of MMP-9 in the pathogenesis of failure in IHR is not well understood.

Innovations and breakthroughs

Initially, the authors hypothesized that MMP-9 plays an important role in parenchymal hemorrhage and necrosis in the small remnant liver. In this study, the authors found significantly less hemorrhagic and necrotic lesions in MMP-9(-/-) remnant livers than in wild-type and tissue inhibitors of metalloproteinases (TIMP)-1(-/-) remnant livers. Similar results were observed with a MMP-9 monoclonal antibody or synthetic inhibitor GM6001.

Applications

The authors demonstrated that MMP-9 plays an important role in the development of hepatic hemorrhage and necrosis in the small remnant liver 6 h after extensive partial hepatectomy. Moreover, the authors showed that infiltrating neutrophils are critical to the process of liver injury.

Terminology

Targeting MMP-9 could provide a clinically useful tool to optimize the function of a small liver volume, i.e., IHR after extended hepatectomy.

Peer review

Using MMP-9(-/-), wild type, and TIMP-1(-/-) mice, the authors demonstrated that in IHR at 6 h postoperatively MMP-9 is upregulated in the infiltrating neutrophils associated with the foci of hemorrhage and necrosis. Blocking MMP-9 expression by using a synthetic inhibitor, specific monoclonal antibodies, or gene deletion significantly ameliorated the formation of parenchymal hemorrhage and necrosis. The authors finally proposed that MMP is tightly associated with liver parenchymal hemorrhage and necrosis for insufficient liver remnant, which always leads a lethal outcome clinically.

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Anti-tumor effect of 5-aza-2'-deoxycytidine by inhibiting telomerase activity in hepatocellular carcinoma cells

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Abstract

AIM: To investigate the effect of the demethylating reagent 5-aza-2'-deoxycitidine (DAC) on telomerase activity in hepatocellular carcinoma (HCC) cell lines, SMMC-7721 and HepG2.

METHODS: The related gene expression in cell lines was examined by real-time reverse transcription-polymerase chain reaction and Western blotting analysis. The telomerase activity was examined by telomeric repeat amplification protocol-enzyme-linked immunosorbent assay and DNA methylation was determined by methylation-specific polymerase chain reaction.

RESULTS: The telomerase activity was significantly reduced in both cell lines treated with DAC, accompanied by downregulation of telomerase reverse transcriptase (hTERT). We also observed the effect of DAC on the methylation status of hTERT promoter and the expression of regulatory genes, such as c-myc, p15, p16, p21, E2F1, and WT1. The methylation status of hTERT promoter could be reversed in SMMC-7721 by DAC, but not in HepG2 cells. However, p16 expression could be reactivated by demethylation of its promoter, and c-Myc expression was repressed in both cell lines. Moreover, DAC could enhance the sensitivity to the chemotherapeutic agents, such as cisplatin, by induction of apoptosis of HCC cells.

CONCLUSION: The DAC exerts its anti-tumor effects in HCC cells by inhibiting the telomerase activity.

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Key words: 5-aza-2'-deoxycitidine; Telomerase; Hepatocellular carcinoma; DNA methylation

INTRODUCTION

The demethylating reagent 5-aza-2'-deoxycitidine (DAC) inhibits DNA methyltransferases and reverses DNA me-

thylation[1]. It has been found that DAC can inhibit cancer cell growth, particularly the leukemia cells, and it has been applied for the treatment of myelodysplastic syndromes. A phase 1 study was finished using DAC as an antineoplastic drug for hematopoietic malignancies[2,3]. However, the mechanisms underlying its anticancer activity and other biological effects are not fully understood. It is believed to reactivate genes, including the tumor suppressor genes p16, E-cadherin, and hMLH1 in cancer cells. Reactivation of these genes is associated with cell cycle arrest and apoptosis, which leads to inhibition of tumor cell growth[4]. However, the mechanisms of telomerase activity and telomerase reverse transcriptase (hTERT) down-regulated by DAC remain unclear. Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA sequences and almost universally provides the molecular basis for tumor cell proliferative capacity[5]. Telomerase reactivation is a critical step in cellular immortality and carcinogenesis[6,7]. The enzyme consists of three major components: hTERT, telomerase associated protein (TEP1), and telomerase RNA (TERC)[8]. Transcriptional regulation of hTERT is believed to be the major mechanism of telomerase regulation in human cells. Transient transfection experiments with hTERT promoter-luciferase reporters showed that the hTERT promoter is inactive in normal and transformed preimmortal cells but, like telomerase, is activated in immortal cells[9]. Expression of hTERT was observed at high levels in malignant tumors and cancer cell lines, but not in normal tissues or telomerase-negative cell lines, and a strong correlation has been found between hTERT expression and telomerase activity in a variety of tumors[10,11]. These findings suggest that expression of hTERT might be a critical event in carcinogenesis. Thus, the mechanisms of hTERT activation are essential for understanding the molecular basis of telomerase activation and carcinogenesis. The hTERT promoter contains binding sites for many transcription factors that could be involved in its regulation. The abundance of these potential transcription factor binding sites was subjected to various factors in different cellular contexts[12,13]. Several transcription factors are known to participate in hTERT gene expression, including positive regulators: c-Myc, Sp1, human papillomavirus 16 E6, and steroid hormones; and negative regulators: Mad1, p53, p15, p16, p21, E2F, pRB, WT1, interferon-α, tumor growth factor-β and myeloid cell-specific zinc finger protein[14,15]. Our previous studies revealed that methylation status of P21, P15, P16, WTI and E2F-1 was significantly associated with cancer tissues in hepatocellular carcinoma (HCC)[16]. In the present study, we observed the effect of DAC on telomerase activity and hTERT expression in HCC cell lines, and found that DAC down-regulated the telomerase activity and hTERT expression by p16 promoter demethylation.

MATERIALS AND METHODS

Cell lines and cell culture

Human hepatoma cell lines SMMC-7721 and HepG2 were maintained in Dulbecco's modified Eagle's medium (high glucose) (Gibco, Invitrogen, United States) and supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, United States), 100 units/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator under 95% air and 5% CO_2 at 37 ℃. Cells from exponentially growing cultures were used in all the experiments. This study was approved by the Institutional Ethics Committee of the Second Military Medical University, China.

DAC

Human hepatoma cell lines (SMMC-7721 and HepG2) were seeded at a density of 5×10^5 cells per well in 6-well tissue culture plates and were allowed to attach over a 24-h period. The demethylating reagent DAC (Merk, Calbiochem, United States) was added to a final concentration of 1 μmol/L, 2 μmol/L and 4 μmol/L and the cells grew for 1 d, 3 d and 5 d. At indicated time intervals, cells were harvested by trypsinization and washed with phosphate-buffered saline.

Assay of telomerase activity by telomeric repeat amplification protocol-enzyme-linked immunosorbent assay

Telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA) was performed using the telomerase kit Telo TAGGG Telomerase PCR ELISA PLUS (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Extracts from HEK293 cells were used as positive controls for the assay, and cell lysates heat-inactivated for 10 min at 85 ℃ were used as negative controls. Absorbance values were reported as the $A_{450 nm}$ reading.

Real-time reverse transcription-polymerase chain reaction

RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA, United States). cDNA was synthesized using 2 μg total RNA and a moloney murine leukemia virus-reverse transcriptase kit with random hexamer primers and an RNase inhibitor (Takara Biotechnology Co. Ltd., Dalian, China). Polymerase chain reaction (PCR) amplifications of the respective genes were carried out with 40 ng complementary DNA, 500 nmol/L forward and reverse primer, and iTaqSYBRGreen Supermix (Bio-Rad Laboratories, Hercules, CA) in a final volume of 20 μL. Primer sequences and annealing temperature are summarized in Table 1. Real-time RT-PCR was performed on a Mastercycler cycler (Eppendorf, Hamburg, Germany), and all experiments were performed twice. Amplification of the housekeeping gene β-actin was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed by the -ΔΔct method.

Methylation-specific polymerase chain reaction

The bisulfite modification of DNA was done as described previously[17]. DNA methylation was determined

Table 1 Primer sequences for reverse transcription-polymerase chain reaction and methylation-specific polymerase chain reaction analysis

Gene	Primer sequence (5'-3')	Annealing (℃)	Product size (bp)
hTERT-F	CGGAAGAGTGTCTGGAGCAA	58	422
hTERT-R	GGATGAAGCGGAGTCTGGA		
c-myc-F	CTTCTCTCCGTCCTCGGATTCT	65	132
c-myc-R	GAAGGTGATCCAGACTCTGACCTT		
p15-F	GAATGCGCGAGGAGAACAAG	65	204
p15-R	CCATCATCATGACCTGGATCG		
p16-F	GCTGCCCACGCACCGAATA	57	179
p16-R	ACCACCAGCGTGTCCAGGAA		
p21-F	GCAGACCAGCATGACAGATTT	60	70
p21-R	GGATTAGGGCTTCCTCTTGGA		
WT1-F	GGCATCTGAGACCAGTGAGAA	62	120
WT1-R	GAGAGTCAGACTTGAAAGCAGT		
E2F1-F	AGCTGGACCACCTGATGAAT	60	95
E2F1-R	GTCCTGACACGTCACGTAGG		
β-actin-F	CCTGTACGCCAACACAGTGC	60	275
β-actin-R	ATACTCCTGCTTGCTGATCC		
hTERT-MF	AGTTTTGGTTTCGGTTATTTTCGC	58	122
hTERT-MR	AACGTAACCAACGACAACACC		
hTERT-UF	AGTTTTGGTTTTGGTTATTTTTGT	58	132
hTERT-UR	AACGTAACCAACGACAACACCT		
p16-MF	TTATTAGAGGGTGGGGCGGATCGC	56	150
p16-MR	GACCCCGAACCGCGACCGTAA		
p16-UF	TTATTAGAGGGTGGGGTGGATTGT	56	151
p16-UR	CAACCCCAAACCACAACCATAA		

by methylation-specific PCR (MSP). Forty ng of bisulfite-modified DNA was subjected to PCR amplification. The PCR reaction mixture contained 2.5 µL of 10 × PCR buffer, 100 pmol of each primer, 2 mmol/L of each dNTPs, and 1 U of Hotstart Taq DNA polymerase (Takara Biotechnology Co. Ltd., Dalian, China) at a final volume of 25 µL. The PCR was performed in a thermal cycler. Primer sequences and reaction conditions are summarized in Table 1. DNA methylated by SssI methylase (Sss DNA) was used as positive control for methylated alleles.

Western blotting analysis

HepG2 and SMMC-7721 cells were lysed in RIPA lysis buffer (Beyotime, China) with 1 mmol/L phenylmethanesulfonyl fluoride (PMSF). An equal amount of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membrane. After blocking with 5% nonfat milk, the membrane was probed with anti-hTERT (Santa Cruz, United States), developed with the BeyoECL Plus substrate system (Beyotime, China). Blots were stripped and re-probed with β-actin antibody (Santa Cruz, United States) to confirm equal protein loading.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay and cell apoptosis analysis

The SMMC-7721 and HepG2 cells were seeded in 96-well plates and cultured with chemotherapeutic drugs and DAC for 3 d. The cells were examined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-

mide (MTT) (5 mg/mL, Sigma) assay. The spectrophotometric absorbance was measured using a plate reader at 570 nm. The morphology of apoptosis was observed by 4',6-diamidino-2-phenylindole (DAPI) staining. The cells were analyzed using a Facial Action Coding System (FACS) Aria flow cytometer (Becton Dickinson, San Jose, CA).

Statistical analysis

All of the experiments were repeated at least three times. The data were expressed as means ± SD. Statistical analysis was performed using Student's *t* test (two tailed). $P < 0.05$ was considered statistically significant.

RESULTS

Telomerase activity in HCC cells with DAC

To investigate the effects of DAC on telomerase activity, SMMC-7721 and HepG2 were cultured with 1 µmol/L, 2 µmol/L and 4 µmol/L DAC. Telomerase activity was measured by TRAP-PCR-ELISA assay after 1 d, 3 d and 5 d of exposure to DAC. Inhibition of telomerase activity was observed in both cell lines in a dose-dependent manner, by maximal repression on day 3 at 4 µmol/L or day 5 at 2 µmol/L DAC (Figure 1). There was a 52.7% reduction of telomerase activity in SMMC-7721 cells treated with 4 µmol/L DAC for 3 d, and a 45.6% reduction of telomerase activity in HepG2 cells. The results revealed that the effect of DAC on telomerase activity varied in different cell lines.

Effect of DAC on telomerase reverse transcriptase expression in HCC cells

Since the expression of hTERT is closely associated with telomerase activity, we examined whether hTERT expression is suppressed in SMMC-7721 and HepG2 cells by DAC. The expression of hTERT mRNA in SMMC-7721 cells was decreased to 82% on day 1, 34% on day 3, and 26% on day 5 after DAC treatment (2 µmol/L) (Figure 2). A decline in hTERT mRNA was also detected in HepG2 cells treated with DAC. hTERT mRNA expression was maximally down-regulated by 4 µmol/L DAC. The complete down-regulation of hTERT mRNA became apparent on day 3 of treatment and maximal on day 5 in both cell lines. Furthermore, we treated SMMC-7721 and HepG2 cells with 1 µmol/L, 2 µmol/L, 4 µmol/L DAC respectively for 3 d and 2 µmol/L for 1 d, 3 d, 5 d, respectively, then detected the hTERT expression in protein level by Western blotting analysis (Figure 3). The hTERT protein in SMMC-7721 and HepG2 cells was also down-regulated by DAC in a dose- and time-dependent manner, with maximal repression at 4 µmol/L on day 5. The hTERT protein was notably suppressed in both HepG2 and SMMC-7721 cells after treated by 2 µmol/L DAC for 3 d; however, the effect was more significant in SMMC-7721 cells. These results were in accordance with hTERT mRNA expression. The results indicated that inhibition of telomerase activity in HCC cells treated with DAC may contribute to a striking decrease in hTERT

Figure 1 Effect of 5-aza-2'-deoxycitidine on telomerase activity in human hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B). Cells were incubated with DAC (1 μmol/L, 2 μmol/L or 4 μmol/L). Cell pellets were collected and subjected to telomeric repeat amplification protocol-enzyme-linked immunosorbent assay. [a]$P < 0.05$ (by unpaired Student's *t* test). Neg: Negative control; Pos: Positive control; DAC: 5-aza-2'-deoxycitidine. All studies are representative of at least three independent experiments.

Figure 2 Effect of 5-aza-2'-deoxycitidine on telomerase reverse transcriptase mRNA in hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B). Cells were incubated with DAC (1 μmol/L, 2 μmol/L or 4 μmol/L). Cell pellets were collected and subjected to real-time reverse transcription-polymerase chain reaction assay. PC: Positive control; DAC: 5-aza-2'-deoxycitidine; hTERT: Human telomerase reverse transcriptase.

Figure 3 Expression of telomerase reverse transcriptase protein in hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B) during exposure to 5-aza-2'-deoxycitidine (1 μmol/L, 2 μmol/L or 4 μmol/L). Total proteins were extracted, and Western blotting analysis was performed. The same blot was reprobed for β-actin as a loading control; hTERT: Human telomerase reverse transcriptase.

Figure 4 Methylation of telomerase reverse transcriptase promoter in 5-aza-2'-deoxycitidine-treated hepatocellular carcinoma cell lines SMMC-7721 (A) and HepG2 (B). M: Methylation; U: Unmethylation; PC: Positive control; DAC: 5-aza-2'-deoxycitidine.

mRNA and protein.

Methylation of telomerase reverse transcriptase promoter in HCC cells by DAC

Since promoter methylation may be involved in hTERT repression in HCC cells, we observed the effects of DAC on promoter methylation of hTERT gene using MSP[18]. According to MSP analysis, the hTERT promoter was found to be hypermethylated in SMMC-7721, but not in HepG2 cells (Figure 4). The demethylation of hTERT was found in SMMC-7721 cells treated with DAC in a dose- and time-dependent manner, and there was complete demethylation after treatment with 5 μmol/L DAC for 3 d or 2 μmol/L for 3 d (Figure 4A). However, DAC showed no effects on hTERT methylation in HepG2 cells (Figure 4B). These data suggested that the demethylation of hTERT promoter by DAC could not play an important role in down-regulation of hTERT expression.

Expression of critical regulatory genes of telomerase reverse transcriptase transcription by DAC

We focused on some regulatory genes of hTERT transcription, such as c-myc, p15, p16, p21, E2F-1, WT1. Using real-time PCR, we examined the mRNA expression of these genes during exposure to DAC at 2 μmol/L for 1 d, 3 d and 5 d. The data showed that c-myc had high expression while p16 had low expression (SMMC-7721) or lost expression (HepG2) in hepatoma cells, and the other genes showed different levels of expression (Figure 5). The down-regulation

of c-myc and up-regulation of p16 mRNA expression were found in HCC cell lines treated with DAC. Western blotting analyses further revealed the significant levels of c-myc and p16 in both SMMC-7721 and HepG2 cells. These results suggested that c-myc and p16 could play an important role in down-regulating the hTERT expression by DAC.

Methylation of p16 promoter and reactivation of its expression by DAC

We reproduced the methylation pattern of p16 (Figure 6) using MSP. With DAC treatment, we detected increased demethylation of p16 promoter. The data suggested that promoter hypermethylation could causatively contribute to transcriptional silencing of p16, which up-regulated the hTERT transcription in HCC cells.

Human hepatoma cells sensitive to chemotherapeutic agents after DAC treatment

To determine whether DAC could effectively inhibit HCC cell growth, we treated SMMC-7721 and HepG2 cells with various doses of DAC for 72 h. We found that DAC inhibited the growth of cell lines in a dose-dependent manner (Figure 7). To determine whether the growth inhibition by DAC can be enhanced by chemotherapeutic agents, SMMC-7721 and HepG2 cells were treated with DAC in combination with cisplatin. Notably, while dramatic morphological changes were caused by combination of DAC with cisplatin, many cells revealed detachment and shrinkage. The growth inhibition was about 76% in

Figure 5 Effect of 5-aza-2'-deoxycitidine on expression of critical regulatory genes of telomerase reverse transcriptase transcription in hepatocellular carcinoma cell lines. A, B: Cells were incubated with 2 μmol/L DAC for 3-5 d, and subjected to real-time reverse transcription-polymerase chain reaction assay, [a]*P* < 0.05 (by unpaired Student's *t* test); C: Western blotting analysis of c-myc and p16 in hepatocellular carcinoma cell lines by DAC. The same blot was reprobed for β-actin as a loading control. DAC: 5-aza-2'-deoxycitidine.

the cells treated with DAC in combination with cisplatin (20 μmol/L) as compared with 54% in the cells treated with cisplatin alone, or 48% treated with DAC alone. These data suggested that DAC could enhance the sensitivity of HCC cells to chemotherapeutic agents such as cisplatin. With the administration of cisplatin and DAC, more cells revealed nuclear condensation and fragmentation of apoptotic cell death. These results were confirmed by Annexin-V and propidium iodide staining and FACS analysis (Figure 8). The cell apoptosis was significantly enhanced by the combined administration of 2 μmol/L DAC and 20 μmol/L cisplatin. These results suggested

that the enhanced effects of the combined treatment on cell death were attributed to the augmented induction of apoptosis.

DISCUSSION

The most widely used demethylating agent, DAC, was first characterized 30 years ago and it functions as a mechanism-dependent suicide inhibitor of DNA methyltransferases, with which genes silenced by hypermethylation can be reactivated[19]. It has been found that telomerase activity in cancer cells was inhibited by differ-

Figure 6 Demethylation of p16 promoter region and reactivation of p16 expression after 5-aza-2'-deoxycitidine treatment. Cells were incubated with 2 μmol/L DAC for 3-5 d and subjected to methylation-specific polymerase chain reaction (MSP) and real-time reverse transcription-polymerase chain reaction assay, respectively. M: Methylation; U: Unmethylation; Pos: Positive control. [a]$P < 0.05$ (by unpaired Student's *t* test). DAC: 5-aza-2'-deoxycitidine.

Figure 7 Sensitivity of SMMC-7721 and HepG2 cells to cisplatin enhanced by 5-aza-2'-deoxycitidine. A: Effects of DAC on growth inhibition. The hepatocellular carcinoma cell lines SMMC-7721 and HepG2 were treated with various doses of DAC for 72 h. Cell viability was determined by cell proliferation assay kit; B: The morphology of the cells was captured under a light microscope; C, D: SMMC-7721 (C) and HepG2 cells (D) cells were treated with 2 μmol/L DAC, 20 μmol/L cisplatin, or in combination for 72 h. Cell viability was determined by MTT assays. DAC: 5-aza-2'-deoxycitidine; Con: Control; CDDP: Cisplatin.

entiation, inducing demethylating agent DAC. We demonstrated that telomerase activity could be inhibited by DAC in HCC cells (HepG2 and SMMC-7721), and the demethylation of p16 promoter could play an important role.

Telomerase reactivation is a critical step in cellular immortality and carcinogenesis and is considered as a target for cancer treatment[8]. Our lab revealed that more than 85% HCC had much stronger telomerase activity than cirrhosis[16]. Drug-induced cell killing of tumor cells is as-

sociated with a decline in detectable telomerase activity[20]. In the present study, DAC inhibited telomerase activity and down-regulated hTERT expression in both human hepatoma cell lines SMMC-7721 and HepG2, and DAC suppressed the transcriptional activity of hTERT genes. Targeting telomerase activity is one of the mechanisms responsible for this reagent's inhibition of cancer cell growth.

On one hand, promoter methylation was associated with transcriptional silencing of the hTERT gene, as treat-

Figure 8 Apoptosis induced by 5-aza-2'-deoxycitidine. SMMC-7721 and HepG2 cells were treated with 2 μmol/L DAC, 20 μmol/L cisplatin, or in combination for 72 h. A: The fluorescent microscopic pictures of apoptotic cells were captured by 4',6-diamidino-2-phenylindole staining of the condensed and fragmented nuclei; B, C: Annexin V-fluorescein isothiocyanate (FITC) staining following facial action coding system analysis was shown for SMMC-7721 (B) or HepG2 cells (C). DAC: 5-aza-2'-deoxycitidine; Con: Control; CDDP: Cisplatin.

ment of cells with demethylating agent DAC resulted in an increase in hTERT transcription in an immortal fibroblast line SUSM-1[21]. On the other hand, DNA hypermethylation was implicated in the positive regulation of the hTERT promoter because demethylation in several telomerase-positive tumor cell lines reduced hTERT expression and telomerase activity accompanied by telomere shortening[22,23]. Our present study showed that hTERT promoter was methylated in SMMC-7721 cells, but not in HepG2 cells, and almost complete demethylation of hTERT promoter only occurred in the former cell line after DAC treatment. The explanation that CpG methylation likely interfered with the binding of transcriptional repressors, thereby positively regulating the hTERT promoter, may be reasonable for SMMC-7721 but not for HepG2. These findings suggested that hTERT promoter methylation is not the sole regulator of hTERT gene expression in HCC cells treated with DAC.

Several transcription factors are known to be responsible for the regulation of hTERT expression, including c-myc, p21, p16, p15, E2F-1 and Wilms' Tumor 1 suppressor gene[24]. This evidence prompted us to examine the effect of DAC on the expression of these genes. The c-myc was over-expressed while p16 expression was low or lost in hepatoma cell lines, and DAC repressed c-myc expression while reactivating p16. c-myc plays a critical role in telomerase activation through up-regulating the hTERT transcription, and this could be one mechanism by which 5-aza-CR represses hTERT transcription. Inactivation of p16-dependent pathways possibly in conjunction with telomerase activation might be a critical step for immortalization[25]. Our findings indicated that up-regulation of p16 and subsequent down-regulation of c-myc could be major pathways for hTERT repression by DAC.

Several evidences indicated that p16 expression could be transcriptionally silenced by CpG island hypermethylation in HCC[26]. The absence of expression and promoter methylation of p16 suggested that aberrant methylation is a major mechanism of the inactivation of p16 expression in HCC. Our data showed that DAC reversed p16 promoter methylation status and reactivated its expression, suggesting that p16 plays an important role in the down-regulation of telomerase activity by DAC.

In conclusion, the demethylating reagent 5-aza-CR represses telomerase activity and down-regulates hTERT expression. p16 could play a key role in this regulation. Our findings may provide insights into one of the mechanisms through which 5-aza-CR exerts growth-inhibitory effects on HCC cells.

COMMENTS

Background
The inactivation of tumor suppressor genes by aberrant DNA methylation plays an important role in the development of malignancies. An inhibitor of DNA methylation, 5-aza-2'-deoxycitidine (DAC), could inhibit telomerase activity in some cancer cell lines, but the molecular mechanism remains unclear.

Research frontiers
DAC can inhibit cancer cell growth, particularly leukemia cells, and it has been

applied for the treatment of myelodysplastic syndromes. Targeting telomerase activity is one of the mechanisms responsible for this reagent's inhibition of cancer cell growth.

Innovations and breakthroughs
The authors demonstrated that the demethylating reagent DAC represses telomerase activity and down-regulates telomerase reverse transcriptase (hTERT) expression. p16 could play a key role in this regulation.

Applications
By understanding the mechanism of DAC repressing telomerase activity, this study may represent a future strategy for the treatment of patients with hepatocellular carcinoma (HCC).

Terminology
Telomerase and hTERT is a critical step in cellular immortality and carcinogenesis and is considered as a target for cancer treatment. And several transcription factors such as c-myc and p16 were responsible for the regulation of hTERT expression. The DNA methylation could play an important role in telomerase activity.

Peer review
This is a nice paper that describes the role of an inhibitor of DNA methylation DAC on telomerase expression and activity in HCC cell lines. Work has been well designed, effects have been proved in more than one HCC cell lines and in general terms, conclusions are supported by the presented results.

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ORIGINAL ARTICLE

Lactobacillus crispatus M206119 exacerbates murine DSS-colitis by interfering with inflammatory responses

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Abstract

AIM: To investigate the role of *Lactobacillus crispatus* (*L. crispatus*) strain China Center for Type Culture Collection (CCTCC) M206119 in intestinal inflammation.

METHODS: Forty 8-wk-old Balb/c mice (20 ± 2 g) were divided into four groups of 10 mice each. Three groups that had received dextran sulfate sodium (DSS) were administered normal saline, sulfasalazine or CCTCC M206119 strain, and the fourth group received none of these. We assessed the severity of colitis using a disease activity index, measured the colon length and weight, collected stools and mesenteric lymph nodes for bacterial microflora analysis. One centimeter of the proximal colon, middle colon and distal colon were collected and fixed in 10% buffered formalin, dehydrated in ethanol, and embedded in paraffin. Interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α expression was detected using reverse transcription polymerase chain reaction. Protective factors zonula occludens (ZO)-1 and β-defensin 2 were detected by immunoblot-

ting. The features of CCTCC M206119 strain were identified based on morphology, biochemical profile, and 16S RNA sequencing.

RESULTS: DSS-colitis animals treated with CCTCC M206119 had markedly more severe disease, with greater weight loss, diarrhea, fecal bleeding, and shortened colon length. In addition, the CCTCC-M206119-treated group had comparatively higher histological scores and more neutrophil infiltration than the controls. Expression of protective factors ZO-1 and β-defensin 2 was downregulated due to destruction of the mucosal barrier after CCTCC M206119 strain treatment. An *in vitro* assay demonstrated that CCTCC M206119 strain increased the nuclear translocation of nuclear factor-κB in epithelial cells. Intestinal proinflammatory or anti-inflammatory cytokine responses were evaluated. Proinflammatory colonic cytokine (IL-1β, IL-6 and TNF-α) levels were clearly increased in CCTCC-M206119-treated animals, whereas anti-inflammatory colonic cytokine (IL-10) level was lowered compared with saline or 5-aminosalicylic-acid-treated DSS-colitis mice. Next, CCTCC M206119 strain was characterized as *L. crispatus* by microscopic morphology, biochemical tests and 16S rRNA gene level.

CONCLUSION: Not all lactobacilli are beneficial for intestinal inflammation, and *L. crispatus* CCTCC M206119 strain is involved in exacerbation of intestinal inflammation in DSS-colitis mice.

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Key words: Colitis; *Lactobacillus crispatus*; Intestine; Dextra sodium sulfate; Mice

INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (UC), are characterized by an abnormal activation of the gut-associated immune system resulting in a chronic inflammation of the digestive tract[1]. It is widely accepted that a combination of genetic factors, immune disorders and environmental factors could be involved in the etiology of IBD[2]. Recent research has shown that some commensal and pathogenic bacteria are closely related to these diseases[3,4].

In acute dextran sulfate sodium (DSS)-induced colitis, bacteria and/or bacterial products play a major role in the initiation of inflammation but not in chronic DSS-colitis[5]. In the neonatal period, *IL-10* gene-deficient mice have decreased levels of colonic *Lactobacillus spp.* and an increase in colonic mucosal adherent and translocated bacteria. Normalizing *Lactobacillus spp.* levels reduces colonic mucosal adherent and translocated bacteria and prevents colitis[6]. Also, it is widely accepted that antibiotic treatment can definitely alleviate the clinical manifestation of UC. Various antibiotics have been shown to exhibit varying abilities to prevent and treat colitis in HLA-B27 rats and IL-$10^{-/-}$ mice[7,8]. Some authors recommended that where there are no bacteria, there is no colitis. Various IBD models in rodents reared under germ-free conditions are free of intestinal inflammation[6]. Increasing evidence supports that luminal microflora, or their products, are probably an important initiating factor in the pathogenesis of IBD[9].

Interestingly, facultative anaerobic Gram-positive bacteria and *Lactobacillus spp.* are decreased in patients with active UC, whereas bifidobacteria are decreased in fecal extracts from patients with Crohn's disease (CD)[10]. Another group has shown that bifidobacteria are reported to be deficient in rectal biopsies from patients with IBD and studies of fecal flora in patients with CD suggest a deficiency in both lactobacilli and bifidobacteria[11]. Furthermore, a marked decrease in intestinal *Lactobacillus spp.* precede the onset of colitis in *IL-10* gene deficient mice, and specific lactobacilli are relevant to disease induction or progression in the IL-10-deficient mouse model of colitis[12]. These studies offer strong evidence to support the view that an imbalance in the intestinal microflora triggers intestinal inflammation.

Microflora considered beneficial to the host includes the genera *Bifidobacterium* and *Lactobacillus*, whereas species potentially pathogenic include Enterobacteriacae and clostridia[4]. Therefore, maintaining or recovering the intestinal microflora or mucosa-associated bacteria in specific compartments has been proposed as a potential therapy to treat IBD[13]. For example, the administration of the VSL#3 probiotic cocktail delays relapse of pouchitis after surgical resection[14,15]. Probiotics have undergone investigation for their capacity to reduce the severity of a number of inflammatory conditions including pouchitis and UC. Also, some natural anti-inflammatory effects have recently been shown for *Lactobacillus salivarius*, *Lactobacillus crispatus* (*L. crispatus*), *Bifidobacterium* and *Lactobacillus plantarum* and *Lactobacillus casei* Shirota based on experimental colitis models[16-18].

Despite the wide usage of probiotics in controlling IBD, their efficacy is still controversial[19,20]. Thus, it is very difficult to draw a definitive conclusion in evaluating the role of microflora in pathogenesis of IBD, and subsequently, the efficacy of controlling IBD. Some published data have also questioned the safety of probiotics. The possibilities include possible adverse effects of probiotics in diseased or debilitated patients, rational bacterial selection, bacterial translocation, and microflora shift among individuals. Some published data have shown that lactobacilli isolated from IL-10-deficient mice failed to decrease tumor necrosis factor (TNF)-α production, whereas six lactobacillus isolates from mice without colitis significantly inhibited TNF-α production. This indicates that multiple strains of lactobacteria may be capable of probiotic activity but, conversely, that not all strains of lactobacteria have *in vitro* immunomodulatory activity[12]. Interestingly, it has been reported that *Lactobacillus rhamnosus* GG exacerbated acute DSS-induced colitis, demonstrated by increased colitic disease activity and histological damage score, however, in a chronic colitis model, a protective effect was observed[21].

In a previous study, we investigated and compared the effect of different *Lactobacillus* and *Bifidobacterium* strains, in a model of DSS-induced colitis[22]. When we screened to find some probiotics that can be potentially therapeutic, we came across a strain *L. crispatus* China Center for Type Culture Collection (CCTCC) M206119 that is capable of aggravating murine DSS-colitis. What was the possible mechanism? Does it mean that probiotics safety should be reconsidered?

In the present study, the promotive effects of strain CCTCC M206119 on the development of the inflammation were analyzed using a DSS-colitis model in BALB/c mice. We compared the effect of CCTCC M206119 administration, saline, and 5-aminosalicylic acid (5-ASA) treatment on DSS-colitis in mice. Thus, we tested *in vivo* and *in vitro* whether and how the CCTCC M206119 strain could aggravate epithelial damage of the colon, and indentified the features of CCTCC M206119 strain based on morphology, biochemical profiles, and 16S RNA sequencing.

MATERIALS AND METHODS

Bacterial strains and growth conditions

L. crispatus strain CCTCC M206119 was isolated from

Table 1 Experimental protocol for induction of dextran sulfate sodium-colitis

Groups	Experimental design
Healthy control	No DSS treatments
Negative control (saline)	5% (w/v) DSS dissolved in drinking water for 7 d
	Saline administered intragastrically once daily for 9 d
Positive control (5-ASA)	5% (w/v) DSS dissolved in drinking water for 7 d
	5-ASA administered intragastrically once daily for 9 d
Investigating group (CCTCC M206119)	5% (w/v) DSS dissolved in drinking water for 7 d
	CCTCC M206119 administered intragastrically once daily for 9 d

DSS: Dextran sulfate sodium; 5-ASA: 5-aminosalicylic acid.

Table 2 Scoring of disease activity index

Score	Weight loss (%)	Stool consistency	Occult/gross fecal bleeding
0	0	Normal	Negative
1	1-5		
2	5-10	Loose stool	Hemoccult
3	10-15		
4	> 15	Diarrhea	Gross bleeding

DAI = (combined score of weight loss, stool consistency, and bleeding)/3. Normal stool, well-formed pellets; Loose stool, pasty and semi-formed stool that does not adhere to the anus; Diarrhea, liquid stool that adheres to the anus.

Table 3 Histological score to quantify the degree of colitis

Score	Inflammation	Depth of lesions	Destruction of crypt	Width of lesions (%)
0	None	None	None	
1	Mild	Submucosa	1/3 basal crypt	1-25
2	Severe	Muscularis	2/3 basal crypt	26-50
3		Sera	Intact epithelium only	51-75
4			Total crypt and epithelium	76-100

human feces in the Division of Digestive Disease of the Second XiangYa Hospital (Changsha, Hunan, China), and kept at China Center for Type Culture Collection (CCTCC, Wuhan, China). *Lactobacillus* strains were grown at 37 ℃ in de Man, Rogosa and Sharpe (MRS) medium (Difco, MD, United States) without shaking.

Animals

Eight-week-old BALB/c mice of either sex were purchased from Hunan Agricultural University [SCXK (Xiang 2002-003)], and then bred under specific pathogen-free conditions. Mice were kept in ventilated and filtered cages, fed an irradiated diet, and housed on irradiated bedding. Food and water were supplied *ad libitum*. All animal experiments were performed in compliance with the guidelines of the Chinese government approved by the College BioResources ethical review board.

Preparation of bacterial strains and administration to mice

Bacterial strains were grown to an optical density at 600 nm of 3 to 4 (early stationary phase) at 37 ℃ in MRS medium anaerobically, harvested by centrifugation, washed with sterile neutral saline, and resuspended at 10^9 colony forming units (CFU)/mL in neutral saline. Three hundred microliters of this daily-prepared suspension was fed daily to the mice *via* intragastric gavage, whereas the controls (both UC-induced and healthy mice) received 300 μL neutral saline *via* the same procedure.

DSS-induced colitis model and experimental design

Eight-week-old Balb/c mice (20 ± 2 g) were divided into a healthy control group, negative control (normal saline) group, positive control (5-ASA, 6 mg/20 g) group, and investigation (CCTCC M206119 strain) group, with 10 mice in each. Normal saline (300 μL), sulfasalazine, CCTCC M206119 strain were administered intragastrically once daily for 2 d before starting DSS and continued for 7 d after DSS induction. Colitis was induced

by 5% (w/v) DSS (molecular weight 35 000-50 000; MP Biomedicals, Solon, OH, United States) dissolved in drinking water for 7 d (Table 1). The healthy control group received no DSS. Severity of colitis was assessed daily using a disease activity index (DAI). After the seventh day of induction of colitis, animals were euthanized by CO_2 asphyxiation. The colon lengths and weight were measured. Under aseptic technique, 300 mg stools and mesenteric lymph nodes (MLNs) were collected for bacterial microflora analysis. All the samples were placed immediately in sterile tubes containing 5 mL of transport medium. One centimeter of the proximal colon, middle colon and distal colon was collected and fixed in 10% buffered formalin, dehydrated in ethanol, and embedded in paraffin. The remaining tissues were immediately snap-frozen and stored at -80 ℃ [23,24].

Assessment of colitis DAI

The severity of colitis was assessed daily using a DAI based on the scoring system of Hamamoto *et al*[25], which scores body weight loss, stool consistency and occult/gross fecal bleeding (Table 2). Occult blood in feces was evaluated by means of Hemoccult II test (Beckman Coulter, Palo Alto, CA, United States).

Histology and histological grading

Tissue fixed with 4% (w/v) paraformaldehyde in phosphate buffered solution (PBS) was prepared for light microscopy, and 5-μm-thick sections were stained with hematoxylin and eosin to study histological changes. Grading of intestinal inflammation was determined as follows (Table 3)[26]. All scores were obtained in a blinded fashion by two independent investigators.

Table 4 Primers used to detect gene expression at mRNA level

Genes		Sequence of primers
GAPDH	Sense	5′-ATCACCATCTTCCAGGAGCG-3′
	Anti-sense	5′-CCTGCTTCACCACCTTCTTG-3′
IL-1β	Sense	5′-TTTTAATCAGCTATCCGGAC-3′
	Anti-sense	5′-TAATGGGAACGTCACACACC-3′
IL-6	Sense	5′-ATGAAGTTCCTCTCTGCAAGAGACT3′
	Antisense	5′-CACTAGGTTTGTTTAATCTC-3′
TNF-α	Sense	5′-ACGTGGAACTGGCAGAAGAG-3′
	Antisense	5′-GGTTGTCTTTGAGATCCATGC-3′

Analysis of colon microflora

Three-hundred-microgram fecal samples were dispersed in 2 mL PBS. Each pooled sample (0.1 mL) was serially diluted *via* 10-fold dilutions (from 10^{-1} to 10^{-10}). Eosin methylene blue agar, KULB agar, TTC azide dextrose agar and mannitol salt agar were used for the enumeration of *Enterobacteriaceae*, *Bacteroides*, *Enterococcus* and *Staphylococcus spp.*, respectively. Standard Nutrient Agar was used for the enumeration of total aerobic bacteria. All the plates were incubated at 37 ℃, aerobically, for 24-48 h and the number of colonies were counted. Brain-heart infusion agar and LAMVAB agar were used to enumerate bifidobacteria and lactobacteria, respectively. Anaerobic incubation was carried out in anaerobic jars (Oxoid, Basingstoke, Hants, United Kingdom) at 37 ℃ for 48-72 h. Anaerobic conditions were obtained using Anaerogen (Oxoid) and were checked using methyl blue strips as oxidation reduction indicator. CFUs were counted and expressed per gram of sample[27,28].

Myeloperoxidase activity

The extent of neutrophil infiltration was determined by myeloperoxidase (MPO) assay[29]. Briefly, 500 mg colon was homogenized in 1 mL iced 0.5% hexadecyltrimethylammonium bromide buffer. The aliquot was centrifuged (4000 *g*, 15 min) and the supernatant was diluted into 5% homogenates. A mixture of 0.1 mL 0.05% hydrogen peroxide and 0.9 mL 5% homogenates was vortexed to release MPO from the tissue. After incubation in 37 ℃ for 15 min, 0.2 mL mixture was added to 3 mL O-dianisidine reaction mixture and incubated for 30 min at 37 ℃. Sodium azide (50 μL, 2.0%) was added and incubated at 60 ℃ for 10 min. Absorbance of the reaction mixture was measured at 460 nm by spectrophotometer. Saline was used as a control. MPO activity was expressed as U/g tissue.

Nuclear translocation of nuclear factor-κB in CCTCC-M206119-stimulated HT-29 cells

HT-29 cells (purchased from Y-Y Chemical Reagent Co. Ltd., Shanghai, China) were cultured in RPMI 1640 medium (Gibco, Rockville, MD, United States) supplemented with 10% fetal calf serum under 5% CO_2 at 37 ℃ and divided into negative control, TNF-α (10 ng/mL) and CCTCC M206119 groups. CCTCC M206119 was diluted

to 10^9 CFU/mL with RPMI 1640 medium. HT-29 (3 × 10^6 cells) were cultured in 24-well tissue culture plates for 72 h and TNF-α or CCTCC M206119 was added. After 4 h of incubation, HT-29 cells were fixed in cold methanol for 5-15 min. The fixed cells were blocked in 5% bovine serum albumin (BSA)/PBS for 1 h on ice. We remove the blocking buffer and added primary antibody anti-nuclear factor (NF)-κB p65 (10 μg/mL) (MBL, Woods Hole, MA, United States; clone 8F11, rat IgG2a) diluted in PBS/0.3% Triton X100/1%BSA/1% serum overnight at 4 ℃ in a humidified chamber. After washing three times for 10 min with PBS on ice, the cells were incubated with anti-rabbit Cy3 (10 μg/mL) (Abcam, Cambridge, United Kingdom; polyclonal rabbit IgG) for 1 h at room temperature. The cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, United States) with 4′,6′-diamidino-2-phenylindole hydrochloride after three washes in PBS at room temperature, and analyzed by a Nikon ECLIPSE E600 fluorescent microscope. The ratio of positive nuclear cells to total cells represented the nuclear translocation of NF-κB. The average number of positive stained cells within at least nine independent high power fields (400 × magnification) were determined microscopically and subjected to statistical analysis as indicated.

Reverse transcription polymerase chain reaction

Total tissue RNA was isolated using TRIzol reagent following the manufacturer's instructions For each sample, first-strand cDNA was synthesized using 1.0 μg total RNA with oligo dT23VN primer and M-MuLVuLV reverse transcriptase (Gibco). Five milliliters cDNA samples were amplified in 25 μL of a reaction mixture containing 10 × Taq buffer, 1.5 mmol $MgCl_2$, 5 μmol dNTPs (each), 10 μmol of each 5′ and 3′ primers, and 2 U TaqGold polymerase (Perkin-Elmer Cetus, Waltham, MA, United States). Polymerase chain reaction (PCR) was performed in a thermal cycler (GeneAmp Model 2400; Perkin-Elmer Cetus) for 22 cycles (94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 1 min), followed by a 10-min extension at 72 ℃. The PCR products (5 μL) were subjected to electrophoresis on 1.5% agarose gels and stained with 0.5 μg/mL ethidium bromide. Primers used in this paper are illustrated in Table 4.

Immunoblotting

Colon extracts (40 μg total protein) from each group of mice were resolved by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, United States). The membrane was blocked with 5% nonfat dry milk overnight and incubated with a rabbit polyclonal antibody to zonula occludens (ZO)-1, β-defensin 2 (Lab Version, Fremont, CA, United States), with a rabbit polyclonal antibody to IL-1β, IL-6 or TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, United States), with a mouse monoclonal antibody to IL-10 (BD Biosciences, Franklin

Lakes, NJ, United States), or with an anti β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Affinity BioRegents, Golden, CO, United States) for loading control. Antigen-antibody binding was detected with horseradish-peroxidase-conjugated anti-rabbit or mouse IgG (Vector Laboratories) using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, United States).

Scanning electron microscopy
To investigate the characterization of CCTCC M206119 strain, 10^8 CFU/mL bacteria were grown for 18 h in MRS (Difco), washed with PBS and fixed with 2.5% glutaraldehyde. After two washings with PBS, samples were dehydrated with ethanol to the critical point, coated with 20 nm of gold in the scanning electron microscopy (SEM) coating unit, and examined by a XL30 ESEM scanning electron microscope (Philips, Eindhoven, Netherlands).

Biochemical profiling
CCTCC M206119 strain were grown on MRS agar and incubated under anaerobic conditions at 37 ℃ for 24-48 h. All isolates were visualized by Gram staining. Biochemical testing was performed with API 20A strips (BioMerieux, Hazelwood, MO, United States) according to the manufacturer's instructions. Catalase and SPOR tests were performed according to the supplier's recommendations (Becton Dickinson). G + C percentage was determined using high-performance liquid chromatography (HPLC) method in the Chemistry College of Central South University (China). Ten microliters base standard and 10 μL DNA hydrolase were injected into the HPLC sample injector and analyzed three times. The separating conditions used were as follows: 250 mm Zonbax-C18 column, 0.05 mol/L $NH_4H_2PO_4$ (pH 4.0) and acetonitrile mixture (20:1) as washing solution, 1 mL/min, moving phase: 2% acetonitrile, 98% 50 mmol/L NaH_2PO_4 buffered solution (pH 3.94), room temperature, detecting wavelength 254 nm and 270 nm, 0.1 mg/mL dA, dT, dG, dC solution used as external standard.

16S rRNA gene sequencing
Approximately 700 bp of the *16S rRNA* gene were amplified with primers 16S-F (5'-AGA GTT TGA TCA TGG CTC AG-3') and 16S-R (5'-CAC CGC TAC ACA TGG AG-3') under the following PCR conditions: 95 ℃ for 5 min; 10 cycles of 94 ℃ for 30 s, 70 ℃ for 30 s, and 72 ℃ for 40 s; 26 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 1 min; and 72 ℃ for 10 min. 16S rDNA amplicons were gel purified by using SDS-PAGE and a gel band purification kit (Amersham Biosciences). The 5' terminus of the *16S rRNA* gene was sequenced with primers 16S-F and 16S-R by using an ABI Prism 3100 (Applied Biosystems) sequencing system and an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (version 2.0, Applied Biosystems). Sequencing traces of amplicons containing ambiguous signals were resubmitted for sequencing. rDNA sequences were ana-

lyzed by using Lasergene (version 5.0, DNAStar, Madison, WI, United States). Contigs were generated by using SeqMan. Isolates were identified by using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) (www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis
Data were analyzed by Student's *t* test or analysis of variance (ANOVA). The Wilcoxon signed-rank test was used to compare data that did not satisfy Student's *t* test. *P* < 0.05 was considered significant.

RESULTS

Colonic inflammation was aggravated in DSS-colitis mice after CCTCC M206119 treatment
Survival rate of DSS-colitis mice reduced to 80% from day 5 after CCTCC M206119 treatment changes, which differed from other groups significantly (Figure 1A). Body weight loss, stool consistency, and occult/gross fecal bleeding were evaluated and scored individually for each animal according (Table 2). The scores were evaluated statistically by χ^2 test and Monte Carlo exact test. CCTCC-M206119-treated DSS-colitis animals showed much higher DAI scores compared with 5-ASA- and saline-treated DSS-colitis mice, starting from day 3 after 5% DSS treatment (Figure 1B).

After 7 d of treatment, all DSS-colitis mice showed a significant reduction in their colon length compared with the healthy controls. CCTCC-M206119-strain-treated DSS-colitis mice had the shortest length (8.2 ± 0.68 cm), followed by saline- (9.8 ± 0.56 cm) or 5-ASA- (10.0 ± 0.89 cm) treated DSS-colitis mice (*P* < 0.05, Figure 1C). CCTCC-M206119-treated DSS-colitis mice had markedly more severe disease, with greater weight loss, diarrhea, fecal bleeding, and shortened colon length.

The histological lesions were examined blindly and the average scores were evaluated statistically by ANOVA. In the acute study, CCTCC-M206119-treated DSS-colitis mice presented scores of 10.9 ± 0.5 that differed statistically from those in the 5-ASA-treated DSS-colitis mice. It became evident that the CCTCC-M206119-treated DSS-colitis group had comparatively higher DAI scores than the saline controls (*P* < 0.01, Figure 1D and E).

Furthermore, there was significant difference in MPO enzymatic activity detected in the colons control mice (1.038 ± 0.012 U/g tissue), DSS plus saline group (18.368 ± 1.226 U/g tissue), DSS plus 5-ASA group (8.369 ± 0.652 U/g tissue), and DSS plus CCTCC M206119 group (24.565 ± 2.006 U/g tissue) (Figure 1F). This indicated that more neutrophils infiltrated DSS-colitis mice after CCTCC M206119 treatment.

CCTCC M206119 administration changed the fecal microflora in DSS-colitis mice
To characterize the role of CCTCC M206119 strain on potential gut flora shifts during DSS-induced barrier damage, we performed a bacterial-culture-based survey

Figure 1 Colonic inflammation was aggravated in dextran sulfate sodium-colitis mice after China Center for Type Culture Collection M206119 treatment. A: Survival rate changes of four age- and sex-matched BALB/c mouse groups (n = 10) [○ : Healthy controls; ● : 5% dextran sulfate sodium (DSS) plus saline treatment; △ : 5% DSS plus 5-aminosalicylic acid (5-ASA) treatment; ▲ : 5% DSS plus China Center for Type Culture Collection M206119] at the indicated time points; B: Disease activity index (DAI) changes among groups in (A) after 7 d 5% DSS treatment at the indicated time points. Each point represents the mean ± SE (n = 10); C: Mean colon length (cm) for the mice in (A); D: Histological analysis of representative colons from the mice in (A) (original magnification, hematoxylin and eosin staining, 100 ×); E: Summarized histological scores from colons of (D); F: Summarized myeloperoxidase (MPO) activities of colons from mice in (D).

of the gut flora in DSS-colitis mice treated with saline, 5-ASA, or CCTCC M206119 strain. No significant differences in total aerobic bacteria or *Enterococcus* were obtained for any of the groups. Bacterial counts of all fecal aerobic [*Escherichia coli* (*E. coli*), *Staphylococcus*, *Bacteroides*, or total aerobic bacteria] cultured in DSS-colitis mice increased significantly compared to the healthy group. Also, both *E. coli* and *Staphylococcus* increased in the saline group, whereas 5-ASA rebalanced the bacterial counts of *Staphylococcus* to normal levels (Figure 2A). In contrast, *Lactobacillus* in the colon lumen was reduced significantly in all DSS-colitis groups, with no significant changes in *Bifidobacterium* or *Enterococcus spp.* (Figure 2B).

To determine whether the changes in gut flora after

CCTCC M206119 treatment were due to alteration of the epithelial junction or antagonistic agents, we measured the expression levels of ZO-1 and β-defensin 2 from colons of DSS-colitis mice treated with saline or CCTCC M206119 and healthy mice, by western blotting. Expression of ZO-1 in healthy mice remained at an abundant level compared to all DSS-colitis mice. Treatment of mice for 5 d with 5% DSS plus saline reduced ZO-1 expression significantly compared to healthy mice, whereas 5% DSS plus CCTCC M206119 strain reduced ZO-1 expression dramatically at day 3. In contrast, no β-defensin 2 expression was detected in the healthy mice, with relatively high expression of β-defensin 2 detected in the colons in mice that were treated with 5 d of either

Figure 2 Alteration of fecal microflora in DSS-colitis mice after China Center for Type Culture Collection M206119 treatment. Bacterial counts of (A) aerobic (*Escherichia coli*, *Staphylococcus*, *Bacteroides*, total aerobic bacteria) and (B) anaerobic (*Bifidobacterium*, *Lactobacillus*, *Enterococcus spp.*) in colon luminal contents from healthy animals and dextran sulfate sodium (DSS)-colitis mice treated by saline, 5-aminosalicylic acid (5-ASA), or China Center for Type Culture Collection (CCTCC) M206119 strain at day 2 and day 7 after DSS-treatment, as determined by culture. The data sets were pooled from two independent experiments. Significance levels were determined by *U* test. Underlined *P* values shown in black were calculated from comparisons of healthy *vs* diseased animals; C: Zonula occludens (ZO)-1 and defensin 2 expression from colons of DSS-colitis mice treated with saline or CCTCC M206119 and healthy mice was measured by western blotting at indicated time points; GAPDH was used as a loading control. The data represent at least three experiments. [a]*P* < 0.05 *vs* healthy group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

5% DSS plus saline or 5% plus CCTCC M206119. After 5 d exposure to 5% DSS plus CCTCC M206119, there was a significant (*P* < 0.05) decrease in β-defensin 2 level in the colons of mice relative to 5% DSS plus saline treatment (Figure 2C).

Effects of CCTCC M206119 strain on epithelial injury and translocation of NF-κB *in vitro*

Nuclear translocation of NF-κB p65 represents increased inflammation at the initiation stage. To investigate whether the aggravation of DSS-colitis after CCTCC M206119 strain treatment was due to the initiation of inflammation-related transcription, we detected the translocation of NF-κB p65 in HT-29 cells inoculated with CCTCC M206119 strain. TNF-α was used as a control. In the blank control, no positive staining for NF-κB p65 was detected. Both TNF-α and CCTCC M206119 strain upregulated the translocation of NF-κB p65 *in vitro*, with more positively stained cells in the latter group (*P* < 0.05, Figure 3A and B).

Colonic cytokines in CCTCC-M206119- or vehicle-treated DSS-colitis mice

To investigate if CCTCC M206119 administration affected the expression of colonic cytokines, we first screened the mRNA levels of the major colonic proinflammatory cytokines by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). We observed increased mRNA levels for IL-1 β, IL-6 and TNF-α from the colons of DSS-colitis mice treated with CCTCC M206119 strain or saline for 3 d. Also, it was evident that mRNA levels of IL-1 β, IL-6 and TNF-α from colons of CCTCC-M206119-treated DSS-colitis mice were significantly higher than those from colons of saline-treated DSS-colitis mice (Figure 4A). Next, we confirmed the changes in expression of the above cytokines at the protein level, suggesting that DSS treatment resulted in increased expression of proinflammatory cytokines, whereas DSS plus CCTCC M206119 strain treatment resulted in significantly higher expression of the cytokines mentioned above (Figure 4B).

Anti-inflammatory cytokine IL-10 expression from colons of DSS-colitis mice treated with saline, 5-ASA, or CCTCC M206119 was measured by western blotting. IL-10 expression was dramatically downregulated in saline- and CCTCC-M206119-treated DSS-colitis mice, with more significant changes in the latter group. In contrast, 5-ASA upregulated IL-10 expression compared to saline-treated DSS-colitis mice (Figure 4C). It indicated that CCTCC M206119 treatment inhibited the expression of anti-inflammatory cytokine in DSS-colitis mice.

Characteristics of CCTCC M206119 strain

Distinct microscopic morphological features were observed after Gram staining of broth-grown CCTCC M206119 strain. Gram-positive, rod-shaped morphology could be distinguished (Figure 5A). Under SEM, CCTCC M206119 strain showed exclusively smooth rod-

Figure 3 Effects of China Center for Type Culture Collection M206119 strain on epithelial injury and translocation of nuclear factor-κB *in vitro*. Immunofluorescence staining for nuclear factor (NF)-κB p65 (red) and 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) (blue) in HT-29 cells treated either by tumor necrosis factor (TNF)-α or China Center for Type Culture Collection (CCTCC) M206119 (scale bars, 10 mm). A: Increased positive NF-κB p65 staining after CCTCC M206119 treatment; B: Positive staining areas were quantified as pixels per high-power microscopic field (400 ×) using Photoshop. Error bars indicate mean ± SD (n = 3).

or fork-shaped morphology, without spores, flagella or capsules (Figure 5A). Based on analysis of the peak and data of base C, G, T and A, G + C mol% of CCTCC M206119 strain was determined as 58.64% (Figure 5B). All CCTCC M206119 strains tested were unable to utilize indepamide, urea enzymes and gelatin. All strains tested were able to utilize glucose, mannitol, lactose, saccharose, maltose, salicin, L-xylose, arabitol, esculin, glycerol, cellobiose, MNC, melezitose, D-raffinose, sorbitol, rhamnose, and trehalose. All strains were found to be catalase negative [at 3% (v/v) H_2O_2]. With discriminant and factorial analyses of data from all biochemical tests, a presumptive identification scheme for CCTCC M206119 strain was formulated based on biochemical properties. In conjunction with Gram stain morphology, biochemical profiling can be used to differentiate among major groups of lactobacilli (up to 95% confidence interval). In our abbreviated identification scheme, CCTCC M206119 strain can be presumptively grouped into lactobacilli when biochemical tests are combined with microscopic morphology (Figure 5C). To verify the identities of the reference strains used in this study, 16S rRNA genes were amplified and sequenced. With BLASTn, CCTCC M206119 strain was determined as *L. crispatus* at the *16S rRNA* gene level (Figure 5D).

DISCUSSION

IBD, including CD and UC, are chronic immune-mediated diseases in which endogenous bacteria are thought to play an important role, as suggested by many clinical observations and experimental studies summarized in recent reviews[1]. Recent studies have also shown that some bacterial strains or mixtures may have the capacity to promote or reduce intestinal inflammation[4].

Although the pathogenesis of IBD remains elusive, the relevance of intestinal luminal bacteria in the initiation and progression of chronic intestinal inflammatory disorders is gaining support[30]. However, no specific microorganism has been associated with the pathogenesis of IBD, suggesting that qualitative/quantitative differences in the intestinal microbiota may play some role in the initiation or perpetuation of intestinal inflammation.

In the current study, we compared the phenotypes of DSS-colitis mice treated by saline, 5-ASA, and CCTCC M206119 strain, trying to determine the role of the probiotic bacterium. We demonstrated that CCTCC M206119 treatment reduced survival rate of DSS-colitis mice. CCTCC-M206119-treated DSS-colitis animals had markedly more severe disease, with greater weight loss, diarrhea, fecal bleeding, and shortened colon length. In addition, the CCTCC-M206119-treated DSS-colitis group had comparatively higher histological scores and more neutrophil infiltration than the controls. To our surprise, CCTCC M206119 strain could be characterized as *L. crispatus* by microscopic morphology, biochemical tests and *16S rRNA* gene level. Hence, our study suggested that not all probiotics are safe for treatment of colitis. Instead, probiotics safety should be carefully evaluated before their application.

Other recent studies also have shown supporting evidence. A strain of *Lactobacillus salivarius* was isolated from blood and bile pus cultures of a 70-year-old man with bacteremic acute cholecystitis[31]. Administration of different lactobacilli and a *Bifidobacterium* strain in an acute liver injury rat model has shown different effects on bacterial translocation and hepatocellular damage. *Bifidobacterium animalis* NM2 increases bacterial translocation to the MLNs but does not affect hepatocellular damage[32]. Together with our data, some probiotics could act as op-

A

IL-1β									498 bp
TNF-α									365 bp
GAPDH									574 bp
5% DSS	-	+	+	-	+	+	-	+	+
Days	3	3	3	5	5	5	7	7	7

B

IL-1β									19 KD
IL-6									25 KD
TNF-α									17 KD
GAPDH									54 KD
5% DSS	-	+	+	-	+	+	-	+	+
Days	3	3	3	5	5	5	7	7	7

C

	Control	Model	Saline	5-ASA	CCTCC M206119	
IL-10						15 KD
β-actin						42 KD
5% DSS	-	+	+	+	+	

Figure 4 Colonic cytokines changes in China Center for Type Culture Collection M206119 or vehicle-treated dextran sulfate sodium-colitis mice. Expression of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α from the colons of dextran sulfate sodium (DSS)-colitis mice treated with China Center for Type Culture Collection (CCTCC) M206119 or saline was measured by (A) RT-PCR and (B) western blotting at indicated time points; GAPDH was used as a loading control; C: IL-10 expression from colons of DSS-colitis mice treated with saline, 5-aminosalicylic acid (5-ASA), or CCTCC M206119 was measured by western blotting at indicated time points; β-actin was used as a loading control. The data represent at least three experiments. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

portunistic pathogens and result in endogenous infection unexpectedly. Also, some researchers have demonstrated that the activities to promote endogenous infections are strain specific.

As we know, most lactobacilli have a remarkable record of safety and have been consumed by humans for decades. However, the possible involvement of certain strains has been described in cases of sepsis, endocarditis, or bacteremia; mostly in association with a severe underlying disease

A

Gram staining. Oil 1000 ×   10 000 ×

B

C

IND	-	ESC	+
URE	-	GLY	+
GLU	+	CEL	+
MAN	+	MNC	+
LAC	+	MLZ	+
SAC	+	RAF	+
MAL	+	SOR	+
SAL	+	RHA	+
XYL	+	TRE	+
ARA	+	CAT	-
GEL	-	SPOR	-

D

http://www.ncbi.nlm.nih.gov/blast
Lactobacillus crispatus

Figure 5 Characteristics of China Center for Type Culture Collection M206119 strain. China Center for Type Culture Collection (CCTCC) M206119 strain was identified by (A) microscopy (Gram staining, oil 1000 ×) and scanning electron microscopy (10 000 ×); (B) Gas chromatography ratio (high performance liquid chromat); (C) biochemical reaction; and (D) sequence analyses of *16S rRNA* gene fragments amplified from DNA eluted from CCTCC M206119 genomic DNA.

Figure 6 Model illustrating how China Center for Type Culture Collection M206119strain leads to aggravated phenotype in dextran sulfate sodium-colitis mice.
DSS: Dextran sulfate sodium; TLR: Toll-like receptor; IL: Interleukin; TNF: Tumor necrosis factor; NOD: Nucleotide-binding oligomerization domain; NK: Natural killer.

or detrimental condition[33]. In view of these facts, the safety of potential probiotic microorganisms in these conditions should be assessed individually. Bacterial translocation (BT) is most likely the first step in the possible passage of viable (indigenous) bacteria to sterile body sites and is thus important for sepsis, endocarditis, and bacteremia caused by the commensal flora. In animal studies, mono association experiments have shown that the type of colitis is dependent on the bacterial species[12,34].

Based on our research, *E. coli* and staphylococci were increased in DSS-colitis mice, whereas lactobacilli in the colon luminal contents were reduced significantly in all DSS-colitis groups, with no significant changes in *Bifidobacterium* or *Enterococcus spp*. Furthermore, our data indicated that changes in gut flora after *L. crispatus* CCTCC M206119 treatment were related to reduced ZO-1 expression and increased β-defensin 2 expression. Our *in vitro* experiment showed that both TNF-α and CCTCC M206119 strain upregulated the nuclear translocation of NF-κB p65, with more positively stained cells in the latter group. Other studies have found that NF-κB can be activated in both intestinal epithelium and intestinal lamina propria mononuclear cells (LPMCs) in acute DSS-colitis mice[35,36]. Intrarectal administration of a*ntisense* oligonucleotides to NF-κB P65 could alleviate the in-

flammation[24,37]. Thus, it is reasonable to conclude that *L. crispatus* CCTCC M206119 strain exacerbated the imbalance of gut flora; changed the pathogen species or colonies adhered to the colonic mucosa; subsequently altered the expression of tight junction proteins and antibacterial molecules; and activated nuclear translocation of NF-κB p65 in epithelium or inflammatory response cells.

It is thought that IBD might be due to complex mucosal immune responses to antigens of resident enteric bacteria[38-40]. IL-1 and IL-6 have been shown to be the major cytokines secreted by lamina propria cells and play a critical role in the development of Th1-cell-mediated chronic colitis[41]. Recombined IL-1 receptor antagonist can alleviate the mucosal inflammation and necrosis in DSS-coltis mice[42]. DSS-induced colitis was less severe in *IL-6* gene knockout mice, whereas transgenic mice with a mutant *CIS/SOCS3* gene, encoding a negative regulator for the IL-6/STAT3 (signal transducer and activator of transcription) signaling pathway, had increased susceptibility to DSS-induced colitis[43]. In our study, we found that both mRNA and protein levels of IL-1 β, IL-6 and TNF-α from colons of CCTCC-M206119-treated DSS-colitis mice were significantly higher and earlier than those from colons of saline-treated DSS-colitis mice. In contrast, we found that expression of anti-inflammatory

cytokine IL-10 was dramatically downregulated in saline- and CCTCC-M206119-treated DSS-colitis mice, with more significant change in the latter group. This indicated that DSS treatment resulted in increased expression of proinflammatory cytokines and decreased anti-inflammatory cytokines, whereas DSS plus CCTCC M206119 treatment resulted in significantly higher and earlier expression of the proinflammatory cytokines and inhibited the expression of anti-inflammatory cytokine in DSS-colitis mice.

IL-10 can inhibit the expression of IL-1α, IL-1β, IL-6, IL-12, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, M-CSF, TNF, leukemia inhibitory factor and platelet activating factor produced by activated monocytes or macrophages. It exerts strong immune inhibitory function[8] and plays a major role in the immune tolerance of intestinal mucosa[9,10]. Thus, it is possible that administration of *L. crispatus* CCTCC M206119 strain interrupts the balance between anti-inflammatory and proinflammatory responses in colonic mucosa or lamina propria after DSS treatment, and leads to subsequently aggravated colonic inflammation. These processes are similar to those observed in UC patients[11].

Thus, our hypothesis in this study is as follows: some bacteria or bacterial products, such as *L. crispatus* CCTCC M206119, may interact directly with colonic epithelial cells or LPMCs after disruption of the mucosal barrier and balance of gut flora by DSS administration. Then neutrophils and mononuclear cells infiltrate the lamina propria and activate NF-κB translocation, which in turn increases proinflammatory cytokines such as IL-1β, IL-6 and TNF-α. Furthermore, TNF-α can augment NF-κB activation in various cell types, and inhibit the production of anti-inflammatory cytokines such as IL-10 (Figure 6).

Overall, human IBD trials to date have suggested that probiotics display no overt side effects, but conflicting reports on probiotic efficacy highlight the importance of selecting well-characterized probiotic strains and in delivering intact pharmaceutical formulations at an appropriate dose level to the inflamed regions of the intestine. These studies have emphasized that certain probiotic strains need to be thoroughly investigated *in vitro* and *in vivo* using appropriate disease models. Also, some probiotics such as *L. crispatus* CCTCC M206119 might act as aggravating factors in the pathogenesis of UC. Future studies to identify associations between microorganisms (and or their genes and gene products) and human physiological or disease processes will lead to the development and testing of hypotheses that address causality as well as a myriad of specific host-microbe interactions.

COMMENTS

Background

It is widely accepted that a combination of genetic factors, immune disorders and environmental factors could be involved in the etiology of inflammatory bowel disease (IBD). Recent research has illustrated that some commensal and pathogenic bacteria are closely related to IBD. However, to date, the role of bacteria in the pathogenesis of IBD, or the efficacy of probiotics is still controversial.

Research frontiers

The efficiency of probiotics in controlling IBD is still controversial. Thus, it is difficult to draw a definitive conclusion in evaluating the role of microflora in the pathogenesis of IBD, and subsequently, the efficacy in controlling IBD. Some published data have also questioned the safety of probiotics.

Innovations and breakthroughs

Microflora considered beneficial to the host include the genera *Bifidobacterium* and *Lactobacillus*. In this context, several candidate strains were screened to find some probiotics as therapeutics using dextran sodium sulfate (DSS)-induced colitis in mice, and China Center for Type Culture Collection (CCTCC) M206119 strain led to an exacerbated phenotype of DSS-colitis mice. Expression of protective factors zonula occludens-1 and β-defensin 2 was downregulated after CCTCC M206119 treatment. There was an increase in the nuclear translocation of nuclear factor-κB in epithelial cells. Then, intestinal proinflammatory and anti-inflammatory cytokine responses were evaluated. Proinflammatory colonic cytokines [interleukin (IL)-1β, IL-6 and tumor necrosis factor-α] levels were clearly increased in CCTCC-M206119-treated animals, whereas anti-inflammatory colonic cytokine (IL-10) level was lowered compared with saline- or 5-ASA-treated DSS-colitis mice. Next, CCTCC M206119 strain was identified as *Lactobacillus crispatus*, so it was concluded that not all lactobacilli strains have beneficial effects on intestinal inflammation and that *Lactobacillus crispatus* (*L. crispatus*) CCTCC M206119 is involved in exacerbation of intestinal inflammation in DSS-colitis mice.

Applications

The study results suggest that some probiotics such as *L. crispatus* CCTCC M206119 strain might act as an aggravating factor in the pathogenesis of ulcerative colitis (UC). They also highlight the importance of selecting well-characterized probiotic strains and in delivering intact pharmaceutical formulations at an appropriate dose level to the inflamed regions of the intestine.

Terminology

IBD is a group of inflammatory conditions of the colon and small intestine, including Crohn's disease and UC, which are characterized by abnormal activation of the gut-associated immune system, resulting in chronic inflammation of the digestive tract. *Lactobacillus* is a genus of Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria. They are a major part of the lactic acid bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid. They are common and usually benign.

Peer review

In this study, the authors investigated the role of CCTCC M206119 strain on intestinal inflammation using a DSS-induced colitis mice model. They showed that CCTCC M206119 strain was identified as *L. crispatus*, but this strain was involved in the exacerbation of intestinal inflammation in DSS-colitis mice. From these results, the authors concluded that not all lactobacilli strains have beneficial effects on intestinal inflammation. This paper has been well written and the results are interesting.

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BRIEF ARTICLE

Endoscopic ultrasonography-guided fine needle aspiration: Relatively low sensitivity in the endosonographer population

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Abstract

AIM: To assess the characteristics and quality of endoscopic ultrasonography-guided fine needle aspiration (EUS-FNA) in a large panel of endosonographers.

METHODS: A survey was conducted during the 13th annual live course of endoscopic ultrasonography (EUS) held in Amsterdam, Netherlands. A 2-page questionnaire was developed for the study. Content validity of the questionnaire was determined based on input by experts in the field and a review of the relevant literature. It contained 30 questions that pertained to demographics and the current practice for EUS-FNA of responders, including sampling technique, sample processing, cytopathological diagnosis and sensitivity of EUS-FNA for the diagnosis of solid mass lesions. One hundred and sixty-one endosonographers who attended the course were asked to answer the survey. This allowed assessing the current practice of EUS-FNA as well as the self-reported sensitivity of EUS-FNA for the diagnosis of solid mass lesions. We also examined which factors were associated with a self-reported sensitivity of EUS-FNA for the diagnosis of solid mass lesions > 80%.

RESULTS: Completed surveys were collected from 92 (57.1%) of 161 endosonographers who attended the conference. The endosonographers had been practicing endoscopy and EUS for 12.5 ± 7.8 years and 4.8 ± 4.1 years, respectively; one third of them worked in a hospital with an annual caseload > 100 EUS-FNA. Endoscopy practices were located in 29 countries, including 13 countries in Western Europe that totaled 75.3% of the responses. Only one third of endosonographers reported a sensitivity for the diagnosis of solid mass lesions > 80% (interquartile range of sensitivities, 25.0%-75.0%). Factors independently associated with a sensitivity > 80% were (1) > 7 needle passes for pancreatic lesions or rapid on-site cytopathological evaluation (ROSE) ($P < 0.0001$), (2) a high annual hospital caseload ($P = 0.024$) and (3) routine isolation of microcores from EUS-FNA samples ($P = 0.042$). ROSE was routinely available to 27.9% of respondents. For lymph nodes and pancreatic masses, a maximum of three needle passes was performed by approximately two thirds of those who did not have ROSE. Microcores were routinely harvested from EUS-FNA samples by approximately one third (37.2%) of survey respondents.

CONCLUSION: EUS-FNA sensitivity was considerably lower than reported in the literature. Low EUS-FNA sensitivity was associated with unavailability of ROSE, few needle passes, absence of microcore isolation and low hospital caseload.

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Key words: Caseload; Community surveys; Cytopa-

thology; Endoscopic ultrasonography; Histopathology; Quality improvement

INTRODUCTION

Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) has become widely available in a variety of endoscopy settings. Due to its high diagnostic accuracy, EUS-FNA plays an important role in the management of masses located in or close to the gastrointestinal tract. For example, median sensitivity of EUS-FNA to differentiate between benign and malignant masses of the pancreas (technically the most difficult location)[1], is reported to be greater than 80%[2]. Such a high sensitivity for malignancy diagnosis has allowed EUS-FNA to significantly impact patient management in various clinical situations, including pancreatic cancer[3], mediastinal lesions[4,5], lung cancer[6], and solid liver lesions[7,8].

Excellent results of EUS-FNA such as those cited above have mostly been reported by dedicated endoscopists, many of them working in academic centers. The quality of EUS-FNA in the endosonographer population is poorly known, despite the fact that the quality of endoscopic procedures has become a central concern for the American and European Societies of Gastrointestinal Endoscopy[9-11].

To characterize the situation of EUS-FNA in the community, we performed a survey in a large audience of endosonographers to assess (1) current modalities of EUS-FNA; (2) self-reported sensitivity of EUS-FNA for the diagnosis of solid masses; and (3) factors associated with a diagnostic yield similar to that reported in the literature.

MATERIALS AND METHODS

Survey design and administration

A 2-page, 30-item, questionnaire (Appendix 1) was developed for the study. Content validity of the survey was determined based on input by experts in the field and a review of the relevant literature. Participants were asked to answer questions pertaining to demographics (6 items), their own current practice for EUS-FNA (16 items), sample processing (3 items), cytopathological diagnosis (3 items) and sensitivity of EUS-FNA for the diagnosis of solid mass lesions.

The survey was conducted during the 13th annual live EUS course held in Amsterdam, Netherlands, June 3rd and 4th 2010, as previously described[12,13]. Briefly, questionnaires were placed in bags distributed to course participants, and attendees were asked to deposit completed surveys in a dedicated box at the registration desk. Consent to participate in this study was inferred from voluntary completion of the survey. Efforts to increase response rates included reminders by the course director and moderators, projection of a reminder slide during breaks, and collection of surveys by staff members. No gift or financial incentive was granted to attendees.

Statistical analysis

Results are expressed as mean ± SD or as a percentage. Answers for each individual question were obtained from all survey respondents except when otherwise stated (each response was included in the analysis, regardless of the completeness of the survey); therefore, the number of respondents for each individual question (i.e., the denominator for percentage calculations) is indicated.

Comparisons between groups were performed with the Pearson χ^2 test or Fisher's exact test (Freeman-Halton extension where applicable) for categorical data and the Wilcoxon signed rank test for continuous variables. We also examined, by using multiple logistic regression analysis, which factors-including years of EUS practice, EUS-FNA annual hospital caseload, availability of rapid onsite cytopathological evaluation (ROSE) of EUS-FNA samples, number of needle passes > 7 or based on ROSE for three lesion types (lymph node, pancreas mass < 25 mm, pancreas mass > 25 mm), method of EUS-FNA sample preservation and routine isolation of microcores from EUS-FNA samples-were associated with a sensitivity of EUS-FNA for the diagnosis of solid mass lesions > 80%. All tests were 2-sided and P values < 0.05 were considered statistically significant. All analyses were performed with JMP software (version 9.0.0; SAS, Cary, NC, United States).

RESULTS

Study population

Completed surveys were collected from 92 (57.1%) of 161 endosonographers who attended the conference (excluding international faculty members, local faculty, fellows, and industry delegates). Survey respondents had been practicing endoscopy and EUS for 12.5 ± 7.8 years and 4.8 ± 4.1 years, respectively; 38 (42%) of them had been performing EUS for more than 5 years (Table 1). Approximately one third of respondents were practicing in a center with an annual caseload > 100 EUS-FNA. Endoscopy practices were located in 29 countries, including 13 countries in Western Europe (Austria, Belgium, Denmark, Germany, France, Greece, Italy, Netherland, Portugal, Spain, Sweden, Switzerland and the United Kingdom) that totaled 75.3% (67 out of 89) of the responses. Most (77.1%) survey respondents were performing FNA or drainage in fewer than 40% of EUS procedures. For patients subjected to both endoscopic biliary drainage

Table 1 Characteristics of survey respondents *n* (%)

Male gender (Nr = 92)	66 (71.7)
Age (yr) (Nr = 92)	43.1 ± 8.0
Years of endoscopy practice (Nr = 92)	12.5 ± 7.8
Years of EUS practice (Nr = 91)	4.8 ± 4.1
Number of respondents practicing in Western Europe (Nr = 89)	67 (75.3)
Proportion of EUS with FNA or drainage (Nr = 92)	
< 20%	35 (38)
20%-40%	36 (39.1)
40%-60%	18 (19.6)
60%-80%	3 (3.3)
> 80%	0
EUS-FNA annual hospital caseload (Nr = 92)	
< 50	32 (34.8)
50-100	26 (28.3)
100-200	25 (27.2)
> 200	9 (9.8)
Sensitivity of EUS-FNA for the diagnosis of solid mass lesions (Nr = 61)	
< 40%	1 (1.6)
40%-60%	14 (23.0)
60%-80%	23 (37.7)
> 80%	23 (37.7)

Numbers are means ± SD except where stated otherwise. Nr: Number of respondents for each question; ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasonography; EUS-FNA: Endoscopic ultrasonography-guided fine needle aspiration; FNA: Fine needle aspiration.

and EUS-FNA, only 27% of survey respondents were performing both procedures during a single endoscopy session. Staging of pulmonary cancer was performed by approximately one third of survey respondents (27 of 86; 31.4%).

Practice of EUS-FNA

A 22G needle was used for all lesion types by the majority of endosonographers; a 25G needle was used by 19.8% of survey respondents for EUS-FNA of the pancreas head (as compared to 6.8% for esophagogastric lesions; *P* = 0.009) (Table 2). ROSE of EUS-FNA samples was available to less than half of survey respondents, routinely (27.9% of answers) or in selected cases (15.1% of answers). Most endosonographers who had ROSE routinely available used it to determine the number of needle passes during EUS-FNA, while a maximum of three needle passes was performed by approximately two thirds of those who did not have ROSE routinely available. Samples were prepared using liquid-based methods plus smearing by approximately half of survey respondents (46.5%); microcores were routinely harvested from EUS-FNA samples by approximately one third (37.2%) of survey respondents. Paraffin-embedded blocks were prepared for histopathological examination of EUS-FNA samples in the laboratory of approximately half of survey respondents (55.8%). Forty-five (52.9%) of 85 survey respondents had regular meetings with the pathologist examining EUS-FNA samples.

If EUS-FNA was repeated after a first inconclusive procedure, survey respondents repeated an identical procedure or referred the patient to another endosonog-

Table 2 Practice of endoscopic ultrasonography-guided fine needle aspiration *n* (%)

Diameter of the needle used for lesions located	
In the esophagus/stomach (Nr = 88)	
19G	12 (13.6)
22G	70 (79.5)
25G	6 (6.8)
In the head of the pancreas (Nr = 86)	
19G	5 (5.8)
22G	64 (74.4)
25G	17 (19.8)
ROSE available (Nr = 86)	
Routinely	24 (27.9)
In selected cases	13 (15.1)
Never	49 (56.9)
Number of needle passes	
Pancreatic mass < 25 mm (Nr = 84)	
≤ 3	41 (48.8)
5-7	19 (22.6)
> 7 or based on ROSE	24 (28.6)
Pancreatic mass > 25 mm (Nr = 83)	
≤ 3	37 (44.6)
5-7	21 (25.3)
> 7 or based on ROSE	25 (30.1)
Lymphadenopathy (Nr = 87)	
≤ 3	51 (58.6)
5-7	14 (16.1)
> 7 or based on ROSE	22 (25.3)
Paraffin-embedded blocks prepared for histopathological analysis (Nr = 86)	
Yes	48 (55.8)
No	12 (13.9)
Do not know	26 (30.2)
Pathologist making routine diagnosis for EUS-FNA samples (Nr = 85)	
Dedicated digestive cytopathologist	25 (29.4)
General cytopathologist	47 (55.3)
Digestive pathologist not specialized in cytology	13 (15.3)
Attitude if EUS-FNA is repeated after a first inconclusive EUS-FNA (Nr = 85)	
Change in the procedure (2 answers allowed)	48 (56.5)
Higher number of needle passes	42 (87.5)
Larger needle	19 (39.6)
Addition of ROSE	12 (25.0)
Tru-Cut needle in the esophagus and rectum	7 (14.6)
Repetition of identical procedure	30 (35.3)
Referral to another endosonographer	7 (8.2)

Nr: Number of respondents for each question; EUS-FNA: Endoscopic ultrasonography-guided fine needle aspiration; G: Gauge; ROSE: Rapid on-site cytopathological evaluation.

rapher in 35.3% and 8.2% of cases, respectively. Those who modified the procedure performed more needle passes, used a larger needle and, in 25% of cases, added ROSE of the EUS-FNA sample. The vast majority of survey respondents performed Doppler examination before EUS-FNA (79 of 88 responses; 89.7%) and administered antibiotic prophylaxis before EUS-FNA of pancreatic fluid collections (82 of 86 responses; 95.3%); antibiotic prophylaxis was less consistently administered in other indications of EUS-FNA (perirectal lesions, 43 (58.1%) of 74 responses; pancreas solid lesions, 10 (11.6%) of 86 responses). For sampling of pancreatic cysts, EUS-guided cyst wall brushing was rarely used (5 of 86 responses, 5.8%).

Table 3 Univariate analysis of variables potentially associated with an endoscopic ultrasonography-guided fine needle aspiration sensitivity > 80% for the diagnosis of solid mass lesions

	P value
Number of needle passes based on ROSE or > 7 (small pancreatic lesions)	< 0.0001
Number of needle passes based on ROSE or > 7 (large pancreatic lesions)	0.0002
ROSE available	0.0017
Number of needle passes based on ROSE or > 7 (lymphadenopathy)	0.0019
High annual hospital caseload	0.0242
Routine isolation of microcores	0.0422
Method of sample preservation	0.1198
Years of EUS practice	0.6475

EUS: Endoscopic ultrasonography; ROSE: Rapid on-site cytopathological evaluation.

Figure 1 Percentages of respondents with sensitivity > 80% according to availability of rapid on-site cytopathology evaluation (rapid on-site cytopathological evaluation) of endoscopic ultrasonography-guided fine needle aspiration samples. The number above each bar represents the total number of respondents in each category. For example, among 10 respondents who had ROSE available in selected cases, five (50%) self-reported a sensitivity > 80% for the diagnosis of solid mass lesions. EUS-FNA: Endoscopic ultrasonography-guided fine needle aspiration; ROSE: Rapid on-site cytopathological evaluation.

Sensitivity of EUS-FNA

Only one third of endosonographers (37.7%) reported a sensitivity > 80% for the diagnosis of solid mass lesions; the interquartile range of self-reported sensitivities (that represents 50% of the answers, centered on the median value) was 25.0%-75.0%. Factors associated in univariate analysis with a sensitivity greater than 80% are shown in Table 3 and specific associations (availability of ROSE and annual hospital caseload of EUS-FNA) are illustrated in Figures 1 and 2. In multivariate analysis, the only independent factors associated with a sensitivity greater than 80% were (1) the usage of ROSE to determine the number of needle passes or, if no ROSE was available, > 7 passes; (2) a high annual hospital caseload of EUS-FNA; and (3) routine isolation of microcores from EUS-FNA samples. The other associations listed in Table 3 became non-significant after including the number of needle passes for small pancreatic lesions based on ROSE or >

Figure 2 Percentages of respondents with sensitivity > 80% according to annual hospital caseload. The number above each bar represents the total number of respondents in each category. For example, among 20 respondents with an annual hospital caseload < 50 EUS-FNA, three (15.0%) self-reported a sensitivity > 80% for the diagnosis of solid mass lesions. EUS-FNA: Endoscopic ultrasonography-guided fine needle aspiration.

7 passes in the stepwise analysis.

DISCUSSION

The main finding of our survey is that the sensitivity of EUS-FNA for the diagnosis of solid mass lesions was strikingly lower than that reported in the literature. Only one third of endosonographers reported a sensitivity > 80% and one fourth of them reported a sensitivity < 60%. For comparison, a median sensitivity of 83% was reported in a large review of 28 studies that reported the performance of EUS-FNA to differentiate benign *vs* malignant pancreatic masses, technically the most difficult location; only one of these 28 studies reported a sensitivity < 60%[1,2]. The relatively poor performance of EUS-FNA reported by endosonographers in this survey is unlikely to be due to reporting bias as respondents would likely have over-, not under-, estimated their sensitivity for the diagnosis of solid mass lesions.

Quality has become a central concern in endoscopy. The large variability in sensitivities reported by endosonographers (interquartile range of 25%-75%) suggests that there is much more room for quality improvement in EUS-FNA compared to other procedures that have long been scrutinized such as colonoscopy and endoscopic retrograde cholangio-pancreatography (ERCP). For comparison, (1) in the case of ERCP for instance, deep cannulation of the desired duct was validated as a measure of quality; it was achieved in 83.6% of 3210 patients undergoing their first ever ERCP in an audit of 76 endoscopy units[14]; and (2) regarding colonoscopy, cecal intubation rates ranged between 88% and 97% for 9 of 10 endoscopists investigated over a 6-year period[15]. No quality indicator has been recommended by the Societies of Gastrointestinal Endoscopy to assess the diagnostic accuracy of EUS-FNA[16]. Recently, the overall yield of malignancy for pancreatic masses was proposed as a benchmark for EUS-FNA[17].

Factors independently associated with a high sensitivity of EUS-FNA in our study included (1) the usage of ROSE to determine the number of needle passes or, if no ROSE was available, > 7 passes; (2) a high annual hospital caseload of EUS-FNA; and (3) routine isolation of microcores from EUS-FNA samples. The number of needle passes reported by approximately half of endosonographers was ≤ 3 for lymph nodes as well as for pancreatic masses, although a minimum of 5 or even 7 passes have been recommended for the pancreas when ROSE is not available (for lymph nodes, three needle passes are sufficient)[18-20]. In a study of pancreas EUS-FNA, sensitivity for malignant diagnoses increased from 16.7% with one needle pass to 86.7% if more than 7 passes were performed and another study showed that tumor differentiation was the single factor associated with the number of needle passes required to make a diagnosis[18,19]. The low number of needle passes performed by many endosonographers in the pancreas could be related to procedure duration (approximately five minutes are required per needle pass). To shorten procedure duration, Möller *et al*[21] have proposed to perform only two needle passes for solid pancreatic masses, to harvest microcores for histopathological examination and to subject the residual sample to cytopathological examination. Using this technique, they obtained a sensitivity of 82.9% in a multicenter retrospective study that included 192 patients. A new EUS-FNA needle could also facilitate acquisition of samples adequate for histopathological evaluation[22].

Of note, routine isolation of microcores was another independent factor associated with a high sensitivity in our survey. Microcores adequate for histopathological evaluation can be obtained in 83.9%-90.9% of EUS-FNA of pancreatic masses performed using a standard 22G needle (the model used by most of our survey respondents)[21,23,24]. Several nonrandomized studies have suggested that microcores are useful: (1) in the study by Möller *et al*[21], combined cytopathological and histopathological examination was more sensitive than cytopathological or histopathological examination alone for discriminating malignant *vs* benign pancreatic lesions using two needle passes (82.9% *vs* 68.1% *vs* 60.0%, respectively; *P* < 0.01); and (2) in a prospective series of 50 patients with lymphoma, a diagnostic accuracy of 96% was reached by examining microcores obtained using a 19G needle as compared with 57% in another series when cytopathological examination alone was considered[25,26]. Paraffin-embedded cell blocks might represent an alternative to microcore isolation, however, cell blocks are made in the laboratory, not in the endoscopy room, and thus out of the control of the endosonographer (one third of survey respondents did not know if cell blocks were made with the samples that they provided to the laboratory); furthermore cell blocks have not been as well studied as microcores.

ROSE was routinely available to 28% of endosonographers only, compared to 90% in a study performed among 21 centers in the United States, of which 81% were academic[17]. Anecdotal evidence suggests that low ROSE availability in EUS-FNA facilities may be related to logistical issues, lack of perceived benefit and cost even though ROSE has been shown to be cost-effective during EUS- and percutaneous-guided FNA in some settings[27-29]. Studies about the usefulness of ROSE to reach a high diagnostic yield are contradictory. A retrospective comparison of EUS-FNA performed by a single endosonographer in two university hospitals, one with ROSE available and the other without ROSE, is traditionally cited to support the usefulness of ROSE, but differences in patient populations and indications for EUS-FNA between the two hospitals preclude definitive conclusion[30]. In another, more recent, retrospective study of EUS-FNA for pancreatic masses with ROSE available for 43.8% of 520 procedures, ROSE was associated with a higher diagnostic yield in multivariate analysis (odds ratio, 3.1; *P* = 0.0001)[31]. Nevertheless, in a multicenter prospective study that evaluated 409 patients with 474 lesions[32], a similarly high diagnostic accuracy was achieved in centers regardless of ROSE availability and, in a prospective series of 108 consecutive EUS-FNAs performed without ROSE by a single endosonographer[29], diagnostic accuracy of EUS-FNA for pancreatic lesions was 97%. The authors of the latest study attributed their results to high caseload, dedicated endosonography and cytopathology. Our finding that a high sensitivity of EUS-FNA was independently associated with ROSE and the annual hospital caseload supports that view.

A significant relationship between hospital caseload and quality has been demonstrated for few endoscopy procedures. For ERCP, a large administrative study showed that procedural failure rates were lower for inpatients undergoing ERCP at high- compared to low-volume US hospitals but the difference, albeit statistically significant because of the large sample size (> 2500 hospitals), was small (4.7% *vs* 6.0% in hospitals with an annual caseload ≥ 300 *vs* < 50 procedures, respectively)[33]. The difference in sensitivities reported by endosonographers in our study was much larger, with 15.0% *vs* 80.0% of endosonographers reporting a sensitivity greater than 80% depending if they worked in a hospital with an annual caseload < 50 *vs* > 200 EUS-FNAs, respectively (*P* = 0.024), as illustrated in Figure 2. This supports the view that centralization should be discussed for at least some of the EUS-FNA procedures, as has recently been proposed for ERCP[34]. Such a change would be important as only 8.2% of our survey respondents were referring patients to another endosonographer after a first inconclusive EUS-FNA.

Limitations of our survey include selection and recall bias: (1) surveyed endosonographers were working mostly in Western Europe and our findings may not apply to other locations; and (2) self-reported data are exposed to recall and goodwill biases because surveyed professionals tend to give the expected answer more than the real one, which indeed strengthens our main conclusion that the performance of EUS-FNA is significantly lower than

assumed from published studies. In particular, sensitivity for the diagnosis of solid mass lesions was reported by 61 (37.8%) of 161 participants in the EUS course. Because of these limitations (recruitment of participants from a course, response rate), our conclusion that sensitivity for the diagnosis of solid mass lesions is strikingly lower in the community than reported in the literature should be confirmed by larger studies.

In conclusion, the quality of EUS-FNA, as assessed in this survey by its most crucial result, *i.e.*, sensitivity for cancer diagnosis, was relatively low. Low quality was associated with unavailability of ROSE, low number of needle passes for pancreatic EUS-FNA, absence of routine microcore isolation and low annual hospital caseload. Efforts should be made to improve the quality of EUS-FNA amongst endosonographers; we suggest that endoscopists review their sensitivity for cancer diagnosis and, if this is < 80%, then they should modify their practice taking into account the factors cited above. These recommendations may be particularly valuable for endoscopists working in hospitals with a low annual caseload of EUS-FNA.

ACKNOWLEDGMENTS

COMMENTS

Background
Endoscopic ultrasonography-guided fine needle aspiration (EUS-FNA) has become widely available and is known for its high diagnostic accuracy in academic centers.

Research frontiers
The quality of several endoscopic procedures (e.g., colonoscopy) has become an area of active improvement. However, for EUS-FNA, simple quality parameters (e.g., diagnostic accuracy) are poorly known in the community. Recently, the overall yield of malignancy for pancreatic masses was proposed as a benchmark for EUS-FNA. This parameter is relatively easy to collect but it does not take into account the prevalence of malignant diseases among pancreatic masses subjected to EUS-FNA by a particular endosonographer.

Innovations and breakthroughs
The authors performed a survey of the current practice of EUS-FNA. The self-reported sensitivity of EUS-FNA for the diagnosis of solid mass lesions was considerably lower than commonly reported in the literature. A high sensitivity for the diagnosis of solid mass lesions was associated with the availability of rapid on-site cytopathological evaluation (ROSE), a high number of needle passes, microcore isolation and a high hospital caseload. The data suggest that there is ample room for improvement of the quality of EUS-FNA and provides several clues for improvement.

Applications
Scientific societies play an important role in helping to improve the quality of endoscopic procedures; particular attention in that field could be paid to EUS-FNA. Centralization of some of the EUS-FNA procedures could be explored as a means of achieving that aim.

Terminology
ROSE of material sampled by EUS-FNA is performed by a cytopathologist or a cytotechnician; preliminary diagnoses resulting from ROSE are highly reliable.

ROSE allows a reduction in the number of needle passes but its cost-efficacy is debated. Microcore isolation consists of examining the sample obtained by EUS-FNA for the presence of small tissue core samples, transferring them into a preservative (e.g., formalin) for histopathological examination and sending the rest of the material collected for cytopathological analysis.

Peer review
This is an interesting paper on a survey study performed during the 13th annual live EUS course in Amsterdam in June 2010.

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BRIEF ARTICLE

Clinical benefit of radiation therapy and metallic stenting for unresectable hilar cholangiocarcinoma

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Abstract

AIM: To determine the efficacy of external beam radiotherapy (EBRT), with or without intraluminal brachytherapy (ILBT), in patients with non-resected locally advanced hilar cholangiocarcinoma.

METHODS: We analyzed 64 patients with locally advanced hilar cholangiocarcinoma, including 25 who underwent resection (17 curative and 8 non-curative), 28 treated with radiotherapy, and 11 who received best supportive care (BSC). The radiotherapy group received EBRT (50 Gy, 30 fractions), with 11 receiving an additional 24 Gy (4 fractions) ILBT by iridium-192 with remote after loading. ILBT was performed using percutaneous transhepatic biliary drainage (PTBD) route. Uncovered metallic stents (UMS) were inserted into non-resected patients with obstructive jaundice, with the exception of four patients who received percutaneous transhepatic biliary drainage only. UMS were placed endoscopically or percutaneously, depending on the initial drainage procedure. The primary endpoints were patient death or stent occlusion. Survival time of patients in the radiotherapy group was compared with that of patients in the resection and BSC groups. Stent patency was compared in the radiotherapy and BSC groups.

RESULTS: No statistically significant differences in patient characteristics were found among the resection, radiotherapy, and BSC groups. Three patients in the radiotherapy group and one in the BSC group did not receive UMS insertion but received PTBD alone; cholangitis occurred after endoscopic stenting, and patients were treated with PTBD. A total of 16 patients were administered additional systemic chemotherapy (5-fluorouracil-based regimen in 9, S-1 in 6, and gemcitabine in 1). Overall survival varied significantly among groups, with median survival times of 48.7 mo in the surgery group, 22.1 mo in the radiotherapy group, and 5.7 mo in the BSC group. Patients who underwent curative resection survived significantly longer than those who were not candidates for surgery ($P = 0.0076$). Cumulative survival in the radiotherapy group was significantly longer than in the BSC group ($P = 0.0031$), but did not differ significantly from those in the non-resection group. Furthermore, the median survival time of patients in the radiotherapy group who were considered for possible resection (excluding the seven patients who were not candidates for surgery due to comorbid disease or age) was 25.9 mo. Stent patency was evaluated only in the 24 patients who received a metallic stent. Stent patency was significantly longer in the radiotherapy than in the BSC group ($P = 0.0165$). Biliary drainage was not eliminated in any patient. To determine the efficacy of ILBT, we compared

survival time and stent patency in the EBRT alone and EBRT plus ILBT groups. However, we found no significant difference in survival time between groups or for stent patencies. Hemorrhagic gastroduodenal ulcers were observed in 5 patients (17.9%), three in the EBRT plus ILBT group and two in the EBRT alone group. Ulcers occurred 5 mo, 7 mo, 8 mo, 16 mo, and 29 mo following radiotherapy. All patients required hospitalization, but blood transfusions were unnecessary. All 5 patients recovered following the administration of anti-ulcer medication.

CONCLUSION: Radiotherapy improved patient prognosis and the patency of uncovered metallic stents in patients with locally advanced hilar cholangiocarcinoma, but ILBT provided no additional benefits.

INTRODUCTION

Hilar cholangiocarcinoma is a relatively rare, slow-growing, late-metastasizing tumor associated with poor patient prognosis[1-7]. The only known effective treatment is surgery, but only a small proportion of patients are suitable candidates. The operation is technically demanding and may be too invasive for high-risk elderly patients.

External beam radiotherapy (EBRT), with or without intraluminal brachytherapy (ILBT), is widely used to treat patients with hilar cholangiocarcinoma. A randomized trial comparing stenting alone with stenting plus radiotherapy showed that both procedures significantly prolonged patient survival and stent patency[8]. However, the relatively long survival time of untreated patients (median, 298 d) suggests that some of these patients may have been resectable. Thus, the effects of radiotherapy alone, and the benefits of ILBT, are not known.

Biliary stenting is a widely-accepted palliative procedure used to treat patients with unresectable hilar cholangiocarcinoma and obstructive jaundice. Patient prognosis, however, is poor, even with the absence of metastasis. A randomized trial showed that uncovered metallic stents remained patent longer than plastic stents in patients with hilar cholangiocarcinoma[9]. The primary cause of uncov-

ered metallic stent occlusion is tumor ingrowth into the mesh[10]. Covered metallic stents prevent tumor ingrowth in patients with distal biliary obstruction and have a longer patency than uncovered metallic stents[11-16]. Although covered metallic stents are not feasible for treating hilar cancer patients because of the complex anatomy of the hilar duct, radiotherapy may be used to prevent tumor ingrowth after placement of an uncovered metallic stent[17].

The aims of this study were therefore twofold: to test the effects of radiotherapy, with or without ILBT, on the survival of hilar cholangiocarcinoma patients who did not undergo surgical resection; and to evaluate whether radiotherapy prolongs stent patency and thus improves patient quality of life.

MATERIALS AND METHODS

Patients

Between 1986 and 2008, 84 patients with hilar cholangiocarcinoma were admitted to the Department of Gastroenterology of Tokyo University Hospital. Of these 84 patients, 20 had metastatic and 64 had locally advanced disease (Figure 1 and Table 1). Of those with locally advanced tumors, 34 fulfilled our resectability criteria, and 25 underwent surgical resection. The remaining 9 patients were not candidates for surgery due to comorbid disease or advanced age. Bile duct cancer was diagnosed by pathological examination, clinical course, or imaging results. Cholangiocarcinoma staging was based on computed tomography, magnetic resonance cholangiopancreatography, or direct cholangiogram using endoscopic retrograde cholangiopancreatography or percutaneous transhepatic biliary drainage (PTBD). In addition, Bismuth's classification was applied[6].

Criteria for resectability

The resectability of each tumor was determined after consultation with the hepato-biliary surgeon. Surgical contraindications included: invasion of the celiac and super mesenteric arteries, biliary invasion of the third branch of the future remnant liver, and invasion of the both hepatic artery and portal vein of the future remnant liver.

Biliary stenting

All patients with locally advanced tumors underwent endoscopic or percutaneous biliary drainage (Table 2). Prophylactic antibiotics were administered intravenously prior to the endoscopic procedure and for 3 d afterwards. An Amsterdam-type plastic stent (7 or 8.5 Fr; Flexima stent, Microvasive, Boston Scientific, Natick, MA, United States) or a nasobiliary drainage tube (Cook Medical Company) was inserted into patients. PTBD using 8 Fr balloon catheters was performed under ultrasonographic and fluoroscopic guidance. Additional stents were inserted or PTBD was performed if jaundice did not improve.

Radiation therapy

Radiotherapy was recommended for patients whose jaun-

Figure 1 Distribution of patients with hilar cholangiocarcinoma from August 1995 to August 2008.

Table 1 Patient characteristics

	Patient group			
	Radiotherapy	**BSC**	**Resection**	***P* value**
Cases	28	11	25	
Gender (M/F)	14/14	4/7	20/5	
Mean age (yr)	70.1 ± 9.7	74.0 ± 9.0	67.0 ± 9.7	
	(52-86)	(61-90)	(55-78)	
Reason for non-resection				> 0.9999
Tumor factor	21	9	-	
Patient factor	7	2	-	
Performance status				
0	12	3	17	
1	15	6	8	
2	1	2	0	
TNM stage				0.4953
1a	0	0	7	
1b	2	2	1	
2a	1	0	3	
2b	1	1	8	
3	24	8	6	
Bismuth classification				0.2332
1	3	0	2	
2	6	3	2	
3	7	6	10	
4	12	2	11	

No statistically significant differences in patient characteristics were observed among the resection, radiotherapy, and BSC groups. The mean age of the BSC patients was higher than that of the other groups, but the difference was not significant. TNM: Tumor, node, metastasis; BSC: Best supportive care.

dice did not improve and who were not candidates for surgery. A total of 28 patients agreed to undergo EBRT (54 Gy, 30 fractions); of these, 11 also underwent percutaneous ILBT (24 Gy, 4 fractions) using a high-dose iridium-192 remote after loading system (Table 2). A metallic stent was inserted into the 11 patients who refused EBRT after their jaundice improved; these patients constituted the best supportive care (BSC) group.

After radiotherapy, a metallic stent was inserted endoscopically or percutaneously, depending on the initial drainage procedure. Some patients received bilateral metallic stents because multiple insertion routes were necessary for internal brachytherapy and unilateral stents produced insufficient drainage.

Follow up
Patient symptoms were assessed and blood tests were performed at monthly intervals in the outpatient clinic. Computed tomography scans were taken every 6 mo (every 2 mo during chemotherapy), with additional scans taken when patients were symptomatic or showed an increase in hepatic-biliary enzymes and bilirubin. Patients received chemotherapy every 2 mo.

Endpoints
The primary endpoints were patient death or stent occlusion through November 2008. Stents were considered patent if the jaundice did not worsen, with or without cholangitis. Patient survival time was measured from the date of diagnosis to the date of death, and stent patency was measured from placement to occlusion or to patient death if the stent remained patent. Survival time of patients in the radiotherapy group was compared with that of patients in the resection and BSC groups. Stent patency was compared in the radiotherapy and BSC groups.

Statistical analysis
Cumulative patient survival and stent patency were ana-

lyzed using the Kaplan-Meier method and compared using the log rank test. Patients whose stents were not obstructed were excluded from stent patency analysis. The Mann-Whitney U-test was used to compare quantitative variables, and Fisher's exact test was used to analyze qualitative variables. All analyses were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC, United States).

RESULTS

Patient survival
Overall survival varied significantly among groups, with median survival times of 48.7 mo in the surgery group, 22.1 mo in the radiotherapy group, and 5.7 mo in the BSC group (Figure 2). Patients who underwent curative resection survived significantly longer than those who were not candidates for surgery ($P = 0.0076$). Cumulative survival in the radiotherapy group was significantly longer than in the BSC group ($P = 0.0031$), but did not differ significantly from those in the non-resection group (Figure 2). Furthermore, the median survival time of patients in the radiotherapy group who were considered for possible resection (excluding the seven patients who were not candidates for surgery due to comorbid disease or age) was 25.9 mo.

Stent patency
Stent patency was evaluated only in the 24 patients who received a metallic stent (Table 3). Stent patency was significantly longer in the radiotherapy than in the BSC

Table 2 Details of biliary drainage and anti-cancer therapy

	Patient group		
	Radiotherapy	BSC	*P* value
Cases	28	11	
Biliary metallic stenting	24	10	
Drainage area			0.0630
Bilateral	14	2	
Unilateral	10	8	
Insertion route			0.0339
Endoscopic	4	6	
Percutaneous	20	4	
PTBD only	3	1	> 0.9999
No need for drainage	1	0	> 0.9999
Anti-cancer therapy			
Radiotherapy	28	-	
Extra corporeal (50 Gy)	28	-	
Intra bile duct (24 Gy)	11	-	
Additional chemotherapy	16	-	
Gemcitabine	1	-	
S-1	6	-	
5FU-based regimen	9	-	

Three patients in the radiotherapy group and one in the BSC group did not receive a metallic stent but underwent PTBD alone; cholangitis occurred after endoscopic stenting, and patients were treated with PTBD. A total of 16 patients were administered additional systemic chemotherapy, with 9 receiving a 5-fluorouracil (5FU)-based regimen, 6 receiving S-1, and 1 receiving gemcitabine. PTBD: Percutaneous transhepatic biliary drainage; BSC: Best supportive care.

	1 yr	2 yr	3 yr	4 yr	5 yr	MST
— Curative resection (*n* = 17)	100	81	75	66	58	NE
⋯ Non-curative resection (*n* = 8)	57	29	29	14	0	19.4M
— Radiotherapy (*n* = 28)	74	45	6	6	6	22.2M
- - BSC (*n* = 11)	18	9	9	0	0	5.7M

Figure 2 Cumulative survival rate and median survival time of patients who received curative or non-curative resection, radiotherapy or best supportive care. Cumulative survival times were calculated using the Kaplan-Meier method and compared using the log rank test. BSC: Best supportive care; MST: Median survival time; NE: Not evaluable.

group (*P* = 0.0165; Figure 3). Biliary drainage was not eliminated in any patient.

Figure 3 Cumulative metallic stent patency. Stents were patent significantly longer in the radiotherapy than in the BSC group (*P* = 0.0165). BSC: Best supportive care.

Figure 4 Cumulative survival times of patients who received external beam radiotherapy alone and those who received external beam radiotherapy plus intra bile ductal radiotherapy (intraluminal brachytherapy). There was no significant difference in survival between groups. EBRT: External beam radiotherapy; ILBT: Intraluminal brachytherapy.

Additive effect of ILBT to EBRT

To determine the efficacy of ILBT, we compared survival time and stent patency in the EBRT alone and EBRT plus ILBT groups. However, we found no significant difference in survival time between groups (Figure 4) or for stent patencies.

Complications of radiotherapy

Hemorrhagic gastroduodenal ulcers were observed in 5 patients (17.9%), three in the EBRT plus ILBT group and two in the EBRT alone group. Ulcers occurred 5 mo, 7 mo, 8 mo, 16 mo, and 29 mo following radiotherapy. All patients required hospitalization, but blood transfusions were unnecessary. All 5 patients recovered follow-

Table 3 Results of metallic stenting

	Patient group		
	Radiotherapy	BSC	*P* value
Cases	23	11	
Mean stent patency (d)[1]	604 ± 78.3	235 ± 53.3	0.0038
Median stent patency (d)[1]	557	358	
Stent obstruction (%)	12 (56.5)	4 (36.4)	0.4646
Mean patent period	329.2 ± 234.8	136.8 ± 147.1	0.1472
Cause			
Tumor ingrowth	9	1	0.1133
Tumor overgrowth	1	1	0.5490
Sludge	2	2	0.5799

[1]Mean and median stent patency were calculated using the Kaplan-Meier method and compared using the log rank test. Stent patency was significantly longer in the radiotherapy than in the BSC group. Mean patent period was longer in the radiotherapy than in the BSC group, but the difference was not significant. BSC: Best supportive care.

ing the administration of anti-ulcer medication.

DISCUSSION

Surgical resection is the only known cure for hilar cholangiocarcinoma. The surgery is highly invasive and recommended for only a small percentage of patients; thus, alternative treatments are necessary. Our results suggest that radiotherapy prolongs survival in patients who cannot undergo resection. Survival was significantly longer in patients who received radiotherapy than in those who received BSC, but was similar to that of patients who underwent non-curative resection.

Radiotherapy has been shown to improve survival times of patients with locally advanced hilar cholangiocarcinoma who cannot undergo curative resection. For example, a randomized trial reported that median survival times were 12.9 mo in patients who received both EBRT and ILBT and 9.3 mo in patients did not receive radiotherapy[8]. A second study found that median survival in patients who received chemoradiotherapy (EBRT plus 5-fluorouracil) was 22 mo, but that study did not include an untreated control group[18]. A retrospective analysis found that the median survival time in patients with extrahepatic cholangiocarcinoma who received EBRT plus ILBT was 9 mo, compared with 5 mo in patients treated with EBRT alone[19]. This wide range in survival times may reflect differences in patient backgrounds. Most reports did not describe the percentage of patients with resectable tumors, the reasons that they were not resected, or the resection criteria; thus, it is difficult to generalize from these results. In contrast, we have stated the inclusion criteria for non-surgical treatment. Moreover, in our radiotherapy group, the median survival of non-resected patients considered for possible resection was 25.9 mo, longer than previously reported survival times.

We observed no significant differences between the radiotherapy and BSC groups in patient background, including the reasons for not undergoing resection and

performance score. We expected that selection bias may have resulted in longer median survival of the radiotherapy than of the BSC group, but we found no significant difference in background between these two groups. Our results therefore provide additional evidence suggesting that radiotherapy increases survival time in patients with locally advanced hilar cholangiocarcinoma.

Although surgery is the only known cure for cholangiocarcinoma, we found that median survival of the radiotherapy group did not differ significantly from that of the non-curative resection group. This finding supports the survival benefits of radiotherapy for patients with locally advanced hilar cholangiocarcinoma. Thus, radiotherapy is a treatment option for patients in poor condition or those with highly invasive tumors.

Biliary stenting is a widely accepted palliative treatment for non-resected patients with biliary obstruction, with uncovered metallic stents maintaining patency longer than plastic stents[9]. The primary cause of obstruction in patients who receive an uncovered metallic stent is tumor ingrowth into the stent mesh. In a previous randomized trial, we prevented distal biliary obstruction by inhibiting tumor ingrowth and found that a covered metallic stent was patent longer than an uncovered stent[11]. However, covered metallic stents cannot be used in patients with hilar obstruction, and it is necessary to develop strategies to prevent tumor ingrowth of hilar lesions. Stents remained patent significantly longer in the radiotherapy group than in the BSC group (stenting alone); suggesting that local tumor control using radiotherapy prolongs stent patency. Previous studies of the efficacy of metallic stents in the treatment of hilar cholangiocarcinoma did not use radiotherapy, and studies investigating the survival benefits of radiotherapy have not reported results of metallic stenting. Thus, no evidence was available to determine the efficacy of radiotherapy in improving stent patency.

Multiple laser-cut-type Nitinol stents were found to have a mean patency of 150 d[20], with Wallstents having a median patency of 169 d[21]. A comparison of the patency of metallic stents inserted endoscopically and percutaneously showed that the median stent patency of these two groups was 9.8 mo and 11.0 mo, respectively[22]. In comparison, we found that median and mean stent patency in our radiotherapy group were 557 d and 604 d, respectively. The length of time the stents remained patent suggests that radiotherapy had a significant effect on patency.

We found that the addition of ILBT to EBRT did not improve patient survival or stent patency. Stent patency is a surrogate indicator of local anti-tumor effectiveness, because the main cause of stent occlusion is tumor ingrowth through the stent mesh. ILBT requires an additional treatment period and multiple PTBD insertions. Since it did not improve patient survival or stent patency, we do not recommend this additional treatment. A retrospective study of 31 patients found that, although additional ILBT improved the 2-year survival rate, it had no effect on cumulative survival time[20]. No randomized studies to date

have compared the effect on survival of radiotherapy with and without ILBT. Thus, the efficacy of additional ILBT remains unclear.

Photodynamic therapy is a promising local anti-tumor therapy for patients with non-resectable hilar cholangiocarcinoma, with median survival times of patients who did and not receive photodynamic therapy of 493 d and 98 d, respectively, a difference that was statistically significant[23]. Although photodynamic therapy can be used to treat local tumors extending along the bile duct, it cannot be used to treat large tumors that have invaded other organs, and it cannot be used to treat loco-regional lymph node metastasis because it is performed in the bile duct. The combination of photodynamic therapy with EBRT may be a more feasible treatment option.

The major limitation of this study was its retrospective design. Moreover, despite all included patients having unresectable, locally advanced tumors, the characteristics of the radiation and BSC groups differed. Since there were no selection criteria for the BSC group, the differences in outcomes between this group and the radiation group may be due not only to the effects of radiation, but to patient condition as well.

In conclusion, radiotherapy may improve the prognosis of patients with non-resected, locally advanced hilar cholangiocarcinoma and may increase the patency of uncovered metallic stents. ILBT provides no benefit for these patients.

COMMENTS

Background
Hilar cholangiocarcinoma is a relatively rare, slow-growing and late-metastasizing tumor associated with poor patient prognosis. Surgery is the only known curative treatment, but it may be technically difficult and too invasive for high-risk elderly patients. The treatment of patients with unresectable tumors has not been established.

Research frontiers
Biliary stenting and radiotherapy, including external beam radiotherapy (EBRT) with or without intraluminal brachytherapy (ILBT), are widely used to treat patients with locally advanced hilar cholangiocarcinoma. These treatments, however, are not well established, although a recent randomized study showed good results for photodynamic therapy.

Innovations and breakthroughs
Several recent studies have shown the efficacy of radiation therapy in patients with locally advanced hilar cholangiocarcinoma. In many of those patients, however, the tumors may have been resectable. The authors clarified the reasons for non-resection of the patients and compared outcomes with resected patients. The authors found that radiation therapy effectively prolonged patient survival and stent patency. They also showed that additional ILBT did not affect survival or stent patency.

Applications
EBRT is indicated for patients with locally advanced hilar cholangiocarcinoma, with similar survival times as patients who underwent non-curative resection. Patients who undergo uncovered metallic stent placement should be considered for EBRT to prolong stent patency and survival.

Terminology
EBRT is a conventional radiation method, and ILBT is performed with iridium wire through a PTBD catheter using an after loading system. A metallic stent is a braided metallic wire or tubular nitinol cut with a laser. Metallic stents have self-expandability and are mounted onto a thin delivery system (6-9Fr). This type of stent, however, tends to be occluded by tumor ingrowth into the stent mesh.

Peer review
As stated by the authors, the limitation of the study is that it is a retrospective study and that the groups are not the results of a randomization but the consequence of different situations such not being suitable for surgery. In any case, the paper is a well presented study on a very difficult subject and is worthy of publication.

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BRIEF ARTICLE

Comparison of percutaneous transhepatic portal vein embolization and unilateral portal vein ligation

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Abstract

AIM: To compare the effect of percutaneous transhepatic portal vein embolization (PTPE) and unilateral portal vein ligation (PVL) on hepatic hemodynamics and right hepatic lobe (RHL) atrophy.

METHODS: Between March 2005 and March 2009, 13 cases were selected for PTPE ($n = 9$) and PVL ($n = 4$) in the RHL. The PTPE group included hilar bile duct carcinoma ($n = 2$), intrahepatic cholangiocarcinoma ($n = 2$), hepatocellular carcinoma ($n = 2$) and liver metastasis ($n = 3$). The PVL group included hepatocellular carcinoma ($n = 2$) and liver metastasis ($n = 2$). In addition, observation of postoperative hepatic hemodynamics obtained from computed tomography and Doppler ultrasonography was compared between the two groups.

RESULTS: Mean ages in the two groups were 58.9 ± 2.9 years (PVL group) *vs* 69.7 ± 3.2 years (PTPE group), which was a significant difference ($P = 0.0002$). Among the indicators of liver function, including serum albumin, serum bilirubin, aspartate aminotransferase, alanine aminotransferase, platelets and indocyanine green retention rate at 15 min, no significant differences were observed between the two groups. Preoperative RHL volumes in the PTPE and PVL groups were estimated to be 804.9 ± 181.1 mL and 813.3 ± 129.7 mL, respectively, with volume rates of 68.9% ± 2.8% and 69.2% ± 4.2%, respectively. There were no significant differences in RHL volumes ($P = 0.83$) and RHL volume rates ($P = 0.94$), respectively. At 1 mo after PTPE or PVL, postoperative RHL volumes in the PTPE and PVL groups were estimated to be 638.4 ± 153.6 mL and 749.8 ± 121.9 mL, respectively, with no significant difference ($P = 0.14$). Postoperative RHL volume rates in the PTPE and PVL groups were estimated to be 54.6% ± 4.2% and 63.7% ± 3.9%, respectively, which was a significant difference ($P = 0.0056$). At 1 mo after the operation, the liver volume atrophy rate was 14.3% ± 2.3% in the PTPE group and 5.4% ± 1.6% in the PVL group, which was a significant difference ($P = 0.0061$).

CONCLUSION: PTPE is a more effective procedure than PVL because PTPE is able to occlude completely the portal branch throughout the right peripheral vein.

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INTRODUCTION

Postoperative liver failure may be induced by major hepatectomy, whereby more than 60%-70% of total liver volume is planned for resection in cases such as hilar bile duct carcinoma, hepatocellular carcinoma and liver metastasis. To reduce the risk of postoperative liver failure due to an insufficient volume of functional liver, the well-established percutaneous transhepatic portal vein embolization (PTPE) procedure can be performed prior to major hepatectomy[1-6]. Unilateral portal vein ligation (PVL) is an alternative method that requires laparotomy[7,8]. Although PTPE has since its introduction become the more popular procedure of the two, PVL remains a viable option during the initial stage of a two-stage hepatectomy procedure (TSHP) for cases such as bilobar multiple liver metastases[9-11]. Both of these techniques occlude the unilateral portal vein, with the aim of inducing atrophy of the ipsilateral liver and thus inducing hypertrophy of the future liver remnant (FLR). In the present study, we retrospectively compared the effectiveness of the two techniques by evaluating the postoperative atrophy rate and hemodynamics of the right hepatic lobe (RHL).

MATERIALS AND METHODS

Patient characteristics of the PTPE and PVL groups

Between March 2005 and March 2009, nine patients were selected for PTPE and four for PVL. The mean patient age was 69.7 ± 3.2 years for the PTPE group and 58.9 ± 2.9 years for the PVL group (*P* = 0.0002). The clinical characteristics of the patients included hilar bile duct carcinoma (*n* = 2), intrahepatic cholangiocarcinoma (*n* = 2), hepatocellular carcinoma (*n* = 2) and liver metastasis (*n* = 3) in the PTPE group, and the PVL group had hepatocellular carcinoma (*n* = 2) and liver metastasis (*n* = 2). The PTPE group included one patient with hepatitis C, one with hepatitis B, and seven positive for hepatitis B virus (HBV) antigen but negative for hepatitis C virus (HCV) antibody. The PVL group included one patient with hepatitis C, one with hepatitis B, and two positive for HBV antigen and negative for HCV antibody.

PTPE cases

Two patients were diagnosed with hilar bile duct carcinoma, based on findings of obstructive jaundice. Endoscopic nasobiliary drainage was first performed. Before conducting an extended right lobectomy, PTPE was performed because the liver volumes to be resected were 70.8% and 70.2% in the two patients.

In the two cases of intrahepatic cholangiocarcinoma, the tumors were 10 cm and 8 cm in diameter, and both were adjacent to the hilar plate. These two patients also underwent a preoperative PTPE because of planned liver resection volumes of 74.2% and 70.5% respectively.

In the two patients with hepatocellular carcinoma, the tumors were 4.0 cm and 3.5 cm in diameter and adjacent to the right Glisson's capsule. In addition, both

the tumors recurred after the transarterial embolization procedure. The liver volume to be resected in these cases was 68.3% and 66.1%, respectively. In addition, indocyanine green retention rate at 15 min (ICG R15) was 17% and 18%, respectively, indicating functional liver impairment. Because of these factors, PTPE was performed prior to resection.

In the three patients with liver metastases, multiple lesions in the right lobes were noted after the initial round of chemotherapy. Preoperative PTPE was scheduled because the liver volumes to be resected in the three patients were 65.2%, 66.6% and 68.8%.

PVL cases

Patients with hepatocellular carcinoma in the PVL group presented with lymph node metastasis. The tumors adjacent to the right Glisson's capsule were 5 cm and 4 cm in diameter with ICG R15 of 38.8% and 15.2%, respectively. The planned liver resection volumes were 65.1% and 69.8%, respectively. Thus, PVL was performed on the right portal branch only during the implementation of lymphadenectomy.

Regarding the two liver metastasis cases, the patients had undergone chemotherapy and developed multiple synchronous liver metastases to both lobes, disseminated from ascending colon cancer. Because the liver resection volumes were 66.9% and 74.8%, the right portal vein was ligated. In addition, right colectomy and partial resection of the left hepatic lobe were also performed in both patients.

Indications for resection

Resectability criteria included an FLR of ≤ 30% of the total liver volume, whereas this criterion was ≤ 35% for the patients who had liver cirrhosis or underwent neoadjuvant chemotherapy. In addition, the following equation established by Yamanaka *et al*[12,13] and Okamoto *et al*[14] was used to predict posthepatectomy liver failure: Y = -110 + 0.942 × resection rate (%) + 1.36 × ICG retention rate (%) + 1.17 × patient's age + 5.94 × ICG maximal removal rate (mg/kg per minute). With this equation, the patients who had a calculated Y value > 50 points were deemed unresectable.

PVL and PTPE techniques

For all patients, PTPE or PVL was performed on the RHL. PVL was indicated for patients who were to undergo laparotomy for lymphadenectomy or right colectomy. In PTPE, the umbilical portion of the portal vein was punctured, and the right branch of the portal vein was embolized using a mixture of fibrin glue (Beriplast P; CSL Behring, Tokyo, Japan) and iodized oil (Lipiodol; Guerbet, Aulnay-sous-Bois, France). In the PVL cases, preoperative multidetector row computed tomography (CT) was routinely performed to check for the presence of anatomical variants of the right portal vein. The right branch of the portal vein was intraoperatively isolated and ligated. After each method, Doppler ultrasonography was used to confirm that portal blood flow had

Table 1 Clinical characteristics of the study population (mean ± SD)			
Variables	PTPE group (*n* = 9)	PVL group (*n* = 4)	*P* value
Age (yr)	69.7 ± 3.2	58.9 ± 2.9	0.0002
Sex (male:female)	7:2	3:1	
Background (HCV:HBV:NBNC)	1:1:7	1:1:2	
Albumin (g/dL)	3.9 ± 0.4	4.0 ± 0.4	0.61
Bilirubin (mg/dL)	0.9 ± 0.4	1.2 ± 0.7	0.35
AST (IU/L)	30.6 ± 12.2	32.3 ± 8.5	0.47
ALT (IU/L)	35.9 ± 29.1	29.3 ± 15.6	0.92
WBC (/mm^3)	6744 ± 3109	5228 ± 1973	0.41
PLT (× 10^4/mL)	22.1 ± 11.0	19.3 ± 9.9	0.76
PT (%)	84.1 ± 10.5	84.5 ± 12.2	0.86
ICG 15 (%)	10.7 ± 7.4	17.5 ± 13.2	0.43
Child pugh (A:B:C)	7:2:0	3:1:0	

PTPE: Percutaneous transhepatic portal vein embolization; PVL: Portal vein ligation; HCV: Hepatitis C virus; HBV: Hepatitis B virus; NBNC: Hepatitis B surface antigen and HCV antibody negative; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cells; PLT: Platelet count; PT: Prothrombin time; ICG 15: Indocyanine green retention rate at 15 min.

been occluded in the ligated lobe and that it had been sustained in the FLR.

Follow-up
No patients had any postoperative complications. Although a slight postoperative decline in liver function was noted in some patients, all improved with conservative treatments.

Statistical analysis
Each patient underwent CT volumetry 1 mo before and after each procedure to evaluate volume changes in the RHL, and the values were compared between the two groups. The RHL atrophy rate was estimated by subtracting the RHL volume rate at 1 mo after PTPE or PVL from the preoperative RHL volume rate. The values in each group were compared using the Mann-Whitney test. All analyses were performed using statistical software (JMP 8.0.2 Macintosh; SAS Institute, Japan). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Clinical characteristics of the study population
The mean age of patients was significantly higher in the PTPE group (58.9 ± 2.9 years *vs* 69.7 ± 3.2 years in the PTPE group), which was a significant difference ($P = 0.0002$). Among the indicators of liver function, including serum albumin, serum bilirubin, asparate aminotransferase, alanine aminotransferase, platelets and ICG R15, no significant differences were observed between the two groups (Table 1).

Volume change of RHL
Preoperative RHL volumes in the PTPE and PVL groups were estimated to be 804.9 ± 181.1 mL and 813.3 ±

Table 2 Right hepatic lobe atrophy rate 1 mo after percutaneous transhepatic portal vein embolization or portal vein ligation (mean ± SD)			
Variables	PTPE group (*n* = 9)	PVL group (*n* = 4)	*P* value
Preoperative RHL volume (mL)	804.9 ± 181.1	813.3 ± 129.7	0.83
Preoperative RHL volume rate (%)	68.9 ± 2.8	69.2 ± 4.2	0.94
Postoperative RHL volume (mL)	638.4 ± 153.6	749.8 ± 121.9	0.14
Postoperative RHL volume rate (%)	54.6 ± 4.2	63.7 ± 3.9	0.0056
RHL atrophy rate 1 mo postoperation (%)	14.3 ± 2.3	5.4 ± 1.6	0.0061

PTPE: Percutaneous transhepatic portal vein embolization; PVL: Portal vein ligation; RHL: Right hepatic lobe.

129.7 mL, respectively, with volume rates of 68.9% ± 2.8% and 69.2% ± 4.2%, respectively. There were no significant statistical differences in RHL volumes ($P = 0.83$) and RHL volume rates ($P = 0.94$).

At 1 mo after PTPE or PVL, postoperative RHL volumes in the PTPE and PVL groups were estimated to be 638.4 ± 153.6 mL and 749.8 ± 121.9 mL, respectively, which was not a significant difference ($P = 0.14$). Postoperative RHL volume rates in the PTPE and PVL groups were estimated to be 54.6% ± 4.2% and 63.7% ± 3.9%, respectively, which was a significant difference ($P = 0.0056$). At 1 mo postoperatively, the liver volume atrophy rate was 14.3% ± 2.3% in the PTPE group and 5.4% ± 1.6% in the PVL group, which was a significant difference ($P = 0.0061$) (Table 2).

With respect to the findings from imaging, postoperative CT and Doppler ultrasonography data confirmed residual peripheral portal inflow in the right branch of the ligated portal vein in two cases in the PVL group. In contrast, portal venous flow was confirmed as completely occluded in the PTPE group.

Liver resection and postoperative course
PTPE successfully facilitated liver resection for all nine patients, whereas among the four patients who underwent PVL, two with hepatocellular carcinoma remained unresectable after the procedure. An extended right lobectomy was performed for two hilar bile duct carcinomas and two intrahepatic cholangiocellular carcinomas. A right lobectomy was performed in all the other resectable cases. The 11 patients showed no postoperative complications.

DISCUSSION

Through experimentation on rabbits in 1920, Rous *et al*[15] proved that PVL could induce atrophy of the ipsilateral hepatic lobe and hypertrophy of the FLR lobe. Since then, this technique has been clinically applied by Honjo *et al*[16] and has recently been adopted for TSHP for bilobar multiple liver metastases. In the first stage, partial resec-

Figure 1 Computed tomography showing a case of hilar bile duct carcinoma where the right hepatic lobe volume rate decreased from 68.3% to 50.0% at 1 mo after percutaneous transhepatic portal vein embolization. A: Before percutaneous transhepatic portal vein embolization (PTPE) [right hepatic lobe (RHL) volume rate: 68.3%]; B: After PTPE (RHL volume rate: 50.0%); C: The portal vein in the RHL was completely occluded after PTPE.

Ligation point

Figure 2 Computed tomography showing a case of liver metastasis where the right hepatic lobe volume rate decreased from 66.9% to 60.4%. A: Before portal vein ligation (PVL) [right hepatic lobe (RHL) volume rate: 66.9%]; B: After PVL (RHL volume rate: 60.4%); C: The right portal branch was intraoperatively ligated (arrow).

Figure 3 Postoperative computed tomography confirming a degree of residual portal venous flow from the periphery to the ligation point (arrow). A: Axial image; B: Multiplanar reconstruction image.

tion or ablation is performed on the FRL, and PVL is subsequently performed to induce atrophy in the hemiliver to be resected, in preparation for the planned major hepatectomy[17-20]. PTPE has been advocated as a technique for inducing atrophy of the ipsilateral liver without the need for a laparotomy, and is commonly performed for cases such as hilar bile duct carcinoma and hepatocellular carcinoma, in which a substantial liver volume is planned for resection[21-23]. Although PVL and PTPE are both techniques that occlude the portal vein, they differ in approach. PTPE is performed by percutaneously injecting embolic materials, whereas PVL is performed by a laparotomy to ligate the first-order branch of the portal vein. To date, several studies have demonstrated the safety and effectiveness of the two techniques individually; however, there are not enough data available from comparative studies reporting the relative efficacies of

the two techniques. In the present study, we compared the atrophic effect and postoperative hepatic hemodynamics associated with PTPE and PVL.

In our study, the PTPE group demonstrated a significantly higher rate of postoperative liver volume atrophy than the PVL group 1 mo after hepatectomy. The mean liver volume atrophy rate was 14.3% ± 2.3% in the PTPE group and 5.4% ± 1.6% in the PVL group (Figures 1 and 2). The CT and Doppler ultrasound findings may explain the reason for this difference. It was confirmed that portal venous flow was completely occluded due to the use of embolic materials acting throughout the peripheral portal vein in the PTPE group. However, portal vein venous flow continued at peripheral sites away from the ligation point in two cases in the PVL group (Figure 3). Residual flow was observed from the anterior to the posterior branch (Figure 4).

Figure 4 Doppler ultrasound findings on hemodynamics. A: Color Doppler ultrasound confirming portal venous flow from the anterior to the posterior branch (arrow); B: Pulse Doppler ultrasound confirming hepatofugal venous flow in the anterior branch (arrow); C: Pulse Doppler ultrasound confirming hepatopetal venous flow in the posterior branch (arrow).

Figure 5 Hemodynamics during portal vein ligation procedure. A: The residual portal venous flow may be explained as backflow from the hepatic vein; B: The presence of an arterioportal shunt may be another reason for the residual portal venous flow.

Two explanations can be proposed for the peripheral portal blood flow observed despite the portal vein being ligated at its central site. First, the observed peripheral portal blood flow could be backflow from the ipsilateral hepatic vein (Figure 5A). Normally, portal vein pressure and hepatic venous pressure are approximately equal at 100-150 mmH$_2$O. However, ligating the first-order branch of the portal vein may decrease peripheral portal vein pressure, causing a relative pressure elevation in the hepatic vein and resulting in backflow from the hepatic vein. This backflow was significant for cases where tumors had compressed or obstructed hepatic veins in the presence of high hepatic venous pressure. A second possible explanation for the observed peripheral portal blood flow is related to the arterioportal shunt (AP shunt) (Figure 5B). Blood inflow *via* the AP shunt diminishes the effectiveness of the technique by allowing some blood to flow in the first-order branch of the portal vein. Consequently, when the AP shunt is preoperatively observed, PVL should not be the preferred treatment option.

In contrast to the PVL group, postoperative CT data confirmed the presence of embolic materials in the peripheral portal vein in all PTPE patients. In addition, postoperative Doppler ultrasound showed no residual blood in the portal vein, leading us to conclude the superiority of PTPE over PVL. However, PVL is currently more often performed in TSHP cases requiring portal vein occlusion. Time between the first stage and PTPE should be minimized because of possible cancer progression[24,25]. Therefore, we recommend PTPE for the first part of TSHP.

In conclusion, the small sample size and the retrospective nature of this study are limitations towards obtaining more conclusive results, compared to a prospective study with a larger population. However, our results show that PTPE can more effectively and rapidly achieve atrophy of ipsilateral liver volume and consequently induce compensatory FLR volume hypertrophy, compared with PVL.

COMMENTS

Background
Postoperative liver failure may be induced because of the insufficient remnant liver volume after major hepatectomy where more than 60%-70% of the total liver volume is resected in such cases as hilar bile duct carcinoma or liver metastasis. To achieve safer major hepatectomy, ligation or embolization of the portal vein is preliminarily performed to induce atrophy of the ipsilateral liver and hypertrophy of the future remnant liver.

Research frontiers
To occlude unilateral portal vein, either portal vein ligation (PVL) ligating the unilateral portal vein or percutaneous transhepatic portal vein embolization (PTPE) injecting embolic agent to the unilateral portal vein through catheter is performed. PTPE is a procedure advocated after PVL, and PVL cases have decreased in number since the introduction of PTPE. However, as two-stage hepatectomy procedure (TSHP) for liver metastasis has recently become a more popular procedure, PVL has been again increasingly performed in combination with the first resection of TSHP.

Innovations and breakthroughs
The liver atrophy rate of unilateral lobe was compared between the PTPE (n = 9) and PVL groups (n = 4) 1 mo after each procedure. The liver atrophy rate was 14.3% ± 2.3% (PTPE) and 5.4% ± 1.6% (PVL), which was a significant difference (P = 0.0061). To date, the two procedures have been regarded equivalent in effectiveness, however, the results suggest the superiority of PTPE over PVL, and to the best of knowledge, this is the first report to demonstrate such difference.

Terminology
PVL is a procedure to ligate the unilateral portal vein to induce ipsilateral liver atrophy and consequent hypertrophy of the contralateral liver. PTPE is a procedure to occlude the unilateral portal vein by percutaneously injecting embolic agent to induce ipsilateral liver atrophy and consequent hypertrophy of the contralateral liver. TSHP is a surgical strategy adopted for severe metastatic liver cases where it is impossible to remove all malignant lesions in a single procedure. In the first procedure, resectable tumors are removed and after a period of time for liver regeneration, the second procedure is performed to remove the remaining tumors.

Peer review
The current study compared PTPE and unilateral PVL. Using small cohorts, the authors concluded that PTPE is a more effective procedure than PVL as the postoperative liver volume atrophy rate was significantly greater in the PTPE group than the PVL group. This study was novel, well written, technically well conducted, and highly clinically important.

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BRIEF ARTICLE

Comparison of sequential and 7-, 10-, 14-d triple therapy for *Helicobacter pylori* infection

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Abstract

AIM: To compare the effectiveness of sequential therapy for *Helicobacter pylori* (*H. pylori*) infection with that of triple therapy of varying durations.

METHODS: The 460 patients enrolled in this study had *H. pylori*-associated gastritis or a gastric or duodenal ulcer. After screening, *H. pylori*-infected patients were randomly assigned to receive either conventional triple therapy for 7, 10 or 14 d, or a new 10-d sequential therapy. Each of the 4 treatment groups included 115 patients. The outcomes of eradication therapy were assessed 4 wk after treatment by the urea breath test and histology.

RESULTS: The overall eradication rate was 81.0%, and eradication rates were 75.7% for 7-d conventional triple therapy, 81.9% for 10-d conventional triple therapy, 84.4% for 14-d conventional triple therapy, and 82.0% for 10-d sequential therapy. Neither intention-to-treat analysis nor per protocol analysis showed significant differences in eradication rates using sequential therapy or the standard triple therapy ($P = 0.416$ and $P = 0.405$, respectively).

CONCLUSION: There are no significant differences between 10-d sequential eradication therapy for *H. pylori* and any duration of standard triple treatment in Korean patients.

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INTRODUCTION

Helicobacter pylori (*H. pylori*), first identified in 1982, is recognized as a risk factor for gastrointestinal ulcers, chronic gastritis, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. The most widely used eradication therapy at this time combines a proton pump inhibitor (PPI) with 2 antibiotics, amoxicillin and clarithro-

mycin. However, the failure rate of this triple therapy is 10%-20%[1-4] and has increased with the emergence of clarithromycin resistance[5-7]. When first-line triple therapy fails, a quadruple regimen of PPI, bismuth, tetracycline, and metronidazole is usually applied. The failure rate of this second-line therapy (20%-30%) is also high, which may stem from metronidazole resistance[6,8,9].

To address an increase in antibiotic resistance in *H. pylori* in Western countries, a 10-d sequential therapy was proposed. This new regimen includes a PPI plus 1 g of amoxicillin twice daily for the first 5 d followed by a PPI, 500 mg of clarithromycin and 500 mg of tinidazole twice daily for the remaining 5 d. Since Zullo *et al*[10]. first developed this therapy, it has produced higher eradication rates than triple therapy in several studies[11-15]. However, few studies have reported outcomes of sequential therapy in East Asian countries. Since antibiotic-resistant strains differ in geographical distribution, an *H. pylori* eradication therapy may vary in effectiveness between regions[16,17].

Furthermore, no universal evidence-based guidelines have been established for the optimal duration of triple therapy. Some countries prefer a 7-d therapy, and in other countries treatment longer than 7-d is deemed mandatory[1,18,19]. Most previous comparative studies tested the sequential regimen against 7-d or 10-d triple therapy.

We conducted this study to prospectively compare the *H. pylori* eradication rate obtained with a 10-d sequential regimen to the rates achieved with the conventional 7-d, 10-d and 14-d triple regimens in a Korean cohort of *H. pylori*-infected patients.

MATERIALS AND METHODS

Study population
From March 2008 to August 2011, we interviewed and enrolled patients who visited Korea University Anam Hospital with *H. pylori*-positive gastritis or peptic ulcer (gastric and/or duodenal ulcer) identified in gastroduodenoscopy. *H. pylori* infection was detected using a urea breath test, rapid urease test, or histopathological investigation. This was a prospective randomized controlled study.

Four hundred and sixty patients tested positive for *H. pylori* during this time. Study inclusion criteria were *H. pylori*-associated gastritis, or gastric or duodenal ulcer, and age 18 years or older. Criteria for exclusion from the study were: (1) serious kidney disease that required drug dosage adjustment; (2) previous treatment with antibiotics within the past 4 wk; (3) PPI treatment during the previous 8 wk; (4) previous *H. pylori* eradication failure; (5) significant cardiopulmonary, endocrine or hepatic disease, or a hematologic disorder; (6) previous surgery of the upper gastrointestinal tract; (7) history of malignancy; (8) history of drug or alcohol misuse; (9) antiulcer medication treatment within previous 4 wk; (10) systemic glucocorticoid or anticoagulation treatment; (11) severe psychiatric or neurological disease; and (12) current pregnancy or lactation. After screening, participants were randomly

assigned to treatment groups using a computer generated list. Patients provided written consent to participate, and the Institutional Review Board of Korea University Hospital approved this study. We conducted the study in agreement with the principles of the Declaration of Helsinki, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use and Guidelines for Good Clinical Practice.

Treatment protocol
Eligible patients who had no documented contraindications were randomly assigned to one of four treatments: (1) standard triple therapy (rabeprazole 20 mg + amoxicillin 1.0 g + clarithromycin 500 mg twice daily) for 7 d; (2) standard triple therapy for 10 d; (3) standard triple therapy for 14 d; and (4) a 10-d sequential treatment (amoxicillin 1.0 g + rabeprazole 20 mg twice daily for the first 5 d, followed by rabeprazole 20 mg + clarithromycin 500 mg + tinidazole 500 mg twice daily for the remaining 5 d).

Confirmation of eradication
At least 4 wk after completion of treatment, a urea breath test and histopathological diagnosis were performed to determine if *H. pylori* had been successfully eradicated. Drug side effects were assessed with a questionnaire.

Statistical analysis
Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS 18.0 for Windows; SPSS Inc., Chicago, IL, United States). Power calculations to determine sample size based on previously published data[20] showed that 420 patients would be required to detect a treatment difference at the 5% level of significance with a power of 80%. Hence the sample size was set at 460 patients to allow for a possible 10% dropout rate.

Univariate analysis, with age, gender, endoscopic diagnosis, smoking, alcohol habits, and medications (NSAID) as variables, was performed using the χ^2 test. The *H. pylori* eradication rate was determined using intention-to-treat (ITT) and per protocol (PP) analyses using the χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics
The mean age of the 460 patients enrolled in this study was 46.8 years. Two hundred and thirty-nine patients were male and 221 were female. Each of the 4 treatment groups included 115 patients. The groups did not differ significantly in gender, previous disease history, endoscopic diagnosis, smoking, alcohol consumption, NSAID and aspirin use, or use of previous medications (Table 1).

Eradication rates
Twenty-five of the 460 patients did not return to hospital for eradication testing and 8 patients discontinued the

Table 1	Clinical characteristics of patients with *Helicobacter pylori* infection					
	7-d eradication	10-d eradication	14-d eradication	10-d sequential eradication	Total	*P* value
Number of patients	115	115	115	115	460	
Gender						0.959
Male	60	58	62	59	239	
Female	55	57	53	56	221	
Endoscopic diagnosis						0.940
GU	48	52	52	48	200	
DU	40	36	32	36	144	
Gastritis as NUD	27	27	31	31	116	
Smokers	40	36	53	44	176	0.117
Alcohol drinkers	42	48	58	56	204	0.123
NSAID/ASA	12	16	19	13	60	0.513

GU: Gastric ulcer; DU: Duodenal ulcer; NUD: Non-ulcer dyspepsia; NSAID: Non-steroidal anti-inflammatory drug; ASA: Acetylsalicylic acid.

Figure 1 Flow diagram for patients enrolled in the study.

medication due to side effects. Eradication results were confirmed in 427 patients; eradication was confirmed in 408 of the 427 patients by the urea breath test and in 19 patients by histopathological examination for *H. pylori*.

Among the 427 patients, 107 patients received the 7-d conventional triple therapy, 105 received the 10-d conventional triple therapy, 109 received the 14-d conventional triple therapy, and 106 received the 10-d sequential therapy (Figure 1).

The overall eradication rate among the 427 patients was 81.0%. The eradication rates according to group were as follows: 70.4%/75.7% (ITT/PP) for the 7-d conventional triple therapy group, 74.7%/81.9% for the 10-d conventional triple therapy group, 80.0%/84.4% for the 14-d conventional triple therapy group, and 75.6%/82.0% for the 10-d sequential therapy group. Neither the ITT nor the PP analysis showed a significant difference in eradication rates between the new 10-d sequential therapy and the standard triple therapy (*P* = 0.416 and *P* = 0.405, respectively) (Table 2).

Compliance and side-effects
The compliance was greater than 95% for all of the triple conventional groups and the 10-d sequential therapy group.

In the latter group, 15 patients showed side effects including problems with taste (*n* = 1), loose stools (*n* = 3), nausea/vomiting (*n* = 4), epigastric discomfort (*n* = 3), abdominal distention (*n* = 3) and itching (*n* = 1) (Table 3). For the triple therapy, reported side effects included problems with taste, loose stools, abdominal discomfort, nausea, vomiting, epigastric discomfort, and itching. Most side effects were not severe (Table 3).

DISCUSSION
We compared the *H. pylori* eradication rates obtained after 10-d sequential therapy with those achieved after 7-, 10- and 14-d conventional triple therapy and found no significant differences.

Triple therapy, which is currently used most widely, combines a PPI with 2 antibiotics such as clarithromycin and amoxicillin[2]. However, resistance to clarithromycin now stands at 13.8%-16.7%[5,21], and recent reports place the rate of *H. pylori* eradication at 74%-83.6%[18,22].

The Maastricht III consensus report published in 2005 recommends a quadruple therapy, which includes a PPI, bismuth, tetracycline, and metronidazole, as a second-line eradication therapy[1]. The effectiveness of

Table 2 *Helicobacter pylori* eradication rates according to treatment group

	7-d eradication	10-d eradication	14-d eradication	10-d sequential eradication	Total	*P* value
ITT	70.4%	74.7%	80.0%	75.6%	75.2%	0.416
	81/115	86/115	92/115	87/115	346/460	
PP	75.7%	81.9%	84.4%	82.0%	81.0%	0.405
	81/107	86/105	92/109	87/106	346/427	

ITT: Intention-to-treat; PP: Per protocol.

Table 3 Adverse events

	7-d eradication	10-d eradication	14-d eradication	10-d sequential eradication
Taste alterations	1	1	1	1
Loose stools	3	3	2	3
Abdominal distention	2	3	3	3
Nausea/vomiting	2	4	3	4
Epigastric discomfort	3	2	3	3
Itching	0	1	0	1
Total	11	14	12	15

quadruple therapy is compromised, however, by resistance to metronidazole in *H. pylori*[8,23,24]. The prevalence of metronidazole resistance in Korea has increased from 33.3% in 1994, to 47.7% in 1999, to 66.2% in 2003[25].

In Western countries, increased resistance to triple therapy fosters much discussion. Notably, an increase in clarithromycin resistance found through large-scale studies in several European countries is linked to extensive use of this antibiotic in both children and adults, and to an increasing rate of failure to eradicate *H. pylori* using conventional therapy[20,26-28]. Nevertheless, European guidelines for *H. pylori* treatment specify triple therapy with a PPI, amoxicillin, and clarithromycin or metronidazole for 14 d, or quadruple therapy with a PPI, amoxicillin, clarithromycin, and metronidazole for 10-14 d[1,29].

Zullo *et al*[10] reported a new 10-d sequential therapy for *H. pylori* in 2000. This regimen includes a PPI plus 1 g of amoxicillin for the first 5 d followed by a PPI, 500 mg of clarithromycin, and 500 mg of tinidazole for the remaining 5 d. Some studies report that this regimen yields a higher *H. pylori* eradication rate than triple therapy[11,30,31] and improves the rate in children as well as in adults[32,33]. The mechanisms underlying the effects of the 10-d sequential treatment are not known; however, the early administration of amoxicillin appears to weaken the cell walls of *H. pylori*, reducing resistance to clarithromycin and enhancing the treatment effects[28,34]. The 5-d PPI and amoxicillin regimen may reduce the numbers of *H. pylori* by 50%, which when followed by 5 d of triple therapy, is thought to improve the overall effectiveness of the regimen[35,36]. In addition, the use of 3 or more antibiotics improves the treatment effects[20].

In our patient cohort, the 10-d sequential therapy did not achieve a higher eradication rate than the standard triple therapy. This discrepancy may reflect in part genetic differences in *H. pylori* strains in the East and West[37]. In addition, the high rate of antibiotic resistance in Korea, in particular to metronidazole, may contribute to geographical variations in treatment effectiveness. Factors in addition to location and antibiotic resistance that may affect *H. pylori* eradication include patient age and compliance, gastric acid concentration, individual response to PPI, and differences in prevalence of *H. pylori* Cag A genotype[38]. We did not investigate gastric acid concentration, antibiotic resistance (or minimal inhibitory concentrations), or *H. pylori* genotypes; and as all subjects were recruited at a single center, geographical differences in *H. pylori* sensitivity and in treatment protocol did not emerge in this study.

The optimal treatment duration using triple therapy is not established. A meta-analysis performed by Fuccio *et al*[39] concluded that extending triple therapy beyond 7 d does not significantly improve outcome compared to the standard 7-d regimen. However, individual studies report higher eradication rates using 10 or 14 d of triple therapy as compared to 7 d[40,41]. Guidelines in the United States and Europe favor longer durations of triple therapy[1,19]. Previous comparative studies of the sequential regimen involved the 7-d and 10-d triple therapy. The present study aimed to clarify the relationship of treatment duration to the *H. pylori* eradication rate.

Data from East Asian countries on use of sequential therapy are limited, and further study is needed to determine the optimal duration of treatment for the triple therapy in Asian populations. Several comparative studies of sequential therapy conducted in Korea differed in outcome. Three of these studies produced higher eradication rates for sequential therapy and another failed to detect a significant difference[38,42-45]. This lack of concordance may be related to known regional variations in prevalence of *H. pylori* resistance to antibiotics, especially clarithromycin, in Korea[17].

In conclusion, we detected no significant differences between the 10-d sequential eradication therapy for *H. pylori* and any duration of the standard triple treatment tested (7-, 10- and 14-d regimens) in a group of Korean patients. More research is needed to determine whether this finding also holds true for patients from other East Asian countries.

COMMENTS

Background

Helicobacter pylori (*H. pylori*), first identified in 1982, has been identified as a risk factor for gastrointestinal ulcer, chronic gastritis, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. To date, the most widely used eradication therapy is administration of a proton pump inhibitor (PPI) and 2 types of antibiotics; amoxicillin and clarithromycin. However, the success rate of these multiple antibiotics to eradicate *H. pylori* has decreased in recent years. An increase in the antibiotic resistance of *H. pylori* has been reported in Western countries, and a 10 d sequential eradication therapy was proposed to address this problem.

Research frontiers

The 10-d sequential therapy includes a PPI plus 1 g amoxicillin for the first 5 d

followed by a PPI, clarithromycin 500 mg, and tinidazole 500 mg for the remaining 5 d. There is no established evidence about the duration of triple therapy. Guidelines for the duration of triple therapy varies according to region. So, a Korean research team evaluated whether *H. pylori* eradication with 10 d of sequential therapy is better than the conventional 7-, 10- and 14-d triple therapy in a Korean cohort.

Applications
The researchers concluded that there was no significant difference between 7-, 10-, 14-d standard triple treatment and 10-d sequential therapy in eradication of *H. pylori* in a Korean cohort of patients. Data from East Asian countries on use of sequential therapy are limited, and further study is needed to determine the optimal duration of treatment for triple therapy in Asian populations.

Terminology
Sequential therapy is a new regimen to eradicate *H. pylori* includes a PPI plus 1 g of amoxicillin twice daily for the first 5 d followed by a PPI, 500 mg of clarithromycin and 500 mg of tinidazole twice daily for the remaining 5 d.

Peer review
This is an interesting study aimed at comparing the eradication rate of a 7, 10 and 14 d triple therapy *vs* sequential therapy in Korean patients. Interestingly, the authors found that the eradication rate provided by sequential therapy is similar to that of standard therapy of the same duration. Therefore, the final message is that the most important factor in the eradication of *H. pylori* is the duration of the therapy instead of the way of administration of antibiotics and PPI.

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BRIEF ARTICLE

Hexahydrocurcumin enhances inhibitory effect of 5-fluorouracil on HT-29 human colon cancer cells

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Abstract

AIM: To investigate the ability of hexahydrocurcumin (HHC) to enhance 5-fluorouracil (5-FU) in inhibiting the growth of HT-29 cells by focusing on cyclooxygenase (COX)-2 expression.

METHODS: Antiproliferative effects of HHC and 5-FU, alone and in combination, on growth of HT-29 human colon cancer cells were assessed using 5-diphenyltetrazolium bromide (MTT) reduction assay. In combination treatment, low doses of 5-FU were used combined with various concentrations of HHC to minimize the toxicity and side effects of 5-FU. The therapeutic effects of these drugs on down-regulation of COX-2 mRNA and protein expression were examined using semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) and Western blotting analysis.

RESULTS: MTT reduction assay indicated that HHC alone markedly decreased the viability of HT-29 human colon cancer cells compared to control. Semi-quantitative RT-PCR analysis indicated that HHC is a selective COX-2 inhibitor. This finding was supported by the observation that HHC significantly down-regulates COX-2 mRNA expression compared to the control (control: $100.05\% \pm 0.03\%$ *vs* HHC: $61.01\% \pm 0.35\%$, $P < 0.05$) but does not alter COX-1 mRNA. In combined treatment, addition of HHC to a low dose of 5-FU exerts a synergistic effect against the growth of HT-29 cells by markedly reducing cell viability to a greater degree than monotherapy. Semi-quantitative RT-PCR indicated that 5-FU at the concentration of 5 μmol/L in combination with HHC at the concentration of 25 μmol/L significantly down-regulates COX-2 mRNA expression when compared with values in cells treated with 5-FU or HHC alone (HHC + 5-FU: $31.93\% \pm 5.69\%$, 5-FU: $100.66\% \pm 4.52\%$ *vs* HHC: $61.01\% \pm 0.35\%$, $P < 0.05$).

CONCLUSION: HHC together with 5-FU exerts a synergistic effect and may prove chemotherapeutically useful in treating human colon cancer.

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Figure 1 Chemical structures of curcumin (A) and hexahydrocurcumin (B).

INTRODUCTION

Colorectal cancer is a major cause of cancer death in many countries. 5-Fluorouracil (5-FU) is a chemotherapy drug widely used in treating colorectal cancer. However, the toxicity of 5-FU towards normal cells and resistance to this drug are major barriers to successful cancer chemotherapy. Therefore, the combination of 5-FU and other regimens is often practiced to enhance the efficacy of 5-FU and also to reduce its toxicity. Previous *in vitro* and *in vivo* studies of colon cancer have reported that 5-FU combined with other regimens, such as genistein[1] and geraniol[2], are more effective than 5-FU treatment alone.

Curcumin (diferuloylmethane, Figure 1), the major yellow pigment in turmeric which is obtained from the rhizome of *Curcuma longa L.*[3], has been traditionally used in cooking in India and Southeast Asia. It has also been used in both *in vitro* and *in vivo* studies as a naturally occurring substance to treat a wide variety of cancers, including ovarian[4], lung[5], skin[6] and colon cancer[7-9]. Curcumin specifically inhibits mRNA and protein expression of cyclooxygenase (COX)-2, which is highly expressed in a variety of human cancers[10-12] including colon cancer, but it does not alter the expression of COX-1, the enzyme that maintains normal gastric mucosa and influences kidney function[13,14]. All these results seem to suggest that curcumin might have minimal toxicity and is safe for the treatment of human colon cancer compared with other traditional chemopreventive agents such as nonsteroidal anti-inflammatory drugs. Although curcumin is a very important agent in preventing and treating colon cancer, its disadvantages include poor solubility and poor absorption in the gastrointestinal tract. Previous reports have indicated that after oral administration of curcumin, about 60% of the dose was absorbed and 38% remained in the large intestine of rats[15] and it is rapidly decomposed in human blood[16]. Curcumin metabolites were synthesized to solve these problems[17].

Hexahydrocurcumin (HHC, Figure 1) is one of the major metabolites of curcumin. Previous studies revealed that this compound exhibits stronger antioxidant activity than curcumin[18]. Moreover, this compound inhibits the biosynthesis of prostaglandin (PGE2) in LPS-stimulated macrophages[19]. PGE2 is a major product of COX-2 enzymes implicated in colorectal carcinogenesis and has been shown to stimulate the growth of human colorectal carcinoma cells. In addition, HHC decreases the level of phorbol ester-induced PGE2 production in human colonic epithelial cells (HCECs) but weakly inhibits COX-2 protein[20].

All these results suggest that HHC down-regulates COX-2 expression, leading us to hypothesize that the HHC-induced suppression of COX-2 expression may improve the effectiveness of the 5-FU conventional chemotherapy drug. The synergistic effect of using 5-FU in combination with curcumin to inhibit the growth of HT-29 human colon cancer cell line has already been demonstrated[21]. The present study aims to evaluate the ability of HHC to enhance 5-FU in inhibiting the growth of HT-29 human colon cancer cells by focusing on the expression of COX-2.

MATERIALS AND METHODS

Materials

The HT-29 human colon adenocarcinoma cell lines were obtained from the American Type Culture Collection. McCoy's 5A Media Modified medium, fetal bovine serum (FBS), trypsin, penicillin and streptomycin were purchased from GIBCO-BRL (Gaitherburg, MD). 5-FU and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, United States). TRIzol® Reagent was purchased from Invitrogen (Carlsbad, CA). InProm-Ⅱ™ reverse transcriptase and GoTaq® Flexi DNA polymerase were obtained from Promega (Madison, United States). COX-1 monoclonal antibody (1:1000 dilutions) and COX-2 polyclonal antibody (1:500 dilutions) were obtained from Cayman Chemical (Ann Arbor, MI, United States).

Preparation of hexahydrocurcumin

Curcumin was obtained from *Curcuma longa* as described previously[22]. HHC was synthesized from curcumin by catalytic hydrogenation reaction in ethanol for 5 h, with palladium on charcoal as a catalyst. The product was isolated from tetrahydrocurcumin and octahydrocurcumin by silica gel column chromatography, followed by recrystallization with dichloromethane-*n*-hexane to give 45% yield of HHC as a white amorphous solid, m.p. 81-82 ℃. Tetrahydrocurcumin and octahydrocurcumin were obtained in 28% and 11% yields, respectively, after repeated column chromatography. The spectroscopic

(IR, ^{1}H-NMR and mass spectra) data of all the synthesized compounds were identical with those obtained from the previous report[22].

Cell culture and treatment

HT-29 cells were cultured in McCoy's 5A Media Modified containing 10% FBS at 37 ℃ in 5% CO_2 and 90% relative humidity. In all experiments, cells were seeded at 1×10^4 cells per well in 96-well plates for cell viability assay, or 1.5×10^5 cells per well in 6-well plates for RNA extraction and Western blotting analysis. After plating, combinations of 5-FU with HHC were added concurrently to the culture medium. Cells were harvested after various times.

Cell viability assay

HT-29 cells were plated in 96-well plates with 100 μL medium and were exposed to various concentrations of HHC, 5FU or combination of 5FU and HHC for 24 and 48 h. After incubation, cells were treated with 100 μL MTT solution (final concentration, 0.5 mg/mL) at 37 ℃ for an additional 4 h. The formazan crystals were solubilized with 100 μL DMSO and then the absorbance was measured at wavelength 540 nm using a microplate reader (Bio-Tek, Instruments, Winooski, VT, United States).

Analysis of combined effects of 5-FU with HHC

The effectiveness of 5-FU and HHC, alone or in combination, to inhibit growth of HT-29 cells was evaluated by measurement of the combination index (CI)[23], adapted from the method described by Chou *et al*[24]. The fractional inhibitory concentrations were calculated by dividing the concentration of the drug in the combination at IC_{50} by the IC_{50} of the individual drugs. In the following equation, the sum of the dose of 5-FU and the dose of HHC give 50% inhibition of cell growth. CI < 1 indicates a synergistic effect; CI = 1, additive effect; and CI > 1, antagonistic effect. CI = (Dose of 5-FU)/[IC_{50} (5-FU)] + (Dose of HHC)/[IC_{50} (HHC)].

RNA extraction and reverse transcription-polymerase chain reaction

After the HT-29 cells were treated for 24 h, total RNA was extracted using TRIzol reagent (Life Technologies, Carlbad, CA, United States). A volume of 1 μg total RNA from each sample was subjected to reverse transcription using ImProm-II™ Reverse Transcription system (Promega Madison, WI, United States). PCR amplification was performed in a reaction volume of 20 μL containing 2 μL of cDNA product and GoTag® DNA polymerase (Promega, United States) using a PerkinElmer 9600 thermal cycler. Amplification of the constitutively-expressed enzyme d-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The oligonucleotide primers used were: human COX-1, 5'-TGCCCAGCTCCTGGCCCGCCGCTT-3' (sense), 5'-GT-GCATCAACACAGGCGCCTCTTC-3' (antisense); human COX-2, 5'-TTCAAATGAGATTGTGGAAAAT-3' (sense), 5'-AGATCATCTCTGCCTGAGTATCTT-3' (antisense); GAPDH, 5'-TCCCTCAAGATTGTGAGCAA-3' (sense), and 5'-AAATGAGCCCCAGCCTTCTCC-3' (antisense). The temperature cycling conditions of amplification were as follows: 15 min at 95 ℃, then 25 cycles at 95 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. The amplification products were electrophoresed on a 1.0% agarose gel, visualized by ethidium bromide staining, and photographed gel images were scanned using an image analysis system (Gel Doc 1000; Bio-Rad, Hercules, CA, United States). The intensities of specific COX-1 or COX-2 bands were quantitated in relation to GAPDH bands amplified from the same cDNA using Gene Tools analysis software (Syngene, Cambridge, United Kingdom).

Western blotting analysis

Western blotting analysis was performed to measure protein expression of COX-1, COX-2 or β-actin. Cell lysates were prepared by treating the HT-29 cells in RIPA lysis buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) for 30 min. The lysates were sonicated twice for 20 s on ice and then were centrifuged at 10 000 × *g* for 10 min to sediment the particulate material. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed under reducing conditions on 8% polyacrylamide gels. The resolved proteins were transferred to PVDF (Millipore, Bedford, MA, United States) and the membranes were incubated with antibodies against human COX-1 or COX-2 (Cayman Chemical Co.) protein in blocking solution. After washing with PBS plus 0.5% Tween-20, the membranes were then incubated with the secondary antibody goat anti-mouse IgG HRP in blocking solution for 2 h at room temperature. After washing, membrane blots were developed using the Immobilon Western (Millipore) chemiluminescence kit and finally exposed to X-ray film.

Statistical analysis

All values are represented as mean ± SE. Statistical comparisons between different treatment results were analyzed by one-way analysis of variance followed by the Dunnett test. The significance was taken when *P*-values were less than 0.05.

RESULTS

Antiproliferative effect of 5-FU, HHC alone and their combination on HT-29 colon cancer cells

After the HT-29 colon cancer cells were exposed to various concentrations of HHC or 5-FU alone for 24 and 48 h, HHC significantly decreased the viability of HT-29 colon cancer cells as compared to control (Figure 2A, upper panel). The growth inhibition ability of HHC on HT-29 cells was time- and concentration-dependent. A similar result was also observed in the 5-FU-treated group (Figure 2B, lower panel). The half-maximal inhibitory concentration of HHC and 5-FU for inhibiting the growth of HT-29 colon cancer cells at 24 and 48 h exposure was

Figure 2 Antiproliferative effect of hexahydrocurcumin and 5-fluorouracil on HT-29 human colon cancer cells. HT-29 cells were exposed to different concentrations of 5-fluorouracil (5-FU) (A) and hexahydrocurcumin (HHC) (B) for 24-48 h and then cell viability was measured by 5-diphenyltetrazolium bromide assay. 5-FU or HHC significantly decreased cell viability concentration-dependently. Data are expressed as mean ± SE. $^aP < 0.05$ *vs* control ($n = 3$).

represented by IC_{50}. The respective IC_{50} values for 24 and 48 h of HHC exposure were 77.05 ± 1.53 and 56.95 ± 2.75, and for 5-FU were 39.13 ± 2.32 and 38 ± 2.21.

To determine the antiproliferative effect of HHC combined with 5-FU, the HT-29 colon cancer cells were exposed to 5-FU at 5 or 25 μmol/L in combination with HHC at 5 or 25 μmol/L concurrently for 24 h and 48 h and then evaluated by MTT assay. After 24 and 48 h of exposure to two doses of HHC (5 and 25 μmol/L) and high dose of 5-FU (25 μmol/L), marked inhibition of the growth of HT-29 colon cancer cells was observed when compared with those treated with 5-FU or HHC alone. Furthermore, exposure to low dose of 5-FU (5 μmol/L) together with high dose of HHC (25 μmol/L) for 24 and 48 h markedly inhibited the viability of HT-29 colon cancer cells when compared with those treated with HHC or 5-FU alone (Figure 3). It was noteworthy that the inhibitory effect of HHC at 25 μmol/L with a low dose of 5-FU was not different from its effect in combination with a high dose of 5-FU. These results indicated that a low dose of 5-FU could effectively be used in combination with high dose of HHC to inhibit the growth of HT-29 colon cancer cells. In addition, the interactions of these two drugs at 25 μmol/L HHC and 5 μmol/L 5-FU for 24 and 48 h were evaluated by the CI at the IC_{50} level as described in materials and methods. The combination of

25 μmol/L HHC with 5 μmol/L 5-FU for 24 and 48 h resulted in a synergistic effect (CI < 1) on the inhibition of the growth of HT-29 colon cancer cells when CI = 0.46 ± 0.01 and 0.57 ± 0.02, respectively.

Combination therapy down-regulation of COX-2 mRNA expression

The MTT results showed that a low dose of 5-FU (5 μmol/L) together with HHC at 25 μmol/L decreased the viability of HT-29 colon cancer cells to a markedly greater extent than 5-FU or HHC exposure alone. Moreover, this treatment showed the quantitative synergistic inhibitory effect; therefore, this combination was chosen to further investigate the effects on COX-2 mRNA expression in HT-29 cells. In this study, we observed that 5-FU alone did not decrease the COX-2 mRNA. However, 5-FU combined with HHC markedly reduced the COX-2 expression compared to HHC or 5-FU alone ($P < 0.05$) (Figure 4). Furthermore, we also observed the combination's effects on the expression of COX-1 mRNA. The result indicated that the level of COX-1 was not altered by treatment with 5-FU or HHC alone, or their combination.

Inhibition of COX-2 protein by the combination of 5-FU with HHC

To investigate the combination's effect on the level of COX-2 protein, HT-29 colon cancer cells were treated with 5-FU (5 μmol/L) together with HHC (25 μmol/L). The results are shown in Figure 5. It is evident that the combination treatment of 5-FU and HHC reduced the COX-2 protein level to a markedly greater degree than did 5-FU or HHC alone ($P < 0.05$). We further examined the combination's effect on the level of COX-1 protein. The results show that 5-FU alone, HHC alone or 5-FU together with HHC did not alter the level of COX-1 protein.

DISCUSSION

The toxicity and the resistance to 5-FU have been major obstacles in successful colorectal cancer chemotherapy. In addition, COX-2 up-regulation is directly involved in colorectal carcinogenesis[25,26]. Therefore, a combined treatment of 5-FU with non-toxic agents that can inhibit COX-2 activity might be useful for treating colon carcinogenesis.

Curcumin, a specific COX-2 inhibitor, is usually used in combination with traditional chemotherapy or other regimens both *in vitro*, *in vivo* and also in clinical studies of colon cancer. Many reports have indicated that curcumin enhanced the cytotoxic effect of celecoxib in several human colon cancer cell lines[27]. This effect has also been observed in colorectal cancer models[28]. Moreover, curcumin enhances the cytotoxic effect of 5-FU and decreases the COX-2 protein expression better than single agent treatment[21]. However, several studies over the past three decades have reported that curcumin exhibits

Figure 3 Combination effects of 5-fluorouracil and hexahydrocurcumin on proliferation of HT-29 colon cancer cells. The HT-29 cells were exposed to 5-fluorouracil (5-FU) (5 and 25 μmol/L), hexahydrocurcumin (HHC) (5 and 25 μmol/L) and their combination for 24 h (A) and 48 h (B) and then harvested for 5-diphe-nyltetrazolium bromide assay. Combination treatment of 5-FU and HHC significantly decreased cell viability compared to the 5-FU or HHC monotherapy. Data are expressed as mean ± SE. [a]*P* < 0.05 *vs* 5-FU or HHC monotherapy (*n* = 3).

poor bioavailability due to poor absorption, low serum levels and rapid metabolism. Therefore, the pharmacological activity of curcumin may be mediated in part by its metabolites[29,30].

In the present study, we investigated the anti-carcinogenic effects of HHC on growth of HT-29 human colon cancer cells. We found that HHC was sufficient for inhibiting the growth of human colon cancer cells by decreasing the viability of HT-29 cells through down-regulation of COX-2 mRNA and protein expression. Moreover, HHC did not alter COX-1 mRNA and protein expression. These results agreed with previous study, which suggested that HHC could sensitize the cancer cells to chemotherapeutic drugs by decreasing phorbol ester-induced PGE$_2$ production in HCECs[20]. PGE$_2$ is a major

product of COX-2 enzymes. Moreover, it has been demonstrated that HHC exhibited higher antioxidant activity than its curcumin progenitor[18] and it has been suggested that the hydrogenation at the conjugated double bonds of the central seven-carbon chain and a keto group of the β-diketone of curcumin to HHC markedly enhances antioxidant activity.

Based on these results, it is reasonable to assume that HHC is a safe and effective agent for the treatment of colon cancer, and that this compound is more stable than curcumin. The present investigation deals with the study of the combination effect of HHC with 5-FU chemotherapy drug. Our results indicated that HHC in combination with even a low concentration of 5-FU (5 μmol/L) was sufficient to decrease the viability of HT-29 colon

A

COX-2 (305 bp)

GAPDH (200 bp)

B

Figure 4 Combination effects of hexahydrocurcumin and 5-fluorouracil on cyclooxygenase-2 mRNA expression in HT-29 colon cancer cells. The HT-29 cells were exposed to 5-fluorouracil (5-FU) (5 μmol/L), hexahydrocurcumin (HHC) (25 μmol/L) and their combination for 24 h. Cells from different experimental groups were subjected to semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). A: RT-PCR showed that the expression of cyclooxygenase (COX)-2 (305 bp) was significantly decreased in HHC or combination of HHC and 5-FU-treated group; B: The expression of COX-2 was determined by normalizing the band intensity of COX-2 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values are expressed as percentage of control (mean ± SE), [a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* 5-FU or HHC monotherapy ($n = 3$).

A

COX-2 (72 kDa)

β-actin (34 kDa)

B

Figure 5 Combination effects of hexahydrocurcumin and 5-fluorouracil on cyclooxygenase-2 protein expression in HT-29 colon cancer cells. A: Western blotting with polyclonal anti-cyclooxygenase (COX) 1 (70 kDa) and anti-COX2 (72 kDa), and anti-β actin (43 kDa) antibodies; B: Fold changes of proteins as represented below, each band was measured by Lab Image software. Combination treatment of 5-fluorouracil (5-FU) and hexahydrocurcumin (HHC) significantly decreased the expression of COX-2 protein with no effect on the expression of COX-1 protein. Values are expressed as percentage of control (mean ± SE), [a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* 5-FU or HHC monotherapy ($n = 3$).

cancer cells. Moreover, this combination showed a synergistic effect on antiproliferation against colon cancer cells (CI < 1) and down-regulated the expression of COX-2 mRNA and protein, compared to either 5-FU or HHC monotherapy. Based on these results, we suggest that the addition of HHC could enhance the inhibiting effects of 5-FU on the growth of HT-29 human colon cancer cells. Moreover, the combination of these drugs that are different in their modes of action could prove much safer and more effective than cancer monotherapy[27]. In addition, this combination did not alter the COX-1 mRNA and protein levels. It is thus reasonable to assume that this therapeutic approach may have no toxic effect and enables the use of 5-FU chemotherapy drug at a lower and safer concentration, which would be highly desirable for long-term treatment of colon cancer.

In conclusion, this study illustrates that HHC is a specific COX-2 inhibitor that plays an important role in carcinogenesis. It has been demonstrated for the first time in this study that the addition of HHC to 5-FU standard chemotherapy in HT-29 human colon cancer cells can enhance the growth inhibition and down-regulation of COX-2. Thus, the combination of these two drugs should have a great potential to be used to treat human colon cancer.

COMMENTS

Background
Colorectal cancer is a major cause of cancer death in many countries. The toxicity of 5-fluorouracil (5-FU) chemotherapeutic drug for normal cells and resistance to this drug are major barriers to successful cancer chemotherapy.

Research frontiers
Combining 5-FU with other regimens and agents, such as curcumin, is a strategy to enhance the efficacy of 5-FU and also to reduce its toxicity. Curcumin down-regulates cyclooxygenase (COX)-2, which is highly expressed in human colon cancer, but it does not alter the level of COX-1, a housekeeping enzyme. However, curcumin is only slightly absorbed in the gastrointestinal tract due to its poor solubility in water. Therefore, hexahydrocurcumin (HHC), one of the major curcumin metabolites, was synthesized to solve these problems.

Innovations and breakthroughs
This report has highlighted the importance of HHC which could improve the effectiveness of the 5-FU chemotherapeutic drug. In this study, the authors demonstrate that HHC is a specific COX-2 inhibitor. HHC down-regulates COX-2 expression, but it does not alter the level of COX-1. This is the first study to report the anti-carcinogenic effects of HHC on growth of human colon cancer cells. Furthermore, HHC has ability to enhance 5-FU in inhibiting the growth of HT-29 human colon cancer cells and down-regulates the expression of COX-2 mRNA and protein.

Applications
This report illustrates the action of HHC combined with 5-FU, and this study may represent a future strategy to provide much safer and more effective treatment for patients with colon cancer.

Terminology
HHC is one of the major curcumin metabolites. Curcumin is the major yellow pigment in turmeric which is obtained from the rhizome of *Curcuma longa* L, traditionally used in cooking in India and Southeast Asia.

Peer review
This is a good descriptive study in which authors investigate ability of HHC to enhance 5-FU in inhibiting the growth of HT-29 cells by focusing on COX-2 expression. The results are interesting and suggest that HHC together with 5-FU exerted a synergistic effect and may prove chemotherapeutically useful in treating human colon cancer.

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BRIEF ARTICLE

A randomized open-label trial of on-demand rabeprazole *vs* ranitidine for patients with non-erosive reflux disease

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Abstract

AIM: To compare the efficacy of the proton-pump inhibitor, rabeprazole, with that of the H$_2$-receptor antagonist, ranitidine, as on-demand therapy for relieving symptoms associated with non-erosive reflux disease (NERD).

METHODS: This is a single center, prospective, randomized, open-label trial of on-demand therapy with rabeprazole (group A) *vs* ranitidine (group B) for 4 wk. Eighty-three patients who presented to the American University of Beirut Medical Center with persistent gastroesophageal reflux disease (GERD) symptoms and a normal upper gastrointestinal endoscopy were eligible for the study. Patients in group A (n = 44) were al-lowed a maximum rabeprazole dose of 20 mg twice daily, while those in group B (n = 39) were allowed a maximum ranitidine dose of 300 mg twice daily. Efficacy was assessed by patient evaluation of global symptom relief, scores of the SF-36 quality of life (QoL) questionnaires, total number of pills used, and number of medication-free days.

RESULTS: Among the 83 patients who were enrolled in the study, 76 patients (40 in the rabeprazole group and 36 in the ranitidine group) completed the 4-wk trial. Baseline characteristics were comparable between both groups. After 4 wk, there was no significant difference in the subjective global symptom relief between the rabeprazole and the ranitidine groups (71.4% *vs* 65.4%, respectively; P = 0.9). There were no statistically significant differences between mean cumulative scores of the SF-36 QoL questionnaire for the two study groups (rabeprazole 22.40 ± 27.53 *vs* ranitidine 17.28 ± 37.06; P = 0.582). There was no significant difference in the mean number of pills used (rabeprazole 35.70 ± 29.75 *vs* ranitidine 32.86 ± 26.98; P = 0.66). There was also no statistically significant difference in the mean number of medication-free days between both groups.

CONCLUSION: Rabeprazole has a comparable efficacy compared to ranitidine when given on-demand for the treatment of NERD. Both medications were associated with improved quality of life.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic, remitting-relapsing medical condition that involves the reflux of gastric contents into the esophagus causing a multitude of unpleasant symptoms including heartburn, sore throat, chest pain, cough, and regurgitation. GERD has been shown to have a significant negative impact on the quality of life (QoL) of affected patients and may even impair their daily activities[1]. The prevalence of GERD has markedly increased over the past two decades affecting 20%-40% of the western population[2-5]. This prevalence is predicted to rise further with time[6].

Non-erosive reflux disease (NERD) has been defined by the Vevey NERD Consensus Group as a subcategory of GERD that is characterized by reflux-related symptoms with the absence of esophageal mucosal erosions or breaks at conventional endoscopy and without recent acid-suppressive therapy[7]. About two-thirds of patients with typical GERD symptoms, such as heartburn, belching, cough, nausea, sore throat and voice changes, have no erosive changes on upper gastrointestinal endoscopic evaluation[2]. The complex pathophysiology of NERD and the exact mechanisms by which the associated symptoms are caused remain unclear[7]. It is highly evident, however, that the degree of acidity and duration of esophageal acid exposure play an essential role in NERD symptomatology. These factors are not different from the precipitants of moderate erosive reflux disease (ERD)[7-11].

The majority of GERD patients use acid-suppressive medications to control their symptoms. Unlike ERD, symptoms of NERD are more difficult to control, and tend to have a lower response rate, even to the most potent proton-pump inhibitors (PPIs)[12,13]. Nonetheless, initial management of NERD is similar to that of GERD, and includes the use of a PPI or H_2-receptor antagonist (H2RA). Around 75% of patients with NERD report a relapse of their symptoms after cessation of the initial therapy. Therefore, long-term management is often needed to maintain symptom control[1]. The preferred maintenance therapeutic strategy is supposed to utilize the least amount of medication. Hence, on-demand therapy should be a reasonable therapeutic mode for managing patients with NERD symptoms. By convention, medications for on-demand therapy should ensure a rapid onset of action in order to give instantaneous relief.

To our knowledge, and after reviewing the literature, no clinical studies have compared a PPI to an H2RA as on-demand therapy for NERD. The objective of our trial is to compare the efficacy of a PPI, rabeprazole, to that of a standard H2RA, ranitidine, as an on-demand therapy for treating patients with NERD. We selected rabeprazole among the various currently available PPIs because it results in a faster and a more prompt acid suppression and has the advantage of a first-dose symptom relief[14-16]. H2RAs including ranitidine are widely used on-demand for the management of GERD symptoms.

MATERIALS AND METHODS

The study was a 4-wk prospective, randomized, single center, open-label trial of on-demand therapy with rabeprazole *vs* ranitidine for patients with NERD. Patients who were considered eligible for enrollment had to be older than 18 years of age, had persistent GERD symptoms (typical or atypical), and a negative upper gastrointestinal endoscopy exam. Exclusion criteria were age below 18 years and older than 75 years, allergy to rabeprazole or ranitidine, any degree of esophagitis or mucosal damage on upper gastrointestinal endoscopy, pregnancy, and any use of PPI or H2RA within 2 wk of enrollment into the study.

Patients who presented to the American University of Beirut Medical Center with persistent GERD symptoms and a normal upper gastrointestinal endoscopy were considered candidates for the study. After signing a written informed consent, patients were asked to complete a baseline SF-36 QoL questionnaire. They were randomized by an independent investigator using a computer-generated random number table to one of two groups: those to receive rabeprazole (group A) and those to receive ranitidine (group B). Patients in group A were allowed a maximum oral rabeprazole dose of 20 mg (10 mg tablets, twice daily), while those in group B were allowed a maximum oral ranitidine dose of 300 mg (150 mg tablets, twice daily). A research fellow was responsible for contacting patients by phone on a daily basis over the 4-wk study period to assess for the number of pills taken, the need for a rescue medication, as well as the occurrence of any side effects. At the end of the 4 wk, patients were asked about their global symptom relief and were also requested to answer the post-treatment QoL questionnaire.

Primary efficacy endpoints were assessed by the subjective global symptom relief, total number of pills used, number of medication-free days, and the need for rescue medications. Secondary endpoints included the scores of the QoL questionnaires and the occurrence of side effects. The study protocol and informed consent were approved by the Institutional Review Board at the American University of Beirut Medical Center.

Statistical analysis

Analysis of the primary end-point (global symptom relief) was done according to intent-to-treat (ITT) basis.

Table 1 Patient baseline characteristics *n* (%)

	Group A (*n* = 44)	Group B (*n* = 39)	*P* value
Age (mean ± SD) (yr)	45.4 ± 15.2	45.1 ± 15.3	0.916
Gender M:F	16:28	16:23	0.663
Duration of symptoms (yr)	4.68 ± 7.79	3.85 ± 4.21	0.552
Esophageal manifestations	28 (63.6)	29 (74.4)	0.293
Regurgitation and heartburn	21 (47.7)	23 (59.0)	0.306
Epigastric pain	8 (18.2)	9 (23.1)	0.581
Nausea	7 (15.9)	6 (15.4)	0.948
Vomiting	0 (0)	1 (2.6)	0.285
Extra-esophageal manifestations	38 (86.4)	33 (84.6)	0.821
Chest pain	16 (36.4)	10 (25.6)	0.293
Globus	24 (54.5)	16 (41.0)	0.219
Hoarseness	15 (34.1)	10 (25.6)	0.402
Metallic taste	22 (50.0)	16 (41.0)	0.413
Nocturnal cough	17 (38.6)	09 (23.1)	0.127
Dyspnea	21 (47.7)	20 (51.3)	0.746
Sore throat	20 (45.5)	13 (33.3)	0.260
Thick sputum production	24 (54.5)	13 (33.3)	0.052
Mixed symptoms	25 (56.8)	24 (61.5)	0.663

Group A, rabeprazole; Group B, ranitidine. M: Male; F: Female.

The association between the drug group and response for binary measures was assessed using the Fischer's Exact test. For continuous measures, either a two sample *t*-test or Wilcoxon two sample rank sum test were used depending on whether normality held or not. Frequency tables and cross-tabulations were derived in order to depict any associations between the different variables. The paired samples *t*-test and the independent-samples *t*-test were used to compare the QoL score before and after treatment. A *P*-value at or below 0.05 was significant. The data were entered and analyzed using SPSS for Microsoft version 18.0 (SPSS Inc, United States).

Sample size calculation: The sample size was estimated based on an expected response rate of 80% for rabeprazole and 50% for ranitidine. Therefore, we had to include 42 patients in each arm of the study to detect a statistical significance using a power of 80% and a margin of error of 5%.

RESULTS

A total of 83 patients with symptoms consistent with GERD and a negative upper gastrointestinal endoscopy were enrolled in the study. The random assignment of patients into two arms resulted in 44 patients (53%) in group A assigned to receive rabeprazole, and 39 patients (47%) in group B assigned to receive ranitidine. Overall, seven patients dropped out of the study; two patients because of mild medication side effects (both of whom were receiving rabeprazole) and five were lost to follow-up. Seventy-six patients completed the 4-wk trial per-protocol, 40 of whom were assigned to the rabeprazole group and 36 to the ranitidine group. The ITT population consisted of 83 patients.

Baseline characteristics between both groups were

Table 2 Subjective global symptom assessment *n* (%)

	Better	Same	Worse	Total
Group A	20 (71.4)	7 (25.0)	1 (3.6)	28
Group B	17 (65.4)	8 (30.8)	1 (3.8)	26
Total	37	15	2	54

P = 0.9; Group A, rabeprazole; Group B, ranitidine.

comparable (Table 1). The mean age of individuals in group A was 45.43 ± 15.16 years *vs* 45.08 ± 15.29 years for those in group B. There was a slight female predominance in both groups: 63.6% in group A and 59% in group B. Most patients had been symptomatic for more than 1 year, with duration ranging from 3 mo to 20 years. The majority of patients (85.5%) suffered from extra-esophageal manifestations, with globus sensation, metallic taste, shortness of breath, as well as thick sputum production being the commonest among these manifestations. Esophageal manifestations were encountered in approximately 69% of patients.

Global symptom improvement was subjectively assessed at the end of the 4-wk treatment phase. Improvement was reported in 71.4% (20 out of 28) of patients in group A and 65.4% (17 out of 26) of patients in group B, while worsening of symptoms was noted in 3.6% and 3.8 % of patients in group A and B, respectively (Table 2). Of the patients in group A, 25% experienced no change in symptoms compared to 30.8% of group B patients (*P* = 0.889). There was no difference in gender distribution between responders and non-responders in either group. However, the mean age of responders to rabeprazole was significantly lower than that of patients who did not respond to this drug (38.90 ± 13.15 years *vs* 53.14 ± 17.68 years; *P* = 0.033). This difference was not noted in patients who received ranitidine.

Regarding the mean number of pills consumed, patients in group A used a mean number of 35.70 ± 29.75 pills of rabeprazole 10 mg, while group B patients consumed a mean number of 32.86 ± 26.98 pills of ranitidine 150 mg, (*P* = 0.66). The mean number of medication-free days was 13.08 ± 9.73 in group A, and 10.78 ± 10.14 in group B (*P* = 0.32).

The SF-36 QoL questionnaire was completed by a total of 65 patients at baseline: 37 in group A (84.1%) and 28 in group B (71.8%). Mean cumulative scores at baseline were comparable between groups A and B, 106.38 ± 30.30 *vs* 104.43 ± 29.82, respectively (*P* = 0.797) (Figure 1). A follow-up SF-36 QoL questionnaire was obtained from 50 patients immediately after the 4-wk treatment phase: 25 were in group A (62.5%) and 25 in group B (69.4%). Mean cumulative scores at follow-up were 135.32 ± 32.19 for group A and 122.76 ± 37.48 for group B (*P* = 0.210). Mean scores of both groups increased significantly (*P* < 0.01 for group A and *P* = 0.028 for group B, compared to respective baseline scores). However, the absolute score differences between baseline and 4 wk for groups A and B were (22.40 ± 27.53

Figure 1 Mean quality of life scores at baseline and immediately after treatment (*n* = 25 at baseline). Group A, rabeprazole; Group B, ranitidine.

vs 17.28 ± 37.06, respectively; *P* = 0.582), indicating that both drugs improve patient QoL to the same extent.

DISCUSSION

The present study compares the efficacy of the PPI rabeprazole to that of the H2RA ranitidine, as an on-demand option in the management of patients with NERD. The impact on QoL was also evaluated as a secondary outcome with both drug regimens.

Analysis of our data showed that rabeprazole is as effective as the routinely and commonly used ranitidine in controlling both the typical and atypical symptoms in patients with NERD. Evidence from several previous studies supports our findings regarding the efficacy of anti-secretory (H2RA and PPI) therapy in patients with NERD[17-24]. However, no head-to-head comparison between any H2RA and PPI had been carried out in the setting of on-demand therapy for NERD.

Our PubMed® search identified a number of clinical trials[14,21-25] that assessed the efficacy of various PPIs (rabeprazole, omeprazole, esomeprazole, lansoprazole) when given as on-demand in patients with NERD. Almost all of these studies were conducted over a period of time ranging from 3 mo to 6 mo and preceded by initial short periods (around 4-8 wk) of daily treatment with the designated PPI to achieve complete symptom resolution. A study by Lind *et al*[21] randomized 424 patients with NERD to one of three groups: omeprazole 20 mg, omeprazole 10 mg, or placebo. After 6 mo of on-demand therapy, it was concluded that omeprazole was effective in the majority of NERD patients. In another placebo-controlled study, Talley *et al*[22] assigned 342 patients to either esomeprazole 20 mg or placebo. On-demand therapy with esomeprazole 20 mg was found suitable for the long-term symptom management of NERD patients. In a separate placebo-controlled trial, Talley *et al*[23] assessed the efficacy of on-demand therapy with esomeprazole 40 mg or 20 mg in patients with NERD and showed that both dosages were superior to placebo in controlling heartburn in those patients. Bytzer *et al*[24] achieved favorable results in

a 6-mo trial of on-demand rabeprazole 10 mg in patients with NERD. Five hundred and twenty-three patients with NERD were given 4 wk of rabeprazole 10 mg once daily. The 432 patients who had complete resolution of their symptoms were then randomized for the on-demand phase of the study to two groups: rabeprazole 10 mg and placebo. Symptom relief was significantly better in the rabeprazole group compared to the placebo group.

Rabeprazole was also investigated as on-demand treatment by Ponce *et al*[25] in patients with NERD and low-grade esophagitis. Symptom control was maintained in over 85% of patients during six months of on-demand rabeprazole 20 mg therapy, following a 4-wk daily run-in period with rabeprazole 20 mg per day. During the study period, PPI consumption was found to be low and patient satisfaction with the treatment was high.

Rabeprazole appeared to be ideal for our study given its rapid onset of action and powerful acid suppression[14-16]. Studies involving NERD patients have documented its superiority over placebo. In addition, on-demand use of rabeprazole for the management of NERD incurs the least cost in comparison with other PPIs[26].

H2RAs have been widely used on-demand for patients with GERD. Clinical studies have demonstrated that, when given on-demand, they are superior to placebo in controlling heartburn in this group of patients[18,19,27]. These findings may be extrapolated to NERD patients who constitute the majority of patients with GERD. H2RAs are known to have a rapid onset, but a short duration of action. They suppress acid for approximately 4 to 8 h and produce incomplete inhibition of post-prandial gastric acid secretion. They inhibit acid secretion by up to 70% over a 24-h period. A major disadvantage of using these drugs is the development of tolerance that occurs within two weeks of uninterrupted daily intake[28]. Thus, tolerance would be less concerning if they are to be used on an on-demand basis[29]. PPIs have the advantage over H2RA in controlling both basal and food-stimulated acid secretion producing a longer-lasting acid suppression in addition to the fact that tolerance has not been observed with PPIs.

Our pilot study has a few limitations. One is the open-label nature of the study. Although patients were randomized to different arms, they were aware of the arm they were randomized to. This may have created some bias especially if those patients were previously treated with the same medication class that they were assigned to. The sample size was also relatively small and further investigation based on a larger number of patients is necessary to corroborate our data. Our study duration was short, then again the purpose of our study was not to prove the efficacy of either of the two drugs, but rather to compare them.

The advantages of our study include the fact that it is the first to compare an H2RA to a PPI in the setting of on-demand therapy for NERD. We also showed response to on-demand therapy for both typical and atypical reflux symptoms. Finally, this is a "pure" on-demand study, in

the sense that it was performed without a preceding continuous anti-secretory treatment period.

In conclusion, rabeprazole and ranitidine have been shown to be comparable in efficacy when given on-demand for the treatment of NERD. Both medications were associated with a statistically significant improved quality of life.

COMMENTS

Background
Non-erosive reflux disease (NERD) is the most prevalent of the subcategories of gastroesophageal reflux diseases (GERD). It is a chronic condition with a significant impact on patients' quality of life. On-demand acid-suppressive agents constitute the mainstay in the management of NERD. The most favorable of such agents should be fast-acting, long-lasting, potent, and safe, in order to reach an effective and timely symptom control.

Research frontiers
Acid-suppressive medications differ in terms of power of acid inhibition, onset and duration of action, and drug interactions. Identification of the ideal agent in the context of chronic and on-demand therapy is of utmost interest to patients and researchers alike.

Innovations and breakthroughs
The authors compared the efficacy of the proton-pump inhibitor (PPI) rabeprazole, to that of the H2-receptor antagonist ranitidine, as on-demand therapy for relieving symptoms associated with NERD. They concluded that both possessed comparable efficacy and were associated with an improved quality of life. Thus, a fast-acting PPI, rabeprazole, is an effective on-demand option for the management of NERD.

Applications
In conditions where an acid-suppressive medication is to be prescribed for a long period of time, recognizing the agent that has the best efficacy and safety profiles would have a prompt and significant clinical impact.

Terminology
NERD is a non-erosive reflux disease, commonly defined as the presence of classic GERD symptoms in the absence of esophageal mucosal injury during upper endoscopy. The majority of patients with GERD fall into the NERD subcategory.

Peer review
The article is novel and interesting. It answers an important research question, which might result in changing clinical practice of NERD management. The design is appropriate, and the manuscript is well written.

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BRIEF ARTICLE

Poorly expandable common bile duct with stones on endoscopic retrograde cholangiography

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in Chang Gung Memorial Hospital. Patients with characteristic PE bile duct on ERC were identified from the database. Data of the patients as well as the safety and technical success of therapeutic ERC were collected and analyzed retrospectively.

RESULTS: A total of 30 patients with CBD stones and characteristic PE segments were enrolled in this study. The median patient age was 45 years (range, 20 to 92 years); 66.7% of the patients were men. The diameters of the widest non-PE CBD segment, the PE segment, and the largest stone were 14.3 ± 4.9 mm, 5.8 ± 1.6 mm, and 11.2 ± 4.7 mm, respectively. The length of the PE segment was 39.7 ± 15.4 mm (range, 12.3 mm to 70.9 mm). To remove the CBD stone(s) completely, mechanical lithotripsy was required in 25 (83.3%) patients even though the stone size was not as large as were the difficult stones that have been described in the literature. The stone size and stone/PE segment diameter ratio were associated with the need for lithotripsy. Post-ERC complications occurred in 4 cases: pancreatitis in 1, cholangitis in 2, and an impacted Dormia basket with cholangitis in 1. Two (6.7%) of the 28 patients developed recurrent CBD stones at follow-up (50 ± 14 mo) and were successfully managed with therapeutic ERC.

CONCLUSION: Patients with a PE duct frequently require mechanical lithotripsy for stones extraction. To retrieve stones successfully and avoid complications, these patients should be identified during ERC.

Abstract

AIM: To describe characteristics of a poorly expandable (PE) common bile duct (CBD) with stones on endoscopic retrograde cholangiography.

METHODS: A PE bile duct was characterized by a rigid and relatively narrowed distal CBD with retrograde dilatation of the non-PE segment. Between 2003 and 2006, endoscopic retrograde cholangiography (ERC) images and chart reviews of 1213 patients with newly diagnosed CBD stones were obtained from the computer database of Therapeutic Endoscopic Center

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INTRODUCTION

Endoscopic retrograde cholangiography (ERC) with endoscopic sphincterotomy (ES) and stone extraction are considered standard therapies for the treatment of common bile duct (CBD) stones[1-3]. After ES, 85% to 90% of CBD stones can be removed with a Dormia basket or balloon catheter[4,5]. However, removal of CBD stones can be challenging in certain cases, such as those involving large stones (> 15 mm), stones above strictures, and impacted stones[6]. In cases involving such difficult stones, fragmentation using mechanical lithotripsy or shock wave lithotripsy is required to facilitate stone extraction[6-8]. However, mechanical lithotripsy for extraction of CBD stones can be unsuccessful in the presence of bile duct stricture, when the size of the stone is large, and when the ratio of stone size to bile duct diameter is greater than 1[9-11]. Impaction of an extraction basket and an entrapped stone in the distal CBD may complicate stone clearance in some cases[12-14].

In contrast, anatomic abnormality of the CBD has been considered to be a contributing factor to difficult stones[15]. Kim *et al*[16] recently reported that complete clearance of CBD stones was technically difficult in patients with acute distal CBD angulation ($\leqslant$ 135 degrees) and a short distal CBD arm ($\leqslant$ 36 mm). However, the current definition of large (or difficult) CBD stone does not include the factor of distal CBD diameter[17]. In our clinical experience, we have encountered a subgroup of patients whose CBD stones were particularly difficult to extract. Further, in some cases, the extraction basket containing the entrapped stone was impacted in the bile duct if mechanical lithotripsy was not applied. The common characteristic feature of these patients was a relatively narrowed distal CBD on ERC: the distal CBD was almost normal in diameter, but it was poorly expandable (PE), as the upstream CBD was disproportionately dilated. The aim of this study was to describe the clinical and imaging features and to examine the safety and technical success of ERC in patients with both CBD stones and a PE bile duct.

MATERIALS AND METHODS

Description of poorly expandable bile duct

A PE bile duct was characterized by a rigid distal CBD with an almost normal diameter on ERC. The non-PE segment of CBD was often more dilated than the PE segment (Figure 1). The rigid characteristic of the PE segment was usually confirmed by increased resistance when the balloon or basket catheter with the stone was pulled from the non-PE segment across the PE segment.

Patients

During the 4-year period between 2003 and 2006, we retrospectively collected data from 1213 patients with CBD stones from the endoscopic computer database of Change Gung Memorial Hospital, Taipei (China). Endoscopic retrograde cholangiopancreatography (ERCP) images of patients with newly diagnosed CBD stones were reviewed, and 30 (2.5%) of the patients with the characteristics of a PE bile duct were enrolled in this study. The subjects included 20 men and 10 women. The median patient age was 45 years (range, 20 to 92 years). Chart records and the safety and technical success of ERC in these patients were reviewed. None of the patients had a medical history of chronic pancreatitis or biliary stricture prior to ERCP. The clinical diagnoses were obstructive jaundice in 13 cases, acute cholangitis in 10 cases, acute cholecystitis in 3 cases, acute pancreatitis in 1 case, and others in 3 cases. The gallbladder status of the patients was as follows: previously resected in 6 (20%) cases, intact with stone(s) in 23 (76.7%) cases, and intact without stones in 1 (3.3%) case. There were 2 (6.7%) patients with low insertion of the cystic duct and 6 (20%) patients with juxtapapillary diverticulum.

Endoscopic procedures

Endoscopic procedures were performed by endoscopists with an ERCP case volume of at least 2 per week, using a duodenoscope (JF-240 or TJF-240; Olympus). The ES procedure was performed as mentioned in our previous study[18]. After ES, a retrieval balloon catheter ($n = 9$), Dormia basket ($n = 5$), or lithotripter (BML-202Q.B or BML-203Q.B, Olympus Corporation, Tokyo, Japan, $n = 16$) was used to extract the CBD stone(s). When the balloon catheter or Dormia basket failed to retrieve the stone(s), a lithotripter was used to crush and extract the stones. After stone removal, a contrast medium was injected, and the inflated balloon catheter was withdrawn along the CBD to the duodenum to confirm bile duct clearance.

Measurements of parameters on cholangiograms

The maximum transverse diameter of the PE segment, non-PE CBD, and largest stones as well as the length of PE segment and the distal CBD angle were assessed on the cholangiography. The length of the PE segment was defined as the length between the duodenal wall and the tapering end of the non-PE CBD (Figure 1). The distal CBD angle was defined as the angle between the axis of the non-PE CBD and the PE segment (Figure 1A). These factors were measured from the ERC scan, which was obtained under the condition of full contrast injection

Figure 1 Endoscopic retrograde cholangiogram showing the poorly expandable distal common bile duct (arrows) and the more dilated upstream bile duct. A: The arrowhead indicates the stone. Line a and line b indicate the length of the non-poorly expandable bile duct and the poorly expandable segment. The actual lengths were measured after correction for the magnification with the known diameter of the duodenoscope on the endoscopic retrograde cholangiography (line c). The distal common bile duct angle was defined as the angle formed by line a and line b; B: The stone (arrowhead) is floating in the non-poorly expandable bile duct.

with the patient in a prone position and the duodenoscope in a shortened configuration. The parameters were measured after correction for the magnification using the known diameter of the duodenoscope on the ERC.

Statistical analysis

Data in the text and tables are expressed as the mean ± SD values. The difference in the ratio of the diameters of the non-PE segment to the PE segment and the stone size to the PE segment were compared using the Mann-Whitney U test. Further, the difference was compared with the two-sample t test for continuous variables and Fisher's exact test for categorical variables. The analyses were performed with SPSS version 17.0 statistical software for Windows. A P value of < 0.05 was considered statistically significant.

RESULTS

A total of 30 patients with PE bile ducts who underwent a combined total of 52 ERC procedures were analyzed. The baseline characteristics of these patients are listed in Table 1. The serum total bilirubin level before ERC was 6.5 ± 3.6 mg/dL (range, 0.6 to 15 mg/dL). The maximal diameter

of the non-PE CBD was 14.3 ± 4.9 mm (range, 7 mm to 26 mm). The diameter and the length of the PE segment were 5.8 ± 1.6 mm (range, 4 mm to 10 mm) and 39.7 ± 15.4 mm (range, 12.3 mm to 70.9 mm), respectively. The distal CBD angle was 159.3 ± 13.9 degree (range, 130 degree to 175 degree). The number of stones was one in 18 cases, two in 4 cases, three in 7 cases, and five in 1 case. The average diameter of the largest stone from each case was 11.3 ± 4.7 mm (range, 6 mm to 24 mm). Five patients had stones > 15 mm in diameter. Cholesterol stones were found in 16 cases, black stones in 11 cases, and brown stones in 3 cases. The ratio of the maximal diameter of the non-PE CBD to the PE segment was 2.4 (range, 1.4 to 3.5). The ratio of the diameter of the largest stone to the PE segment was 1.8 (range, 0.9 to 4).

In all patients, the CBD stones were eventually retrieved successfully. The number of ERC procedures needed to completely remove the CBD stone(s) was one in 16 (53.3%) cases, two in 8 (26.7%) cases, three in 5 (16.7%) cases, and five in 1 (3.3%) case. The reasons for performing more than one ERC procedure to completely extract the CBD stones were as follows: incomplete clearance of the CBD stones during the first ERC procedure (n = 8), referral from other institutions due to failure to extract the CBD stones (n = 3), recurrent CBD stones due to migration of the cystic duct stones (n = 2), and occurrence of Mirizzi syndrome (n = 1).

Overall, mechanical lithotripsy was performed in 25 (83.3%) patients, including 12 out of the 16 patients undergoing a single session of ERC and 13 out of the 14 patients undergoing multiple (≥ 2) sessions of ERC (4 of the 13 patients did not undergo lithotripsy during the first ERC procedure). Five patients did not require mechanical lithotripsy. Comparative data for the patients with and without lithotripsy for CBD stone clearance are given in Table 1. The factors used for analysis were age; gender; total serum level of bilirubin; stone size, number, and characteristics; history of cholecystectomy; presence of juxtapapillary diverticulum; diameter of the non-PE segment and the PE segment; length of the PE segment; distal CBD angle; and the diameter ratio of the non-PE segment to the PE segment and of the stone to the PE segment. Of the various factors analyzed, the stone size and the ratio of the diameter of the stone to the PE segment were significantly greater in patients who needed lithotripsy (P = 0.04 and 0.02, respectively).

Post-ERC complications occurred in 4 (13.3%) cases, including pancreatitis in 1 (3.3%), cholangitis in 2 (6.7%), and impacted Dormia basket with cholangitis in 1 (3.3%). One patient died of acute myocardial infarction 2 d after ERCP. Twenty-eight patients had long-term follow-up with a mean period of 50 ± 14 mo (range, 29 mo to 80 mo). Two (6.7%) patients developed recurrent CBD stones and were successfully managed with therapeutic ERC.

DISCUSSION

In this study, we describe the ERC findings of patients with concomitant CBD stones and a PE bile duct and the

Table 1 Characteristics of patients with a poorly expandable bile duct and comparisons between the patients with and without mechanical lithotripsy

Parameters	Overall (*n* = 30)	Need lithotripsy (*n* = 25)	Without lithotripsy (*n* = 5)	[1]*P* value
Age (yr)	47.4 ± 19.4 (20-92)	45.0 ± 19.3 (20-92)	59.0 ± 19.4 (29-76)	0.15
Gender (men) *n* (%)	20 (66.7)	17 (68)	3 (60)	1
Serum total bilirubin (mg/dL)	6.5 ± 3.6 (0.6-15)	6.7 ± 3.8 (1.1-15)	5.5 ± 3.6 (0.6-10.1)	0.55
History of cholecystectomy *n* (%)	6 (20)	5 (20)	1 (20)	1
Juxtapapillary diverticulum *n* (%)	7 (23.3)	5 (20)	2 (40)	0.57
CBD stones				
Size of the largest stone (mm)	11.3 ± 4.7 (6-24)	12.0 ± 4.8 (6-24)	7.2 ± 1.6 (6-10)	0.04
Characteristic (cholesterol/black/brown)	16/11/3	13/9/3	3/2/0	0.72
Number (≥ 2) *n* (%)	12 (40)	11 (44)	1 (20)	0.62
Bile duct diameter (mm)				
Non-PE CBD	14.3 ± 4.9 (7-26)	14.8 ± 4.9 (7-26)	11.4 ± 5.0 (7-20)	0.17
PE segment	5.8 ± 1.6 (4-10)	5.9 ± 1.6 (4-10)	5.6 ± 1.8 (4-8)	0.73
Length of the PE segment (mm)	39.7 ± 15.4 (12.3-70.9)	38.0 ± 14.8 (12.3-70.9)	45.1 ± 17.5 (22.5-70.2)	0.44
Distal CBD angle (degree)	159.3 ± 13.9 (130-175)	160 ± 12.1 (135-175)	157 ± 18.9 (130-175)	0.75
Diameter ratio of non-PE segment to PE segment	2.4 ± 0.6 (1.4-3.5)	2.5 ± 0.6 (1.5-3.5)	2.2 ± 0.4 (1.4-2.5)	0.05
Stone to PE segment	1.8 ± 0.7 (0.9-4)	2.1 ± 0.7 (1.2-4)	1.4 ± 0.4 (0.9-1.8)	0.02

CBD: Common bile duct; PE: Poorly expandable. [1]*P* value was the basis of comparison between patients with and without lithotripsy. A *P* value of < 0.05 indicated a significant difference.

impact of these findings on the retrieval of the stones. This series showed that 2.5% of patients with CBD stones had PE bile ducts. The mean diameter and length of the PE duct were 5.8 mm and 39.7 mm, respectively. The non-PE CBD was more dilated than the PE segment, with a mean diameter ratio of 2.4. Post-ERC complications occurred in 4 (13.3%) of the patients.

After ES, CBD stones up to 15 mm in diameter can usually be completely extracted with a basket or a retrieval balloon catheter[8,15]. In this study, the average diameter of the largest CBD stones was 11.3 mm, and there were only five patients (16.7%) with stones > 15 mm in diameter. However, mechanical lithotripsy was frequently performed (overall, 83.3%) to clean the CBD stones for patients with a PE bile duct. This rate was much higher compared with the rate reported in the literature (9.4% to 21.7%) for patients with CBD stones who needed lithotripsy[9,10]. In the subsequent analysis, we found that stone size or the ratio of stone size to the PE segment diameter were associated with the need of lithotripsy. Since the stones were relatively small, the major factor associated with the need of lithotripsy was the ratio of stone size to the PE segment diameter. Sharma and Jain reported that 6 of 304 patients (2%) with small CBD stones (7-9 mm) had stones extraction with mechanical lithotripsy due to a narrowed distal CBD (3-4 mm)[19]. These 6 patients might also have the PE bile duct.

The endoscopists frequently chose a lithotripter as the first tool to extract the CBD stones (16/30 or 53.3%), which might be one of the reasons for the high rate of lithotripsy in this study. This practice was adopted because of several cases in our early experience that featured an impaction of the extraction basket and an entrapped stone in the PE segment. Since then, we have often used a lithotripter rather than a Dormia basket to entrap the stone, even when the stone was small. Hence, the authors had only 1 case of biliary-basket impaction in this study.

Biliary-basket impaction often occurred upstream of the PE segment. Therefore, we suggest using Conquest through the channel lithotriptor cable for emergency lithotripsy when this complication occurs. In our experience, the Soehendra lithotripter cable is more difficult to pass through the relatively narrowed PE segment.

An impacted CBD stone or a large stone (≥ 30 mm) poses a high risk of mechanical lithotripsy failure[11]. In this series, CBD stones were successfully removed in all patients for whom mechanical lithotripsy was indicated, although some patients needed more than one endoscopic session. This high success rate was probably due to the small stone size, and there was no case in which the stone was impacted in the bile duct. The non-PE segment was dilated to a greater extent than the PE segment, providing the space for the opening of the lithotripter to capture the stone(s).

Eight (26.7%) of the 30 patients had incomplete clearance of the CBD stones during the first procedure of ERC; therefore, they needed multiple sessions of ERC to clean the stones completely. There might be 2 possible reasons for performing multiple sessions. First, a high proportion of patients underwent mechanical lithotripsy. After lithotripsy, small stone fragments may be left undetected after completion of the ERC procedure with bile duct clearance[20-22]. Second, the distal end of the non-PE segment tapered abruptly at its point of attachment to the PE segment. Similar to the bile duct stricture, some stone fragments might be left in the junction of the non-PE segment and the PE segment during extraction[11].

The cause of PE bile duct remains unknown. Physiologically, the intrapancreatic CBD can be entirely free within the pancreatic capsule, or less commonly, it is partially or completely enclosed by a strip of pancreatic tissue up to 20 mm wide and 3 to 5 mm thick, i.e., the lingula pancreatic[23]. The diameter of the PE segment was 5.8 ±

1.6 mm (range, 4 mm to 10 mm), which was compatible with the reported duct widths for an intrapancreatic portion of CBD[24]. Therefore, the PE bile duct may result from an intrapancreatic CBD that is entirely enclosed by the lingula pancreatis, resulting in the rigid characteristic. However, the length of PE segment had a wide range in this study (12.3 mm to 70.9 mm; mean 39.7 mm), which was not compatible with the length of the lingula pancreatis (range, 10 mm to 25 mm)[23]. Further study may be needed to investigate the nature of the PE bile duct.

The PE bile duct might not increase the risk of the recurrence of CBD stones. Tsuchiya *et al*[20] reported a recurrence rate of 13.2% in patients with CBD stones during a 3-year follow-up study. In a study conducted in Taiwan, the recurrence rate of CBD stones was 18%[25]. In the present series, CBD stone recurred in 7.8% of the patients during the 3-year follow-up period. This rate is no higher than that reported in the literature.

In conclusion, PE bile duct occurred in a small proportion of patients with CBD stones. Lithotripsy or multiple ERC procedures were frequently needed in patients with a PE bile duct. To avoid the complication of biliary-basket impaction, we suggest the use of a lithotripter rather than a Dormia basket to entrap and extract the stone when a PE bile duct is noted on ERC during the initial procedure.

COMMENTS

Background
There were several cases in early experience that featured an impaction of the extraction basket and an entrapped stone in the distal common bile duct (CBD) rather than in the papillary orifice after endoscopic sphincterotomy. Since then, the authors found that the common characteristic of these patients was a poorly expandable (PE) distal CBD on endoscopic retrograde cholangiography (ERC). Therefore, the authors conducted this study to describe the ERC findings of patients with CBD stones and a PE bile duct and evaluate the impact of these findings on the retrieval of the stones.

Research frontiers
CBD stones in the PE bile duct have not been reported in the literature. To the knowledge, this is the first study to describe the clinical and imaging features and to examine the safety and technical success of ERC in patients with both CBD stones and a PE bile duct.

Innovations and breakthroughs
The study showed that the diameters of the PE segment and the widest non-PE CBD segment were 5.8 ± 1.6 mm and 14.3 ± 4.9 mm, respectively. The length of the PE segment was 39.7 ± 15.4 mm (range, 12.3 mm to 70.9 mm). To remove the CBD stone(s) completely, mechanical lithotripsy was required in 83.3% of the patients, even though the stone size was not as large (11.2 ± 4.7 mm). The stone size and stone/PE segment diameter ratio were associated with the need for lithotripsy.

Applications
Patients with CBD stones and a PE duct frequently require mechanical lithotripsy during therapeutic ERC, especially when the ratio of the stone diameter to the PE segment diameter is high. To achieve successful stone retrieval and avoid complications, the authors suggest the use of a lithotripter rather than a Dormia basket to entrap and extract the stone when a PE bile duct is noted on ERC during the initial procedure.

Terminology
A PE bile duct was characterized by a rigid distal CBD with an almost normal diameter on ERC. The non-PE segment of CBD was often more dilated than the PE segment. The rigid characteristic of the PE segment was usually confirmed by increased resistance when the balloon or basket catheter with the stone was pulled from the non-PE segment across the PE segment.

Peer review
The authors have performed a study in which they identified all patients with CBD stones and a poorly expandable bile duct by means of a retrospective search in a computerized database. A total of 30 patients were found and the authors attempted to describe their characteristics. They concluded that the majority of these patients needed mechanical lithotripsy, especially those with a high ratio of stone diameter to the poorly-expandable CBD segment diameter. This a well-written paper and an interesting contribution to the understanding of factors related to difficult CBD stone extraction.

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BRIEF ARTICLE

Prognosis of HER2 over-expressing gastric cancer patients with liver metastasis

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Abstract

AIM: To study the risk factors for liver metastasis and the prognosis in patients with human epidermal growth factor receptor 2 (HER2) over-expressing gastric cancer (GC).

METHODS: A total of 84 GC patients recruited from the General Hospital of the People's Liberation Army (PLA) between 2003 and 2010 were randomly enrolled in this study. HER2 expression was detected by immunohistochemistry in 84 GC patients with liver metastases. The study group consisted of 66 men and 18 women, with an average age of 54 years (range: 19-74 years). Liver metastasis was diagnosed by magnetic resonance imaging or computed tomography. Patients were followed-up and predictive factors of liver metastasis were evaluated.

RESULTS: The median follow-up period was 47 mo (range: 6-85 mo). The characteristics of 35 (25.7%) patients with HER2 over-expression of liver metastatic GC are presented. HER2 over-expression was detected in 23 out of 49 (46.9%) patients with intestinal GC, and 9 out of 35 (25.7%) patients with diffuse GC. 29 out of 59 (49.2%) patients aged < 60 years were HER2-positive, while 8 out of 25 (32%) patients aged ≥ 60 were HER2-positive; a significant difference ($P < 0.05$). Univariate analysis (log-rank test) showed that HER2 over-expression, sex, Lauren classification, differentiation and disease-free interval were correlated with poor survival ($P < 0.05$). Survival analysis with a survival curve showed that HER2 over-expression was significantly relevant, with a reduced survival time in GC patients with liver metastases ($P < 0.01$). 2-year survival was not associated with the patient's age. A disease-free survival longer than 12 mo has a significant association with extended overall survival (OS) in GC patients with liver metastases. The median survival time after the diagnosis of liver metastases was 18 mo [95% confidence interval (CI): 9.07-26.94] among HER2 positive GC patients with liver metastases. In comparison, for 49 (69.4%) out of 84 HER2 negative patients with liver metastatic GC, the median survival time was 47 mo (95% CI: 19.37-74.63). In patients with HER2 positive liver metastatic GC, the median OS was significantly shorter than in HER2 negative patients (median, 20.32 mo; 95% CI: 16.51-24.13 *vs* median, 50.14 mo; 95% CI: 37.83-62.45; $P < 0.01$).

CONCLUSION: HER2 over-expressing GC patients with liver metastases have a poor prognosis. Overall survival was significantly lower in HER2 positive patients. HER2-overexpression is correlated with a lower survival rate.

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Key words: Human epidermal growth factor receptor 2;

INTRODUCTION

Although the incidence of gastric cancer (GC) has slightly declined in the past several decades, it remains the second leading cause of cancer death worldwide, especially in Asia[1]. GC with human epidermal growth factor receptor 2 (HER2) over-expression accounts for 9%-38% of GC, and HER2 is mainly expressed on the cell membrane. Yan et al[2] studied HER2/neu protein expression and gene amplification in gastric carcinoma and their relationship. They recommended that all samples with immunohistochemistry (IHC) as HER2 expression should be analyzed with fluorescence *in situ* hybridization and found that the detection of *HER2* gene amplification can assess the malignant biological behaviors and prognosis of gastric cancer. Zhang et al[3] found HER2 over-expression in 18.6% of GC and HER2 located mostly on the membrane, which was consistent with what was previously reported. Yu et al[4] examined the expressions of Grb2 and HER2 in normal gastric mucosa, primary gastric cancer, and lymph node metastatic foci in 1143 GC patients by using tissue microarray assay, which suggested the importance of Grb2 and HER2 in GC development and progression. Matsubara et al[5] investigated 86 patients who received first-line chemotherapy for advanced GC. Their results showed that patients with high HER2 expression had a longer survival time than those with low HER2 expression. However, there is controversy with regard to the hypothesis that HER2 expression serves as a factor in predicting the prognosis of GC patients[6-10]. In a prospective study of 63 patients with resectable CG (in which follow-up was performed for 40.7 mo), membranous epidermal growth factor receptor (EGFR) was detected by random and double blind assays, and cytosolic HER2 by immuno-enzymatic assay. Results showed that high expression of EGFR and HER2 in cancers were associated with a poor outcome in patients with resectable GC[11].

Detection of micrometastatic foci may allow for a more accurate assessment of the prognosis, along with aiding in the selection of candidates for intensive chemotherapy among GC patients. Bone marrow micrometastasis has been shown to influence the prognosis of GC patients[12,13]. Matsunami et al[14] used ion mobility spectrometry to improve detection sensitivity in their study, and their results showed that only 30% of patients showed the development of bone marrow micrometastasis. The positive rate was lower than that in Western patients[15], but similar to those of the Japanese[15]. In the present study, we investigated the prognosis of GC patients with HER2 over-expression in the liver metastatic foci in Chinese patients.

MATERIALS AND METHODS

Patient selection and clinicopathologic parameters

A total of 84 GC patients recruited from the General Hospital of the People's Liberation Army (PLA) between 2003 and 2010 were randomly enrolled in this study. All patients underwent radical dissection and chemotherapy, and/or radiotherapy. The study group consisted of 66 men and 18 women, with an average age of 54 years (range: 19-74 years). GC tissue and adjacent normal tissue were collected for pathologic examination. The detailed pathologic results were obtained from the Department of Pathology of the General Hospital of the PLA.

The histologic type of GC was defined according to Lauren's classification. This classification divides GC into intestinal (in which well-formed tubules were found) and diffuse (in which diffuse tumor infiltration and signet ring cells were noted, but well-formed tubules were absent) types. In our study, 53 patients were pathologically diagnosed with intestinal GC and 31 with diffuse GC.

Patients were followed-up for 6-85 mo. Informed consent was obtained before the study, and the protocol was approved by the Clinical Research Ethics Committee of the General Hospital of the PLA.

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections (5 μm) were deparaffinized in xylene, rehydrated through an ethanol series, and treated for 10 min in a microwave oven at 98 ℃ for antigen retrieval. Sections were then blocked in 3% hydrogen peroxidase, followed by incubation with a protein-blocking agent. Sections were treated with fetal bovine serum (10%), with or without the HER2 antibody (1:100), for 30 min at 37 ℃ then counterstained with hematoxylin and mounted. Omission of the primary antibody was used as a negative control for tissue sections. Anti-HER2 monoclonal antibody (Dako Herceptin Test kit; Dako, Glostrup, Denmark), and Dako EnVisionTM Kit (Dakocytomation, Dako) were used for IHC. The sections were examined microscopically and interpreted in a blinded fashion by two pathologists. HER2 protein expression on the cell membrane was scored according to the following criteria: 0: No staining or < 10% of tumor cells; 1+: Faint/barely perceptible partial staining in > 10% of tumor cells; 2+: Weak to moderate staining of the entire membrane or cytoplasm in > 10% of tumor cells; 3+: Strong staining in > 10% of tumor cells. Tumors with scores of 0 and 1+ were evaluated as negative while those with scores 2+ and 3+ were positive.

Table 1 Human epidermal growth factor receptor 2 expression in the liver metastatic foci in gastric cancer patients

Parameter	Patient (*n*)	HER2 + patients (*n*)	Ratio (%)	*P* value
Age (yr)				
< 60	59	29	49.2	< 0.05
⩾ 60	25	8	32	
Gender				
Male	66	28	42.4	> 0.05
Female	18	7	38.9	
Tumor type				
Intestinal	49	23	46.9	< 0.05
Diffuse	35	9	25.7	

The proportion of patients with human epidermal growth factor receptor 2 (HER2) over-expression was higher in patients with gastric cancer (GC) of the intestinal-type than in those with GC of the diffuse-type.

Table 2 Clinicopathologic parameters and survival

Parameter	Patient (*n*)	2-yr survival (%)	*P* value
Age (yr)			> 0.05
< 60	56	66.1	
⩾ 60	28	60.7	
Gender			< 0.05
Male	66	78.6	
Female	18	21.4	
HER2 over-expression			< 0.05
Absent	49	58.3	
Present	35	41.7	
Lauren classification			< 0.05
Intestinal	49	58.3	
Diffuse	35	41.7	
Differentiation			< 0.05
High/middle	4	80	
Low/null	50	63.3	
Disease-free interval (*n* = 84)			< 0.05
⩾ 12 mo	63	75	
< 12 mo	19	22.6	

Disease-free survival longer than 12 mo has significant association with extended overall survival in gastric cancer patients with liver metastasis. HER2: Human epidermal growth factor receptor 2.

Statistical analysis

A total of 35 GC patients with HER2-positive liver metastases were included in this study. Liver metastatic GC was pathologically diagnosed by pathologists at the National Comprehensive Cancer Network. Patients with clinical symptoms suggesting liver metastasis were diagnosed by abdominal magnetic resonance imaging (MRI) or computed tomography (CT) clinical information was collected from medical records, including the date of initial diagnosis of GC, the development of liver metastases, the date of chemotherapy, and death or the last follow-up, as well as disease sites at the start of chemotherapy and the details of treatment. We also evaluated the clinical response of other metastatic diseases according to the Response Evaluation Criteria in Solid Tumors at the time of diagnosis of liver metastasis. The baseline characteristics and prognostic factors for GC were reviewed, including age, sex, differentiation and GC type, and disease-free interval.

Figure 1 Kaplan-Meier survival curve in patients positive and negative for human epidermal growth factor receptor 2 in the liver metastatic gastric cancer. HER2: Human epidermal growth factor receptor 2.

Overall survival (OS) served as a main outcome.

SPSS version 17.0 software package (SPSS, Chicago, IL, United States) was used for statistical analysis. Frequency comparisons of HER2 expression status and clinicopathologic variables were performed with the *F* test (two-sided). Log-rank test was used for survival analysis. Survival curves were computed according to the Kaplan-Meier method. A value of *P* < 0.05 was considered statistically significant.

RESULTS

HER2 expression in the liver metastatic in gastric cancer patients

The median follow-up period was 47 mo (range: 6-85 mo). The characteristics of 35 (25.7%) patients with HER2 over-expression in liver metastatic GC are presented in Table 1. The results showed no significant differences between the sexes, although significant associations were observed between intestinal and diffuse (*P* < 0.05) types of GC. Over-expression of HER2 was observed as being higher for patients younger than 60 (*P* < 0.05).

HER2 over-expression was detected in 23 out of 49 (46.9%) patients with intestinal GC and 9 out of 35 (25.7%) patients with diffuse GC. The proportion of patients with HER2 over-expression was higher in patients with GC of the intestinal-type than in those with GC of the diffuse-type (*P* < 0.05). In addition, 29 out of 59 (49.2%) patients aged < 60 years were HER2-positive, while 8 out of 25 (32%) patients aged ⩾ 60 were HER2-positive, which was a significant difference (*P* < 0.05). However, there was no significant difference with regards to gender for HER2 over-expression (Table 1).

HER2 over-expression and overall survival

Univariate analysis (log-rank test) showed that HER2 over-expression, sex, Lauren classification, differentiation and disease-free interval were correlated with poor sur-

vival ($P < 0.05$), while age was not correlated with 2-year survival ($P > 0.05$) (Table 2). Survival analysis with a survival curve (Figure 1) showed that HER2 over-expression was significantly relevant with decreased survival time in GC patients with liver metastases ($P < 0.01$).

2-year survival was not associated with a patient's age. A disease-free survival (DFS) longer than 12 mo had significant association with an extended OS in GC patients with liver metastasis (Table 2).

The median survival time after diagnosis of liver metastases was 18 mo [95% confidence interval (CI): 9.07-26.94] among those HER2 positive with liver metastases in GC. In comparison, for 49 (69.4%) out of 84 HER2 negative patients with liver metastatic GC, the median survival time was 47 mo (95% CI: 19.37-74.63).

In patients with HER2 positive liver metastatic GC, the median OS was significantly shorter than in HER2 negative patients (median, 20.32 mo; 95% CI: 16.51-24.13 mo *vs* median, 50.14 mo; 95% CI: 37.83-62.45 mo; $P < 0.01$).

DISCUSSION

The HER2, an important member of the HER family, is encoded by a gene located on chromosome 17q21. During tumorigenesis, HER2 has been identified to act as an oncogene to modulate the proliferation, invasion and apoptosis of tumor cells. In cancer formation, HER2 acts as an oncogene to regulate the proliferation, invasion, and apoptosis of cancer cells. HER2 over-expression was reported in 9%-38% of GC, and was mainly found on the cell membrane[16]. The concordance between protein and mRNA over-expression of HER2 was recently elucidated in tumorigenesis, especially in cancers scored 3+ by IHC[17].

In approximately 25.7% of invasive GC, the HER2 tyrosine kinase receptor is over-expressed. HER2 consists of four different receptors and is associated with cell proliferation, differentiation, and survival. The HER2 over-expressing GC is more aggressive and has a poor prognosis. With respect to the importance of HER2 heterodimer in tumorigenesis, the signaling pathways and downstream effectors of the HER family have become key molecules in exploring strategies for anti-cancer therapy. For example, herceptin, a humanized monoclonal antibody targeting HER2/neu, has been used as a first-line anti-cancer drug in the treatment of breast cancer over-expressing HER2/neu[18,19]. Evidence from preclinical trials also indicated that herceptin could benefit GC patients[20,21].

To date, a high incidence of liver metastases has been observed in patients with HER2 over-expressing GC, which may be attributed to the biological characteristics of GC and the treatments for GC. A retrospective analysis has identified HER2 as a risk factor for the development of liver metastases and relapse. The 5-year incidence of liver metastases is significantly higher in patients with HER2 over-expressing GC. In particular, improvements in the treatment of systemic diseases have enabled

patients with HER2 over-expressing metastatic GC to survive for a relatively long period of time. MRI and CT have been routinely used for the diagnosis of liver metastases in these patients and found that the incidence of liver metastases is increasing. However, the association between liver metastases and HER2 expression remains largely unclear.

The results of the present study demonstrated that 25.7% of GC patients with liver metastases had HER2 over-expression, and that HER2 was mostly expressed in the membrane, which is consistent with what has been previously reported. The present study aimed to investigate the predictive factors in GC patients with HER2 over-expressing liver metastases.

GC of the two histologic types (intestinal and diffuse) differs in their epidemiology, pathogenesis, clinical outcome, and even genetic profiling[22]. The study showed tumors of two Puerto Rican (PR) patients with overexpressed Her2/neu and the resulting partial clinical responses motivated us to compare Her2/neu expression in PR ($n = 101$) and Caucasian non-Hispanic ($n = 95$) patients. The immunohistochemistry of tumors showed overexpression of *P*-Stat3, Cyclin D1, and Her2/neu, compared to non-neoplastic mucosa. Her2/neu and EGF-R protein levels were statistically significantly different, with higher levels of both proteins in the PR group. Importantly, Her2/neu expression was strong and diffuse in tumors with signet-ring morphology, while other histo-pathological subtypes showed higher intra-tumoral Her2/neu heterogeneity than typically observed in breast cancer. Targeted therapies in gastric cancer directed at EGF-R and Hers-2/neu pathways warrant further investigation. These therapies may be especially effective in PR patients and in patients with signet-ring cell morphologies with a dismal prognosis[23]. The study showed that overexpression of HIF-1a had no association with clinicopathological status, patient prognosis, or chemosensitivity; the expression of HIF-1a mRNA is up-regulated by a signal transduction pathway from a tyrosine kinase receptor, such as HER2[24]. Our results showed HER2 positivity differed significantly by histologic subtype (intestinal: 46.9%; diffuse: 25.7%). The mechanisms of HER2 over-expression in intestinal-type GC are complex and still largely unclear. Depending on the depth of invasion (T), the involvement of lymph nodes (N), and the presence of distant metastasis (M), the TNM stage is the most important prognostic factor for GC in clinic practice[25]. The role of HER2 over-expression as a prognostic factor in GC is still controversial[26,27]. Recently, increasing evidence has showed a direct correlation between HER2 over-expression and poor survival[28]. In a series of 260 GC patients, Okegawa *et al*[29] found that HER2 over-expression was an independent factor and correlated with serosal invasion and lymph node metastases. Our results demonstrated that HER2 over-expression was closely associated with a lower survival rate in GC patients with liver metastases ($P < 0.01$). HER2 may become a novel molecule in tumorigenesis of GC and a potential candi-

date for molecule-targeted therapy, especially in diffuse-type GC.

In our study, liver metastases in GC patients with HER2-overexpression had a shorter DFS (75% *vs* 22.6%, $P < 0.05$), which was consistent with what had previously been reported[30]. Tumor type was significantly associated with liver metastases (intestinal 46.9% *vs* diffuse 25.7%, $P < 0.05$), but age was not associated with GC. The association between liver and other location metastases suggests that high tumor load contributes to the development of hematogenous metastases, and supports the results in the present study that GC in half of patients was refractory to systemic chemotherapy.

Patients with liver metastases had a shorter survival time than those without, and the median survival time of 18 mo was comparable with previously reported results. In our study, HER2 negative GC patients with liver metastases remained stable, in contrast to other location metastases at the time of diagnosis of liver metastases.

Furthermore, HER2 positive patients with liver metastases exhibited progressive bone and other metastases and 75% of them had a general condition that allowed further systemic therapy, from which most of them benefited. Therefore, although liver metastases seem to appear at the late stage in patients with GC, other metastatic foci are still more or less chemosensitive, and thus treating liver metastases has an important influence on prognosis.

In our study, HER2 over-expressing GC patients seemed to develop symptomatic liver metastases at about 2.5 years after the diagnosis of recurrent or metastatic GC, which poses the question as to whether liver metastases should be monitored. If surveillance is carried out, who would be the candidates and what timing for surveillance should be further investigated? To date, there has been no evidence to suggest that the surveillance of liver metastases in GC patients is beneficial for patient survival or cost-effectiveness.

In conclusion, our findings reveal that the liver metastases in GC patients with HER2-overexpression had a poor prognosis. The low survival was correlated with sex, HER2 over-expression, Lauren classification, differentiation, and disease-free interval. In our future studies, the optimal regimens for chemotherapy will be studied in GC patients with liver metastases who acquire prolonged OS.

ACKNOWLEDGMENTS

COMMENTS

Background

Gastric cancer (GC) is the second leading cause of cancer death worldwide, especially in Asia. There are many risk factors used for predicting the prognosis of GC patients. A high incidence of liver metastases has been reported among patients with human epidermal growth factor receptor 2 (HER2) over-expressed GC. However, there is some controversy regarding the hypothesis that HER2 expression serves as a factor for predicting the prognosis of GC patients.

Research frontiers

HER2, an important member of the HER family, is encoded by a gene located on chromosome 17q21. In cancer formation, HER2 acts as an oncogene to regulate the proliferation, invasion, and apoptosis of cancer cells. The current research hotspot is to study the risk factors for liver metastasis and the prognosis in patients with HER2 over-expressing GC.

Innovations and breakthroughs

Although HER2 is over-expressed about 38% of gastric cancer patients, few studies are available on HER2 status in the liver metastases of gastric carcinoma patients and there is also little data on the evaluated prognosis for Chinese patients. A total of 84 GC patients recruited from the General Hospital of the People's Liberation Army in China between 2003 and 2010 were randomly enrolled in this study. All patients underwent radical dissection and chemotherapy, and/or radiotherapy. HER2 over-expression was detected with immunohistochemistry in gastric cancer with liver metastasis patients. Liver metastasis was diagnosed by magnetic resonance imaging or computed tomography. The authors also evaluated the clinical response of other metastatic diseases according to the Response Evaluation Criteria in Solid Tumors at the time of diagnosis of liver metastasis. The baseline characteristics and prognostic factors for GC were reviewed, including age, sex, differentiation and GC type, and disease-free interval. Overall survival (OS) served as a main outcome. From the results, the authors drew the conclusion that HER2 over-expressing GC patients with liver metastases have a poor prognosis, and the overall survival was significantly lower in HER2 positive patients.

Applications

Immunohistochemistry may be used to screen the HER2 status in gastric carcinoma patients with liver metastasis patients, and to study the risk factors for liver metastasis in gastric cancer. Since the sample size of our study was not large enough, that limited this study's ability to provide robust evidence. Therefore, larger-scale, multicentric studies are needed to test the results. Even so, the authors believe the current study provides preliminary and powerful data of evaluating the prognosis in patients with HER2 over-expressing GC, and contributes to determining whether target therapy is necessary for gastric carcinoma with liver metastasis.

Terminology

HER2 is the human epidermal growth factor receptor 2, an important member of the HER family, that is encoded by a gene located on chromosome 17q21. During tumorigenesis, HER2 has been identified to act as an oncogene to modulate the proliferation, invasion and apoptosis of tumor cells.

Peer review

The authors provide a retrospective study which reviewed 84 patients with HER2 expressing liver metastases in gastric cancer between 2003 and 2010. The study shows that HER2-overexpression has no correlation with liver metastases in gastric cancer. However, OS was obviously lower for HER2 negative patients. HER2-overexpression had correlation to a lower survival rate. Overall, this is an interesting and good study.

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Glypican-3 expression and its relationship with recurrence of HCC after liver transplantation

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Abstract

AIM: To investigate the diagnostic value of glypican-3 (GPC3) and its relationship with hepatocellular carcinoma (HCC) recurrence after liver transplantation.

METHODS: HCC tissue samples (n = 31) obtained from patients who had undergone liver transplantation were analyzed. GPC3 mRNA and protein expression were analyzed by TaqMan real-time reverse transcription-polymerase chain reaction and immunohistochemistry. Correlation between the GPC3 expression and clinicopathological features was analyzed. The potential prognostic value of GPC3 was investigated by comparing recurrence-free survival between HCC patients with and without GPC3 expression.

RESULTS: Using a cutoff value of 3.5×10^{-2}, 20 of 31 cancerous tissues had expression values of > 3.5×10^{-2}, whereas 3 of 31 adjacent non-neoplastic parenchyma and 0 of 20 control liver tissues had expression values of > 3.5×10^{-2} ($P < 0.001$). GPC3 protein was immunoexpressed in 68% of cancerous tissues, but not in adjacent non-neoplastic parenchyma and control liver tissues. Vascular invasion was significantly related to GPC3 expression ($P < 0.05$). Recurrence-free survival was significantly longer for patients without GPC3 mRNA overexpression (> 3.5×10^{-2}) and those without vascular invasion ($P < 0.05$ for both).

CONCLUSION: GPC3 expression may serve as a valuable diagnostic marker for HCC. GPC3 mRNA overexpression may be an adverse indicator for HCC patients after liver transplantation.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumors in the world. More than 600 000 deaths globally per year have been reported, with 82% of cases (and deaths) occurring in "developing" countries (with 55% in China)[1,2]. HCC is up to four-times more common in men than in women, and 60%-90% of these tumors develop in

a cirrhotic liver[3]. About 200 000 patients die from HCC every year in China.

Liver transplantation offers the potential to resect the entire tumor-bearing liver and eliminate the cirrhosis. Liver transplantation therefore holds great theoretical appeal in treating this disease. Furthermore, the liver is entirely removed at liver transplantation, so recurrence must derive from extrahepatic dissemination that occurred before or during transplantation, reflecting highly aggressive tumor biology. Given the current shortage of organs and the risk of aggressive recurrence, selection of candidates for liver transplantation is crucial and controversial[4-6]. Therefore, predicting outcomes for patients is a challenge for clinicians and researchers.

Glypican-3 (GPC3) is a heparan sulfate proteoglycan and locates on the cell surface by a mechanism involving the glycosylphosphatidylinositol anchor[7]. GPC3 has been discovered to be a potential serologic and immunohistochemical diagnostic marker for HCC in general[8-10] and to promote the growth of HCC cells through stimulation of the canonical Wnt signaling pathway[11]. Zhu et al[12] and Sung et al[13] reported that GPC3 mRNA was significantly elevated in most HCC compared with normal liver as well as in livers with focal nodular hyperplasia and liver cirrhosis. This result was later confirmed by Libbrecht et al[14] using reverse transcription-polymerase chain reaction (RT-PCR), who mainly studied small HCC. Despite this clinical interest in GPC3, until now, the relationship between GPC3 expression and post-transplant HCC recurrence has not been clarified.

In our previous study, GPC3 mRNA-expressing cells in peripheral blood had no clinical value in the diagnosis of HCC[15]. The present study is an extension of the study mentioned above. We designed the study to assess the diagnostic value of GPC3 mRNA and protein expression in HCC tissues and to investigate their relationship with HCC recurrence after orthotopic liver transplantation (OLT).

MATERIALS AND METHODS

Patients

HCC tissue samples (*n* = 31) were obtained from patients who underwent OLT at the Department of Transplantation Surgery, Tianjin First Central Hospital (Tianjin, China) during 2008. Histological types were assigned according to the classification set by the World Health Organization. The clinicopathological features of OLT patients with HCC are summarized in Table 1. All recipients had undergone successful OLT (livers were from cadaveric donors who had agreed to donate their organs in the event of death). Liver-tissue specimens were obtained intraoperatively from cancerous tissues and adjacent non-neoplastic parenchyma. The controls were normal liver tissues from 20 patients (15 men, 5 women; mean age, 48 years; age range 30-65 years) with hepatic hemangioma. There were no statistically significant differences in age and sex between the study groups ($P > 0.05$). Liver-tissue

Table 1 Clinicopathological features of the study population (*n* = 31)

Gender (Male/female)	29/2
Age (mean/range) (yr)	49/37-62
Primary liver disease (HBV cirrhosis/HCV cirrhosis)	30/1
TNM stage (Ⅰ - Ⅱ / Ⅲ -Ⅳa)	12/19
Milan criteria (within/beyond)	15/16
Tumor diameter (< 5 cm/≥ 5 cm)	16/15
Tumor number (< 3/≥ 3)	26/5
Vascular invasion (present/absent)	16/15
Serum AFP (≤ 20 μg/L/> 20 μg/L)	11/20
Histological differentiation (good/moderate and poor)	6/25

HBV: Hepatitis B virus; HCV: Hepatitis C virus; TNM: Tumor, node and metastasis; AFP: α fetoprotein.

specimens were fixed in 10 % formalin and embedded in paraffin for routine histological examination. The remaining tissue was immediately snap-frozen in liquid nitrogen and stored at -80 ℃ until examination.

All patients received standard post-liver transplantation care in the Intensive Care Unit. They received the same immunosuppressive therapy: tacrolimus, daclizumab, mycophenolate mofetil, and methylprednisolone. The diagnosis of HCC recurrences (follow-up of 24 mo) was based on elevation of serum levels of alpha-fetoprotein (AFP) and ultrasonography, hepatic arteriography, or computed tomography. Fine-needle aspiration cytology was used for confirmation of recurrence if necessary.

The present study was approved by our institutional ethics committee. Informed consent was obtained from each patient. The procedure met all applicable guidelines of our institute as well as governmental regulations concerning the ethical use of donated organs.

Real time reverse transcription-polymerase chain reaction assay

Total RNA was extracted from frozen liver tissues using Trizol reagents according to manufacturer instructions (Invitrogen, Carlsbad, CA, United States). Reverse transcription to complementary DNA (cDNA) was undertaken using random primer and Superscript RNase H-reverse transcriptase (Invitrogen).

A total of 1 μL of cDNA was mixed with the TaqMan probe[15], RNase-free water, and TaqMan Universal PCR Master Mix. Real-time PCR amplification and data analyses were carried out using an ABI PRISM 7300 Sequence Detector System (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's protocol. β-actin was used as an internal control. Each sample was assayed in duplicate in a MicroAmp optical 96-well plate. The thermo-cycling condition was 2 min at 50 ℃ and 15 min incubation at 95 ℃, followed by 40 two-temperature cycles of 20 s at 95 ℃ and 1 min at 60 ℃. Data were analyzed with Sequence Detection Software. Mean and standard-deviation values were calculated from the data obtained. The level of expression was

Figure 1 Receiver operating characteristic curve of glypican-3 mRNA levels for the detection of hepatocellular carcinoma. The area under the receiver operating characteristic curve is 0.878 (95% confidence interval: 0.802-0.953) ($P < 0.001$).

calculated using the formula: Relative expression = (copy number of target molecule/copy number of β-actin)[16].

Immunohistochemical analyses

Sections of formalin-fixed, paraffin-embedded tissue cell blocks (4 μm) were tested for the presence of monoclonal antibody directed against GPC3 (Santa Cruz Biotechnology, Santa Cruz, CA, United States). The method used for the immunohistochemical analysis has been described previously[17].

For the immunohistochemical analysis of GPC3, we evaluated the area of GPC3-positive staining in one slide for each patient. At first, to analyze GPC3 expression, the results of immunohistochemical staining were classified according to the area of GPC3-positive stained cells as follows: -, Negative (< 10%); ±, Weakly positive (10%-30%); and +, Positive (> 30%). Finally, we classified two groups between GPC3-negative (< 10%) and GPC3-positive (> 10%). The expression of GPC3 was judged to be positive if the percentage of immunoreactive cells was assessed semi-quantitatively as being ≥ 10% in focal lesions.

Statistical analysis

Data are mean ± SD. For comparisons of continuous variables, one-way analysis of variance with the Bonferroni *post-hoc* test was used to compare differences among the three groups. We generated receiver operating characteristic curves for GPC3 mRNA levels to determine the cutoff points that yielded the sensitivity and specificity for predicting the diagnosis of HCC. Correlations between GPC3 results and various clinicopathological parameters in the tissue samples were evaluated using the chi-square test. The distribution of time to recurrence was estimated according to the Kaplan-Meier method, and differences assessed using log-rank statistics. $P < 0.05$ was considered significant. Statistical analyses were done using SPSS ver12 (SPSS, Chicago, IL, United States).

Table 2 Correlation between glypican-3 expression and clinicopathological features in 31 orthotopic liver transplantation patients with hepatocellular carcinoma				
Variable	**GPC3 protein expression (n/N) (%)**	**P value**	**GPC3 mRNA overexpression (n/N) (%)**	**P value**
TNM stage				
I - II	7/12 (58)		6/12 (50)	
III-IVa	14/19 (74)	0.373	14/19 (74)	0.179
Milan criteria				
Within	11/15 (73)		8/15 (53)	
Beyond	10/16 (62)	0.519	12/16 (75)	0.208
Tumor diameter				
< 5 cm	11/16 (69)		9/16 (56)	
≥ 5 cm	10/15 (67)	0.901	11/15 (73)	0.320
Tumor number				
< 3	18/26 (69)		17/26 (65)	
≥ 3	3/5 (60)	0.686	3/5 (60)	0.818
Vascular invasion				
Present	14/16 (87)		14/16 (87)	
Absent	7/15 (47)	0.015	6/15 (40)	0.006
Serum AFP				
≤ 20 μg/L	6/11 (54)		6/11 (54)	
> 20 μg/L	15/20 (75)	0.244	14/20 (70)	0.390
Histological differentiation				
Good	3/6 (50)		2/6 (33)	
Moderate and poor	18/25 (72)	0.301	18/25 (72)	0.075

TNM: Tumor, node and metastasis; AFP: α fetoprotein; GPC3: Glypican-3.

RESULTS

Glypican-3 mRNA expression in hepatocellular carcinoma tissues

GPC3 mRNA expression in adjacent non-neoplastic parenchyma ($n = 31$, $2.00 \pm 1.91 \times 10^{-2}$) and in control liver tissues ($n = 20$, $1.41 \pm 1.57 \times 10^{-2}$) was much lower than in cancerous tissues ($n = 31$, $5.64 \pm 3.06 \times 10^{-2}$) ($P < 0.001$). There were no significant differences between adjacent non-neoplastic parenchyma and normal liver tissues with respect to GPC3 mRNA expression ($P = 0.270$). A cutoff value for GPC3 mRNA expression of 3.5×10^{-2} was diagnostic of HCC with a sensitivity of 68%, specificity of 94%, positive predictive value (PPV) of 87%, and negative predictive value (NPV) of 83% [area under the curve, 0.878; 95% confidence interval (CI): 0.802-0.953, $P < 0.001$] (Figure 1). Twenty of 31 (64%) cancerous tissues had expression values of $> 3.5 \times 10^{-2}$, whereas 3 of 31 (10%) adjacent non-neoplastic parenchyma and 0 of 20 control liver tissues had expression values of $> 3.5 \times 10^{-2}$ ($P < 0.001$).

The expression of GPC3 mRNA was evaluated in relation to clinicopathological features. GPC3 mRNA overexpression ($> 3.5 \times 10^{-2}$) was closely associated with vascular invasion ($P = 0.006$). No significant correlation was found between GPC3 mRNA overexpression and tumor, node, metastasis (TNM) stages, Milan criteria, tumor diameter, tumor number, serum AFP levels, and histological differentiation (Table 2).

Figure 2 Hepatocellular carcinoma immunohistochemical staining (× 400). Glypican-3 expression in hepatocellular carcinoma by immunohistochemical analyses. A: Negative expression in HCC tissue; B: Weakly positive expression in HCC tissue; C: Positive expression in HCC tissue; D: Negative expression in adjacent non-neoplastic parenchyma. HCC: Hepatocellular carcinoma.

Figure 3 Kaplan-Meier survival curve for recurrence-free interval in 31 patients who underwent liver transplantation.

GPC3 protein expression in hepatocellular carcinoma tissues

GPC3 protein was immunoexpressed in 21 of 31 (68%) cancerous tissues, but not in any adjacent non-neoplastic parenchyma and control liver tissues ($P < 0.001$). In GPC3-positive cases, protein expression was localized in the cytoplasm and membrane of cells (Figure 2). The sensitivity, specificity, PPV, and NPV of GPC3 protein expression was 0.68, 1.0, 1.0, and 0.84. Vascular invasion was significantly related to GPC3 protein expression ($P = 0.015$) (Table 2).

Association between glypican-3 expression and hepatocellular carcinoma recurrence

Up-to-date analyses after a follow-up of 24 mo revealed that 10 (32%) patients developed HCC recurrence. The median duration of HCC recurrence was 8.1 (range, 3-18 mo) after OLT (Figure 3). Assessment of Kaplan-Meier curves showed that patients without GPC3 mRNA overexpression had significantly longer recurrence-free survival than those with GPC3 mRNA overexpression ($P = 0.034$, log-rank test) (Figure 4A). Recurrence-free survival was significantly longer in patients without evidence of vascular invasion compared with those with vascular invasion ($P = 0.016$, log-rank test). Patients who met the Milan criteria had longer recurrence-free survival than those who did not, though the difference was not statistically significant ($P = 0.077$, log-rank test). There was no significant difference in recurrence-free survival between patients with positive and negative GPC3 protein expression ($P = 0.231$, log-rank test) (Figure 4B).

DISCUSSION

OLT is an excellent therapeutic choice for patients with cirrhosis complicated by HCC because it provides simultaneous treatment for both diseases. However, due to the limited availability of organs, prior selection of candidates most likely to benefit from OLT is very important. Thus, screening for potential early prognostic markers and therapeutic targets is very urgent for better selection

Figure 4 Kaplan-Meier survival curves for recurrence-free survival after liver transplantation for hepatocellular carcinoma. A: with or without glypican-3 mRNA overexpression (> 3.5 × 10^{-2}) (P = 0.034, compared by log-rank test); B: with or without glypican-3 protein expression (P = 0.231, compared by log-rank test).

of HCC patients for liver transplantation.

More recent studies have demonstrated upregulation of GPC3 in HCC at the protein level. GPC3 expression has been noted in 57% to 90% of HCC cases using immunohistochemistry[17-20]. In the present study, immunohistochemical analyses revealed that HCC expressed GPC3 protein in 68% of HCC tissues tested. RT-PCR analyses showed that, with a cutoff value of 3.5 × 10^{-2}, 20 of 31 HCC tumors showed much stronger expression of GPC3 mRNA than adjacent non-neoplastic parenchyma. It was proposed that GPC3 expression could be a useful tissue marker to distinguish between benign and malignant hepatocellular lesions. Nevertheless, in some studies, HCC and non-malignant liver originated from different patients, so these studies could not exclude the possibility that HCC and adjacent non-neoplastic parenchyma have similar GPC3 expression[12,21]. We can presume that retained carcinoma cells in the adjacent non-neoplastic parenchyma of surgical specimens with large tumors and tumor invasion may be the cause of GPC3 protein expression or GPC3 mRNA overexpression. This suggests that detection of GPC3 expression in adjacent non-neoplastic parenchyma could indicate whether or not the tumor is completely resected. It may also serve as a reference value in deciding if further treatment after resection is required.

Although GPC3 mRNA and protein show expression in HCC tissues, in our previous study, we examined GPC3 mRNA-expressing cells in peripheral blood in liver-transplant recipients during the transplant period, and found no clinical diagnostic value for HCC. Studies showed that GPC3 was a potential serologic diagnostic marker for HCC. However, the diagnostic value of GPC3 protein in sera remains questionable. Yasuda *et al*[22] found that GPC3 is not a serologic marker for detection of HCC. We speculate that GPC3 serves as a potential tissue-specific diagnostic marker for HCC. Some liver-transplant candidates undergo locoregional tumor treatments, so obtaining tissue samples for preoperative molecular marker analyses would not be problematic.

In the clinicopathological analysis, TNM stages, Milan criteria, tumor diameter, tumor number, or serum AFP levels were not clearly associated with increase in GPC3 expression. GPC3 may therefore play an important part in the malignant transformation of cells in HCC. Up until now, AFP has been regarded as the most useful marker of HCC, although its sensitivity is limited[23]. Our data show that GPC3 mRNA and protein expression in HCC without serum AFP elevation ($\leqslant$ 20 μg/L) were 54% and 54%, respectively, and in HCC of diameter < 5 cm were 56% and 69%, respectively. These findings suggest that GPC3 is a sensitive marker in small HCC and negative-serum AFP patients not only in resection[24,25] but also in OLT. Vascular invasion has been consistently reported to be a significant factor for a poor prognosis after liver transplantation for HCC[15,26,27]. Interestingly, in the present study, vascular invasion was significantly related to GPC3 expression. Zhu *et al*[12] using *in-situ* hybridization signals, showed that pushing HCC expressed significantly more GPC3 mRNA than the invading HCC. Their observations suggested that GPC3 may promote the growth of local cancer cells, and may also inhibit tissue invasion and metastasis. Their finding is different to ours. In addition, expression of GPC3 was lower in well-differentiated HCC than in moderately and poorly differentiated HCC, though the difference was not statistically significant, a finding consistent with previous reports[17,19]. The present study suggests that GPC3 expression may facilitate the growth of cancer cells, the degree of malignancy, and contribution to HCC progression.

Recurrence of HCC after transplantation remains a formidable problem despite refined selection criteria and exhaustive preoperative staging. In the present study, recurrence-free survival was longer in patients who had tumors with no evidence of vascular invasion. Microscopic vascular invasion in the explant is known to be a risk factor for HCC recurrence after transplantation. In addition, recurrence-free survival was longer in patients who met the Milan criteria, though the difference was not statistically significant. This could be because (1) the sample size in the present study was relatively small to allow apprecia-

tion of significant differences in the frequency of HCC recurrence in the groups; and (2) the patients in the Milan group had worse pathological stages. In our previous study, GPC3 mRNA-expressing cells in peripheral blood carried no predictive value for HCC recurrence after liver transplantation[15]. Hence, we used liver tissue instead of peripheral blood to determine if GPC3 expression in tumor tissues was associated with HCC recurrence after liver transplantation. In the present study, HCC patients with GPC3 mRNA overexpression had a significantly shorter recurrence-free survival than those with lower GPC3 mRNA expression after liver transplantation. This finding indicated that GPC3 mRNA overexpression could be an independent factor of a poor prognosis in HCC. Thus, we hypothesized that GPC3 may have a role in promoting carcinogenesis and in the development of HCC.

In general, GPC3 has been reported to interfere with different pathways and growth factors, and has a tissue- and stage-specific role in the development and tumor growth[28]. Ishiguro *et al*[29] showed that anti-GPC3 antibody can be used as a potential antitumor agent for human liver cancer, and can provide a novel treatment option for liver-cancer patients with GPC3-positive tumors. Evaluation of GPC3 as a diagnostic and immunotherapeutic target may be worthwhile for the prevention and treatment of HCC. Further studies must be completed to clarify these issues.

In conclusion, GPC3 is a valuable diagnostic marker sensitive and specific for HCC in liver-transplant patients. The most valuable finding in the present study was that GPC3 mRNA overexpression may act as a prognostic factor for recurrence-free survival in HCC after liver transplantation. The number of samples used in the present study was small. Confirmation of the findings of our study using more samples is warranted.

COMMENTS

Background
Given the current shortage of organs and the risk of aggressive recurrence, selection of candidates for liver transplantation is crucial and controversial. Establishment of direct and accurate methods to detect hepatocellular carcinoma (HCC) and predict HCC recurrence after liver transplantation is needed.

Research frontiers
Glypican-3 (GPC3) has been discovered to be a potential serologic and immunohistochemical diagnostic marker for HCC. Despite clinical interest in GPC3, until now, the relationship between GPC3 expression and post-transplant HCC recurrence has not been clarified.

Innovations and breakthroughs
In the previous study, the level of GPC3 mRNA in peripheral blood had no clinical value in assessing tumor recurrence after liver transplantation. Therefore, the present study further examined the diagnostic value of GPC3 expression as retrospectively analyzed by real-time reverse transcription- polymerase chain reaction and immunohistochemistry in explant tumor samples. The principal finding was that GPC3 mRNA overexpression may act as an adverse indicator for HCC patients after liver transplantation because it was associated with significantly shorter recurrence-free survival.

Applications
The results of the present study suggest that GPC3 is a valuable diagnostic marker sensitive and specific for HCC in liver-transplant patients. Also, GPC3 mRNA overexpression may act as a prognostic factor for recurrence-free survival in HCC after liver transplantation.

Terminology
GPC3 is a heparan sulfate proteoglycan which is bound to the cell surface by a mechanism involving the glycosylphosphatidylinositol anchor. GPC3 is highly expressed in fetal but not in normal adult liver.

Peer review
Authors focused on the role of GPC3 mRNA and protein expression in the diagnosis of recurring HCC after liver transplantation. For this purpose, patient samples were analyzed according to clinicopathological features, mRNA expression levels, protein expression, and recurrence-free survival. They concluded that GPC3 expression could serve as a diagnostic marker for HCC and as an adverse indicator for HCC patients after liver transplantation.

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BRIEF ARTICLE

Salvage liver transplantation in the treatment of hepatocellular carcinoma: A Meta-analysis

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Abstract

AIM: To evaluate survival and recurrence after salvage liver transplantation (SLT) for the treatment of hepatocellular carcinoma (HCC) compared with primary liver transplantation (PLT) using a meta-analysis.

METHODS: Literature on SLT versus PLT for the treatment of HCC published between 1966 and July 2011 was retrieved. A meta-analysis was conducted to estimate pooled survival and disease-free rates. A fixed or random-effect model was established to collect the data.

RESULTS: The differences in overall survival and disease-free survival rates at 1-year, 3-year and 5-year survival rates were not statistically significant between SLT group and PLT group ($P > 0.05$). After stratifying the various studies by donor source and Milan criteria, we found that: (1) Living donor liver transplantation recipients had significantly higher 1-year survival rate, lower 3-year and 5-year survival rates compared with deceased-donor liver transplantation (DDLT) recipients. And in DDLT recipients they had better 1-year and 5-year disease-free survival rate in SLT group; and (2) No difference was seen in 1-year, 3-year and 5-year survival rates between two groups who beyond Milan criteria at the time of liver transplantation.

CONCLUSION: SLT can be effectively performed for patients with recurrence or deterioration of liver function after hepatectomy for HCC. It does not increase the perioperative mortality and has a similar long-term survival rates compared to PLT.

INTRODUCTION

In a normal liver, liver resection for hepatocellular carcinoma (HCC) is the primary treatment of choice. But in cirrhotic livers, the presence of HCC and the limited liver capacity are the two intertwined issues rendering the HCC unresectable. Primary liver transplantation (PLT) is the most effective treatment for such HCC patients, especially for those who meet Milan criteria (solitary liver nodule not exceeding 5 cm in maximum diameter, or 2 or 3 tumors not exceeding 3 cm in diameter)[1]. It has been manifested to provide a considerable disease-free

survival and to be the first choice for these patients. Due to shortage of available donors, long waiting times may harm the benefit that might be acquired from PLT. Salvage liver transplantation (SLT) has been proposed and performed for those who undergo primary liver resection for HCC or HCC recurrence or deterioration of liver function[2]. SLT proposes liver resection as a bridge to prevent tumor progression in the waiting list. Although SLT might be an alternate choice for HCC patients as the preferred treatment, long-term results are difficult to ascertain. Moreover, few data are available on the overall and disease-free survival of patients. A few researches concern on the comparison between the result of SLT and PLT. In order to reduce research bias and difference, we did a meta-analysis to compare survival and recurrences for SLT strategy versus PLT in the treatment of HCC patients, in order to provide a reference for clinical practice.

MATERIALS AND METHODS

Literature search strategy
Search was applied to the following electronic databases: PubMed (1966 to July 2011), Embase (January 1996 to July 2011), CNKI (January 1996 to July 2011) and Cochrane database. The following key words were used: "liver resection" or "hepatectomy"; "liver transplantation" or "transplantation" or "salvage liver transplantation" or "salvage transplantation"; "hepatocellular carcinoma" or "HCC". The search was limited to the English language and humans. The relevant reference lists of reviews were also searched at the same time. Abstracts or unpublished studies were not considered. If more than 1 study was published by the same author using the same case series, only the most detailed study was included. And if necessary, authors were contacted to obtain more data on their study.

Definitions
SLT was defined as a liver transplantation performed for recurrent HCC or deterioration of liver function after primary liver resection.

Inclusion, exclusion criteria and quality of the studies
Inclusion criteria were as follows: (1) having definition of SLT; (2) follow-up 12 mo at least; (3) case-control or cohort design; and (4) sufficient data were obtained to calculate odds ratio (OR) with confidence interval (CI). Reasons for exclusion were: (1) no-control; (2) duplicate; and (3) no useable data reported. We also excluded articles published before 1996 because there was no definition for "SLT".

The scoring system was adapted from Stahl, the Cochrane Collaboration and others[3-5]. This system suits not only randomized control trial (RCT) but controlled trial or other studies well. Questions were placed on a 3 point scale: unclear/inadequate (0), adequate (1), good (2). Articles were considered for inclusion if their summary score exceeded 30.

Table 1 Details of studies included in the meta-analysis

Author	Year	Country	Study design	SLT (cases)	PLT (cases)	Score
Adam *et al*[6]	2003	France	Case-control	17	200	32
Belghiti *et al*[7]	2003	France	Case-control	18	70	33
Concejero *et al*[8]	2008	China	Case-control	7	28	31
Del Gaudio *et al*[9]	2008	Italy	Case-control	16	147	32
Facciuto *et al*[10]	2008	USA	Case-control	5	32	30
Hwang *et al*[11]	2007	Korea	Case-control	17	200	31
Kim *et al*[12]	2008	Korea	Case-control	15	31	30
Margarit *et al*[13]	2005	Spain	Case-control	5	36	31
Sapisochin *et al*[14]	2010	Spain	Case-control	17	34	33
Shao *et al*[15]	2008	China	Case-control	15	62	30
Vennarecci *et al*[16]	2007	Italy	Case-control	9	37	30

SLT: Salvage liver transplantation; PLT: Primary liver transplantation.

Data extraction
All data were extracted independently by 2 reviewers according to the selection criteria. We resolved disagreement through discussion. The following data were extracted: the last name of the first author, study design, publication year, definition of SLT, the type of population described [adults or children (< 18 years)], country of transplant center, number of SLT cases and control (PLT) studies, overall survival, overall recurrence and assessment of risk factors.

Statistical analysis
Meta-analysis was performed using fixed-effect or random-effect methods, depending on the absence or presence of significant heterogeneity. Statistical heterogeneity between trials was evaluated by the Cochran χ^2 test and was considered significant when $P < 0.10$. In the absence of statistically significant heterogeneity, the Mantel-Haenszel method in the fixed-effect model was used for the meta-analysis. Otherwise, the DerSimonian and Laird method in the random-effect model was selected.

The OR with 95% CI was used to assess treatment efficacy. The combined result was an average OR and 95% CI weighted according to the standard error of the OR of the trial. $P < 0.05$ was considered statistically significant. We used funnel plots to assess the publication bias, and tested for funnel plot asymmetry using Egger's test and Begg's test. All analyses were performed with Review Manager version 5.0.23 (RevMan, Cochrane Collaboration, Oxford, England).

RESULTS

Studies included in the meta-analysis
There were 410 papers relevant to the search words. Via steps of screening the title, abstract reviewing and article reviewing, 11 studies which included 141 SLT cases and 872 PLT cases were identified to match our inclusion criteria[6-16]. Studies had been carried out in France, Italy, USA, China, Spain, Korea and Chinese Taiwan. Details of studies and the methodological quality of the studies assessed according to a score system described above are described in Table 1.

A Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 1-year survival rates

Study or subgroup	SLT Events	SLT Total	PLT Events	PLT Total	Weight %	Odds ratio M-H, fixed, 95% CI	Odds ratio M-H, fixed, 95% CI
Adam *et al*[6]	12	17	156	195	20.9	0.60 (0.20, 1.80)	
Belghiti *et al*[7]	15	18	64	70	12.4	0.47 (0.11, 2.09)	
Concejero *et al*[8]	7	7	27	28	2.1	0.82 (0.03, 22.20)	
Del Gaudio *et al*[9]	15	16	129	147	4.5	2.09 (0.26, 16.81)	
Facciuto *et al*[10]	5	5	28	32	2.1	1.74 (0.08, 37.08)	
Hwang *et al*[11]	15	17	175	200	9.2	1.07 (0.23, 4.97)	
Kim *et al*[12]	13	15	26	31	6.4	1.25 (0.21, 7.34)	
Margarit *et al*[13]	4	5	28	36	3.9	1.14 (0.11, 11.72)	
Sapisochin *et al*[14]	10	17	29	34	22.6	0.25 (0.06, 0.95)	
Shao *et al*[15]	12	15	56	62	12.4	0.43 (0.09, 1.96)	
Vennarecci *et al*[16]	8	9	29	37	3.6	2.21 (0.24, 20.35)	
Total (95% CI)		141		872	100.0	0.74 (0.46, 1.21)	
Total events	116		747				

Heterogeneity: $\chi^2 = 6.42$, df = 10 ($P = 0.78$), $I^2 = 0\%$
Test for overall effect: $Z = 1.20$ ($P = 0.23$)

0.01　0.1　1　10　100
Favours experimental　Favours control

B Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 3-year survival rates

Study or subgroup	SLT Events	SLT Total	PLT Events	PLT Total	Weight %	Odds ratio M-H, fixed, 95% CI	Odds ratio M-H, fixed, 95% CI
Adam *et al*[6]	9	17	133	195	22.9	0.52 (0.19, 1.42)	
Belghiti *et al*[7]	14	18	53	70	11	1.12 (0.33, 3.87)	
Concejero *et al*[8]	7	7	27	28	1.7	0.82 (0.03, 22.20)	
Del Gaudio *et al*[9]	13	16	119	147	10	1.02 (0.27, 3.82)	
Facciuto *et al*[10]	5	5	22	32	1.3	5.13 (0.26, 101.70)	
Hwang *et al*[11]	11	17	156	200	19.7	0.52 (0.18, 1.48)	
Margarit *et al*[13]	3	5	22	36	4.9	0.95 (0.14, 6.45)	
Sapisochin *et al*[14]	9	17	26	34	18.6	0.35 (0.10, 1.19)	
Shao *et al*[15]	12	15	42	62	7.5	1.90 (0.48, 7.52)	
Vennarecci *et al*[16]	8	9	23	37	2.3	4.87 (0.55, 43.18)	
Total (95% CI)		126		841	100.0	0.89 (0.58, 1.37)	
Total events	91		623				

Heterogeneity: $\chi^2 = 9.37$, df = 9 ($P = 0.40$), $I^2 = 4\%$
Test for overall effect: $Z = 0.51$ ($P = 0.61$)

0.01　0.1　1　10　100
Favours experimental　Favours control

C Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 5-year survival rates

Study or subgroup	SLT Events	SLT Total	PLT Events	PLT Total	Weight %	Odds ratio M-H, fixed, 95% CI	Odds ratio M-H, fixed, 95% CI
Adam *et al*[6]	7	17	119	195	23.8	0.45 (0.16, 1.22)	
Belghiti *et al*[7]	10	18	37	70	14.3	1.11 (0.39, 3.16)	
Concejero *et al*[8]	7	7	25	28	1.5	2.06 (0.10, 44.48)	
Del Gaudio *et al*[9]	10	16	107	147	16.7	0.62 (0.21, 1.83)	
Hwang *et al*[11]	9	17	144	200	22.5	0.44 (0.16, 1.19)	
Margarit *et al*[13]	1	5	11	36	4.5	0.57 (0.06, 5.69)	
Sapisochin *et al*[14]	9	17	22	34	14.6	0.61 (0.19, 2.00)	
Vennarecci *et al*[16]	8	9	23	37	2.1	4.87 (0.55, 43.18)	
Total (95% CI)		106		747	100.0	0.72 (0.46, 1.11)	
Total events	61		488				

Heterogeneity: $\chi^2 = 6.05$, df = 7 ($P = 0.53$), $I^2 = 0\%$
Test for overall effect: $Z = 1.50$ ($P = 0.13$)

0.01　0.1　1　10　100
Favours experimental　Favours control

Figure 1　Fixed-effect model of odds ratio for 1-year survival rates (A), 3-year survival rates (B) and 5-year survival rates (C) after salvage liver transplantation and primary liver transplantation (Experimental: Salvage liver transplantation; Control: Primary liver transplantation). SLT: Salvage liver transplantation; PLT: Primary liver transplantation.

Meta-analysis

1-year survival after SLT and PLT: A total of 1013 patients were included in 11 articles. According to χ^2 test of heterogeneity ($P = 0.78$), a fixed-effect model was used. No difference between SLT group (82.3%) and PLT group (85.7%) were seen in the 1-year survival rate (OR: 0.74, 95% CI: 0.46-1.21, $P = 0.23$, Figure 1A).

3-year survival after SLT and PLT: A total of 967 patients were included in 10 articles. According to χ^2 test of heterogeneity ($P = 0.40$), a fixed-effect model was used. No difference between SLT group (72.2%) and PLT

A Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 1-year disease-free survival rates

	SLT		PLT			Odds ratio	Odds ratio
Study or subgroup	Events	Total	Events	Total	Weight %	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Adam *et al*[6]	8	17	148	195	45.8	0.28 (0.10, 0.77)	
Concejero *et al*[8]	7	7	27	28	2.7	0.82 (0.03, 22.20)	
Del Gaudio *et al*[9]	13	16	125	147	16.8	0.76 (0.20, 2.90)	
Facciuto *et al*[10]	5	5	28	32	2.7	1.74 (0.08, 37.08)	
Margarit *et al*[13]	4	5	28	36	5	1.14 (0.11, 11.72)	
Sapisochin *et al*[14]	14	17	33	34	14.2	0.14 (0.01, 1.48)	
Shao *et al*[15]	13	15	54	62	10.2	0.96 (0.18, 5.08)	
Vennarecci *et al*[16]	8	8	33	37	2.6	2.28 (0.11, 46.66)	
Total (95% CI)		90		571	100.0	0.56 (0.31, 1.00)	
Total events	72		476				

Heterogeneity: $\chi^2 = 5.48$, df = 7 ($P = 0.60$), $I^2 = 0\%$
Test for overall effect: $Z = 1.95$ ($P = 0.05$)

B Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 3-year disease-free survival rates

	SLT		PLT			Odds ratio	Odds ratio
Study or subgroup	Events	Total	Events	Total	Weight %	M-H, random, 95% CI	M-H, random, 95% CI
Adam *et al*[6]	5	17	125	195	15.8	0.23 (0.08, 0.69)	
Concejero *et al*[8]	7	7	27	28	8.1	0.82 (0.03, 22.20)	
Del Gaudio *et al*[9]	10	16	115	147	15.8	0.46 (0.16, 1.37)	
Facciuto *et al*[10]	4	5	23	32	11.2	1.57 (0.15, 15.97)	
Margarit *et al*[13]	3	5	22	36	12.7	0.95 (0.14, 6.45)	
Sapisochin *et al*[14]	11	17	32	34	13.4	0.11 (0.02, 0.65)	
Shao *et al*[15]	13	15	15	62	13.9	20.37 (4.12, 100.69)	
Vennarecci *et al*[16]	8	8	27	37	9.1	6.49 (0.34, 122.71)	
Total (95% CI)		90		571	100.0	0.98 (0.27, 3.47)	
Total events	61		386				

Heterogeneity: Tau^2 = 2.34; $\chi^2 = 29.43$, df = 7 ($P = 0.0001$), $I^2 = 76\%$
Test for overall effect: $Z = 0.04$ ($P = 0.97$)

C Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 5-year disease-free survival rates

	SLT		PLT			Odds ratio	Odds ratio
Study or subgroup	Events	Total	Events	Total	Weight %	M-H, random, 95% CI	M-H, random, 95% CI
Adam *et al*[6]	12	17	113	195	25.5	1.74 (0.59, 5.14)	
Concejero *et al*[8]	7	7	25	28	7.7	2.06 (0.10, 44.48)	
Del Gaudio *et al*[9]	8	16	104	147	26.1	0.41 (0.15, 1.17)	
Margarit *et al*[13]	1	5	11	36	11.9	0.57 (0.06, 5.69)	
Sapisochin *et al*[14]	10	17	30	34	20.6	0.19 (0.05, 0.79)	
Vennarecci *et al*[16]	8	8	27	37	8.3	6.49 (0.34, 122.71)	
Total(95% CI)		70		477	100.0	0.75 (0.29, 1.96)	
Total events	46		310				

Heterogeneity: Tau^2 = 0.63; $\chi^2 = 9.81$, df = 5 ($P = 0.08$), $I^2 = 49\%$
Test for overall effect: $Z = 0.59$ ($P = 0.56$)

Figure 2 Fixed-effect model of odds ratio for 1-year disease-free survival rates (A); random-effect model of odds ratio for 3-year disease-free survival rates (B) and 5-year disease-free survival rates (C) after salvage liver transplantation and primary liver transplantation (Experimental: Salvage liver transplantation; Control: Primary liver transplantation). SLT: Salvage liver transplantation; PLT: Primary liver transplantation.

group (74.1%) were seen in the 3-year survival rate (OR: 0.89, 95% CI: 0.58-1.37, $P = 0.61$, Figure 1B).

5-year survival after SLT and PLT: A total of 853 patients were included in 8 articles. According to χ^2 test of heterogeneity ($P = 0.53$), a fixed-effect model was used. No difference between SLT group (57.5%) and PLT group (65.3%) were seen in the 5-year survival rate (OR: 0.72, 95% CI: 0.46-1.11, $P = 0.13$, Figure 1C).

1-year disease-free survival after SLT and PLT: A total of 620 patients were included in 8 articles. According to χ^2 test of heterogeneity ($P = 0.60$), a fixed-effect model was used. No difference between SLT group (80.0%)

and PLT group (83.4%) were seen in the 1-year disease-free survival rate (OR: 0.56, 95% CI: 0.31-1.00, $P = 0.05$, Figure 2A).

3-year disease-free survival after SLT and PLT: A total of 620 patients were included in 8 articles. According to χ^2 test of heterogeneity ($P = 0.0001$), a random-effect model was used. No difference between SLT group (67.8%) and PLT group (67.6%) were seen in the 3-year disease-free survival rate (OR: 0.98, 95% CI: 0.27-3.47, $P = 0.97$, Figure 2B).

5-year disease-free survival after SLT and PLT: A total of 506 patients were included in 6 articles. According

A Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 1-year disease-free survival rates for DDLT recipients

Study or subgroup	SLT Events	SLT Total	PLT Events	PLT Total	Weight %	Odds ratio M-H, fixed, 95% CI
Adam *et al*[6]	8	17	148	195	45.9	0.28 (0.10, 0.77)
Del Gaudio *et al*[9]	13	16	125	147	16.8	0.76 (0.20, 2.90)
Facciuto *et al*[10]	5	5	28	32	2.7	1.74 (0.08, 37.08)
Margarit *et al*[13]	4	5	28	36	5	1.14 (0.11, 11.72)
Sapisochin *et al*[14]	14	17	33	34	14.2	0.14 (0.01, 1.48)
Shao *et al*[15]	13	15	54	62	10.2	0.96 (0.18, 5.08)
Vennarecci *et al*[16]	8	9	33	37	5.2	0.97 (0.09, 9.90)
Total (95% CI)		84		543	100.0	0.53 (0.29, 0.95)
Total events	65		449			

Heterogeneity: $\chi^2 = 4.76$, df = 6 ($P = 0.58$), $I^2 = 0\%$
Test for overall effect: $Z = 2.12$ ($P = 0.03$)

Odds ratio M-H, fixed, 95% CI
0.01 0.1 1 10 100
Favours experimental   Favours control

B Review: SLT *vs* PLT; Comparison: SLT *vs* PLT; Outcome: 5-year disease-free survival rates for DDLT recipients

Study or subgroup	SLT Events	SLT Total	PLT Events	PLT Total	Weight %	Odds ratio M-H, fixed, 95% CI
Adam *et al*[6]	5	17	113	195	37	0.30 (0.10, 0.89)
Del Gaudio *et al*[9]	8	16	104	147	29.5	0.41 (0.15, 1.17)
Margarit *et al*[13]	1	5	11	36	6.2	0.57 (0.06, 5.69)
Sapisochin *et al*[14]	10	17	30	34	23.8	0.19 (0.05, 0.79)
Vennarecci *et al*[16]	8	9	27	37	3.4	2.96 (0.33, 26.79)
Total (95% CI)		64		449	100.0	0.42 (0.23, 0.74)
Total events	32		285			

Heterogeneity: $\chi^2 = 4.62$, df = 4 ($P = 0.33$), $I^2 = 13\%$
Test for overall effect: $Z = 2.96$ ($P = 0.003$)

Odds ratio M-H, fixed, 95% CI
0.01 0.1 1 10 100
Favours experimental   Favours control

Figure 3 Fixed-effect model of odds ratio for 1-year (A) and 5-year (B) disease-free survival rates for deceased-donor liver transplantation recipients after salvage liver transplantation and primary liver transplantation (Experimental: Salvage liver transplantation; Control: Primary liver transplantation). DDLT: Deceased-donor liver transplantation; SLT: Salvage liver transplantation; PLT: Primary liver transplantation.

to χ^2 test of heterogeneity ($P = 0.08$), a random-effect model was used. No difference between SLT group (65.7%) and PLT group (65.0%) were seen in the 5-year disease-free survival rate (OR: 0.75, 95% CI: 0.29-1.96, $P = 0.56$, Figure 2C).

When stratifying for the donor source, compared with deceased-donor liver transplantation (DDLT) recipients, we found that living-donor liver transplantation (LDLT) recipients had significantly higher 1-year survival rate (OR: 1.02, 95% CI: 0.26-4.10, $P = 0.97$), lower 3-year survival rate (OR: 0.54, 95% CI: 0.20-1.47, $P = 0.23$) and lower 5-year survival rate (OR: 0.54, 95% CI: 0.21-1.35, $P = 0.19$). DDLT recipients had significantly lower 1-year survival rate (OR: 0.66, 95% CI: 0.38-1.15, $P = 0.14$), higher 3-year survival rate (OR: 0.99, 95% CI: 0.62-1.59, $P = 0.97$) and higher 5-year survival rate (OR: 0.77, 95% CI: 0.47-1.26, $P = 0.30$). No useable data about disease-free survival rates can be extracted from LDLT researches. And in DDLT recipients they had better 1-year disease-free survival rate (OR: 0.53, 95% CI: 0.29-0.95, $P = 0.03$, Figure 3A) and better 5-year disease-free survival rate (OR: 0.42, 95% CI: 0.23-0.74, $P = 0.003$, Figure 3B) in SLT group. No difference between SLT group and PLT group were seen in the 3-year disease-free survival rate (OR: 0.95, 95% CI: 0.26-3.52, $P = 0.94$).

When stratifying for Milan criteria, we found that no difference was seen in 1-year survival rates (OR: 0.26, 95% CI: 0.01-4.94, $P = 0.37$), 3-year survival rates (OR: 0.41, 95% CI: 0.01-24.54, $P = 0.67$) and 5-year survival rates (OR: 0.55, 95% CI: 0.07-4.48, $P = 0.57$) between SLT group and PLT group who beyond Milan criteria at the time of liver transplantation (LT). No usable data for patients who met Milan criteria at the time of LT.

Publication bias

Publication bias may exist when no significant findings remain unpublished, thus artificially inflating the apparent magnitude of an effect. Funnel plots of our study results are shown in Figure 4. The funnel plots on survival and disease-free survival following SLT or PLT for the treatment of HCC showed basic symmetry, which suggested no publication bias.

DISCUSSION

As one of the radical treatments for HCC, LT is nowadays limited by organ shortage. Due to the prolonged waiting times before transplantation, tumor progression and deterioration of liver function may counteract its benefit[2]. The outcome of liver resection is mainly influenced by a high rate of recurrence that limits long-term survival rates. But previous research noted that most of patients with recurrence after primary liver resection were still eligible for LT[2]. Hence, hepatectomy and LT should be considered as complementary, not competitive, treatments for HCC in cirrhotic patients with well-preserved

Figure 4 Funnel plot. A: 11 articles in the meta-analysis of 1-year survival after treatment; B: 10 articles in the meta-analysis of 3-years survival after treatment; C: 8 articles in the meta-analysis of 5-years survival after treatment; D: 8 articles in the meta-analysis of 1-year disease-free survival after treatment; E: 8 articles in the meta-analysis of 3-year disease-free survival after treatment; F: 6 articles in the meta-analysis of 5-year disease-free survival after treatment. SE: Standard error; OR: Odds ratio.

liver function. Resection of the liver tumor is an optional bridge treatment[17-19]. SLT was proposed in order to reduce the impact of a long waiting times, donor shortage and tumor recurrence after resection in HCC patients.

The increased technical difficulty during SLT and the risk for impaired posttransplant survival worried most of surgeons. Heavy adhesions and portal hypertension are often encountered after prior liver resection. Inattentive dissection of perihepatic adhesions could result in uncontrollable bleeding at the dissection surface. Also due to heavy adhesions, the relationship between hepatic vein and inferior vena cava are hard to identify. Hwang *et al*[11] found that SLT did not increase the operative risks or postoperative complications. The two major techni-

cal concerns-bleeding and reconstruction of the hepatic vein outflow can be solved successfully by steady and meticulous sharp dissection and sufficient dissection of the recipient inferior vena cava. Kim *et al*[12] showed that end-to-end anastomosis for bile ducts and hepatic artery was feasible, too. Our study showed that SLT had no bad effect on overall survival and disease-free survival in comparison with PLT.

Considering different surgical methods may have an effect on survival rates, the whole patients were stratified to LDLT recipients and DDLT recipients. In each subgroup, no difference between SLT group and PLT group were seen in 1-year survival rates, 3-year survival rates and 5-year survival rates. But LDLT recipients may have

a significant higher 1-year survival rates than DDLT recipients, while a significant lower 3-year survival rates and 5-year survival rates. This may be a result of improvement of surgical technique and perioperative management. However, because of a relatively higher incidence (up to 30%) of biliary complication after LDLT[20-23], LDLT recipients may have a lower long-term survival rates than DDLT recipients. Different results in DDLT recipients' disease-free survival rates seem hard to explain. We consider a better tumor stage at the time of transplantation (meet Milan criteria) may contribute to better 1-year disease-free survival rates and 5-year disease-free survival rates. A lager sample and more randomized controlled studies may resolve this conflict and draw a right conclusion.

The tumors' stage at the time of resection and LT is another risk factor for postoperative overall survival and disease-free survival. Some studies were theoretical and assessed the salvage transplantability according to the pattern of recurrence after resection for HCC within Milan criteria and found that 76% to 87% of recurrences were considered eligible for SLT on imaging grounds[2,12,24,25]. For HCC patients not meeting Milan criteria, SLT could be applied for those cases with less aggressiveness, namely tumor size less than 6 cm and pathological well differentiation. For those cases meeting Milan criteria, PLT seems to be the first option. SLT could be performed for those patients with recurrence within Milan criteria after primary resection and without delay before recurrence with advanced disease manifestations. But there is no consensus about the survival rates for patients with recurrence beyond Milan criteria. Our result reveals that SLT group has similar survival rates compared with PLT group beyond Milan criteria at the time of LT. Unfortunately, data extracted from our including studies are not enough to do further meta-analysis on patients' survival rates meeting Milan criteria at the time of LT and the corresponding disease-free survival rates.

Moreover, in countries with a higher incidence of HCC, a higher proportion of HCC patients on the waiting list and/or a longer median time-to-transplant, SLT could offer a gain in life-expectancy to the remaining waiting-list patients[26].

This review has some limitations. Although funnel plots may be suggestive of publication bias with lack of negative small RCTs, a firm conclusion about bias is difficult to make as the asymmetry of the funnel plots is minimal. And funnel plots can show asymmetry for other reasons. Therefore, our pooled OR might be an overestimate of the true effect. Due to data constraints, this meta-analysis could not analyze the quality of life score and was unable to carry out stratified analyses of other possible confounding factors. The method need to be more effective. Larger samples and randomized controlled studies with longer follow-up are required. Our conclusions also need more detailed data to confirm the results. The search language was limited. The integrity of the data was affected to a certain extent.

In conclusion, this new strategy SLT can be effectively performed for patients with recurrence or deterioration of liver function after hepatectomy for HCC. It does not increase the perioperative mortality and has a similar long-term survival rates compared to PLT. When surgical technique is no longer a problem for SLT, more patients will benefit from it.

COMMENTS

Background
Due to shortage of available donors, salvage liver transplantation (SLT) has been proposed and performed for the patients who undergo primary liver resection for hepatocellular carcinoma (HCC) or HCC recurrence or deterioration of liver function. This meta-analysis was designed to evaluated survival and recurrence after SLT for the treatment of HCC compared with primary liver transplantation (PLT).

Research frontiers
The study evaluated survival and recurrence after SLT for the treatment of HCC compared with PLT using a meta-analysis of all relevant controlled studies.

Innovations and breakthroughs
This is the first systematic review and meta-analysis on the survival and recurrence after SLT for the treatment of HCC compared with PLT. The author made a comprehensive search of studies. Several important conclusions might be used for future selection in SLT or PLT for HCC patients' treatments.

Applications
This meta-analysis shows that SLT has a similar survival rates in comparison with PLT. SLT offers an alternative treatment method for HCC patients in facing a shortage of available donors.

Terminology
SLT was defined as a liver transplantation performed for recurrent HCC or deterioration of liver function after primary liver resection.

Peer review
The article should be published as it is a nice overview on the topic after some revisions are performed.

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BRIEF ARTICLE

Reduced Popdc3 expression correlates with high risk and poor survival in patients with gastric cancer

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Abstract

AIM: To investigate the expression of Popeye domain containing 3 (Popdc3) and its correlation with clinicopathological features and prognosis of gastric cancer.

METHODS: The method of immunohistochemistry was used to investigate the expression of Popdc3 in 306 cases of human gastric cancer and 84 noncancerous gastric tissues. Simultaneously, the relationship between Popdc3 expression and the survival of the patients was retrospectively analyzed.

RESULTS: Popdc3 was detected in 72 (85.71%) of 84 human nontumor mucosa. High expression of Popdc3 protein was detected in 78 (25.49%) of 306 human gastric cancer cases, and low expression was detected in 228 (74.51%). Low expression of Popdc3 correlated with depth of invasion ($P < 0.0001$), regional lymph nodes ($P < 0.0001$) and distant metastasis ($P = 0.02$), and tumor, nodes, metastasis (TNM) stages ($P < 0.0001$). On multivariate analysis, only the patient's gender, regional lymph node metastasis, distant metastasis, TNM stages, and the expression of Popdc3 were independent prognostic factors in patients with gastric cancer. The Kaplan-Meier plot showed that low Popdc3 expression had a much more significant effect on the survival of those patients with early-stage tumors ($\chi^2 = 104.741$, $P < 0.0001$), with a > 51.9% reduction in the three-year survival compared with high Popdc3 expression. In late stages, the difference was also significant ($\chi^2 = 5.930$, $P = 0.015$), with a 32.6% reduction in the three-year survival.

CONCLUSION: Reduced expression of Popdc3 may play a significant role in the carcinogenesis and progression of gastric cancer. Popdc3 may be an independent prognostic factor.

Luo D, Lu ML, Zhao GF, Huang H, Zheng MY, Chang J, Lv L, Luo JB. Reduced Popdc3 expression correlates with high risk and poor survival in patients with gastric cancer. *World J Gastroenterol* 2012; 18(19): 2423-2429 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i19/2423.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i19.2423

INTRODUCTION

Gastric cancer is the second leading cause of cancer-related deaths worldwide because advanced or metastatic gastric cancer constitutes the majority of patients in clinical practice[1]. The incidence of gastric cancer has been falling globally in terms of both incidence and mortality rates since World War Ⅱ. In the United States in 2009, 21 130 new diagnoses of gastric cancer were estimated and 10 620 deaths expected[2]. Although there has been a noticeable decrease in gastric cancer mortality rate, there is a higher prevalence of gastric cancer in China than in the Western countries[3-7]. The prognosis of advanced gastric cancer is apparently poor, and the 5-year survival rate is less than 30% in the patients after surgery[8,9]. Therefore, it is of great clinical value to further understand the molecular mechanisms involved in gastric cancer and to find valuable diagnostic markers as well as novel therapeutic strategies.

The Popeye domain containing (Popdc) gene family consists of Bves/Popdc1, Popdc2 and Popdc3[10]. They were discovered in 1999 by two independent laboratories using screens to identify novel genes that were highly expressed in the developing heart[11,12]. Several studies have illuminated that within one species, Bves was 24% and 28% identical to Popdc2 and Popdc3, respectively, while Popdc2 and Popdc3 were approximately 50% identical[13-15]. In 2004, the expression pattern of Popdc2 in the heart of chick was reported, but no study had been conducted to test the function of this protein[14]. Until now, Bves is still the most studied member of the Popdc family[10]. After that, Kim *et al*[16] found that frequent silencing of Popdc3 was associated with promoter hypermethylation in gastric cancer. Although questions about Popdc3 remain unanswered, general trends are beginning to emerge. Previous studies have led us to the hypothesis that Popdc3 may play a role in cell adhesion, cell motility, DNA methylation and tumorigenesis. Nevertheless, further work is required to elucidate its molecular mechanisms in the biology of cancer.

As far as we know, no report is available on the actual expression level of Popdc3 and the correlation between clinicopathologic features and prognosis of gastric cancer patients. Therefore, in this study, we investigated the Popdc3 protein expression profile in 306 primary gastric cancer patients, and found that Popdc3 was lowly expressed in 228 (74.51%) of 306 human gastric cancer cases, suggesting that the low expression level of Popdc3 may be a reliable indicator for the poor prognosis of gastric cancer patients.

MATERIALS AND METHODS

Patients and tissue samples

A total of 306 patients with primary gastric cancer, who underwent routine surgery at the Department of Surgery, the Second Affiliated Hospital of Kunming Medical University from February 1996 to March 2007, were enrolled in this study. The study was approved by the hospital's ethics committee. Patient diagnosis was established pathologically, and no patient had received any treatment before admission. All patients had follow-up records for over 5 years. The follow-up deadline was April 2011. The survival time was counted from the date of surgery to the end of the follow-up or date of death, which was mostly caused by recurrence or metastasis. According to the tumor, nodes, metastasis (TNM)-7th edition 2009 (UICC/AJCC) and Japanese Classification 2010 in Gastric Cancer[17,18], there were 8 papillary adenocarcinomas, 209 tubular adenocarcinomas, 52 mucinous adenocarcinomas, 37 signet ring cell carcinomas, and 17 highly differentiated adenocarcinomas; 100 were classified as well or moderately differentiated adenocarcinomas, 177 as poorly differentiated adenocarcinomas, and 12 as undifferentiated adenocarcinomas or others. Seventy-three cases were categorized as stage Ⅰ, 109 were stage Ⅱ, 92 were stage Ⅲ, and 32 were stage Ⅳ. Eighty-four cases of noncancerous human gastric tissues were obtained from gastrectomies of adjacent gastric cancer margins greater than 5 cm and served as controls.

Immunohistochemistry

Immunohistochemical analysis was undertaken to study altered protein expression in 84 cases of noncancerous human gastric tissues and 306 cases of human gastric cancer tissues[19,20]. According to the protocol for immunohistochemistry on paraffin-embedded tissue sections, slides were baked at 60 ℃ for 2 h followed by deparaffinization with xylene and rehydrated. The sections were submerged into ethylene diamine tetraacetic acid antigenic retrieval buffer and microwaved for antigenic retrieval, after which they were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block nonspecific binding. Sections were incubated with rabbit anti-Popdc3 polyclonal antibody (ProteinTech Group, Chicago IL) overnight at 4 ℃. Normal goat serum was used as a negative control. After rinsing twice for 5 min with Tris buffered saline tween-20, tissue sections were treated with secondary antibody in Tris buffered saline solution for 1 h at room temperature, developed with chromogen at room temperature, and observed under microscope. After that, all tissue sections were counterstained with hematoxylin, dehydrated and mounted. The cytoplasm with strong Popdc3 expression was stained as buffy, whereas weak expression was associated with cell membranes.

Assessment of Popdc3 staining in the tissue sections

The degree of immunostaining was reviewed and scored independently by at least two observers based on the proportion of positively stained tumor cells and intensity of staining[21,22]. Tumor cell proportion was scored as follows: 0 (≤ 5% positive tumor cells), 1 (6%-25% positive tumor cells), 2 (26%-50% positive tumor cells), and 3 (>

Figure 1 Immunohistochemical staining for Popeye domain containing 3 in gastric cancer lesions and noncancerous tissues. A: Popeye domain containing 3 (Popdc3) was highly expressed in noncancerous tissues, × 400; B: Popdc3 was highly expressed in intestinal metaplasia cells, × 400; C, D: Popdc3 was lowly expressed in tubular adenocarcinoma, × 200 and × 400, respectively.

51% positive tumor cells). Staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown). Staining index was calculated as the staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated Popdc3 expression in benign gastric epithelia and malignant lesions by determining the staining index with scores of 0, 1, 2, 3, 4, 6, or 9. The cutoff value for high and low expression levels was chosen based on the heterogeneity measured using the log-rank test with respect to overall survival. An optimal cutoff value was identified as follows: a staining index score of ≥ 4 was used to define tumors with high Popdc3 expression, and a staining index score of ≤ 3 was used to indicate low expression.

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software. Correlation of Popdc3 expression with immunohistochemistry and clinicopathologic parameters was evaluated by χ^2 test or Fisher's exact probability test. Overall survival rate was calculated by the Kaplan-Meier method and the difference in survival curves was analyzed by the log-rank test. The follow-up time was calculated from the date of surgery to the date of death, or the last known follow-up. Independent prognostic factors were analyzed by the Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Popdc3 in gastric cancer and noncancerous mucosa

In our current study, immunohistochemical analysis was done to examine the Popdc3 expression in 306 gastric cancer lesions and 84 noncancerous tissues. Popdc3 was detected in 72 (85.71%) cases of 84 human nontumor mucosa. High expression of Popdc3 protein was detected in 78 (25.49%) cases of 306 human gastric cancer, and low expression was detected in 228 (74.51%). Popdc3 staining was detected mainly in the majority of normal cells, especially in chief cells and smooth muscle. Besides, we also found Popdc3 expression in intestinal metaplasia. However, Popdc3 was mainly localized in the cytoplasm of tumor cells and its weak staining in cell membranes. The differences of Popdc3 expression between gastric cancer and noncancerous mucosa were also statistically significant ($\chi^2 = 1.010E2$, $P < 0.0001$, Figure 1).

Reduced Popdc3 expression and clinicopathologic features

Reduced expression of Popdc3 correlated with depth of invasion, regional lymph node and distant metastasis, and TNM stages ($P < 0.05$). Popdc3 expression did not correlate with age, gender, location of tumor, size of tumor, histologic type and histologic differentiation ($P > 0.05$, Table 1). The factors for possible prognostic effects in gastric cancer were analyzed by Cox regression analysis.

Table 1 Patient characteristics and Popeye domain containing 3 expression in gastric cancer *n* (%)

| Clinical parameters | *n* | Popdc3 | | χ^2 | *P* value |
		Low	High		
Age (yr)					
< 50	105	72 (68.6)	33 (31.4)		
≥ 50	201	156 (77.6)	45 (22.4)	2.968	0.085
Gender					
Male	199	148 (74.4)	51 (25.6)		
Female	107	80 (74.8)	27 (25.2)	0.006	0.940
Size (cm)					
< 5	154	120 (77.9)	34 (22.1)		
≥ 5	152	108 (71.1)	44 (28.9)	1.901	0.168
Histology					
Papillary adenocarcinoma	8	5 (62.5)	3 (37.5)		
Tubular adenocarcinoma	209	151 (72.2)	58 (27.8)		
Mucinous adenocarcinoma	52	42 (80.8)	10 (19.2)		
Signet ring cell carcinoma	37	30 (81.1)	7 (18.9)	3.084	0.379
Histologic differentiation					
Well	17	13 (76.5)	4 (23.5)		
Moderate	100	67 (67)	33 (33)		
Poor	177	140 (79.1)	37 (20.9)		
Undifferentiated	12	8 (66.7)	4 (33.3)	4.439	0.109
Invasion depth					
T1	24	10 (41.7)	14 (58.3)		
T2	63	32 (50.8)	31 (49.2)		
T3	181	149 (82.3)	32 (17.7)		
T4a	28	24 (85.7)	4 (14.3)		
T4b	10	7 (70.0)	3 (30.0)	48.566	< 0.0001
Regional lymph nodes					
N0	163	94 (57.7)	69 (42.3)		
N1	41	39 (95.1)	2 (4.9)		
N2	54	49 (90.7)	5 (9.3)		
N3a	20	14 (70.0)	6 (30.0)		
N3b	27	19 (70.4)	8 (29.6)	52.504	< 0.0001
Distant metastasis					
M0	274	197 (71.9)	77 (28.1)		
M1	32	31 (96.9)	1 (3.1)	9.412	0.002
TNM stages					
I	73	30 (41.1)	43 (58.9)		
II	109	81 (74.3)	28 (25.7)		
III	92	86 (93.5)	6 (6.5)		
IV	32	31 (96.9)	1 (3.1)	70.624	< 0.0001

TNM: Tumor, nodes, metastasis; Popdc3: Popeye domain containing 3.

The multivariate analysis suggested that the patient's gender ($P = 0.016$), regional lymph node metastasis ($P < 0.0001$), distant metastasis ($P < 0.0001$), TNM stages ($P = 0.002$), and the expression of Popdc3 ($P < 0.0001$) were independent prognostic factors in patients with gastric carcinoma. However, patient's age, tumor location, size of tumor, histologic type, histologic differentiation and depth of invasion had no prognostic value (Table 2).

Correlation between Popdc3 expression and patient prognosis

In stages I and II, the 3-year survival rate of patients with a low expression of Popdc3 was significantly lower than in patients with high Popdc3 expression. The survival estimates showed a dramatic difference in median survival between the high and low Popdc3 expression: the former averaged 55 mo [95% confidence interval (CI): 53.515-56.485], whereas the latter 23 mo (95% CI:

Table 2 Multivariate analysis for disease-related deaths (Cox regression model)

Variables	*P* value	Hazard ratio	95% CI
Popdc3	< 0.0001	0.193	0.133-0.282
Gender	0.016	1.388	1.063-1.814
Regional lymph nodes	< 0.0001	1.344	1.156-1.564
Distant metastasis	< 0.0001	11.591	6.131-21.915
TNM stages	0.002	1.486	1.159-1.905

CI: Confidence interval; TNM: Tumor, nodes, metastasis; Popdc3: Popeye domain containing 3.

Figure 2 Kaplan-Meier curves with univariate analyses (log-rank) for patients with low Popeye domain containing 3 expression *vs* high Popeye domain containing 3 expression tumors in all gastric cancer. The cumulative and 3-year survival rates were 88.46% and 84.6%, respectively in the high Popeye domain containing 3 (Popdc3) protein expression group, but was only 76.75% and 16.7% in the low expression group ($\chi^2 = 145.095$, $P < 0.0001$).

21.201-24.799). For patients with low Popdc3 protein expression, 1- and 3-year survival rates were 76.75% and 16.7%, respectively, which were significantly lower than in patients with high Popdc3 expression (88.46%, 84.6%, respectively, $\chi^2 = 145.095$, $P < 0.0001$). Based on this result, we suggested that diminished expression of Popdc3 is a prognostic indicator of poor survival for patients with gastric cancer. In addition, we further compared the survival between the patients in early TNM stage (stages I and II) or late stages (stages III and IV) with different Popdc3 expression. The results showed that low Popdc3 expression had a much more significant effect on the survival of the patients with early stage tumors ($\chi^2 = 104.741$, $P < 0.0001$), with a > 51.9% reduction in the 3-year survival compared with that of patients with high Popdc3 expression. In late stages, the difference was also significant ($\chi^2 = 5.930$, $P = 0.015$), with a 32.6% reduction in the 3-year survival. Therefore, these data suggested that Popdc3 expression was as an independent prognostic variable for gastric cancer in early stage and late stage (Figure 2 and Table 3).

DISCUSSION

The cause of cancer remains elusive and may be multifac-

Table 3 Survival estimates and 95% confidence intervals for disease-related deaths

Groups	Median survival (mo)	1-yr survival rate (%)	3-yr survival rate (%)	χ^2	*P* value
Low Popdc3 expression	23 (21.20-24.79)	175 (76.75)	38 (16.7)		
High Popdc3 expression	55 (53.51-56.48)	69 (88.46)	66 (84.6)	145.095	< 0.0001
Stage Ⅰ-Ⅱ with low Popdc3 expression	29 (21.00-38.00)	97 (87.39)	44 (39.64)		
Stage Ⅰ-Ⅱ with high Popdc3 expression	55 (45.00-61.00)	65 (91.55)	65 (91.55)	104.741	< 0.0001
Stage Ⅲ-Ⅳ with low Popdc3 expression	15 (10.00-21.50)	78 (66.67)	12 (10.26)		
Stage Ⅲ-Ⅳ with high Popdc3 expression	30 (12.00-44.00)	4 (57.14)	3 (42.86)	5.93	0.015

Popdc3: Popeye domain containing 3.

torial[23,24]. Work by many researchers has illuminated that epigenetic alterations, like mutations and chromosomal abnormalities, can be causally involved in carcinogenesis[25,26]. Previous publications discovered that aberrant DNA methylation was more frequently present in cancers than mutations[27,28].

As a prototypical member of the Popdc family, Bves is mostly studied over the past several decades. Many reports have shown that Bves is highly conserved and has been identified in a wide variety of vertebrates and invertebrates[29,30]. Both mRNA and protein of Bves are highly expressed in striated and smooth muscles and in various forms of epithelial cell types in the embryo and adult[31,32]. It seems that Bves plays a critical role in cell-cell adhesion and in maintaining epithelial integrity, suggesting that loss of Bves function could result in abnormal cell behavior and disease[33-35]. In 2008, Feng *et al*[36] used MethyLight assays to analyze DNA methylation status of 27 genes on 49 paired cancerous and noncancerous tissue samples from non-small cell lung cancer (NSCLC) patients and found that Bves were methylated significantly more frequently in tumor tissues than in noncancerous tissues. Methylation of Bves was present in 80% of NSCLC tissues but only in 14% of noncancerous tissues. It is, so far, the first report of a modification of Bves in cancer.

In 2010, Kim *et al*[16] conducted the first detailed research on the Popdc family and discovered that frequent silencing of Bves and Popdc3 was associated with promoter hypermethylation in gastric cancer. Expression of Bves and Popdc3 was downregulated in 73% of the gastric cancer cell lines and in 69% (Bves) and 87% (Popdc3) of the gastric cancer tissues. Bves and Popdc3 were hypermethylated in 69% (Bves) and 64% (Popdc3) of the gastric cancer tissues. They also found that combined treatment with a DNA methylation inhibitor and a histone deacetylase inhibitor strongly induced Bves and Popdc3 expression. These observations suggested that frequent methylation and inactivation of Bves and Popdc3 in early-stage gastric cancer might predispose cells to other critical changes that cause cancer metastasis.

These results are similar to our study. However, the clinical impact of Popdc3 expression or the prognostic value for gastric cancer was not completely clarified because the number of gastric cancer patients was too small. Actually, it is the first study to explore the correlation between Popdc3 expression and clinical and prognostic factors in gastric cancer. Our study demonstrated

that Popdc3 was frequently downregulated in gastric cancer tissues in comparison with those in normal gastric tissues. We examined the relationship between Popdc3 expression and clinicopathological factors in gastric cancer. As a result, reduced level of Popdc3 protein expression in gastric cancer lesions was found mainly associated with depth of invasion, regional lymph node and distant metastasis, and TNM stages. Bves is required for maintenance of E-cadherin in the membrane and plays an important role in cell adhesion and in maintaining epithelial integrity[32]. In development of tumor tissues or diseases, downregulation or mislocalization of E-cadherin is associated with epithelial-mesenchymal transition (EMT)[37]. EMT is considered to be essential for proper development and underlies embryonic processes such as chick gastrulation and coronary vasculature formation[38]. When spontaneously or aberrantly induced in the adult, however, EMT as a hallmark of cancer, may result in loss of epithelial organization and cell tumor tissues invasion of previously normal tissues[39]. Therefore, interfering with E-cadherin function, loss of Bves could result in abnormal cell behavior and disease by promoting EMT programs[40]. Bves was 28% identical to Popdc3 among Popeye family members[12], indicating that Popdc3 may play a role in tumor suppression and interact with Bves.

In this study, multivariate analysis revealed that patient's gender, regional lymph node and distant metastasis, TNM stages, and the expression of Popdc3 were independent prognostic factors for the disease. Although abnormal expression of Popdc3 in gastric cancer might play an important role in the process of tumorigenesis, its biochemical mechanism and potential impact on patient's survival is unknown. So we further analyzed and assessed the impact of expression of Popdc3 on patient's survival. The result indicated that low levels of Popdc3 protein were closely correlated with the prognosis of gastric cancer. A survival curve plotted by the Kaplan-Meier method showed that in patients with low Popdc3 protein expression, the 1- and 3-year survival rates were 76.75% and 16.7%, respectively, which were significantly lower than in patients with high Popdc3 expression (88.46% and 84.6%, respectively). Besides, we further compared the survival between the patients with Popdc3 expression in early TNM stage (stages Ⅰ and Ⅱ) or late stage (stages Ⅲ and Ⅳ). We found that low Popdc3 expression had a much more significant effect on the survival of those patients with early stage tumors, with a > 51.9% reduc-

tion in the 3-year survival as compared with high Popdc3 expression. In late stages, the difference was also significant, with a 32.6% reduction in 3-year survival. These results suggested Popdc3 expression is an independent prognostic variable for gastric cancer in early stage and late stage. In this regard, routine detection of methylation of Popdc3 in blood might be useful in monitoring and detecting tumor recurrence in early-stage gastric cancer after curative surgical resection.

In conclusion, our study suggests that degradation of Popdc3 is a common feature in gastric cancer that might play an important role in the progression and metastases of gastric cancer. In addition, the potentially important consequence of our work is that Popdc3 may be an attractive therapeutic candidate for gastric cancer. Thus, we believe that more researches on Popdc3 will further provide a basis for the development of potential biomarkers for the diagnosis and prognosis of gastric cancer.

COMMENTS

Background
Although many molecular and biological studies have shown risk factors for gastric cancer, the exact molecular mechanism of gastric cancer has not been clarified completely. There is an urgent need to find special markers closely related to tumor outcome and therapy.

Research frontiers
The Popeye domain containing (Popdc) gene family consists of Bves/Popdc1, Popdc2 and Popdc3. As a prototypical member of the Popdc family, Bves is mostly studied over the past decades. However, the clinical impact of Popdc3 expression on cancers is still unknown. This study was carried out to investigate the alterations in the expression of Popdc3 in surgical specimens of gastric cancer, to explore the possible correlation between Popdc3 expression and clinicopathologic variables, to correlate expression of Popdc3 with lymph node metastasis and distant metastasis.

Innovations and breakthroughs
This is the first study attempting to elucidate the role of Popdc3 in gastric cancer and its prognostic significance. The findings suggest that Popdc3 is downregulated in gastric cancer tissues in comparison with those in normal gastric tissues. This study further demonstrates that degradation of Popdc3 is associated with gastric cancer progression and survival of the patients.

Applications
The results will open an avenue for further research to evaluate the role of Popdc3 in gastric cancer. Popdc3 expression may represent a potential prognostic marker and therapeutic target for gastric cancer.

Terminology
Popdc3, as a member of the Popdc family, was firstly detected in developing and adult striated muscle in vertebrates. Chromosomal mapping indicates that Popdc3 gene is clustered on mouse chromosome 10. Frequent methylation and inactivation of Popdc3 are observed in early-stage gastric cancer might predispose cells to other critical changes that cause cancer metastasis.

Peer review
This is a very well written article about the clinical significance of Popdc3 protein expression observed by IHC in gastric cancer. The message of this article was very clear but the author only did IHC to see the expression of Popdc3. This weakened the strength of this article.

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Giardia infection: Protein-losing enteropathy in an adult with immunodeficiency

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Abstract

The case of a 52-year-old woman with a past history of thymoma resection who presented with chronic diarrhea and generalized edema is the focal point of this article. A diagnosis of *Giardia lamblia* infection was established, which was complicated by protein-losing enteropathy and severely low serum protein level in a patient with no urinary protein loss and normal liver function. After anti-helmintic treatment, there was recovery from hypoalbuminemia, though immunoglobulins persisted at low serum levels leading to the hypothesis of an immune system disorder. Good's syndrome is a rare cause of immunodeficiency characterized by the association of hypogammaglobulinemia and thymoma. This primary immune disorder may be complicated by severe infectious diarrhea secondary to disabled humoral and cellular immune response. This is the first description in the literature of an adult patient with an immunodeficiency syndrome who presented with protein-losing enteropathy secondary to giardiasis.

INTRODUCTION

Protein-losing enteropathy (PLE) is a rare syndrome of gastrointestinal protein loss that may complicate a variety of diseases. The diagnosis of PLE should be considered in patients with hypoproteinemia, especially hypoalbuminemia, after other causes have been excluded, such as malnutrition, proteinuria, and impaired protein synthesis. The diagnosis is most commonly based on the determination of fecal α-1 antitrypsin clearance, though scintigraphy (technetium-99m-labeled human serum albumin scan-^{99m}Tc-HSA) aids in localizing the site and quantifying enteric protein loss[1].

The pathogenic mechanism can be divided into erosive gastrointestinal disorders, nonerosive gastrointestinal disorders, and disorders involving increased central venous pressure or mesenteric lymphatic obstruction[1]. Al-

though not extensively studied, certain enteric infections (*Salmonella*, *Shigella*, *Rotavirus*, and *Giardia lamblia*) can also damage the intestinal mucosa leading to excessive protein loss, particularly in an immunocompromised host[2,3].

Giardia lamblia is a protozoan that frequently infects the gut and plays an important role in the public health of developing countries, such as Brazil[4]. Transmission occurs through oral-fecal contact or intake of contaminated water and food by the infecting forms (cysts or oocysts). Although normally presenting with mild symptoms, such as cramps and chronic diarrhea, more severe complications can take place and malabsorptive syndrome may develop[5]. In this case report, severe giardiasis is illustrated in an adult patient with a past history of thymoma who developed PLE, a rare complication that particularly affects infants.

CASE REPORT

A 52-year-old black woman was referred to our hospital because of chronic diarrhea and generalized edema. She had gained 10 kg over the last year because of peripheral edema, and complained about asthenia and fatigue. She had no abdominal pain, fever or stool bleeding. Two years before the event, while under investigation for chronic cough and chest pain, the diagnosis of a type AB thymoma was made according to World Health Organization classification and she underwent a sternal thymectomy. Anatomopathological analysis showed no signs of capsular, vascular or lymphatic invasion.

The patient presented mild steatorrhea on a semi-quantitative stool analysis, negative proctoparasitologic examination and severe hypoproteinemia on serum protein electrophoresis (Figure 1A). A hepatic cause of hypoproteinemia was ruled out by clinical and laboratory parameters and urinary sedimentary analysis was normal with no proteinuria. There was altered coagulation testing related to vitamin K deficiency, normocytic normochromic anemia, and normal leucocyte and platelet count.

She was submitted to ^{99m}Tc-HSA which demonstrated mild protein loss from the small bowel though no specific site was able to be localized. She had a normal colonoscopy and an upper digestive endoscopy that showed moderate duodenitis. On duodenal biopsy, there were structures identified as *Giardia lamblia*, moderate non-specific active chronic duodenitis, and preserved villi:crypt ratio. Giardiasis was not attributed at first to be the cause of the enteric protein loss. In subsequent investigations, anterograde enteroscopy was undergone, demonstrating only non-specific duodenal and jejunal lesions. No remarkable findings were verified on histological samples.

Nevertheless, after anti-helmintic treatment with oral metronidazole, she ended up recovering from diarrhea and edema. No signs of enteric protein loss were verified by scintigraphy and serum albumin level normalized, though hypogammaglobulinemia persisted (Figure 1B).

On evaluation of intermittent asthma and rhinitis since childhood and recurrent pneumonia, antibody de-

ficiency was diagnosed, including immunoglobulins IgA, IgE, IgG and IgM. Cytometry analysis of peripheral blood lymphocytes revealed a marked decrease in CD19+ B-cells. Bone marrow biopsy demonstrated a chromosome 9 inversion with chromosome 16 deletion [*46,X X,inv(9),del(16)(q22)*] with unknown significance and no additional important findings. Based on previous history of thymoma and current hypogammaglobulinemia, the diagnosis of Good's syndrome (GS) was made even though no evidence of cell-mediated immune defects was verified.

DISCUSSION

Giardia lamblia is a flagellated intestinal protozoan with oral-fecal transmission. Infection takes place in the proximal small gut, especially duodenum, where motile trophozoites live adhered to enterocytes[5].

Examination of concentrated, iodine-stained wet stool preparations and modified-trichrome-stained permanent smears has been the conventional approach to the diagnosis. Despite three negative stool samples, the diagnosis of giardiasis could be made by duodenal biopsy in this case report. In fact, cysts and trophozoites are present only intermittently in feces, offering a low sensitivity of approximately 50%, even with examination of multiple specimens. Identification of trophozoites within small intestinal biopsy specimens requires careful examination of multiple microscope fields to ensure accuracy[6], although on direct sampling of duodenal contents (e.g., duodenal aspiration or the "string test"), sensitivity can be improved to approximately 80%. Therefore molecular tests based on enzyme linked immunosorbent assay or direct immunofluorescent antibody microscopy should be the first diagnostic test to be performed because of high accuracy, with sensitivities greater than 90% and specificities approaching 100%.

Worldwide, *Giardia* affects infants more commonly than adults and has different pathogenicity in experimental human infections. Infected patients, especially those immunocompromised, may present malabsorptive diarrhea of yet unknown mechanism. Trophozoites adhere (perhaps by suction) to the epithelium of the upper small intestine, using a disk structure located on their ventral surface. There is no evidence that trophozoites invade the mucosa. On biopsy, pathologic changes range from an entirely normal-appearing duodenal mucosa (except for adherent trophozoites) to severe villous atrophy with a mononuclear cell infiltrate that resembles celiac sprue[7].

The severity of diarrhea appears to correlate with the severity of the pathologic change[6]. In fact, the host immune response plays a critical role in limiting the severity of giardiasis; this is mediated by humoral immune response, both systemic (IgM and IgG) and mucosal (IgA). Certain populations, including children younger than 2 years of age and patients with hypogammaglobulinemia, are more likely to develop serious disease. When infected with *Giardia*, individuals with common variable immuno-

A

ALB	A1G	A2G	BETA	GG	Total
1.23	0.16	0.41	0.61	0.09	2.26

B

ALB	A1G	A2G	BETA	GG	Total
3.73	0.21	0.59	1.09	0.17	5.79

Figure 1 Serum protein electrophoresis analysis before treatment (A) and after treatment (B). ALB: Albumin; A1G: α-1 globulin; A2G: α-2 globulin; BETA: β globulin; GG: γ globulin.

deficiency develop severe, protracted diarrhea and malabsorption with sprue-like pathologic changes that resolve with treatment[3] such as immunoglobulin replacement[6]. The importance of a cellular immune response is yet to be determined. On immune reconstitution, severe inflammatory changes and villous atrophy develop in the intestine, suggesting that the immune response to infection may contribute to pathology, although severe infections that are resistant to treatment or an increased frequency of giardiasis have been noted only rarely in patients with acquired immune deficiency syndrome[7].

PLE is a rare complication associated with Giardia infection, as formerly described in infants[1-3]. As illustrated in this article, this infection may cause severe enteric mucosal damage that leads to an inflammatory process and exudative protein loss. Sometimes, especially when there is associated lymph leakage, secondary immunodeficiency may develop as decreases in serum lymphocytes and hypogammaglobulinemia occur[1].

As previously mentioned, although PLE is better diagnosed by the determination of fecal α-1 antitrypsin clearance because of high accuracy of the method, scintigraphy was the option chosen for the diagnosis in this case and it could localize protein loss from the small gut at non-specific sites. ^{99m}Tc-HSA scan possesses high sensitivity and specificity; it may demonstrate the site and semi-quantify protein loss, despite some false positives resulting from active gastrointestinal bleeding and *in vivo* breakdown of ^{99m}Tc-HSA, yielding free pertechnetate from radiolabeling[8]. The former is easily excluded by fecal occult blood examination and the latter by the absence of stomach or thyroid visualization.

At first, the hypothesis of hypogammaglobulinemia secondary to gut protein loss was put forward for this patient since severe universal hypoproteinemia was verified in pre-treatment serum protein electrophoresis. Nevertheless, after metronidazole was instituted, every serum protein fraction increased but gammaglobulin remained very low. This led to the belief that she possessed an

immune disorder, which could explain the advanced enteropathy caused by *Giardia* infection and the persistent hypogammaglobulinemia in spite of medical treatment.

Patients with thymoma may suffer from specific types of paraneoplastic syndrome or remote effects of cancer which have underlying autoimmunity origins. They include myasthenia gravis, pure red cell aplasia, and hypogammaglobulinemia (Good's syndrome), with approximate occurrence of 30%, from 1.6% to 5%, and from 3% to 6%, respectively. Autoimmunity to B lymphocyte lineage causes severe deficiency in B lymphocytes and hypogammaglobulinemia, resulting in acquired immunodeficiency vulnerable especially to bacterial infection. Although most paraneoplastic syndromes have been documented to recover after tumor resection, some previous case reports seem to indicate that hypogammaglobulinemia without improvement can last even after tumor resection for as long as 9 years[9].

GS is a rare association of thymoma and immunodeficiency first described more than 50 years ago by Dr. Good. The diagnosis of thymoma usually precedes the diagnosis of hypogammaglobulinemia and other clinical manifestations, such as infection or diarrhea, in almost half of the cases. Recurrent sinopulmonary infection is commonly the first clinical manifestation followed by chronic diarrhea, the mechanism of which is not identified in the majority of cases, although bacteria, such as *Salmonella spp.*, and other pathogens, like cytomegalovirus and *Giardia lamblia* might the etiological factor[9,10].

GS, in contrast to other primary immunodeficiency disorders such as common variable immunodeficiency disease, usually affects adults in the fourth or fifth decade of life, equally male and female, and almost always associated with hypogammaglobulinemia. Although there are no formal diagnostic criteria for this disorder, GS is characterized by low to absent B cells in the peripheral blood, hypogammaglobulinemia, and variable defects in cell-mediated immunity; with a CD4 lymphopenia, an inverted CD4/CD8+ T-cell ratio and reduced T-cell mitogen pro-

liferative responses[4]. In clinical practice, as illustrated in this report, serum flow lymphocytometry shows absence of B lymphocytes, discrete low CD4 T lymphocyte count and inverted CD4/CD8 ratio[9-11].

It has long been known that giardiasis is a frequent infection of the gastrointestinal tract, especially in infants and in developing countries, typically manifesting as chronic diarrhea, abdominal cramps and fatigue. The diagnosis may be challenging as conventional methods of stool examination can lack sensitivity. In cases of strong clinical suspicion, serum serology is of great value when available, though upper digestive endoscopy is a reliable tool to demonstrate degree of duodenal damage and direct diagnosis can be made through histological sample. As far as we know, this is the first time in the literature that Giardia infection complicated with PLE has been reported in an adult patient. In fact, whenever severe giardiasis occurs in adulthood, immunodeficiency should always be kept in mind because of humoral IgA-mediated immunological pathogenicity, and clinical investigation should be directed towards this suspicion, even though rare in cause.

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CASE REPORT

An unusual enteropathy-associated T-cell lymphoma with MYC translocation arising in a Japanese patient: A case report

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Abstract

Enteropathy-associated T-cell lymphoma (EATL) is a rare peripheral T-cell lymphoma classified into 2 types, with or without celiac disease, based on histology. Type 2 EATL is less commonly associated with celiac disease, in which cells are characterized by being monomorphic and small- to medium-sized. Cells are characterized by CD8 and CD56 expression and c-MYC oncogene locus gain. We present an atypical case of type 2 EATL in the jejunum, with human T-lymphotropic virus-1 that was CD4- CD8+ CD56- CD30- CD25- TIA-1+ and granzyme B+ on immunohistological staining. It also displayed translocation of chromosome 8p24 (c-MYC), as de-termined by fluorescent *in situ* hybridization. Mucosal spreading and intraepithelial invasion by lymphoma with villous atrophy were detected adjacent to the mucosal layer. The lymphoma may be derived from in-traepithelial CD8+ T cells, similar to celiac disease.

INTRODUCTION

Gastrointestinal lymphomas account for 4%-20% of all non-Hodgkin's lymphomas. Enteropathy-associated T-cell lymphoma (EATL) is a primary extranodal T-cell lympho-ma arising in the gastrointestinal tract, a rare subtype of peripheral T-cell lymphoma, and accounts for less than 1% of all non-Hodgkin's lymphomas[1-3]. There are two dif-ferent sub-classifications: type 1 EATL (with) and type 2 EATL (without) celiac disease[1,4]. The disease is seen with greater frequency in areas with a high prevalence of celiac disease, but it is rare in Japan[5]. Type 2 EATL accounts for 10%-20% of cases, and is less commonly associated

with celiac disease[1,4]. The tumor cells are monomorphic and small- to medium-sized, with infiltration of the intestinal crypt epithelium without inflammation[1,4]. Type 2 EATL often occurs without a history of celiac disease and shows strong expression of CD56 (> 90%) and CD8 (80%) based on immunohistochemical staining[1]. EATL generally has a poor prognosis because it is often diagnosed late, has spread, and is therefore refractory to treatment. We report a case with human T-lymphotropic virus-1 (HTLV-1) in which a type 2 EATL was CD8+, CD56-, T cell restricted intracellular antigen-1 (TIA-1)+ on immunohistological staining, and possessed a unique chromosomal change as determined by fluorescent *in situ* hybridization (FISH).

CASE REPORT

A 66-year-old woman with a past medical history of hypertension presented to the emergency department with a sudden onset of severe abdominal pain. Her weight was 43.5 kg and body mass was 19.7 kg/m^2. A physical examination revealed a high fever (39 ℃) with chills; diffuse, severe, constant abdominal pain; and muscular guarding. She had experienced abdominal distension for a month, but did not have diarrhea, weight loss, a history of malnutrition, or a food intolerance. We could not detect any palpable peripheral lymph nodes. The laboratory data on admission showed mild leukocytosis (white blood cell count of 10 400/mm^3) and hypoproteinemia (total protein 5.4 g/dL). Persistent hypoproteinemia was recognized at 4 mo prior, based on previous medical records. No atypical lymphocytes were detected in the peripheral blood. A computed tomography showed free air and wall thickening in the pseudo-aneurysmally dilated small bowel within the pelvic area, with a defect of the intestinal wall (Figure 1). Laparotomy was performed and showed a pseudo-aneurysmally dilated and perforated jejunum 80 cm from the ligament of Treitz, with massive purulent ascites fluid. We performed a segmental resection of the lesion and a side-to-side anastomosis. The resected jejunum was generally thickened and there was diffuse transmural infiltration by monomorphic and medium-sized atypical lymphoid cells, with villous atrophy of the intestinal glands (Figure 2A). Intramucosal spreading of lymphoma was found in an adjacent mucosal layer, and many atypical intraepithelial lymphocytes were detected (Figure 2B and C). Immunohistochemical staining was positive for CD3, CD7, CD8, TIA-1 granzyme B, c-MYC and Ki-67, and negative for CD4, CD5, CD20, CD25, CD30, CD56, and CCR4 (Figure 2D and E). FISH analysis revealed translocation of chromosome 8q24 (c-MYC) (Figure 2F). Polymerase chain reaction analysis of *TCRγ* gene rearrangements were performed using the BIOMED-2 procedure[5]. Rearrangement bands were detected in 4 VγIf, Vγ9, Vγ10, Vγ11, and Jγ1.1/2.1, and Jγ1.3/2.3 consensus primers.

Based on these findings, we made a diagnosis of type 2 EATL. On postoperative day 10, the patient was

Figure 1 Computed tomography scan of the dilated and perforated small bowel.

discharged without any complications. After the diagnosis, we performed additional laboratory tests. Serum soluble interleukin-2 receptor was elevated (822 U/mL), and serum HTLV-1 antibodies were positive. 67 Gallium scintigraphy and fluorodeoxyglucose positron-emission tomography did not show any site of involvement. Bone marrow biopsy did not reveal malignant cells. Based on these results, we diagnosed clinical stage 2E. The patient received high-dose chemotherapy with autologous hematopoietic cells 6 mo after surgery.

DISCUSSION

EATL is a rare subtype of peripheral T-cell lymphoma, with an incidence of 0.25% of all lymphomas in Japan[3,6]. Since gluten allergy is uncommon in Japan, it is extremely rare for celiac disease to be a basal disorder, and type 2 EATL is more common than type 1 EATL[5]. EATL most commonly arises in the proximal jejunum, and typically presents with abdominal pain. It is often associated with intestinal obstruction, perforation, or bleeding, and is diagnosed by histology tests. Type 2 EATL consists of monomorphic and small- to medium-sized tumor cells that frequently express CD8 and CD56[3]. The prognosis of EATL is usually poor based on results of conventional combination chemotherapy, although high-dose chemotherapy with autologous hematopoietic cell transplantation may have longer survival rates[2,7].

The pathological findings of this case showed monomorphic, small- to medium-sized tumor cells with villous atrophy of the mucosa. Combined with the mucosal spreading and increased intraepithelial lymphocytes (IELs), we made a diagnosis of type 2 EATL. Type 2 EATL is usually positive for CD8 and CD56 (> 90%), but this patient was CD56 negative[3]. Several studies have suggested that the Epstein-Barr virus plays an etiological role in EATL pathogenesis, but this is still a subject of debate. In this case, Epstein-Barr encoded RNAs and latent membrane proteins were not expressed, suggesting that Epstein-Barr virus was not involved in tumor proliferation. Test results showed HTLV-1 infection based on serological antibodies, but the tumor cells were different from adult T cell lymphoma/leukemia immunopheno-

Figure 2 Pathological findings of the specimen. A: Microscopic findings of the specimen with villous atrophy, crypt hyperplasia, and proliferation of intraepithelial lymphocytes; B: Mucosal and submucosal invasion by lymphoma cells; C: Lymphoma cells with the characteristics of intraepithelial lymphocytes; D: Expression of CD8 by intraepithelial lymphocytes; E: Expression of T cell restricted intracellular antigen-1 by intraepithelial lymphocytes; F: Examination of chromosome 8q24 (c-MYC region) using break point rearrangement probe by *in situ* hybridization.

type features with respect to CD4, CD8, CD25, CCR4 and cytotoxic proteins[8]. FISH analysis, however, showed gene translocation at 8q24, which is the c-MYC oncogene locus. It has been suggested that c-MYC protein plays a role in lymphoid proliferation and the apoptotic pathway in malignant lymphomas[9]. In type 2 EATL pathogenesis, about 70% of cases show a gain of 8q24, suggesting c-MYC is an important transcription factor in EATL[4]. We present here the first EATL case with translocation of chromosome 8q24 (c-MYC region).

Some studies suggested chronic inflammation might contribute to the development of neoplastic lymphocyte growth in EATL[4,10]. Celiac disease is characterized by villous atrophy, crypt hyperplasia, and an increased invasion of CD8+ IEL. Celiac disease may progress to type 1 EATL[10], especially in patients with refractory celiac disease, in which IELs have aberrant phenotypes and genetic alterations due to persistent inflammation[4,10]. This is supported by the fact that refractory celiac disease and type 1 EATL rarely show aberrant CD8 expression[4,10]. This case showed protein-losing enteropathy and lymphoma cells with IEL-like features in the mucosa, and villous atrophy. We considered the possibility of persistent enteropathy, which is consistent with hypoproteinemia 4 mo before the surgery. Celiac-like enteropathy, rare in Japan, is consistent with the clinical symptoms, and in this case, persistent enteropathy might cause CD8+ IELs to transform to neoplastic cells *via* c-MYC. The immunophenotype of this

case was different from typical type 2 EATL cases, regarding CD3+ CD4- CD8+ CD30- CD56-.

In summary, we report the unique case of a 66-year-old HTLV-1 woman diagnosed with type 2 EATL, which was CD3+ CD4- CD8+ CD30- CD56- on immunostaining, and displayed translocation of the c-MYC region.

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CASE REPORT

Spontaneous perforation of an intramural rectal hematoma: Report of a case

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Abstract

Spontaneous hematomas are rare and most occur secondary to hematologic disorders or during anticoagulant therapy. Most spontaneous hematomas occur above the sigmoid colon, and rarely in the rectum. Herein we present the case of a patient with a spontaneous perforating hematoma of the rectum who presented with severe abdominal pain after a bloody stool. The hemoglobin level decreased by 33 g/L within 20 h. An abdominal sonogram showed a hydrops in the lower abdomen with a maximum depth of 7.0 cm. A hematoma, 8 cm × 6 cm × 5 cm in size, was noted intra-operatively in the rectosigmoid junction, with a 1.5-cm perforation in the hematoma and active hemorrhage. Thus, a partial rectectomy and sigmoidostomy were performed. Three months later, a second operative procedure to re-establish intestinal continuity was performed. The patient is in good condition 12 mo after the last surgery. In addition to this case, the causes of spontaneous perforating hematomas and the treatment are discussed.

INTRODUCTION

Intramural hematomas may occur in every portion of the alimentary tract from the esophagus to the sigmoid colon, but rarely occur in the rectum. Intramural hematomas most often result from blunt trauma[1]. Non-traumatic spontaneous hematomas usually reflect an underlying blood dyscrasia, anticoagulant therapy, and hematologic diseases[2]. Spontaneous rectal hematomas have rarely been reported. Herein we present this unusual case of a spontaneous perforating hematoma.

CASE REPORT

A 52-year-old male was admitted to our hospital with a 20-h history of severe abdominal pain after passing a small amount of bloody stool. The patient had no history of trauma before admission and clearly related the acute onset of pain with straining to have a bowel movement. The patient had 5-year history of oral warfarin sodium use after mitral valve replacement and coronary stent implantation. The physical examination revealed diffuse abdominal tenderness, rebound tenderness, widespread perianal ecchymosis, and muscular tension without na-

Figure 1 Pre-operative photograph of the perineum (knee-chest position) revealing widespread perianal ecchymosis (marked with black arrows).

Figure 2 Intra-operative photograph of a large intramural hematoma arising beneath the rectosigmoid junction.

Figure 3 Extensive oozing of blood in the muscular layer of the resected rectal and fibrinoid necrosis within the vascular wall (hematoxylin and eosion stain, × 20).

usea and vomiting (Figure 1). There was a persistent drop in hemoglobin from 94 g/L to 61 g/L over a 20-h interval. The prothrombin time was prolonged (44.40 s; approximately four times the normal prothrombin time). An abdominal sonogram showed a large hydrops in the lower abdomen with a maximum depth of 7.0 cm. After admission, active resistance to shock treatment was given, and the abdominal symptoms did not remit, thus emergent surgery was performed. A palpable hematoma, 8 cm × 6 cm × 5 cm in size, was noted in the rectosigmoid junction intra-operatively (Figure 2). Furthermore, there was a perforation in the hematoma, 1.5 cm in diameter, and active hemorrhage. After confirming that the perforating hematoma of the rectum was the cause of the generalized peritonitis and the extensive oozing of blood could not be stopped, a partial rectectomy and sigmoidostomy were performed. A histopathologic examination of the removed specimen revealed extensive oozing of blood in the muscular layer of the rectum and fibrinoid necrosis within the vascular wall (Figure 3). The patient recovered without complications and was discharged on the 8th post-operative day. Three months later, the patient returned for re-establishment of intestinal continuity. The patient was in good health and completely asymptomatic during the 12-mo follow-up period after the second surgical procedure.

DISCUSSION

Intramural hematomas of the alimentary tract are unusual. Intramural hematomas mainly occur above the sigmoid colon, and rarely in the rectum. The duodenum is the most common site for intramural hematomas[1]. In a review of the medical literature, most rectal intramural hematomas occur following blunt trauma. Spontaneous perforating hematomas of the rectum have rarely been reported[3].

Of all intramural hematomas, 15%-36% are spontaneous in patients with underlying hematologic diseases or on anticoagulant therapy[2]. As shown in the current case, the patient had a long history of oral warfarin sodium use, and the prothrombin time was prolonged to 44.40 s, which is approximately four times the normal prothrombin time. Anticoagulation-induced intramural hematomas can occur in patients with therapeutic prothrombin times, but the majority of intramural hematomas occur in patients with abnormally prolonged prothrombin times[2]. Thus, anticoagulant therapy was thought to be closely related with the hematoma in this patient. In addition, increased abdominal pressure during straining may play an important role in hematoma formation. Specifically, when a patient strains to have a bowel movement, the abdominal pressure increases and the rectum contracts. The contracted rectum increases the intramural pressure which leads to decreased vascular compliance, and even rupture, thus causing a rectal intramural hematoma. When the pressure of the intramural hematoma is greater than the capacity of the hematoma wall, the hematoma breaks and clinical symptoms appear.

Most patients with intramural hematomas present with signs of intestinal obstruction[3]. Conservative therapy is usually sufficient because the hematomas will undergo spontaneous resorption[4], but treatment decisions depend on the symptoms and clinical findings[4,5]. Relief of the tamponade effect during conservative therapy could lead to further bleeding[1]. In patients in whom the cause of obstruction is not known, or in patients with complete obstruction or who have failed medical management, surgical intervention is required[6]. Draining the

hematoma might increase the risk of serious infection, as reported previously[7]. According to the decreasing hemoglobin concentration and failed conservative therapy, we performed a partial rectectomy and sigmoidostomy. The patient recovered without complications.

Therefore, testing prothrombin times regularly and avoiding severe abdominal pressure in a patient who has a long-term oral anticoagulant drug history might help prevent complications associated with intramural hematomas, such as perforation.

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Does antioxidant therapy influence every aspect of quality of life?

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Abstract

To present problems that might severely impact the conclusions drawn by the authors of an article on antioxidant treatment in chronic pancreatitis (*World J Gastroenterol* 2010; 16: 4066-4071). We analyzed and discussed this paper by Shah *et al*, and found that promising as it is, this study has some methodological shortcomings, such as: cross-sectional nature of the study, lack of initial evaluations of quality of life and regular follow-ups to determine the dynamics and real directions of changes in quality of life. We therefore concluded that the results of the study by Shah *et al* are biased and, although very promising, should not be considered as scientifically relevant.

Stefaniak T, Vingerhoets A, Sledzinski Z. Does antioxidant therapy influence every aspect of quality of life? *World J Gastroenterol* 2012; 18(19): 2441-2442 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i19/2441.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i19.2441

TO THE EDITOR

We have read with great interest an article by Shah *et al*[1] (*World J Gastroenterol* 2010; 16: 4066-4071). Exciting news about the clinical use of antioxidant therapy seem to open an entirely new chapter in treatment of chronic pancreatitis. We have already published information on possibilities concerning the role of oxygen free radicals in experimental acute pancreatitis as well as the potential role of antioxidants in treatment the disease[2]. As a team of scientists and clinicians intensively involved in the treatment of pancreatic chronic pain, we have found the study presented by Shah *et al*[1] very promising.

Unfortunately, despite very good rationale and biochemical background, the manuscript cannot be considered as free of significant bias, that most probably severely impacts the final conclusions. The pain and quality of life (QoL) scores were obtained only after a 6-mo period of follow-up, there was no initial score at the beginning of the therapy. Moreover, the authors presented only measurement of pain and QoL, which does not seem to be sufficient in context of complexity of interactions (such as social and emotional) that chronic pancreatitis patients may experience[3,4]. In that circumstances, we would recommend that follow-up measurements should be made every month, as presented in other studies on pain in chronic pancreatitis and/or pancreatic cancer[3,4]. Lack of initial information, randomization and pain and QoL dynamics assessment leaves a suspicion that the patients treated with antioxidants suffered from less pain, had better QoL and consumed less opioids from the very beginning of this doubtlessly non-prospective, uncontrol-

led trial. Significant differences in almost every variable of QLQ-C30 seem to confirm our point of view. It is difficult to explain in any psychological paradigm or biochemical formulae, what possible influence could antioxidant therapy exert on cognitive or emotional functioning or, most interestingly on body image, alcohol-related guilt or even financial problems.

To sum up, we believe that antioxidants may offer a very interesting and promising supplementation to the treatment of chronic pancreatitis, but unfortunately, methodological problems presented in the article of Shah *et al*[1] completely disqualify the conclusions of the authors. We would be most happy to see a randomized prospective trial with dynamic evaluations that would confirm the results presented in this article.

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World Journal of Gastroenterology

MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
United States

April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

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World Journal of Gastroenterology

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Organization as author

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Both personal authors and an organization as author

5   **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Author(s) and editor(s)

12   **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13   **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

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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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Contents

Contents

ABOUT COVER

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T cell immunopathogenesis and immunotherapeutic strategies for chronic hepatitis B virus infection

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Abstract

Hepatitis B is caused by the host immune response and T cells play a major role in the immunopathogenesis. More importantly, T cells not only destroy hepatocytes infected by hepatitis B virus (HBV), but also control HBV replication or eradicate HBV in a noncytolytic manner. Therefore, analysis of T cell immune response during acute and chronic HBV infection is important to develop a strategy for successful viral control, which could lead to immunotherapy for terminating persistent HBV infection. There have been many attempts at immunotherapy for chronic HBV infection, and some have shown promising results. High viral load has been shown to suppress antiviral immune responses and immunoinhibitory signals have been recently elucidated, therefore, viral suppression by nucleos(t)ide analogs, stimulation of antiviral immune response, and suppression of the immunoinhibitory signals must be combined to achieve desirable antiviral effects.

INTRODUCTION

Hepatitis B virus (HBV) is not cytopathic, and hepatitis B is caused by the host immune response, mainly T-cell-mediated, against virus-related peptides expressed on hepatocytes in conjunction with human leukocyte antigens (HLAs). In acute self-limiting hepatitis, a broad T-cell immune response occurs that is strong enough to eradicate the virus or suppress viral replication[1]. However, there are many mechanisms that hamper the antiviral immune response, leading to persistent infection. To develop an optimal strategy to stimulate antiviral immune response with therapeutic potential, extensive analyses of immune mechanisms for successful viral eradication and immunosuppressive mechanisms induced by viral infection during persistent infection are required. In this review, I focus on T cell immune response during HBV infection, and summarize attempted immunotherapeutic approaches against persistent HBV infection.

T CELL RESPONSE IN ACUTE HBV INFECTION

Immunological analysis has been extensively performed in transgenic and chimpanzee models of acute HBV infection. In one model, transgenic mice, in which infectious HBV virions replicate in the liver with expression of all HBV-related antigens, were injected with hepatitis B surface antigen (HBsAg)-specific cytotoxic T lymphocytes (CTLs) that had been induced in nontransgenic mice. The injected CTLs produced interferon (IFN)-γ and tumor necrosis factor (TNF)-α, which purged viral

RNA and DNA without destroying infected hepatocytes[2-4]. Importantly, this noncytolytic clearance of intracellular HBV is more efficient at controlling HBV replication than the killing of infected hepatocytes. In this sense, hepatitis is not only a harmful event but also represents an effective mechanism by which CTLs suppress HBV. Noncytolytic viral eradication can account for recovery from acute HBV infection in that most HBV is cleared from hepatocytes with only a fraction of the hepatocytes being destroyed. This was confirmed in a chimpanzee infection model; HBV DNA level was markedly decreased in the liver and blood of acutely infected chimpanzees before peak serum alanine aminotransferase (ALT) concentrations were reached[5], suggesting that this noncytopathic T cell effector mechanism results in early viral inhibition or eradication, whereas a cytopathic T cell effector mechanism is required to eliminate the remaining virus by destroying infected hepatocytes.

In humans, the HBV-specific T cell response during incubation phase of acute hepatitis B has been analyzed extensively using HLA class I tetramer and cytokine staining[6]. The data showed that maximal reduction in HBV DNA in the serum occurred before the peak of ALT elevation; again indicating that suppression of HBV replication occurs without hepatocyte injury. Moreover, infiltration of HBV-specific CD8+ T cells into the liver has been observed several weeks before the peak of liver injury, suggesting that HBV-specific T cell infiltration occurs at an early stage of infection, resulting in suppression of HBV replication. Thereafter, recruitment of mostly nonspecific cells induced by cytokines or chemokines produced by HBV-specific T cells contributes to significant liver damage. Interestingly, in the HBV transgenic mouse model of acute hepatitis, administration of antibodies against the chemokines, IFN-inducible protein (IP-10) and monokine induced by interferon-(Mig), reduced the recruitment of mostly antigen-nonspecific mononuclear cells into the liver that had been induced by cytokines and chemokines produced by injected CTLs, leading to a reduction in the severity of hepatitis without affecting the antiviral activity of the CTLs[7]. These observations have important therapeutic implications, because suppression of antigen-nonspecific mononuclear cell recruitment may suppress hepatitis, while retaining the antiviral function of the CTLs.

The overall data from studies in chimpanzees and humans are essentially the same, and indicate that a sufficient T cell response to HBV at an early phase of infection is important for eradication of virus infection, and that an insufficient T cell response may lead to persistent viral infection.

The contributions of CD4+ and CD8+ T cells to the control of viral infection have been analyzed in a chimpanzee model of acute hepatitis B by depleting either T cell population with monoclonal antibodies. The data show that CD8+ T cells are the main effector cells responsible for virus elimination[8].

Antigen specificity of T cell response in acute HBV infection

The antigen specificity of the T cell response to HBV in acute hepatitis has been analyzed, and it is clear that acute viral hepatitis involves a vigorous CTL response to multiple epitopes in the viral nucleocapsid, envelope, and polymerase proteins, whereas these are not seen in patients with chronic hepatitis[1]. Although multispecificity of the CTL response is characteristic in acute hepatitis, there is known to be a hierarchy of epitope-specific CD8+ T cell responses determined by cytokine production after peptide stimulation. In acute hepatitis B, CD8+ T cell response to HBc18-27 (HLA-A2 restricted epitope) is dominant followed by the response to polymerase epitope (455-463), whereas envelope epitopes are always subdominant[9]. The hierarchy is clearly distinct from that observed in chronic hepatitis, in which the CD8+ T cell response to envelope epitope (183-191) is always dominant. Interestingly, chronic hepatitis patients with lower HBV DNA levels in the serum show greater responses to HBc18-27 than those with high HBV DNA. These findings imply that the T cell response to hepatitis B core antigen (HBcAg) is important for viral control, which is important for designing peptide vaccines for the treatment of chronic HBV infection.

Long-lasting T cell immune response after resolution of acute hepatitis B

In humans, most HBV is cleared after resolution of acute hepatitis. However, it has been shown that trace amounts of HBV DNA can be detected for several years after resolution of acute hepatitis, and the long-lasting memory T cell response is maintained by persistent replication of HBV[10], indicating that low levels of HBV replication could continue in most patients even in the convalescent phase of acute hepatitis in balance with immunological pressure.

T CELL RESPONSE IN CHRONIC HBV INFECTION

In peripheral blood, HBV-specific helper T lymphocytes and CTLs are barely detectable in patients with chronic hepatitis B (CHB)[11], possibly due to exhaustion by high viral load or tolerance to HBV.

In contrast, several studies have characterized intrahepatic CD4+ and CD8+ T lymphocytes in CHB. Intrahepatic CD4+ T lymphocytes in patients with CHB have been found to contain T helper (Th)0 cells, which produce not only IFN-γ, but also interleukin (IL)-4 and IL-5, thus differing from cells in the livers of patients with chronic hepatitis C, which are mostly Th1 cells[12]. CD4+ T lymphocytes that produce IL-17 infiltrate into the livers of patients with CHB and are involved in liver inflammation[13].

Livers of patients with low HBV replication contain intralobular CD8+ T lymphocytes[14], and the percent-

ages of virus-specific T lymphocytes in the liver have been clarified by immunohistochemical staining with peptide-MHC tetramer. The proportion of CD8^{+} T lymphocytes in the livers of patients with chronic HBV specific for HBc18-27, a major HBV epitope, has been found to range from 0.18% to 1.28%[15]. Maini *et al*[16] have reported that the number of HBc18-27-specific CD8^{+} T cells, detected using tetramers, was the same in livers with low HBV DNA/ALT as in those with high HBV DNA/ALT. Hence, HBV-specific T cells recognize HBV antigens and carry out immune surveillance in the liver. Thus, they have an important role in controlling HBV replication in the liver without causing hepatic necroinflammation in low DNA/ALT anti-HBe[+] HBV carriers. It remains unknown why HBV-specific T cells fail to control effectively HBV replication in the liver with chronic hepatitis. However, recent advances in immunology have given some insight into the mechanism as described below.

IMMUNOSUPPRESSIVE MECHANISM RESPOSIBLE FOR PERSISTENT HBV INFECTION

Regulatory T cells

Regulatory T (Treg) cells expressing the forkhead family transcription factor, FoxP3, are specialized cells that exert negative control on a variety of physiological and pathological immune responses, resulting in maintenance of immunological self-tolerance[17]. They show diverse phenotypes, occurring in both CD4^{+} and CD8^{+} T cell subsets, and express CD25 (IL-2 receptor chain) and/or cytotoxic T-lymphocyte antigen 4 (CTLA-4) in addition to Foxp3.

In HBV infection, hepatitis B e antigen (HBeAg)-positive patients with high HBV DNA levels in the serum show elevated numbers of CD4^{+}CD25^{+} Treg cells in the blood compared to patients with acute and chronic hepatitis C virus (HCV) infection[18]. Significant accumulation of CD4^{+}CD25^{+}FoxP3^{+} Treg cells in the liver is found in patients with chronic HBV infection. Moreover, patients with high viral load have a higher proportion of Treg cells in the liver[19], suggesting that intrahepatic Treg cells suppress antiviral immune responses in the liver in chronic HBV infection.

Th cells that produce IL-17 (Th17 cells) have recently been identified as the third subset of effector T cells[20], which produce IL-17A, IL-17F, IL-22 and IL-21[21]. Recently, IL-6 has been shown to induce the generation of Th17 cells from naïve T cells together with transforming growth factor (TGF)-β and inhibits TGF-induced Treg cell differentiation[22]. Importantly, there is a reciprocal relationship between Th17 and Treg cells; not only in development, but also in their effector function, indicating that the Treg/Th17 balance may determine the quality and magnitude of immune responses in the liver[20]. Unexpectedly, the increases in circulating and intrahepatic Th17 cells are positively correlated with HBV DNA in the serum, serum ALT levels, and histological activity index of the livers with CHB, suggesting that activation of Th17 cells does not exert antiviral function in CHB[23].

Programmed death-1

Programmed death-1 (PD-1) is a surface receptor critical for the regulation of T cell function[24,25]. Binding to PD-1 by its ligands PD-L1 and PD-L2 results in the antigen-specific inhibition of T cell proliferation, cytokine production, and cytolytic function, leading to exhaustion of T cells. In the liver, PD-1 is expressed on lymphocytes; PD-L1 is expressed on lymphocytes, hepatocytes and sinusoidal endothelial cells; and PD-L2 is expressed on Kupffer cells and dendritic cells (DCs)[26]. HBeAg-positive patients with high HBV DNA levels in the serum show increased PD-1 and CTLA-4 expression on HBV-specific CD8^{+} T cells[27]. Moreover, PD-1 expression on CD4^{+} T cells is correlated positively with serum HBV DNA load in CHB patients[28]. Intrahepatic HBV-specific CD8^{+} T cells express higher levels of PD-1, and upregulation of intrahepatic PD-1/PD-L1 is associated with liver inflammation and ALT elevation[29]. Although the mechanism underlying the upregulation of PD-1 on CD8^{+} T cells in the inflamed liver is unknown, signals from PD-1 inhibit HBV-specific T cells, resulting in insufficient antiviral responses, leading to failure of viral control and persistent liver inflammation. Importantly, PD-1/PD-L1 blockade increased CD8^{+} T cell proliferation and enhanced IFN-γ and IL-2 production by intrahepatic lymphocytes[29]. These findings suggest that inhibition of PD-1/PD-L1 may have therapeutic potential for the control of hepatitis B.

IL-10

IL-10 is an important cytokine with anti-inflammatory properties, and is produced by activated monocytes/macrophages and T cell subsets, including Treg and Th1 cells[30]. Immunosuppression by IL-10 is associated with functional exhaustion of memory T cells in chronic lymphocytic choriomeningitis virus (LCMV) infection, and blockade of IL-10 receptors could terminate chronic LCMV infection[31]. In chronic HBV infection, HBcAg stimulates the production of IL-10, which negatively regulates HBcAg-specific Th17 cell responses in CHB patients[32].

T cell immunoglobulin- and mucin-domain-containing molecule-3

It has been reported that not all exhausted T cells show upregulation of PD-1 and downregulation of CD127 (IL-7 receptor), and blockade of the PD-1/PD-L1 signaling pathway does not always restore proliferation and cytokine production[33]. Recently, another inhibitory molecule, T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3), has been reported. A high frequency of Tim3-expressing CD4^{+} and CD8^{+} T cells are found in chronic HBV infection, and the frequency of Tim-3^{+} T cells is positively correlated with the severity of liver inflammation, and negatively correlated with plasma IFN-γ levels[34].

Table 1 Immunotherapeutic approaches for animal models of hepatitis B virus infection

Animal model	Immunotherapy	Results	Ref.
Peptide vaccination			
HBV transgenic mice	A synthesized fusion peptide, consisting HBcAg18-27 and HIV Tat49-57	Decrease in serum HBV DNA levels and the expression levels of HBsAg and HBcAg in the liver	[40]
Protein vaccination			
HBV transgenic mice	HBsAg vaccine	Most of the mice showed reduction of HBV DNA levels and disappearance of HBeAg and HBsAg	[41]
Woodchuck hepatitis virus infection	Combination of vaccine of HBV large surface protein and clevudine	Restoration of T-cell response to Pre-S and S region	[42]
DNA immunization			
Acute DHBV infection	DNA vaccine expressing DHBc and Pre-S/S and entecavir Boosted with fowl poxvirus vectors expressing DHBc and Pre-S/S	Clearance of DHBV infection at a rate of 100%	[43]
Chronic DHBV infection	DNA vaccine encoding the HBV large envelope and/or core protein with or without lamivudine	Reduction of viremia and liver DHBV cccDNA in 33% of ducks Seroconversion to anti-pre S in 67% of ducks showing cccDNA clearance	[44]
DC immunization			
HBV transgenic mice	Activated bone marrow-derived DCs	Break CTL tolerance to HBsAg	[45]
HBV transgenic mice	Anti-CD40 agonistic monoclonal Ab	Induction of noncytopathic inhibition of HBV replication mediated by antiviral cytokines (IL-12 and TNF-α) produced by activated intrahepatic APCs	[46]
HBV transgenic mice	HBV-specific peptide-pulsed DCs	Reductions in the serum HBsAg and HBV DNA	[47]
Cytokines and adjuvants			
HBV transgenic mice	Recombinant IL-12	Marked inhibition of HBV replication in the liver	[48]
HBV transgenic mice	α-galactosylceramide that can activate NK T cells	Complete inhibition of HBV replication	[49]
HBV transgenic mice	Recombinant IL-18	Inhibition of HBV replication noncytopathically, mediated by activation of resident intrahepatic NK cells and NK T cells	[50]
Gene therapy			
HBsAg transgenic mice	Lentivectors expressing HBsAg and IgFc fusion Ag	Induction of seroconversion to anti-HBs	[51]

HBV: Hepatitis B virus; DHBV: Duck HBV; DC: Dendritic cell; HBsAg: Hepatitis B surface antigen; HBcAg: Hepatitis B core antigen; HIV: Human immunodeficiency virus; APC: Antigen-presenting cell; IL: Interleukin; NK: Natural killer; CTL: Cytotoxic T lymphocyte; TNF: Tumor necrosis factor; cccDNA: Covalently closed circular DNA; Ab: Antibody; Ag: Antigen.

Dysfunction of DCs

DCs are specialized antigen-presenting cells that orchestrate immune responses. They stimulate innate and acquired immune responses, but also act as tolerogenic cells for immune responses in a variety of situations. In viral hepatitis, dysfunction of DCs from peripheral blood has been reported. In patients with CHB, maturation of DCs from peripheral blood of patients after incubation with cytokines is lower than that of normal subjects with lower expression of HLA-DR and co-stimulatory molecules in the former population[35], leading to low allostimulatory function of DCs from CHB patients. The mechanism of impairment of DC function in patients with CHB is unclear, but both HBV particles and purified HBsAg may have immunomodulatory capacity and may directly contribute to the dysfunction of myeloid DCs[36]. Interestingly, impaired function of monocyte-derived DCs from patients with CHB could be reversed by inhibiting viral replication with nucleos(t)ide analogs such as lamivudine[37]. Type 2 precursor plasmacytoid dendritic cells (pDCs), which are the most important cells in antiviral innate immunity, are also reported to have quantitative and qualitative impairment in patients with chronic HBV infection[38]. Recently, HBV itself was shown to inhibit the functions of pDCs[39]. These data indicate that DCs in patients with CHB have impaired function leading to insufficient T cell response to HBV, which could be the mechanism responsible for persistent viral infection.

IMMUNOTHERAPY FOR VIRAL HEPATITIS

In chronic HBV infection, strong long-term viral suppression can now be achieved with various nucleoside or nucleotide analogs. However, there are some problems that must be solved in the near future. One of the problems with treatment with nucleos(t)ide analogs is a low rate of HBe seroconversion even after long-term administration in HBeAg$^+$ patients. Moreover, reactivation rate of HBV replication is high in both HBeAg$^+$ and HBeAg$^-$ patients after cessation of treatment, although drug-free viral controls would be better than long-term administration of the drugs in terms of control of medical costs and avoidance of adverse effects of these agents. It could be possible to achieve long-term viral eradication even after cessation of nucleos(t)ide analogs, if viral suppression with nucleos(t)ide analogs could be combined with efficient immunotherapies.

Previous animal studies and human trials in HBV infection are listed in Tables 1 and 2, respectively.

IMMUNOTHERAPEUTIC APPROACHES FOR HBV INFECTION

Immunotherapeutic strategies for CHB include suppres-

Table 2 Immunotherapeutic trials for chronic hepatitis B virus infection in humans

Immunotherapy	Results	Ref.
Peptide vaccination		
A vaccine with HBc18-27 peptide comprised of a T-helper cell epitope and two palmitic acid residues	Low levels of CTL activity were induced but no significant changes in liver biochemistry or viral serology were observed	[52]
Protein vaccination		
PreS2/S (GenHevac B) or S (Recombivax)	HBe/anti-HBe seroconversion in 13% and HBV DNA negativity in 16% of the treated patients	[53]
Intradermal HBsAg vaccine and laimvudine in combination with IL-2	Induction of significant HBV DNA loss in the serum in two of five the treated patients	[54]
Oral administration of HBV envelope proteins (HBsAg + preS1 + preS2)	Induction of histological improvement in 30%, HBeAg negativity in 26.3% and HBsAg-specific T cell proliferation in 78% of the treated patients	[55]
IFN-α-2b monotherapy (9 mo) or IFN-α-2b plus pre-S2/S vaccine	Greater reduction in HBV DNA in patients with combination HBV therapy than those who received IFN-α-2b monotherapy	[56]
The combination with lamivudune and HBsAg vaccine in HBeAg$^+$ cases	No improvement of HBe seroconversion rate in comparison with lamivudine therapy alone	[57]
Combination of lamivudine and HBsAg vaccine	Induction of sustained negativity of HBV DNA in 1/4 of patients	[58]
Combination of lamivudine and HBsAg vaccine	HBV DNA became undetectable in 64% of the patients, and was decreased in the remaining patients	[59]
DNA immunization		
DNA vaccine encoding HBV envelope protein	Induction of an increase in HBV-specific IFN-γ-secreting T cells in nonresponders to conventional therapies, and HBV DNA levels were transiently decreased in 50% of vaccinated patients	[60]
DNA vaccine encoding PreS and S in patients with lamivudine breakthrough	Development of IFN-γ-producing T cells specific for preS or S antigen; Two of 10 patients showed seroconversion to anti-HBe	[61]
DC immunization		
Peripheral blood-derived DCs, activated with GM-CSF and IL-4 pulsed with HBsAg	Both patients with normal and elevated ALT responded equally to DC vaccine and 53% of the patients showed induction of HBeAg negativity	[62]
Activated DCs from PBL with GM-CSF and IL-4, pulsed with two peptides, HBc18-27 and PreS2 44-53	Undetectable HBV DNA was achieved in 46.3% and 3.1% of HBeAg$^-$ and HBeAg+ patients, respectively. ALT normalization was observed in 69% and 30.5% of HBeAg- and HBeAg+ patients, respectively	[63]
Cytokines		
GM-CSF	Safe and tolerable up to 1.0 μg/kg body weight, and induced HBV DNA negativity in 4/8 patients	[64]
Combination therapy with GM-CSF and HBsAg vaccine in HBV carrier children	Significant reduction of serum HBV DNA	[65]
High dose of IL-12 (0.5 μg/kg)	HBV DNA clearance was observed in 25% of the patients	[66]
Combination of IL-12 and lamivudine	Stimulation of T cell response to HBV with IFN-γ production. However, IL-12 was unable to suppress re-elevation of HBV DNA after cessation of lamivudine	[67]
Combination of IL-12 and IL-18	Stimulation of IFN-γ production by CD4+ T cells isolated from peripheral blood in response to HBcAg, and the effect was greater than those observed with either cytokine alone	[68]
α-galactosylceramide	Poorly tolerated and showed no clear suppressive effect on serum HBV DNA or ALT levels	[69]
Tα1		
Combination of Tα1 and IFN-α	No statistically significant differences as compared with IFN-α monotherapy with respect to HBeAg seroconversion, changes in histology, normalization of ALT or loss of HBV DNA	[70]
Tα1 alone	At 12 mo after cessation of therapy, 36.4% of patients treated with 1.6 mg of Tα1 achieved ALT normalization, 15% achieved HBV DNA clearance by transcription-mediated amplification, and 22.8% achieved clearance of HBeAg	[71]
Comparative effect of Tα1 and IFN-α	Tα1 treatment was more effective in achieving ALT normalization and HBV DNA negativity at the end of the follow-up period than IFN-α	[72]
Combination of Tα1 and lamivudine	No any additional antiviral effect compared with lamivudine monotherapy as determined by HBe seroconversion and the emergence of viral breakthrough	[73]
Combination therapy with lamivudine and Tα1	Induction of significantly higher rates of ALT normalization, virological response, and HBeAg seroconversion than lamivudine monotherapy	[74]

HBV: Hepatitis B virus; Tα1: Thymosin α1; IFN: Interferon; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IL: Interleukin; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; HBsAg: Hepatitis B surface antigen; HBcAg: Hepatitis B core antigen; PBL: Peripheral blood lymphocytes.

sion of viral replication, stimulation of T cell immune response to hepatitis virus, activation of nonspecific cells, and administration of cytokines with antiviral activity (Tables 1 and 2).

Suppression of viral replication
High viral load has been shown to suppress CD4$^+$ and CD8$^+$ T cells in addition to induction of Treg cells, which could be reversed by antiviral therapy in CHB[75].

Therefore, immunotherapy followed by restoration of virus-specific T cell response with antiviral therapy could be more efficient in CHB.

Stimulation of immune response to HBV

Peptide immunization: A peptide vaccine containing highly immunogenic HBc18-27 has been developed and administered to CHB patients[52], but the results were disappointing because there was no induction of a significant antiviral T cell response.

Protein immunization: In a model of HBV in transgenic mice, vaccine on the base of surface antigen in complete Freund's adjuvant once monthly for 1 year induced reduction in HBV DNA, and the disappearance of HBeAg and HBsAg in most mice treated[41]. Moreover, it is important to note that some mice developed anti-HBs in the sera. However, several human trials with HBsAg vaccine showed limited efficacy if used as monotherapy.

Recently, hepatitis B vaccine containing not only S protein but also preS has been used with increased immunogenicity[53,55], or has been combined with lamivudine or IFN-α[56], leading to potential improvement of clinical efficacy. However, analysis of the T cell epitope hierarchy has indicated that the most important epitope for viral control is HBc18-27, and not the HBsAg epitope in HLA-A2 patients[9], suggesting the necessity to reconsider antigen selection for vaccination that could lead to better viral control.

DNA immunization: Injection of plasmid DNA has been shown to elicit strongly both cellular and humoral immune responses, and is now known to be safe and well-tolerated both in mice and humans. In a model of duck HBV infection, DNA vaccine encoding HBV large envelope and/or core protein was shown to induce reduction in not only viremia but also covalently closed circular DNA (cccDNA) in the liver in one thirds of ducks receiving DNA monotherapy or combination treatment with lamivudine[44]. This finding is encouraging because clearance of cccDNA from the liver is the goal of treatment for HBV infection, but is difficult to achieve using IFN-α or nucleos(t)ide analogs. Clinical trials have also been performed in HBV infection with some encouraging results, which remain to be confirmed by future randomized large-scale trials.

DC immunization: DCs are specialized antigen-presenting cells that can induce strong immune responses in T and B cells. We have previously shown that activated bone-marrow-derived DCs can break CTL tolerance to HBsAg in HBV transgenic mice[45]. Thereafter, several immunotherapies with activated DCs have been applied in both animals and humans. In a recent study performed in HBV transgenic mice, peptide-pulsed DCs were shown to reduce significantly the concentrations of serum HBsAg and HBV DNA[47], indicating therapeutic potential in chronic HBV infection. Recently, DCs treated with peptide inhibitors of IL-10 have been shown to induce strong anti-HCV T cell responses in HCV transgenic mice[76], suggesting a strategy to augment the immunogenic function of DCs. Moreover, when intrahepatic antigen-presenting cells, including DCs, are activated by injection of an anti-CD40 agonistic antibody, HBV replication is inhibited by a noncytopathic mechanism, possibly through production of antiviral cytokines such as TNF-α and IL-12[46]. Although no CTL response against HBV antigens was reported in this study, the *in vivo* activation of DCs could be an alternative way for inducing antiviral immune responses, including possible activation of CTLs against HBV. In humans, injection of activated DCs loaded with HBV peptide or protein has achieved a reduction in HBV DNA level in some patients[62,63]. HBeAg negativity was achieved in more than half of the treated patients in one study[62]. Although preparation of activated and mature DCs incurs financial costs and requires experienced researchers, immunotherapy with DCs is a promising method.

Natural killer T cells: A single injection of α-galactosylceramide abolished HBV replication by activating natural killer (NK) T cells in the liver in HBV transgenic mice[49]. However, α-galactosylceramide was poorly tolerated in humans and showed no clear antiviral effect[69], possibly due to smaller numbers of NKT cells in the human liver than in the mouse liver.

Cytokines and thymosin-1: Cytokines such as IL-12[48] and IL-18[50] have been shown to inhibit HBV replication noncytopathically in HBV transgenic mice. In humans, granulocyte–macrophage colony-stimulating factor[64,65] and IL-12[66,67] have been used for treatment with some antiviral effects. They have been used as monotherapy or in combination with hepatitis B vaccine or lamivudine.

Thymosin (T)α1, a synthetic 28-amino acid peptide, is able to enhance the Th1 immune response and also exerts a direct antiviral mechanism of action. It has been used for the treatment of chronic HBV infection in humans[70-73], and has shown some antiviral efficacy. Although antiviral effect by the addition of Tα1 to lamivudine or IFN-α therapy was controversial, a meta-analysis has demonstrated that combination therapy with lamivudine and Tα1 shows significantly higher rates of ALT normalization, virological response, and HBeAg seroconversion as compared with lamivudine monotherapy[74]. It is of note that HBeAg seroconversion rate was 45% in the combination group, which was significantly higher than that with lamivudine monotherapy (15%).

Blockade of immunoinhibitory signals

Recently, there have been several basic attempts to improve the efficacy of immunotherapy. Among these reports, augmentation or restoration of T cell response by blocking the inhibitory signals has been extensively analyzed *in vitro*. It has been demonstrated that exhausted T

cells express not only PD-1, but also CTLA-4[77], CD244[78] or Tim-3[33], and blocking of these molecules in combination could be better than blocking any single molecule to achieve full activation of the exhausted T cells.

CONCLUSION

There have been several attempts to apply immunotherapy for the control of chronic HBV infection, and some of the data are promising. Viral suppression, stimulation of antiviral immune response with cytokines or immunization with peptide, protein, DNA or DCs, and suppression of the immunoinhibitory signals must be combined to achieve desirable antiviral effects, although further studies are required to explore the best protocols and their most efficient combinations.

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TOPIC HIGHLIGHT

Luis Bujanda, PhD, Professor, *Series Editor*

Serrated polyposis syndrome: Molecular, pathological and clinical aspects

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Abstract

Hyperplastic polyps have traditionally been considered not to have malignant potential. New pathological classification of serrated polyps and recent discoveries about the serrated pathway of carcinogenesis have revolutionized the concepts and revitalized the research in this area. Until recently, it has been thought that most colorectal cancers arise from conventional adenomas *via* the traditional tumor suppressor pathway initiated by a mutation of the *APC* gene, but it has been found that this pathway accounts for only approximately 70%-80% of colorectal cancer (CRC) cases. The majority of the remaining colorectal cancer cases follow an alternative pathway leading to CpG island methylator phenotype carcinoma with BRAF mutation and with or without microsatellite instability. The mechanism of carcinomas arising from this alternative pathway seems to begin with an activating mutation of the *BRAF* oncogene. Serrated polyposis syndrome is a relatively rare condition characterized by multiple and/or large serrated polyps of the colon. Clinical characteristics, etiology and relationship of serrated polyposis syndrome to CRC have not been clarified yet. Patients with this syndrome show a high risk of CRC and both sporadic and hereditary cases have been described. Clinical criteria have been used for diagnosis and frequent colonoscopy surveillance should be performed in order to prevent colorectal cancer. In this review, we try to gather new insights into the molecular pathogenesis of serrated polyps in order to understand their possible clinical implications and to make an approach to the management of this syndrome.

INTRODUCTION

Colorectal cancer (CRC) is a common and lethal disease. It is a major health issue in western countries where it represents the second most common fatal malignancy after lung cancer[1]. Until recently, it has been thought that most CRCs arise from conventional adenomas *via* the traditional tumor suppressor pathway initiated with a mutation of the *APC* gene, but it has been found that this pathway accounts for only approximately 70%-80% of CRC cases[2-4]. The majority of the remaining CRC cases follow an alternative pathway leading to CpG island methylator phenotype (CIMP+) carcinoma with *BRAF* mutation and with or without microsatellite instability. This pathway is called the serrated pathway of colorectal carcinogenesis[3]. The mechanism of carcinomas arising from this alternative pathway seems to begin with an activating mutation of the *BRAF* oncogene. This *BRAF* mutation provokes the development of serrated lesions that are mainly microvesicular hyperplastic polyps or sessile serrated polyps[5]. These lesions are prone to methylation of CpG islands in the promoter regions of genes resulting in their epigenetic silencing. The best characterized gene silenced by this mechanism is *MLH1*. This gene is one of the mismatch repair genes and its epigenetic silencing results in sporadic tumors with microsatellite instability (MSI). However, other genes such as *P16*, *MGMT*, or *IGFBP7* may also be epigenetically inactivated. The serrated polyposis syndrome (SPS) is a relatively rare condition characterized by multiple and/or large serrated polyps of the colon. Diagnosis of this disease is made by the fulfillment of any of the World Health Organization's (WHO) clinical criteria[6] (Table 1). SPS exhibits an increased risk of CRC[7], which occurs on average in subjects aged between 50 to 60 years. There is a high incidence of synchronous cancers[8] and CRC shows a trend to be located in the proximal colon[9]. These patients and their relatives should receive strict surveillance strategies because of the high risk of CRC. This review focuses on the SPS, its genetics and management.

SERRATED POLYPOSIS SYNDROME

Serrated polyposis syndrome is the paradigm of the serrated pathway of carcinogenesis and an excellent and interesting human model for the study of the features that drive progression from hyperplastic polyps (HP) to serrated carcinoma (Figure 1). These patients show clinical, pathological and molecular features that are very useful for expanding the knowledge of this particular and alternative carcinogenetic pathway.

Diagnostic criteria

Diagnostic criteria of SPS were first defined by Burt and Jass in 2000 for the WHO. These criteria have been recently redefined and this entity is now called Serrated Polyposis[6]. A patient is diagnosed with SPS if at least one of the following criteria is met: (1) At least five serrated polyps proximal to the sigmoid colon, two of

Table 1 The World Health Organization's clinical criteria for the identification of serrated polyposis

Criterion A	At least five serrated polyps proximal to the sigmoid colon, two of which are greater than 10 mm in diameter
Criterion B	Any number of serrated polyps occurring proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis
Criterion C	More than 20 serrated polyps of any size distributed throughout the colon

A diagnosis of serrated polyposis syndrome can be made if a patient fulfils any of these criteria.

Table 2 Summary findings from publications including patients that fulfil World Health Organization criteria of serrated polyposis syndrome

Author	Patients (*n*)	Age at diagnosis (median, yr)	CRC (%)	CRC family history (%)
Lage *et al*[12]	14	54	43	36
Ferrández *et al*[10]	15	52	7	0
Rubio *et al*[13]	10	61	70	10
Chow *et al*[9]	38	44	26	50
Boparai *et al*[7]	77	56	35	NR

CRC: Colorectal cancer; NR: Not reported.

which are greater than 10 mm in diameter; (2) Any number of serrated polyps occurring proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis; and (3) More than 20 serrated polyps of any size distributed throughout the colon (Table 1). This arbitrary definition has been considered over the years somewhat restrictive. Moreover, SPS probably comprises a heterogeneous group of patients that includes several phenotypes of serrated polyposis. However, until the molecular basis of this syndrome is better understood, this clinical definition is applicable.

Clinical characteristics

Characteristics of patients with SPS have been defined mainly based on the publication of series of cases[7,9-13] (Table 2). There is no sex predominance and the mean age at diagnosis is around 55 years. SPS has largely been considered a genetic disease, but the pattern of inheritance remains unknown: both autosomal recessive and autosomal dominant patterns have been suggested. Published case series report that between 10%-50% of patients meeting SPS criteria have a family history of CRC[9,11-13]. In this way, Boparai *et al*[14] have recently described an increased risk of CRC [relative risk (RR) = 5.4] and SPS (RR = 39) in first-degree relatives of probands diagnosed with SPS compared to the general population.

It is important to point out that conventional adenomas may coexist with serrated polyps in patients with SPS[7,9-13]. Some authors have suggested the existence of various phenotypes within the SPS definition. Kalady *et al*[11] described three phenotypic patterns in a series of 115 patients with multiple serrated polyps: (1) The patients presented a right-sided phenotype with large sessile serrated adenomas (SSAs)

Figure 1 Model of serrated pathway of colorectal carcinogenesis. MVHP: Microvesicular hyperplastic polyp; SSA: Sessile serrated adenoma; MGMT: Methylguanine methyltransferase; MSI: Microsatellite instability; MSS: Microsatellite stable; CRC: Colorectal cancer; CIMP: CpG island methylator phenotype; GCSP: Goblet cell serrated polyp; TSA: Traditional sessile adenomas.

and with a CRC onset in younger individuals (48%); (2) Left-sided phenotype with a greater amount of small polyps (16%); and (3) Mixed phenotype with shared features of the previous phenotypes (37%). These different patterns should be revised in future studies.

Environmental factors could be partially responsible for the phenotypic differences and model the unknown pattern of inheritance. Smoking, being overweight and some drugs have been postulated as potential risk factors of HPs. Samowitz et al[15] described a statistically significant dose-response association between CIMP+ CRC and smoking. Moreover, Walker et al[16] found a strong association between cigarette smoking and SPS (odds ratio = 8.3; 95% CI: 3.0-22.9) in a case-control study comparing SPS patients with a population-based registry. Wallace et al[17], using the data of multicenter chemoprevention trials, came upon the association of some environmental factors with an increased risk of colonic serrated polyps (not necessary SPS criteria). On the one hand, in the left colon, obesity, smoking, increased dietary fat and red meat intake were linked with serrated polyps. On the other hand, in the right colon, the risk factors were folate intake and family history of polyps, whereas aspirin treatment was shown as a protective factor. These results should be confirmed by targeted studies.

Somatic molecular characteristics of polyps in patients with serrated polyposis syndrome

Molecular heterogeneity among polyps from patients with SPS has been described[18]. In fact, although a mixed phenotype has been identified[11], SPS patients can be molecularly classified into two defined groups[19]. The first group is characterized by the presence of relatively few large right-sided polyps which show BRAF mutation while the other group presents with many small left-sided polyps associated with KRAS mutation[11,19]. Mutations in KRAS and BRAF are more common in HPs from SPS patients as well as in younger cases. The frequency of BRAF mutations in SPS patients is higher than KRAS mutations[19].

The combined incidence of BRAF and KRAS mutations in serrated polyps ranges from 64% to 75%[19,20]. The presence of epithelial dysplasia is associated with higher rates (90%) of mutation in either BRAF or KRAS, indicating the importance of the activation of the RAS-RAF-MAP kinase pathway in the pathogenesis of the serrated lesions[20]. Furthermore, nearly 90% of all CIMP+ CRCs have either BRAF or KRAS mutations[21]. Serrated polyps from patients with SPS have different frequencies of BRAF mutation and it is higher in those lesions that show typical features of SSA[21-23]. However, there are differences between studies due to the methodology used for the detection of BRAF mutation[22] or because of the lack of consensus about the diagnostic terms for serrated lesions[24,25].

Carvajal-Carmona et al[19] proposed molecular criteria that could complement the clinical WHO criteria for SPS. They recommended that SPS should be diagnosed

Figure 2 Segment of colectomy in a case of serrated polyposis. Polyps are frequently small (arrows) and flat, making their endoscopic detection difficult.

if *BRAF* or *KRAS* mutations are present at a significantly higher frequency in a patient's polyps than in sporadic HPs. In addition, SPS could be excluded if both *BRAF* and *KRAS* mutations are present in less than 10% of HPs from one patient, or if less than 5% of HPs are MSI.

Genetic predisposition in patients with serrated polyposis syndrome

SPS is a very heterogeneous condition[26] and it has been suggested that each phenotype may result from different underlying genetic causes[11]. Familial cases of SPS have been reported[19,26]. Although the genetic basis of SPS remains unknown, both recessive and dominant transmission patterns have been proposed[9,23,26]. Young *et al*[27] provided evidence for a syndrome of familial CRC distinct from hereditary nonpolyposis colorectal cancer by describing 11 families, of which 6 met the Amsterdam I criteria, with multiple members across several generations with CRC with variable MSI phenotype, *BRAF* mutation in 70% and hypermethylation of MINT31 in 80%. Moreover CRCs showed early age at diagnosis and were more likely to show a serrated architecture. Frazier *et al*[28] observed that patients whose CRC show methylation in *p16*, *MINT1*, *MINT31* and *MLH1* are 14 times more likely to have a family history of cancer than patients with methylation at none of the four loci. Taking into account these studies and the fact that extensive DNA methylation in normal colorectal mucosa has been described in patients with SPS[19,29,30], it has been postulated that the hypermethylation of gene promoters is due to genetic predisposition[23].

On the other hand, patients meeting criteria for hereditary nonpolyposis colorectal cancer may also fulfil criteria of SPS[31]. Occasional HPs have also been described in MYH-associated polyposis (MAP) patients and some of them met the criteria for SPS. Moreover HPs and SSAs can also be considered a phenotypic expression of MAP[32] and pathogenic biallelic *MYH* mutations were detected in 1 patient with SPS[9]. For that reason *MYH* mutations should be studied in SPS patients, especially when adenomas occur simultaneously with HPs in the same patient[9]. *PTEN* mutations have also been identified in patients with a combination of hyperplastic and adenomatous polyps[33].

A recent study from Roberts *et al*[34] has showed linkage to 2q32.2-q33.3 in approximately half of the SPS families studied. Sequencing of coding regions and exon-intron boundaries of five potential candidate genes in this region did not reveal any variants segregating with disease.

Together these data support the existence of more than one genetic cause of SPS. Identification of the underlying genetic defect of SPS will help to improve management of these patients and may identify therapeutic targets for the treatment of CRC associated with this disease.

Risk of cancer in serrated polyposis syndrome

Serrated polyposis syndrome has been associated with an increased incidence of CRC. In the published series[7,9,10,12,13], about 25%-70% of patients with SPS had CRC at time of diagnosis or during follow-up. In the largest series with patients meeting WHO criteria for SPS[7], 35% of patients had CRC (28.5% at initial endoscopy and 6.5% during the mean follow-up of 5.6 years). In this study, increased number of polyps and the presence of serrated adenomas were associated with CRC. The results of the larger published series are summarized in Table 2. In addition, first degree relatives of SPS patients have an increased risk for both CRC and SPS compared to the general population[14].

Recommendations for treatment and surveillance

The management of patients with SPS should be based on regular screening colonoscopies in order to remove potential premalignant lesions. It is important to point out that it could be difficult to detect these serrated polyps and colonoscopy should be done under high quality conditions (Figure 2). Serrated polyps are less likely than adenomas to bleed, so fecal occult blood test could be less suitable for an early diagnosis. Surveillance recommendations can be done as follows[4]: (1) Colonoscopy with pancolonic chromoendoscopy every 1-2 years with removal of all polyps. It is recommended that this resection be performed at a tertiary centre, if possible; (2) If colonoscopy does not allow the total control of colonic polyps because of their size or number or the patient does not wish to have such frequent colonoscopies or cancer is detected, colectomy with ileorectal anastomosis should be indicated; and (3) First-degree relatives should be offered 1-2 years screening colonoscopy from 10 years younger than the index case and if it is possible by pancolonic chromoendoscopy.

Pedunculated polyps can be removed by conventional electrocautery snare polypectomy. The technique of choice for removal of flat and large HPs is endoscopic mucosal resection. Besides, it may be advisable to apply argon plasma coagulation in the lesion borders in order to reduce the risk of recurrence[35].

SERRATED PATHWAY OF CARCINOGENESIS

In 1999, Iino *et al*[36] suggested that a proportion of

hyperplastic polyps may serve as precursors of some CRC cases. Now, there is increasing evidence showing that, in some conditions, hyperplastic polyps can be the initial premalignant lesion in the serrated pathway of carcinogenesis. Some studies have reported the existence of *BRAF* mutations in sporadic MSI CRCs which show CIMP[21,23,27,37-41] suggesting the existence of this alternative pathway. *BRAF* mutations and DNA methylation would be early events in this pathway with serrated polyps as precursor lesions[4,21,27,41]. The lack of adenoma-specific mutations such as *APC, KRAS* and *TP53* in sporadic MSI CRCs, and the fact that *BRAF* mutation and methylation of CpG islands are exceptional in classic adenomas[42] supports the existence of this pathway[43]. Tumors following this pathway show some specific characteristics, being more frequent in females and located in the right colon[4]. Moreover, some preliminary studies suggest that these tumors could be unresponsive to 5-fluorouracil chemotherapy[44].

Molecular characteristics of serrated polyps

As mentioned above, *BRAF* mutations and DNA methylation would be early events in this pathway. In fact, epigenetic changes in normal mucosa in patients with SPS have been described[29]. The first role of *BRAF* in the serrated pathway is probably to allow the apoptosis evasion[21,45]. Then, under normal conditions, these cells are eliminated by regular senescence. However, the silencing of key cell cycle regulatory genes such as *p16*, *IGFBP7* or *p53* through promoter methylation allows the cell to escape from senescence[4], facilitating its proliferation (Figure 1). When cells acquire other mutations, activated *BRAF* itself could also drive proliferation[21] and facilitate the maintenance of an invasive phenotype[45]. *BRAF* mutation has even been observed in serrated hyperplastic aberrant crypt foci, suggesting that these lesions are probably the earliest histological evident lesions in the serrated pathway[4,46].

There are several lines of evidence suggesting the existence of two parallel serrated pathways depending on the oncogene involved: *BRAF* or *KRAS* (Figure 1). The serrated pathway that involves *BRAF* mutations usually leads to CIMP tumors[4,29,43,47] and tumors are located in the proximal colon[38,47,48]. These tumors will be MSI or microsatellite stable (MSS) depending on the involvement of *MLH1*. As has been already stated, SSA seems to be the precursor lesion in the *BRAF* serrated pathway. In contrast, serrated tumors with *KRAS* mutations are more frequently MSI-low or MSS and are frequently associated with *MGMT* silencing[47,49]. These tumors are predominantly located in the left colorectum[4,29,47,48]. Differently, traditional sessile adenomas (TSA) would be the intermediate lesion in the *KRAS* serrated pathway.

CpG island methylator phenotype in colorectal cancer

CpG islands are 0.5- to 2-kb regions rich in cytosine guanine dinucleotides and are present in the 5' region of approximately 50% of human genes[21,29]. CIMP is characterized by methylation of CpG islands within the promoter regions of multiple genes resulting in the silencing of gene expression[18,21,29,45]. It might be assumed that methylation of CpG islands in most cancers arises stochastically[18,23,50]. This phenomenon can alter the expression of genes which are known to be important in neoplastic development, such as *p16*, *MGMT*, and the mismatch repair gene *MLH1*. However, the role of some genes affected by hypermethylation is not associated with colorectal carcinogenesis suggesting that not all de novo events are subject to growth selection. Taking into account that particular sequence motifs are significantly overrepresented among promoters vulnerable to CIMP, it is not surprising that some CpG islands are more likely to undergo hypermethylation than others[23]. The balance between DNA methyltransferases and the transcriptional machinery will determine the extent of methylation. Moreover, an active transcription may provide protection from de novo methylation[50]. The CIMP pathway is heterogeneous with respect to MSI status[27] and appears to be responsible for approximately 30% of all sporadic CRC[27,39].

There are different studies showing that a high proportion of polyps in SPS are CIMP+[18,29]. Chan *et al*[29] also observed that 75% of serrated polyps from patients with SPS showed CIMP frequently and had methylation of the *p16* gene. Moreover, extensive DNA methylation in normal colorectal mucosa has been described in patients with SPS[19,29,30], suggesting a field defect in epigenetic regulation and, consequently, a possible underlying genetic predisposition to extensive and early onset of DNA methylation. Furthermore, this phenomenon would be associated with a predisposition to CRC that would arise through the serrated pathway[51].

Endoscopic characteristics of serrated polyps

Serrated and hyperplastic polyps present endoscopic features that could help to differentiate them from adenomatous polyps. HPs appear pale, glistening, and very similar to the surrounding mucosa and usually covered by mucus. The vascular network is weak, in contrast to that of hypervascular adenomas. In addition, serrated polyps, mainly SSAs, are typically sessile or flat, making their detection even more difficult[48] (Figures 2 and 3, panels A and B). Since the malignant potential of these lesions, particularly in the context of SPS, has been shown, early endoscopic detection becomes more important. In this regard, the new advanced endoscopic techniques such as chromoendoscopy and narrow-band imaging (NBI) (Figure 3, panels C and D) become significant.

Chromoendoscopy in the SPS should be carried out by spraying contrast over the entire surface of the colon and using a magnification endoscope. The most widely used contrast is indigo carmine, which accumulates in pits and innominate grooves of the colonic mucosa, outlining the limits of flat lesions and drawing the described Kudo patterns[52]. Hyperplastic and serrated polyps typically show Kudo type I (normal) or type II ("stellate" or

Figure 3 Endoscopic appearance of serrated polyps. A and B: Sessile serrated adenoma (SSA) (arrows) as flat polyp on conventional optical colonoscopy; C: Narrow-band imaging appearance of polyp (arrow) seen in panel A; D: Chromoendoscopy image of SSA revealing Kudo II pattern (Images courtesy of Dr. Adolfo Parra, Hospital Central de Asturias, Oviedo, Spain).

Figure 4 Confocal endomicroscopy of (A) tubular adenoma, low-grade dysplasia and (B) serrated polyp. Both types of polyps show different shape as well as differences in the cellular structures (Images courtesy of Dr. Maria Pellisé, Hospital Clinic, Barcelona, Spain).

"papillary") (Figure 3, panel D). Published randomized trials have shown that pancolonic chromoendoscopy almost doubles the rate of detection of sporadic serrated polyps compared to conventional endoscopy[53-56]. In these studies, HPs were found in 20% of patients using chromoendoscopy *vs* 10% of patients with conventional endoscopy, and this difference was statistically significant. New endoscopic technologies, such as NBI and confocal laser endomicroscopy (CLE) should also be taken into consideration (Figure 4).

It is accepted that the vascular pattern evaluation by chromoendoscopy or NBI could be an appropriate method to differentiate adenomas from HPs[57,58], but it has not been specifically studied in the SPS. In this way, Boparai *et al*[59] ran a prospective series including 7 patients with SPS who underwent a colonoscopy with trimodal imaging (high resolution, AFI and NBI): they obtained an unsatisfactory diagnostic accuracy for differentiate between HPs and SSAs but distinguishing adeno-

mas from HPs was possible with NBI (accuracy 94%). Highest accuracy (76%) was achieved by the combination of a size of 3 mm or larger and a proximal location. Comparing CLE with virtual chromoendoscopy, it was shown that CLE demonstrated higher sensitivity (91% *vs* 77%; *P* = 0.010) with similar specificity in histological classification of colorectal polyps. However, further studies are needed to implement the CLE in clinical practice. The limited field of view and the horizontal sections of CLE hinder the detection of architectural distortion of sessile polyps (Figure 4).

Pathological characteristics of serrated polyps

Confirmation of the serrated character of polyps can only be made by pathological study. Serrated polyps are defined as epithelial lesions that show serrated appearance on histological section due to infolding of crypt epithelium. There are different types of serrated polyps (Table 3). HPs, considered for a long time as a benign and non-

Table 3 Characteristics of serrated polyps

Polyp name	Alternative terminology	Morphology and significance	Predominant location	Molecular features
Hyperplastic polyp, goblet type	Type 1 hyperplastic polyp	Subtype of hyperplastic polyp with conspicuous goblet cells and showing the least morphologic deviation from normal; Described as goblet-cell rich type	Distal colon: Sigmoid and rectum	Frequent *KRAS* mutation (54%)
Hyperplastic polyp, microvesicular type	Type 2 hyperplastic polyp	Variant of hyperplastic polyp in which columnar cells have mucin-filled vesicles within the apical cytoplasm and goblet cells are relatively inconspicuous	Right colon and distal colon	Frequent *BRAF* mutation (76%) and CIMP (68%)
Sesile serrated adenoma	Sessile serrated polyp; Serrated polyp with atypical proliferation	Advanced type of serrated polyp with abnormalities of architecture and proliferation but lacking the classic features of epithelial dysplasia (intraepithelial neoplasia)	Right colon	Frequent *BRAF* mutation (75%-82%) and CIMP (92%)
Sessile serrated adenoma with cytological dysplasia	Mixed polyp	Rare serrated polyp that includes two separate components: Nondysplastic (usually SSA) and either traditional adenoma or serrated adenoma	Right and left colon	Frequent *BRAF* mutation, (89%)
Serrated adenoma	Mixed hyperplastic adenomatous polyp; Atypical hyperplastic polyp; TSA	Relatively rare neoplastic polyp having a serrated architecture reminiscent of hyperplastic polyp but with unequivocal traditional adenomatous dysplasia; Comprises < 5% of serrated polyps	Left colon	Marked molecular heterogeneity; May have either *KRAS* or *BRAF* mutation

SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; CIMP: CpG island methylator phenotype.

Figure 5 Pathological types of serrated polyps. A: Microvesicular serrated polyp; B: Sessile serrated adenomas; C: Traditional serrated adenoma; D: Mixed polyp.

premalignant colorectal lesion, SSA, mixed polyps (MP), and TSA are included in this group[4,48,60,61] (Figure 5).

HPs are the most common colorectal polyps. Sporadic HPs are usually small (2-5 mm)[10], multiple and mainly distributed in the rectum and sigmoid colon[10,47]. HPs have been divided into two main histological subtypes: microvesicular serrated polyps (MVSPs) (Figure 5, panel A), in which columnar cells have mucin-filled vesicles within atypical cytoplasm, and goblet cell serrated polyps (GCSPs) with conspicuous goblet cells that are predominantly found in the distal colon[4,23,47]. MVSPs seem to be the precursor lesion of SSA, especially when located in the right colon. In fact, both have the same molecular genetic abnormalities such as mutations in *BRAF* and CIMP. MVSPs show large and regular stellate pit openings. However, the large GCSPs are likely to have *KRAS* mutation, which is infrequently found in SSA. There is some evidence that large GCSPs are potential precursors of dysplastic serrated polyps which show *KRAS* mutations[47,61,62]. A third type of HP has been added, mucin poor type, but its frequency and importance is lower than the two main HP types[6].

SSA is an atypical HP variant described by Torlakovic and Snover in 1996[63]. SSAs are larger than (usually greater than 1 cm) HPs and more frequently located in the right colon[10]. Histologically, SSAs are distinguished from

typical HPs by the presence of inverted T- or L-shaped crypt bases that reflect disordered proliferation (Figure 5, panel B). Other features include dilated crypts and serration extending into the lower third of the crypt. Focal nuclear stratification, mild nuclear atypia, or dystrophic goblet cells may be seen in the crypt bases[47,60,61]. Moreover, SSAs show increased mucin production, absence of enteroendocrine cells, and absence of a thickened basement membrane under the surface[43]. Other less common features include small foci of pseudostratification and eosinophilic change (identical to that seen in TSAs) of the surface epithelium. Small prominent nucleoli, open chromatin, and irregular nuclear contours also might be present, along with mitoses in the upper third of the crypts or on the surface itself[61]. SSAs are thought to represent approximately 2% of all colonoscopically removed polyps, over 8% of all polyps that were previously diagnosed as HPs and around 18% of all serrated polyps[60].

MP, also called SSA with cytological dysplasia include two separate hyperplastic and adenomatous components (Figure 5, panel C)[21,23]. One component is usually SSA (nondysplastic) whereas the second dysplastic component is either adenoma or TSA.

TSAs, usually present on distal location, are dysplastic serrated polyps which lack SSA patterns and more closely resemble conventional adenoma with tubulovillous architecture (Figure 5, panel D)[4,24,47,60]. Ectopic crypt formation, defined by the presence of crypts with bases not seated adjacent to the muscularis mucosae, is a feature that makes it possible to distinguish between TSAs and SSAs[4]. Columnar cells from the epithelium show eosinophilic cytoplasm, centrally placed elongated nuclei that are hyperchromatic and display pseudostratification[46].

There is no strong morphological evidence suggesting that SSAs are the precursor of TSAs, otherwise there are some histological and epidemiologic differences for keeping these lesions apart in different categories[4,61]. SSAs have been associated with proximal CRCs, high level of CIMP, *BRAF* mutations and MSI-high[47,48]. TSAs have been associated with distal location and MSS, CIMP-low CRCs with KRAS mutations[48]. SSAs, TSAs and MPs are described as "advanced serrated polyps" and represent approximately 5%-15% of all serrated polyps found in colonoscopy patients[23].

FUTURE DIRECTIONS

Advances in the knowledge about the serrated pathway of carcinogenesis are making it possible to differentiate a new type of CRC with different natural history, prognosis and response to chemotherapy treatment. For this reason it is important to be able to easily identify this kind of colorectal tumor and its precursors. Identification of molecular markers in both polyps and cancers that follow this pathway will provide the opportunity of a better understanding of how these tumours grow and how we could explain differences in clinical presentation, evolution and symptoms in different types of CRC. These molecular markers will also allow improvement

in the identification of patients with serrated polyposis, moving forward the currently used clinical criteria, and will give us better rationale for appropriate management and surveillance intervals for patients and their relatives.

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REVIEW

Globus pharyngeus: A review of its etiology, diagnosis and treatment

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Abstract

Globus is a persistent or intermittent non-painful sensation of a lump or foreign body in the throat. It is a commonly encountered clinical condition that is usually long-lasting, difficult to treat, and has a tendency to recur. Furthermore, due to the uncertain etiology of globus, it remains difficult to establish standard investigation and treatment strategies for affected patients. As a first step for managing globus, careful history taking and nasolaryngoscopy are essential. Given the benign nature of the condition and the recent notion that gastroesophageal reflux disease is a major cause of globus, empirical therapy with a high dose of proton pump inhibitors is reasonable for patients with typical globus. If patients are nonresponsive to this therapy, definitive assessments such as endoscopy, multichannel intraluminal impedance/pH monitoring, and manometry should be considered. Speech and language therapy, anti-depressants, and cognitive-behavioral therapy can be helpful in patients whose symptoms persist despite negative investigations.

INTRODUCTION

Globus, a persistent or intermittent non-painful sensation of a lump or foreign body in the throat, is a well-defined clinical symptom that is usually long-lasting, difficult to treat, and has a tendency to recur. This symptom frequently improves with eating and is generally unaccompanied by dysphagia or odynophagia[1]. It is a common condition that accounts for approximately 4% of new referrals to ear, nose and throat (ENT) clinics, and it is reported by up to 46% of apparently healthy individuals, with a peak incidence in middle age[2,3]. This condition is equally prevalent in men and women, though the latter are more likely to seek health care for

this symptom[4].

Hippocrates first noted globus pharyngeus approximately 2500 years ago[5]. In 1707, Purcell[6] was the first to accurately describe the condition; he believed that globus resulted from pressure on the thyroid cartilage due to contraction of the strap muscles of the neck. In the past, globus was described as "globus hystericus" because of its frequent association with menopause or psychogenic factors. However, Malcomson[7] coined the more accurate term "globus pharyngeus" in 1968 after discovering that most patients experiencing globus did not have a hysterical personality. The etiology of globus is still unknown but appears to be multifactorial (Table 1). Although data are limited, recent studies have focused on gastroesophageal reflux disease (GERD), abnormalities of the upper esophageal sphincter (UES), psychological and psychiatric disorders, and stress as major factors contributing to the globus sensation. The variety of potential etiologies has made it difficult to establish standard investigation and treatment strategies for affected patients.

The aim of this review is to present the current literature on globus and to discuss its natural history and potential causes, current trends in its diagnosis, and methods for its treatment.

NATURAL HISTORY

Since few long-term follow-up studies have been conducted on patients with globus, the natural history of this condition has not been fully elucidated. In one study that followed 74 globus patients for an average of 7 years and 7 mo, 45% of the patients had persistent symptoms during the follow-up period[8]. An in-depth analysis of the features at clinical presentation failed to reveal any reliable prognostic indicators. In another long-term follow-up study, 60% of the patients had improved or resolved symptoms over a 5-year period, and the male patients with a history of globus less than 3 mo and who did not complain of any associated throat symptoms were reported to have the greatest chance of becoming asymptomatic or symptomatically improved[9].

POTENTIAL CAUSES OF GLOBUS

Gastroesophageal reflux disease

Although there is still considerable debate about the causative role of GERD in patients with globus, gastroesophageal reflux (GER) has been suggested to be a major etiology of this symptom, potentially accounting for 23%-68% of globus patients[10-18].

Malcomson[7] was the first to link GERD to the globus sensation through the use of barium swallow to uncover the presence of reflux in over 60% of patients with globus. Moreover, Koufman[16] found that 58% of patients with globus had abnormal pH results, and Cherry *et al*[19] demonstrated that 10 out of 12 subjects complained of globus when acid was infused into the

Table 1 Potential cause of globus
Gastroesophageal reflux disease
Abnormal upper esophageal sphincter function
Esophageal motor disorders
Pharyngeal inflammatory causes including: pharyngitis, tonsillitis and chronic sinusitis
Upper aerodigestive malignancy
Hypertrophy of the base of the tongue
Retroverted epiglottis
Thyroid diseases
Cervical heterotopic gastric mucosa
Rare laryngopharyngeal tumors
Psychological factors and stress

distal esophagus. In a study that performed 24-h double-probe pH monitoring on 25 patients with globus and hoarseness, 72% of the patients exhibited pathologic reflux[20], and the globus symptom score was significantly higher in patients with GERD than in those without[21]. Additionally, globus sensation improved after 8 wk of proton pump inhibitor (PPI) therapy[22]. Several population-based surveys have supported such a potential link between GERD and the globus symptom by demonstrating an increased risk of globus among patients with GERD symptoms[23-25]. In a study by Dore *et al*[26], 38.7% of patients with GERD had the globus symptom, and the globus sensation was more prevalent in the non-erosive reflux disease group. Discordant data have also been reported[4,27-31]. However, it is clear that many patients with globus have concomitant GERD and that there is a true association between GERD and globus.

Two basic mechanisms have been proposed to explain the association between GERD and the globus sensation[14,32,33]: (1) Direct irritation and inflammation of the laryngopharynx by retrograde flow of gastric contents, also known as laryngopharyngeal reflux (LPR)[15,16,34]; (2) Vagovagal reflex hypertonicity of the UES triggered by acidification or distention of the distal esophagus[18].

Abnormal upper esophageal sphincter function

Abnormal UES function has also been suggested to be a cause of globus sensation[20,35-38]. Elevated UES pressure has been found to be much more frequent in patients with globus sensation than in controls (28% *vs* 3%), suggesting that hypertensive UES is a background factor for globus[30]. Additionally, injection of botulinum toxin into the cricopharyngeal muscle in a patient with both globus and extremely high UES pressure led to a resolution of the globus symptom and a decrease in UES pressure[35]. In a study of high-resolution manometry in patients with globus sensation, normal controls, and GERD patients without globus, hyperdynamic respiratory UES pressure changes were most prevalent in patients reporting globus[38]. However, other studies have reported contrary results[39-41].

Esophageal motor disorders

The prevalence of esophageal motor disorders has been

reported to be 6%-90% in patients with globus, suggesting that esophageal motor disorders are a possible cause of, or a contributing factor in the development of globus[27,28,31,42]. Esophageal manometry has revealed abnormalities in as many as 67% of globus patients, with nonspecific esophageal motility disorder being the most frequent finding[31]. Moser et al[43] noted that esophageal motor disorders might, before giving rise to dysphagia, be sensed more vaguely and induce the globus sensation. However, to infer an etiological significance of this disorder in globus, it must be shown that the sensation resolves after treatment for the motor disorder.

Pharyngeal inflammatory causes
Many conditions that cause irritation and inflammation of the pharynx, such as pharyngitis, tonsillitis, and chronic sinusitis with postnasal drip, can be the cause of globus sensation by producing increased local sensitivity[28,44].

Upper aerodigestive tract malignancy
The presence of pharyngolaryngeal or upper esophageal malignancy must be excluded in patients with globus sensation, particularly in cases with "high risk" symptoms, such as weight loss, dysphagia, throat pain, and lateralization of pathology[5,45].

Hypertrophy of the tongue base
Globus can be induced by severe hypertrophy of the tongue base, probably due to the follicles touching the posterior wall of the pharynx. Mamede et al[46] demonstrated that hypertrophied follicles were frequent in patients with signs and symptoms of GER and that the symptoms of hypertrophy of the tongue base could be confused with those of GER.

Retroverted epiglottis
Through contact with the tongue base or the posterior pharyngeal wall, retroverted epiglottis may cause globus sensation. Symptom relief has been observed after partial epiglottectomy[47,48].

Thyroid diseases
Impalpable, ultrasound-detectable abnormalities in the thyroid are known to be more common in patients with globus sensation than in controls[32]. Burns et al[49] noted that as many as one-third of patients with a thyroid mass complained of globus-like symptoms. Post-thyroidectomy patients may also complain of globus pattern symptoms, but these frequently diminish with time. Although the exact mechanism of the association between globus and thyroid diseases is poorly understood, some reports have concluded that a thyroidectomy could improve the globus symptom[49-51].

Cervical heterotopic gastric mucosa
Globus sensation has also been linked to the presence of cervical heterotopic gastric mucosa (CHGM)[52-54],

and acid secretion from CHGM appears to cause symptoms similar to those of GERD, including globus sensation. Patients with CHGM who complained of globus sensation and/or sore throat experienced a significant decrease in their symptoms after argon plasma ablation of CHGM[55,56]. Recently, it has been suggested that the globus symptom may be related to Helicobacter pylori infection of the CHGM[57].

Rare tumors
Smooth muscle tumors of the pharynx and post cricoid lymphangioma, as well as oropharyngeal metastasis of Merkel cell carcinoma, have been reported in patients complaining of globus[58-60]. These cases illustrate that patients with persistent globus should be further investigated to exclude rare lesions[32].

Psychological factors and stress
Psychogenic problems have often been thought to cause or trigger the globus sensation. Personality studies have found higher levels of alexithymia, neuroticism, and psychological distress (including anxiety, low mood, and somatic concerns) and lower levels of extraversion in patients presenting with globus[61,62]. In addition, several studies have reported increased numbers of stressful life events preceding symptom onset, suggesting that life stress might be a cofactor in symptom genesis and in exacerbation. Indeed, up to 96% of patients with globus report symptom exacerbation during periods of high emotional intensity[63,64]. However, some reports have found no differences in the psychological states of patients with globus compared to normal controls[4,10,65]. In actuality, psychiatric diagnoses are prevalent in subjects seeking health care for globus, but an explanation distinct from ascertainment bias has not been established, causing the etiological significance of these psychological characteristics to remain uncertain[1,65]. Two recent studies reported that psychological status might be different between LPR-positive and LPR-negative patients with globus[15,66]. Globus patients with LPR exhibited weaker psychological symptoms than non-LPR globus patients[15], and globus patients who did not respond to PPI had significantly higher anxiety scores[66].

Others
There have been numerous isolated case reports that have suggested an association of globus with cervical osteophytes[67], temporomandibular joint disorders[68], hyperviscosity of the nasopharyngeal mucosa[69], Eagle's syndrome[70], excessive laryngeal and pharyngeal tension[61], and salivary hypofunction[33].

DIAGNOSIS
There has been no consensus regarding how best to diagnose and manage globus. A study of United Kingdom-based ENT specialists found that 14% performed no

tests on globus patients but rather simply prescribed antacid medication if clinically indicated[71]. The remaining 86% investigated globus symptoms in a variety of ways, including rigid endoscopy (61%), barium swallow (56%), or a combination of these methods (17.5%).

Since globus is essentially a benign disorder, investigation is primarily aimed at identifying those few cases with upper aerodigestive malignancy. Thus, the first step of an investigation of globus symptoms should be to take a detailed patient history, paying particular attention to the presence of "high risk" symptoms, associated reflux symptoms, and psychological problems. Additionally, physicians should perform a physical examination of the neck followed by nasolaryngoscopic examination of the laryngopharynx, although the routine use of nasolaryngoscopy in patients with typical globus symptoms remains controversial[1]. Patients with typical globus symptoms usually require no further investigation beyond an outpatient nasolaryngoscopy[5]. However, patients with "alarm signs", such as dysphagia, odynophagia, throat pain, weight loss, hoarseness, and lateralization of pathology, should undergo more extensive evaluation[1].

Reflux symptom index and reflux finding score

The symptoms and physical findings of LPR are nonspecific and can be confused with other laryngeal conditions caused by smoking, allergies, infections, vocal abuse, postnasal discharge, or neurogenic mechanisms as well as non-pathological variations[72]. Belafsky *et al*[73,74] proposed a useful self-administered tool, the reflux symptom index (RSI), for assessing the degree of LPR symptoms and developed the reflux finding score (RFS) based on 8 endolaryngeal signs for documenting the physical findings and severity of LPR. However, Park *et al*[15] demonstrated that RFS and RSI have low specificity in globus patients, suggesting that these may not be valid diagnostic tools for LPR in patients with globus.

Barium swallow

Barium swallow studies have been reported to identify benign lesions in up to one-third of patients with globus, and the most common findings include hiatal hernia and/or reflux (8%-18%), cervical osteophytes (0.4%-23%), and cricopharyngeal spasm (2.2%)[27,29,75,76]. However, given the prevalence of these findings in the general population, it is difficult to link these disorders to globus[45]. Two studies demonstrated that barium swallow did not identify any malignancy in typical globus patients[5,29]. Additionally, no pharyngeal or esophageal malignancy was found in a study that reviewed 1145 barium swallows in patients presenting with globus, prompting the authors to conclude that barium swallow should not be systematically requested for the exclusion of malignancies in patients with globus[77]. Thus, this test seems to have limited diagnostic value in the investigation of patients with globus.

Videofluoroscopy

Of 23 globus patients who received videofluoroscopy, 8 patients showed abnormal results; 5 had laryngeal aspiration, 2 had barium stasis in the vallecula and pyriform sinuses, and 4 had poor pharyngeal elevation[78]. Although it is unlikely that this indicates a causal relationship, videofluoroscopy may help to identify pharyngeal dysfunction in a substantial proportion of globus patients.

24-h dual-probe ambulatory pH monitoring

Whereas dual-probe ambulatory pH monitoring has been widely used in the clinical assessment of supraesophageal GERD, this technology is not yet standardized, and its usefulness in the definition of a clinically relevant association with GERD is under debate. This technique has been used to show abnormal esophageal acid exposure in some globus patients[4,11]. However, reflux symptoms such as acid regurgitation and/or heartburn were also noted in these study populations. In a study of globus patients without reflux-like symptoms, all 24 focal individuals had normal dual-probe pH results[78]. Therefore, ambulatory pH monitoring seems to be less helpful for the evaluation of globus without reflux-like symptoms.

24-h multichannel intraluminal impedance monitoring

The results from several trials indicate that the best way to detect GER in patients with extraesophageal manifestations of GERD is to conduct multichannel intraluminal impedance (MII)/pH monitoring. In patients experiencing persistent globus during PPI therapy, MII/pH monitoring increased the diagnostic yield of standard pH testing in the identification of positive symptom indices through the detection of nonacid reflux; furthermore, proximal reflux was a significant predictor of the globus symptom[79]. In studies investigating the utility of MII/pH monitoring in patients displaying atypical symptoms while "off PPI", MII/pH monitoring increased the diagnostic yield for objective detection of atypical manifestations of GERD[80-82]. Thus, this technique appears to be a more promising method of obtaining reliable data for the detection of LPR than 24-h dual probe monitoring, as it can monitor acid as well as nonacid reflux events and can distinguish between liquid and gaseous events. Therefore, MII/pH monitoring appears to be useful for ruling out GERD and for redirecting management of patients with suspected extraesophageal manifestations of GERD.

Flexible esophagogastroscopy

Endoscopy has been shown to be superior to barium swallow as a principal means of diagnosing upper aerodigestive tract malignancy[83]. Excellent views of the pyriform fossa and the postcricoid area can be achieved by insufflating air *via* flexible esophagogastroscopy[75]. Moreover, this procedure enables full esophageal evalu-

Figure 1 Algorithm for management of globus. GERD: Gastroesophageal reflux disease; PPI: Proton pump inhibitor; MII: Multichannel intraluminal impedance.

ation and diagnosis of reflux esophagitis and/or upper esophageal malignancy as a cause of globus. However, in general, endoscopy is known to have low sensitivity and to be of limited value for the diagnosis of extra-esophageal GERD. A study of 58 patients with pH-documented LPR found that only 19% had esophagitis or Barrett's metaplasia[84]. In another study of patients with suspected LPR symptoms, esophagitis was generally prevalent[85] but occurred least in patients with globus and throat symptoms. Due to the association between globus and CGHM, it is necessary to carefully evaluate the cervical esophagus[54].

Manometry

If abnormal UES function and esophageal motor disorder are suspected to be the potential cause of globus, manometry is a useful tool for assessing UES and lower esophageal sphincter pressure, esophageal body contraction amplitude, and peristaltic sequence. However, the etiological significance of such a disorder is difficult to define.

TREATMENT

A suggested algorithm for the management of globus patients based on available evidence is shown in Figure 1. Since there is a paucity of controlled studies on the treatment of globus, evidence-based treatment concepts are currently not available, and a review of the litera-

ture reveals that there is no single effective treatment. Given the benign nature of the condition, the likelihood of long-term symptom persistence, and the absence of highly effective pharmacotherapy, the mainstays of treatment are explanation and reassurance[1]. Other established treatment options include anti-reflux therapy, speech and language therapy, anti-depressants, and cognitive-behavioral therapy[86].

Anti-reflux treatment

Since GER has been suggested to be a major cause of globus[10-18], it seems practical that anti-reflux treatment should be the first attempted method for managing patients with globus. As diagnostic tests for GERD are somewhat invasive and costly and because a negative study result does not definitively rule out GER, it seems reasonable to use empirical PPI therapy as a combined method of diagnosis and treatment[87-92]. Although there are no controlled trials looking at the role of PPIs specifically for the treatment of globus, there is a variety of literature addressing the role of PPIs in LPR management[34,91,93-95]. Current evidence shows that the clinical response to PPIs in LPRD is variable[96,97] and that LPR symptoms improve more slowly than esophageal symptoms following acid-suppression therapy[98]. It is now widely accepted that extraesophageal GERD requires more aggressive and more prolonged therapy than typical GERD[32]. Empirical twice-daily therapy with PPIs for at least 3 mo is recommended; this can be extended

for a maximum period of 6 mo[87-92,99-102]. A PPI should be taken 30-60 min before meals so that it has reached its highest concentration by the time food intake stimulates the proton pumps. After 3-6 mo, responders can be weaned, whereas non-responders should undergo a definitive assessment, such as endoscopy, pH monitoring, or MII/pH monitoring. If available, MII/pH monitoring is preferable to simple pH monitoring because it facilitates the detection of nonacid reflux. Nocturnal acid breakthrough (NAB) may cause incomplete treatment response[103,104]. The addition of h.s. histamine-2 receptor antagonists to twice-daily PPI therapy has been suggested to control NAB[103], but it is currently unclear whether this method offers any additional benefit to the long-term control of LPRD[105,106]. Prokinetics are utilized when it is necessary to speed up esophageal and gastric emptying; they can be useful when the clinical response to PPIs is unsatisfactory[16]. Diet and behavioral modification can also decrease the amount of reflux. Recommended dietary modifications include a reduction in the intake of chocolate, fats, citrus fruits, carbonated beverages, spicy tomato-based products, red wines, caffeine, and late-night meals. Additionally, patients should make more general behavioral modifications, including exercising regularly, avoiding smoking and alcohol, elevating the head of the bed (10-15 cm), avoiding tight clothes around the waist, and losing weight. Sleeping on one's left-hand side also helps to decrease reflux. Steward *et al*[107] demonstrated that lifestyle modification was an independently significant variable in determining the response to pharmacological therapy. An alternative therapeutic strategy is anti-reflux surgery, with some authors having reported good improvement rates of LPR symptoms after laparoscopic nissen fundoplication[108-114]. To achieve a better patient outcome, a surgical approach must be taken into consideration in a carefully selected patient population, especially for patients who respond to treatment but are unable to tolerate PPIs due to side effects, those with confirmed pathological GER who do not respond to maximal medical treatment, and those in whom nonacid reflux has been demonstrated by a MII study[115]. However, if symptoms do not improve in the 4 mo following aggressive acid suppression, laparoscopic fundoplication may be unlikely to yield additional benefits[116]. Previous clinical responses to pharmacological acid suppression and abnormal pharyngeal pH results are preoperative predictors of relief from atypical symptoms[117].

Speech and language therapy/relaxation techniques

Speech therapy/relaxation techniques, including neck and shoulder exercises, general relaxation techniques, voice exercises, and voice hygiene to relieve vocal tract discomfort and tension, have successfully been used to treat patients with persistent globus symptoms[61]. In one uncontrolled study using these techniques on 25 globus patients, 92% experienced improvement following treatment. Khalil *et al*[118] randomly allocated 36 globus pa-

tients to either a speech therapy group or a reassurance group. Those in the speech therapy group used a number of exercises to relieve pharyngolaryngeal tension, including yawning, adopting a "giggle posture" (which helps retract the false vocal cords), and a "wet swallow" (as opposed to a "dry" or "check swallow," which patients often perform habitually and which tends to aggravate the globus symptom). Patients also attempted to eliminate throat clearing and promote adequate hydration by avoiding smoking, excess tea, and coffee. At the end of 3 mo, patients in the speech therapy group demonstrated significantly better globus symptom scores compared with those recorded prior to the intervention. Individuals in the speech therapy group also experienced significant improvements in globus symptoms when compared with controls. However, further research is needed to distinguish whether speech therapy has a specific effect or whether patients simply benefit from general attention and reassurance[119].

Cognitive-behavioral therapy/antidepressants

Globus is the fourth most common symptom of somatization disorder after vomiting, aphonia, and pain in the extremities[120]. Cognitive-behavioral therapy has emerged as the best treatment for a variety of somatoform disorders and medically unexplained symptoms[121]. Although there has not yet been a substantial trial of cognitive-behavioral therapy in globus patients, it is likely to be a promising treatment for repeat attenders whose symptoms remain refractory[76].

A small series of anti-depressants have been found to be beneficial for some globus patients with concomitant psychiatric disorders, such as panic, somatization, major depression, and agoraphobia[122,123].

Other treatment strategies

Thyroidectomy in patients with thyroid disorder[49-51] or partial epiglottectomy in selected cases whose retroverted epiglottis made contact with the tongue base[47,48], were both reported to significantly relieve the globus symptom. In addition, ablation of CHGM by argon plasma coagulation has shown some promise in improving chronic globus symptoms[55,56]. Although additional research of these techniques is needed, these approaches would provide some benefit to patients with unexplained chronic globus who are refractory to any medical treatments.

CONCLUSION

Although globus is a common clinical condition, its etiology remains uncertain, and there is no standard protocol for its diagnosis and management. The results of recent studies have strongly suggested that GERD is a major cause of globus, though this remains under considerable debate. Numerous other disorders, such as abnormal UES function, esophageal motility disorders, structural head and neck diseases, and psychological factors, have

been suggested as potential causes of globus. However, it has been rather difficult to establish a causal relationship between globus and these disorders because most of the reported studies were uncontrolled, had a small sample size, or were case reports. Currently, careful history taking and nasolaryngoscopy are essential as a first step in managing globus. Given the benign nature of the condition, patients with typical globus do not appear to need further investigation; rather, a 3-mo treatment with high-dose PPIs seems to be a reliable treatment option. If patients are nonresponsive to PPI therapy, they should undergo a definitive assessment, such as endoscopy, pH monitoring, or MII/pH monitoring; MII/pH monitoring in particular may increase the diagnostic yield of GER in globus patients. In cases with negative clinical investigations and consistent globus symptom, other treatment strategies, including speech therapy, antidepressants, and cognitive-behavioral therapy, should be considered. In the future, well-designed, randomized controlled studies are needed to definitively determine the effect of PPI treatment on globus. In addition, it is necessary to ascertain the etiology of globus *via* large-scale studies.

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ORIGINAL ARTICLE

Lactobacillus plantarum B7 inhibits *Helicobacter pylori* growth and attenuates gastric inflammation

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Abstract

AIM: To determine the anti-*Helicobacter* property of *Lactobacillus plantarum* B7 (*L. plantarum*) B7 supernatants *in vitro* and the protective effects of *L. plantarum* B7 on serum tumor necrosis factor-alpha (TNF-α), gastric malondialdehyde (MDA) level, apoptosis, and histopathology in *Helicobacter pylori* (*H. pylori*)-induced gastric inflammation in rats.

METHODS: *In vitro*, the inhibition of *H. pylori* growth was examined using *L. plantarum* B7 supernatants at pH 4 and pH 7 and at the concentration of 1×, 5× and 10× on plates inoculated with *H. pylori*. The inhibitory effect of *H. pylori* was interpreted by the size of the inhibition zone. *In vitro*, male Sprague-Dawley rats were randomly divided into four groups including group 1 (control group), group 2 (*H. pylori* infected group), group 3 (*H. pylori* infected with *L. plantarum* B7 10^6 CFUs/mL treated group) and group 4 (*H. pylori* infected with *L. plantarum* B7 10^{10} CFUs/mL treated group). One week after *H. pylori* inoculation, *L. plantarum* B7 10^6 CFUs/mL or 10^{10} CFUs/mL were fed once daily to group 3 and group 4, respectively, for one week. Blood and gastric samples were collected at the end of the study.

RESULTS: *In vitro*, at intact pH 4, mean inhibitory zone diameters of 8.5 mm and 13 mm were noted at concentrations of 5× and 10× of *L. plantarum* B7 supernatant disks, respectively. At adjusted pH 7, *L. plantarum* B7 supernatants at concentrations of 5 × and 10× yielded mean inhibitory zone diameters of 6.5 mm and 11 mm, respectively. In the *in vitro* study, in group 2, stomach histopathology revealed mild to moderate *H. pylori* colonization and inflammation. The level of gastric MDA and epithelial cell apoptosis were significantly increased compared with group 1. The serum TNF-α level was significant decreased in group 3 compared with group 2 (*P* < 0.05). In addition, *L. plantarum* B7 treatments resulted in a significant improvement in stomach pathology, and decreased gastric MDA level and apoptotic epithelial cells.

CONCLUSION: *L. plantarum* B7 supernatant inhibits *H. pylori* growth. This inhibition was dose-dependent and greater at pH 4. Moreover, *L. plantarum* B7 attenuated *H. pylori*-induced gastric inflammation.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral shaped bacterium that has the unique ability of being able to colonize the human gastric mucosa and infects more than half of the world's population. *H. pylori* causes chronic gastritis, plays an etiologic role in peptic ulcer disease and is considered a risk factor in the development of gastric cancer and gastric lymphoma[1]. In 1994, *H. pylori* was classified as a type I carcinogen by the World Health Organization[2].

H. pylori infection is characterized by enhanced production of proinflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-2, IL-6 and IL-8 and the infiltration of lamina propria with inflammatory cells. *H. pylori* lipopolysaccharides (LPS) and released surface proteins stimulate lamina propria mononuclear cells and macrophages to produce proinflammatory cytokines such as TNF-α, IL-1β, and the generation of reactive oxygen species (ROS)[3]. TNF-α and IL-1β are potent inducers of IL-8 expression in many cell types. Furthermore, *H. pylori* is capable of interacting with epithelial cell surfaces to produce IL-8. The release of these inflammatory mediators results in the expression of CD11b/CD18 on leukocytes and intercellular adhesion molecule-1 on endothelial cells, the migration of leukocytes to a site of inflammation, and finally the generation of ROS[4].

ROS can react with the double bonds of polyunsaturated fatty acids (PUFAs), present in the membranes of phospholipids, resulting in lipid peroxidation. One of the major secondary oxidation products of peroxidized PUFAs is malondialdehyde (MDA)[5]. *H. pylori* also induces gastric epithelial cell apoptosis both *in vitro*[6] and *in vivo*[7]. Studies have shown that the *H. pylori* colonized stomach contains more apoptotic epithelial cells than normal epithelial cells. Moreover, the increased numbers of apoptotic epithelial cells decrease to normal after eradication of *H. pylori*[7].

H. pylori eradication is suboptimal because current treatment regimens result in adverse side effects, poor compliance, and an increasing prevalence of antibiotic resistance[8]. Therefore, alternative treatments are of interest.

Lactobacilli are probiotics which, when administered in adequate amounts, may confer a benefit to the host[9]. The most commonly used organisms in probiotic products are *Lactobacillus sp.* and *Bifidobacterium sp.*[10]. *L. plantarum* is commonly found in the human gastrointestinal tract (GI-tract). It is important in the production of a variety of fermented foods such as sauerkraut, Korean kimchi, cheese, sausages and stockfish, and is also used as a probiotic. Moreover, there is increasing evidence that *L. plantarum* has anti-*Helicobacter* activity and shows modulatory effects on the immune system[11,12]. Importantly, *L. plantarum* is acid and bile tolerant, survives passage through the GI-tract, and is safe in humans and animals.

The aim of this study was to examine the *in vitro* anti-*Helicobacter* activity of *L. plantarum* B7 supernatants using the disk diffusion method and the effects of *L. plantarum* B7 on gastric histopathology, serum TNF-α, gastric MDA level, and cell apoptosis in *H. pylori* infection *in vivo*.

MATERIALS AND METHODS

In vitro study

The disk diffusion method was used to assess the anti-*H. pylori* activity of *L. plantarum* B7 supernatants at intact and neutralized pH and various concentrations of 1×, 5× and 10× against *H. pylori*.

Bacterial strains and culture conditions: *H. pylori* ATCC 43504 was grown on Columbia agar (Oxoid, Basingstoke, United Kingdom) containing 7% sheep blood and 7% horse serum. Plates were incubated at 37 ℃ under microaerophilic conditions (10% CO_2, 5% O_2 and 85% N_2) produced by a gas generating system, Anaero-Pack (MGC, Japan), for 72 h in an anaerobic jar (Oxoid, Basingstoke, United Kingdom).

L. plantarum B7, isolated from Thai dyspeptic patients, was stored in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom) with 20% glycerol at -80 ℃. This strain was recovered from frozen stock and cultivated twice on MRS agar anaerobically (10% CO_2, 10% H_2 and 80% N_2) at 37 ℃ in an anaerobic jar for 48 h. A single colony of *L. plantarum* B7 was then inoculated into 10 mL of MRS broth and grown at 37 ℃ under anaerobic conditions for 24 h in a 15 mL conical centrifuge tube (Corning, New York, United States). The OD600 of the culture was determined using a spectrophotometer (Bio-Rad Smart SpecTM Plus), adjusted to OD600 with 0.1 in 10 mL of MRS broth and incubated for 48 h. After incubation, the culture supernatant was collected by centrifugation at $1000 \times g$ for 10 min at 4 ℃ and then filtered using a 0.22 μm pore size filter unit (Minisart, Germany). The supernatant of *Lactobacillus* without the cell pellet was called the *Lactobacillus* condition media (LCM). The concentration and pH of LCM were adjusted to 1×, 5× and 10× by speed-vacuum drying (speed-vacuum, Savant Instruments, United States) and resuspending in an appropriate volume of intact pH 4 and adjusted pH 7 MRS broth. Sterile 6 mm-membrane disks (Whatman, Maidstone, United Kingdom) were then dipped into the resuspended LCM for at least 1 h at room temperature.

Disk diffusion method: The various concentrations of *L. plantarum* B7 supernatants were evaluated at two pH values, intact pH 4, and adjusted pH 7 with NaOH.

H. pylori was spread on Columbia blood agar plates, and *L. plantarum* B7 (LCM) disks were placed directly on the surface of the agar. The plates were incubated under microaerophilic conditions at 37 ℃ for 72 h, after which the diameters of the inhibition zones were measured in millimeters. In this study, the MRS broth was used as a negative control. The experiments were carried out in duplicate and mean values of the growth inhibition zones were measured.

In vivo study

Bacteria preparation: *H. pylori* was subcultured twice on Columbia blood agar. Plates were incubated at 37 ℃ under microaerophilic conditions for 72 h. *L. plantarum* B7 was originally obtained from Thai dyspeptic patients who visited King Chulalongkorn Memorial Hospital. This strain was cultivated twice on MRS agar anaerobically at 37 ℃ for 48 h.

Animal preparation: Thirty-two male Sprague-Dawley rats (Salaya Research Animal Center, Mahidol University, Bangkok, Thailand), weighing about 150-250 g at the beginning of the experiment, were used. The experimental protocol was approved by the Ethical Committee of Medicine Faculty, Chulalongkorn University, Thailand. The animals were housed in Macrolon cages (5 animals per cage), given food and tap water *ad libitum* at room temperature (18 ℃-22 ℃), humidity 55%, and a 12/12 h-light/dark cycle.

Experimental protocol: The rats were randomly divided into four experimental groups (eight rats each group) as follows. Group 1: Rats were fed phosphate buffered saline (PBS) 1 mL/rat by gavage twice a day at an interval of four hours for three consecutive days. Then, they were housed with free access to water and standard food for 1 wk. After that, the animals were treated with PBS 1 mL/rat by gavage once daily for 1 wk. Group 2: Rats were inoculated with *H. pylori* using the method of Thong-Ngam *et al*[13]. Briefly, the rats were pre-treated with streptomycin suspended in tap water (5 mg/mL) for three days before *H. pylori* inoculation. The *H. pylori* suspension (5×10^{10} CFUs/mL) in PBS was administered (1 mL/rat) by gavage twice daily at an interval of four hours for three consecutive days. One week after the inoculation, the animals were treated with PBS (1 mL/rat) by gavage once daily for one week. Group 3: One week after *H. pylori* inoculation, the rats were treated by gavage with *L. plantarum* B7 10^6 CFUs/mL suspended in PBS once daily for 1 wk. Group 4: One week after *H. pylori* inoculation, the rats were treated by gavage with *L. plantarum* B7 10^{10} CFUs/mL suspended in PBS once daily for 1 wk.

At the end of the experiment, animals were sacrificed by an overdose of intraperitoneal thiopental sodium. Blood samples were then collected for TNF-α determination using enzyme-linked immunosorbent assay (ELISA). The stomach was removed. One-half of the stomach was frozen in liquid nitrogen, and stored at -80 ℃ for MDA analysis. The remainder of the stomach was fixed in 4% paraformaldehyde in phosphate buffer solution to determine histopathology and epithelial cell apoptosis.

Determination of serum cytokine levels: Blood samples were taken by cardiac puncture, allowed to clot for two hours at room temperature before centrifuging for 20 min at approximately $1000 \times g$. Then, the serum was removed and stored at -80 ℃ for determination of TNF-α level using an ELISA kit (R and D Systems, United States).

Assessment of *H. pylori* infection and examination of histopathology: The presence of *H. pylori* infection in the rats was determined by the urease test and histopathological examination by a blinded pathologist. After completing the experiment, the rats were sacrificed. The stomach was removed and 2 mm² of gastric mucosa from the antrum was immediately dissected and placed in the urease tube to examine urease activity.

The remaining tissue from the gastric antrum biopsy was fixed in 4% paraformaldehyde in phosphate buffer solution at pH 7.4 and room temperature. The tissue was processed and stained with hematoxylin-eosin. The slides were observed by light microscopy and the presence of *H. pylori* was detected by Warthin-Starry staining in unclear cases. The level of bacterial colonization was evaluated using a grading system as follows. Score 0: No bacteria detected; Score 1: Mild colonization in some gastric crypts; Score 2: Mild colonization in most gastric crypts; Score 3: Moderate colonization in all gastric crypts. The results are presented as the bacterial colonization scores for each group. In addition to *H. pylori* colonization, the gastric inflammation level was estimated and scored following the updated Sydney System[14]. The infiltration of polymorphonuclear leukocytes in the gastric mucosa, defining the inflammatory scores, was recorded. Scores from 0 to 3 represented normal, mild, moderate and marked histopathological changes, respectively.

Determination of gastric malondialdehyde: Gastric MDA level was measured using the thiobarbituric acid (TBA) reactive substances assay kit (Cayman, United States). The principle is that the reaction of one molecule of MDA and two molecules of TBA form a red MDA-TBA complex under high temperature (90 ℃ -100 ℃) and acidic conditions, which can be quantitated using a spectrophotometer at 532 nm. The assay procedures were performed as described. The content of MDA was expressed in terms of nmol/mg protein.

Determination of gastric epithelial cell apoptosis: Apoptosis was measured by the identification of apoptotic nuclei in sections of stomach using fragment end labeling of DNA (Apoptosis detection kit, Chemicon, United States). In brief, the DNA fragments were al-

Concentration of *L. plantarum* B7 supernatant	Diameters of inhibition zone (mm)	
	Intact pH 4	Adjusted pH 7
MRS (negative control)	0	0
1×	0	0
5×	8.5 ± 0.7	6.5 ± 0.7
10×	13 ± 0	11 ± 1.4

L. plantarum: *Lactobacillus plantarum*; MRS: Man-Rogosa-Sharpe.

Figure 1 A bar graph shows the mean ± SD of inhibitory zone diameters (mm) of all *Lactobacillus plantarum* B7 supernatant concentrations at intact pH 4 and adjusted pH 7 (*n* = 2). *L. plantarum*: *Lactobacillus plantarum*; MRS: Man-Rogosa-Sharpe.

lowed to bind an antidigoxigenin antibody that was conjugated to a peroxidase. Diaminobenzidine was applied to develop a dark brown color and the slides were counterstained with hematoxylin. The positive stained cells showed dark brown nuclei under light microscopy. To verify the incidence of apoptosis, the dark brown-stained cells were counted. One thousand gastric epithelial cells were counted for each rat. The data were shown as a percentage (%) of apoptotic cells calculated as: the percentage of apoptotic cells (%) = (numbers of positive stained cells × 100)/1000.

Statistical analysis

All data are presented as mean ± SD. The means were compared by one-way analysis of variance followed by least significant different post hoc test. All statistical tests were performed using SPSS for Windows version 13.0 (SPSS Inc, Chicago, IL, United States). Differences were considered statistically significant at *P* < 0.05.

RESULTS

In vitro study

Disk diffusion method: At intact pH 4, mean inhibitory zone diameters of 8.5 ± 0.7 mm and 13 ± 0 mm were noted at the concentrations of 5× and 10× of *L. plantarum* B7 supernatant disks, respectively. At adjusted pH 7, mean inhibitory zone diameters of 6.5 ± 0.7 mm and 11 ± 1.4 mm were noted at the concentrations of 5× and 10× of *L. plantarum* B7 supernatant disks, respec-

Figure 2 A bar graph shows the mean ± SD of serum tumor necrosis factor-alpha level (pg/mL) in all groups. CON: Control group; HP: *Helicobacter pylori* (*H. pylori*) infected group; HP + L (L): *Lactobacillus plantarum* (*L. plantarum*) B7 10^6 CFUs/mL treated group; HP + L (H): *L. plantarum* B7 10^{10} CFUs/mL treated group. Each group is represented by the mean of 8 rats. [a]*P* < 0.05 *vs H. pylori* infected group. TNF-α: Tumor necrosis factor-alpha.

tively (Table 1). Both intact pH 4 and adjusted pH 7 of *L. plantarum* B7 supernatants showed dose-dependent anti-*H. pylori* activity. The supernatant of pH 4 *L. plantarum* B7 at the concentration of 10× showed the clearest inhibition (Figure 1).

In vivo study

Changes in TNF-α level: The serum TNF-α level was not significantly different between the control group and *H. pylori* infected group. However, in the *L. plantarum* B7 10^6 CFUs/mL treated group, a significant decrease in serum TNF-α level was noted compared with the *H. pylori* infected group (*P* = 0.019). The average concentrations of serum TNF-α were 17.22 ± 0.63 pg/mL, 18.05 ± 1.94 pg/mL, and 16.52 ± 0.84 pg/mL in the control, *H. pylori* infected, and in the *L. plantarum* B7 10^6 CFUs/mL treated group, respectively. The average serum TNF-α levels in all groups are shown in Figure 2.

Histopathological examination: *H. pylori* infection in rats was determined by the urease test and histopathology. Histopathology in the control group was normal, while in the *H. pylori* infected group there was moderate *H. pylori* colonization and inflammation. The *L. plantarum* B7 10^6 CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups showed reduced *H. pylori* colonization and improved stomach inflammation (Figures 3 and 4). The histology scores for *H. pylori* colonization and gastric inflammation are summarized in Table 2.

Determination of gastric malondialdehyde: The level of gastric MDA increased significantly in the *H. pylori* infected compared with the control group (3.46 ± 1.25 nmol/mg *vs* 1.05 ± 0.41 nmol/mg protein, *P* = 0.000, respectively). After one week of 10^6 CFUs/mL or 10^{10} CFUs/mL of *L. plantarum* B7 suspension, there was a significant decrease in elevated gastric MDA level in both *L. plantarum* B7 treated groups compared with the *H. pylori* infected group (1.28 ± 0.69, 1.37 ± 0.66 nmol/mg *vs* 3.46 ± 1.25 nmol/mg protein, *P* = 0.000, respectively) (Figure 5).

Determination of gastric epithelial cell apoptosis: The percentage of apoptotic cells was significantly in-

Figure 3 Hematoxylin-eosin stained gastric sections (×40). A: Control group showed no *Helicobacter pylori* (*H. pylori*); B: *H. pylori* infected group showed colonization (arrows) of *H. pylori*; C and D: *Lactobacillus plantarum* (*L. plantarum*) B7 10^6 CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups showed decreased *H. pylori* colonization. GE: Gastric epithelium.

Figure 4 Hematoxylin-eosin stained gastric sections (×20). A: Control group showed normal gastric histopathology; B: *Helicobacter pylori* infected group showed infiltration of inflammatory cells (arrows); C and D: *Lactobacillus plantarum* (*L. plantarum*) B7 10^6 CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups showed improvements in gastric inflammation. GE: Gastric epithelium; LP: Lamina propria; MM: Muscularis mucosae; SM: Submucosa; ML: Muscularis.

Table 2 Summary of the scores for bacterial colonization levels and gastric inflammation in all groups

Group	Number	Level of *H. pylori* colonization[1]				Gastric inflammation[2]			
		0	1	2	3	0	1	2	3
Control group	8	8	-	-	-	8	-	-	-
H. pylori infected group	8	1	5	2	-	-	3	5	-
L. plantarum B7 10[6] CFUs/mL treated group	8	4	4	-	-	-	8	-	-
L. plantarum B7 10[10] CFUs/mL treated group	8	3	5	-	-	-	8	-	-

[1]The stomach samples were evaluated for *Helicobacter pylori* (*H. pylori*) colonization by the pathologist using the following scoring system. Score 0: No bacteria detected; Score 1: Mild colonization in some gastric crypts; Score 2: Mild colonization in most gastric crypts; Score 3: Moderate colonization in all gastric crypts. [2]The gastric inflammation level was estimated and scored by the pathologist following the updated Sydney System[16]. The infiltration of polymorphonuclear leucocytes in the gastric mucosa defining the inflammatory scores was recorded. Scores from 0 to 3 represented normal, mild, moderate and marked histopathology changes, respectively. *L. plantarum*: *Lactobacillus plantarum*.

creased in the *H. pylori* infected group when compared with the control group (7.44 ± 2.65 *vs* 0.58 ± 0.13, $P = 0.0001$, respectively). After treatment with 10[6] CFUs/mL or 10[10] CFUs/mL of *L. plantarum* B7 suspension, the percentage of apoptotic cells was significantly decreased at 10[6] CFUs/mL ($P = 0.027$) and 10[10] CFUs/mL ($P = 0.038$) compared with the *H. pylori* infected group. The average percentages of apoptotic cells in all the groups are shown in Figure 6. Figure 7 shows gastric sections processed for apoptosis by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction.

DISCUSSION

The *in vitro* study with intact pH 4 and adjusted pH 7 of *L. plantarum* B7 supernatants showed concentration-dependent anti-*H. pylori* activity, however, the culture supernatants of intact pH 4 *L. plantarum* B7 supernatant showed higher inhibition. This implied that low pH values are important for anti-*H. pylori* activity. In a study by Boyanova *et al*[15], the anti-*Helicobacter* activity of *L. delbrueckii subsp. bulgaricus* cultures was strain-dependent and better at their native pH.

It is known that *Lactobacillus* secretes metabolic products such as lactic acid which exerts activity against *H. pylori*[16]. Lactic acid inhibits the urease activity and viability of *H. pylori*. Several studies have reported that bacteriocin, peroxide, proteinase, exopolysaccharide and cell wall components, called *Lactobacillus*-inhibitory factors, have antibacterial effects[17,18]. In addition, Coconnier *et al*[19] showed that a heat-stable antimicrobial substance secreted by *L. acidophilus* LB was active against *H. pylori* infection.

In summary, our *in vitro* study found that *L. plantarum* B7 supernatant inhibited *H. pylori* growth in a dose-dependent manner and was better at intact pH 4 indicating that the amount of antimicrobial substance released by

Figure 5 A bar graph shows the mean ± SD of gastric malondialdehyde levels (nmol/mg protein) in all groups. CON: Control group; HP: *Helicobacter pylori* (*H. pylori*) infected group; HP + L (L): *Lactobacillus plantarum* (*L. plantarum*) B7 10[6] CFUs/mL treated group; HP + L (H): *L. plantarum* B7 10[10] CFUs/mL treated group. Each group is represented by the mean of 8 rats. [a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs H. pylori* infected group. MDA: Malondialdehyde.

Figure 6 A bar graph shows the mean ± SD of apoptotic cells (%) in all groups. CON: Control group; HP: *Helicobacter pylori* (*H. pylori*) infected group; HP + L (L): *Lactobacillus plantarum* (*L. plantarum*) B7 10[6] CFUs/mL treated group; HP + L (H): *L. plantarum* B7 10[10] CFUs/mL treated group. Each group is represented by the mean of 8 rats. [a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs H. pylori* infected group.

L. plantarum B7 correlated with the intensity of the inhibitory effect against *H. pylori*. Furthermore, the anti-*H. pylori* activity of this substance was supported by low pH values.

The present *in vivo* study showed that the gastric histopathology in the *H. pylori* infected group revealed mild to moderate *H. pylori* colonization and inflammation as well as increased gastric MDA and gastric epithelial cell apoptosis.

H. pylori induces a host inflammatory response including production of cytokines, resulting in mucosal damage. The produced cytokines lead to infiltration of inflammatory cells, namely polymorphonuclear neutrophils (PMNs), lymphocytes and macrophages, at the site of infection. These inflammatory cells then release large amounts of ROS, causing tissue injury. Wilson *et al*[20] showed that the gastric mucosal levels of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, were significantly higher in *H. pylori* positive patients than in *H. pylori* negative patients. Crabtree *et al*[21] showed that increased gastric mucosal production of TNF-α and IL-6 was associated with *H. pylori* gastritis. Moreover, they implied that inflammatory cytokines generated locally within the gastric mucosa can be relevant to the gastric physiology of *H. pylori* infection.

As mentioned above, infection with *H. pylori* in the

Figure 7 Representative gastric sections processed for the apoptosis assay by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling reaction (×20). A: Control group; B: *Helicobacter pylori* infected group; C and D: *Lactobacillus plantarum* (*L. plantarum*) B7 10⁶ CFUs/mL treated and *L. plantarum* B7 10¹⁰ CFUs/mL treated groups showed a decrease in gastric epithelium apoptosis. The arrows indicate terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive gastric epithelial cell apoptosis.

gastric mucosa is known to activate the production of many proinflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8. The production of these proinflammatory cytokines is not limited to the local site of infection, as these cytokines are produced in numbers and contribute to the systemic circulation. In 2006, Prabjone *et al*[22] investigated the effects of chronic *H. pylori* infection on serum TNF-α level in rats. They found a significant increase in serum TNF-α in the *H. pylori* infected groups compared with the control groups. In the present study, no significant increase in serum TNF-α level was observed in the *H. pylori* infected group.

Several studies have shown that *H. pylori* strains with the *cagA*⁺/*vacAs1* genotype are more virulent than strains with other genotypes[23]. Similarly, Azuma *et al*[24] reported that *H. pylori cagA*⁺ strains were involved in more intense tissue responses than *cagA*⁻ strains. Moreover, epidemiological studies have shown that colonization with *cagA*⁺ *H. pylori* is associated with an increased risk for the development of both peptic ulcer disease and gastric cancer. In an *in vitro* study, Zhang *et al*[25] demonstrated that *H. pylori cagA*⁺ strains induced an increased oxidative burst in PMNs with higher ROS production. Recently, studies have shown that ROS production in gastric mucosa is enhanced by infection with *cagA*⁺ *H. pylori* species with an extensive accumulation of neutrophils in both patients with chronic gastritis and gastric ulcer[4,5]. In this study, rats infected with *H. pylori cagA*⁺, *vacA*⁺ strains were found to have significantly increased gastric MDA levels,

suggesting that oxidative stress may be associated with the *cagA*⁺ status of *H. pylori*.

Furthermore, we demonstrated that *H. pylori cagA*⁺, *vacA*⁺ strains can induce epithelial cell apoptosis in rats. The *cagA* gene or expression of VacA might be involved in gastroduodenal diseases by affecting apoptosis. The *cagA* gene is a marker of the presence of the pathogenicity island that encodes disease-associated virulence factors and is associated with the expression of VacA[26]. In 2006, Cabral *et al*[27] showed that the expression of pro-apoptotic proteins such as Bax and Bak was higher than anti-apoptotic proteins including Bcl-2 and Bcl-XL in most gastric biopsies from patients with *H. pylori* gastritis and was significantly higher in patients infected by *cagA*⁺ strains than in those infected by *cagA*⁻. Moreover, they found that Bak expression was higher at the lesser curvature (antrum and incisura) than in the other regions and was correlated with atrophy. These results suggest that in addition to *cagA, vacA* plays a crucial role in the induction of apoptosis. In the present study, our data also showed that infection with *H. pylori cagA*⁺, *vacA*⁺ strains leads to elevated gastric MDA levels, as previously mentioned. MDA, a major product of lipid peroxidation, can react with DNA to form MDA-DNA adducts, resulting in DNA damage.

Several previous investigations have shown the anti-inflammatory properties of *Lactobacillus*. A study by Johnson-Henry *et al*[28] found that the probiotic combination containing *L. rhamnosus* R0011 and *L. acidophilus*

R0052 decreased the effects of *H. pylori* infection in a C57BL/6 mouse model of infection by reducing *H. pylori* colonization and alleviating *H. pylori*-induced gastric mucosa inflammation. In 2003, Peña *et al*[29] showed that *L. rhamnosus* GG was able to antagonize *H. pylori* LPS-induced TNF-α production in murine macrophages *in vitro* by a contact-independent mechanism. Ko *et al*[12] reported that *L. plantarum* was capable of inhibiting epithelial barrier dysfunction and reducing IL-8 secretion induced by TNF-α. In addition to anti-inflammatory activity, several studies have shown that *Lactobacillus* also has effective antioxidative and anti-apoptotic properties. Truusalu *et al*[30] found that *L. fermentum* ME-3 suppressed excessive oxidative stress-associated inflammation induced by *S. typhimurium* infection in a mouse model. Using the same experimental typhoid fever model, they also showed that treatment with *L. fermentum* ME-3 alone or in combination with an antimicrobial quinolone (ofloxacin) leads to a significant decrease in lipid peroxidation and the glutathione redox ratio (GSSG/GSH). In 2010, Zhang *et al*[31] reported that oral *L. plantarum* treatment in rats with obstructive jaundice increased GSH levels in the liver and stimulated GSH biosynthesis, resulting in attenuated oxidative damage. Using the TUNEL assay, they also showed that treatment with *L. plantarum* significantly decreased hepatic apoptosis. In addition, Lam *et al*[32] showed that pre-treatment of rats with *L. rhamnosus* GG markedly reduced ethanol-induced mucosal lesion area and gastric cell apoptosis.

Interestingly, all of these studies were concordant with our results. In the current study, we found that *L. plantarum* B7 treatment resulted in improved stomach pathology, and decreased serum TNF-α level, gastric MDA level, and apoptotic epithelial cells. However, the mechanisms of action are unclear and require further investigation.

In conclusion, the present study showed that *H. pylori* infection induced gastric injury by increasing levels of *H. pylori* colonization and inflammation, gastric MDA and epithelial cell apoptosis. *L. plantarum* B7 may have anti-*H. pylori* activity *in vitro* and anti-inflammatory effects on *H. pylori* infection by improving stomach histopathology, and reducing serum TNF-α levels, gastric MDA and epithelial cell apoptosis.

COMMENTS

Background
Helicobacter pylori (*H. pylori*) infection induces the production of proinflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-2, IL-6 and IL-8, and infiltration of the lamina propria with inflammatory cells as well as the generation of reactive oxygen species (ROS). However, these *H. pylori*-induced inflammatory responses do not appear to confer protective immunity, and may lead to the excess production of ROS, oxidative bursts caused by phagocytic cells, and gastric tissue damage. *Lactobacillus plantarum* (*L. plantarum*) B7 has anti-*H. pylori* activity *in vitro* and anti-inflammatory properties resulting in the alleviation of gastric injury in *H. pylori*-induced gastritis in rats.

Research frontiers
L. plantarum is a non-pathogenic gram-positive bacterium that exerts anti-*H. pylori* activity and immunomodulatory effects. *H. pylori* infection can cause gastric mucosal damage by increasing *H. pylori* colonization and inflammation levels, gastric malondialdehyde (MDA) and epithelial cell apoptosis. The hallmark of this study was the interesting results which showed an inhibitory effect of *L. plantarum* B7 supernatant on *H. pylori* growth *in vitro*, and an improvement in stomach pathology, reduction in serum TNF-α level, gastric MDA and epithelial cell apoptosis following treatment with *L. plantarum* B7.

Innovations and breakthroughs
A previous study showed that *L. plantarum* B7 has anti-inflammatory properties *in vitro*. However, it is not clear whether *L. plantarum* B7 has *in vivo* effects on *H. pylori*-induced gastric inflammation. Therefore, in this study, the authors examined the anti-inflammatory effect of *L. plantarum* B7 in rats and found that *L. plantarum* B7 ameliorated *H. pylori*-induced gastritis by improving stomach pathology, and decreasing TNF-α production, gastric MDA level and epithelial cell apoptosis. Moreover, supernatants of *L. plantarum* B7 showed anti-*H. pylori* activity *in vitro*.

Applications
L. plantarum B7 may be beneficial in clinical application, and can be used as an adjunct to antibiotics to decrease *H. pylori*-induced gastric inflammation and reduce side effects of triple therapy.

Peer review
This is an experimental study on the effect of *H. pylori* infection in gastric inflammation. This study shows the efficacy of *L. plantarum* B7 in treatment of *H. pylori*-induced gastritis reflects in attenuated levels of *H. pylori* colonization, gastric inflammation, cytokine production, gastric MDA, and apoptotic cells. Also, the results from *in vitro* study demonstrates the inhibitory effect of *L. plantarum* B7 supernatants on *H. pylori* growth.

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ORIGINAL ARTICLE

Key factors in developing the trinitrobenzene sulfonic acid-induced post-inflammatory irritable bowel syndrome model in rats

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Abstract

AIM: To investigate the key factors in developing the trinitrobenzene sulfonic acid (TNBS)-induced post-inflammatory irritable bowel syndrome (PI-IBS) model in rats.

METHODS: TNBS was administered to rats at the following conditions: (1) with different doses (20, 10, 5 mg/0.8 mL per rat); (2) with same dose in different concentrations (20 mg/rat, 25, 50 mg/mL); (3) in different ethanol percentage (25%, 50%); and (4) at depth either 4 cm or 8 cm from anus. At 5 d and 4 wk after TNBS administration, inflammation severity and inflammation resolution were evaluated. At 4 and 8 wk after TNBS application, visceral hyperalgesia and enterochromaffin (EC) cell hyperplasia were assayed by abdominal withdrawal reflex test, silver staining and capillary electrophoresis.

RESULTS: Our results showed that: (1) TNBS induced dose-dependent acute inflammation and inflammation resolution. At 5 d post TNBS, the pathological score and myeloperoxidase (MPO) activity in all TNBS treated rats were significantly elevated compared to that of the control (9.48 ± 1.86, 8.18 ± 0.67, 5.78 ± 0.77 *vs* 0, and 3.55 ± 1.11, 1.80 ± 0.82, 0.97 ± 0.08 unit/mg *vs* 0.14 ± 0.01 unit/mg, $P < 0.05$). At 4 wk post TNBS, the pathological score in high and median dose TNBS-treated rats were still significantly higher than that of the control (1.52 ± 0.38 and 0.80 ± 0.35 *vs* 0, $P < 0.05$); (2) Intracolonic TNBS administration position affected the persistence of visceral hyperalgesia. At 4 wk post TNBS, abdominal withdrawal reflex (AWR) threshold pressure in all TNBS-treated groups were decreased compared to that of the control (21.52 ± 1.73 and 27.10 ± 1.94 mmHg *vs* 34.44 ± 1.89 mmHg, $P < 0.05$). At 8 wk post TNBS, AWR threshold pressure in 8 cm administration group was still significantly decreased (23.33 ± 1.33 mmHg *vs* 36.79 ± 2.29 mmHg, $P < 0.05$); (3) Ethanol percentage affected the TNBS-induced inflammation severity and visceral hyperalgesia. In TNBS-25% ethanol-treated group, the pathological score and MPO activity were significantly lowered compared to that of the TNBS-50% ethanol-treated group, while AWR threshold pressure were significantly elevated (36.33 ± 0.61 mmHg *vs* 23.33 ± 1.33 mmHg, $P < 0.05$); and (4) TNBS (5 mg/0.8 mL per rat, in 50% ethanol, 8 cm from anus)-treated rats recovered completely from the inflammation with acquired visceral hyperalgesia and EC cell hyperplasia at 4 wk after TNBS administration.

CONCLUSION: TNBS dosage, concentration, intraco-

Ionic administration position, and ethanol percentage play important roles in developing visceral hyperalgesia and EC cell hyperplasia of TNBS-induced PI-IBS rats.

Key words: Post-inflammatory; Irritable bowel syndrome; Rat model; Trinitrobenzene sulfonic acid; Key factors

INTRODUCTION

Post-infectious irritable bowel syndrome (PI-IBS) is a subgroup of IBS in which the IBS patients developed their symptoms after recovery from an acute gastrointestinal infection[1,2]. The features of PI-IBS, such as urgency, loose stool, and abdominal pain, are very similar to those of the diarrhea-predominant IBS[3,4]. Prospective studies indicate that 3%-36% of enteric infections may lead to the generation of new IBS symptoms; the precise incidence mainly depends on the infecting organism and the host responses[1]. Though the term "PI-IBS" was first coined by Chaudhary and Truelove in 1962[5], little attention has been paid to it until recently[1]. The causes and/or underlying mechanisms of PI-IBS are still not fully understood, although it is believed that altered gut flora, changed intestinal permeability, activated gut immunity, and functional or structural changes in enteric nervous system are important factors[1,6].

Validated animal models, which aim to mimic one or more features of human disease, play important roles in the studies of mechanisms and potential therapeutic agents for diseases. Based on the current understanding of the clinical features and underlying mechanisms of PI-IBS, the ideal model for PI-IBS should bear the character with one or more features of IBS, such as visceral hypersensitivity, motility dysfunction, alterations in permeability or secretion, and enterochromaffin (EC) cell hyperplasia, and complete recovery from initial infection/inflammation[7]. The chemical agent-induced post-inflammatory IBS animal models have been widely used in mechanistic studies of PI-IBS because the chemical agent-induced inflammatory response and host immune activation can, to some extent, mimic the characteristics of PI-IBS[8,9], and these alterations are consistent with the findings from clinical studies[10,11]. Our previous systematic review showed that the trinitrobenzene sulfonic

acid (TNBS)-induced model is the most commonly used PI-IBS model[7], and the major features of IBS (i.e., visceral hypersensitivity, motility dysfunction, and alteration in permeability or secretion), can be developed in this model. However, there are large variations in the protocol of model development, such as TNBS dosage and concentration, position of intracolonic TNBS administration, ethanol percentage, and the optimal time after intracolonic TNBS administration for model application, *etc*.[7]. These variations make comparison between studies to be very difficult, if not impossible.

As a chemical hapten, TNBS is capable of binding to tissue proteins and stimulating T helper 1 cell mediated immunity; thus, it has been widely used to induce acute colitis by intracolonic administration[12]. It has been reported that TNBS-induced acute inflammation and damage becomes maximal from 3 d to 1 wk after instillation[9]. Previous studies have shown that TNBS had dose-dependent effects on mucosal inflammation[13,14], suggesting dosage is an important factor in TNBS-induced inflammation severity and colonic recovery from such inflammation. However, in previous studies on the TNBS-induced PI-IBS model, the dosage of TNBS varied from 20 mg per rat to 30 mg per rat[7]. Further, there is no consensus on how long it will take for TNBS-induced inflammation recovery after TNBS administration. Some studies, using TNBS (30 mg/rat), took 4 or 6 wk for inflammation recovery after intracolonic TNBS administration[15,16], while another study took at least 8 wk[17]. In addition, TNBS is generally administered into the colon lumen at either 4 cm or 8 cm from the anus[7]. Given the anatomical and physiological differences between these two sites (descending colon *vs* transverse colon), it is possible - but not known - whether the position of TNBS administration influences the results. Furthermore, ethanol is routinely used as a breaker to the mucosal barrier in TNBS-induced PI-IBS studies, and previous studies have shown that 30%-50% ethanol alone induced acute inflammation and hyperemia[18,19]. The commonly used ethanol percentage in developing TNBS-induced PI-IBS model are 25% and 50%[14,20]; and whether the ethanol percentage influences the development of TNBS-induced PI-IBS rat model is still not clear.

Concerning the above variations in developing protocol of the TNBS-induced PI-IBS rat model, the present study aimed to investigate the effects of some impact factors (i.e., TNBS dosage and concentration, position of TNBS administration, and ethanol percentage) on TNBS-induced acute inflammation, inflammation resolution, and later acquired features of IBS (i.e., visceral hyperalgesia and EC cell hyperplasia).

MATERIALS AND METHODS

Materials

TNBS (2,4,6-trinitrobenzenesulfonic acid solution), hexadecyltrimethylammonium bromide, o-dianisidine dihydrochloride, sodium hyposulfite, and silver nitrate were

Table 1 Study design and the treatments

Groups	Treatment (mg/mL per rat, i.col.)	Ethanol (%)	Position (cm to anus)	Time points for evaluation		
				5 d	4 wk	8 wk
1	TNBS, 20/0.4 per rat	50	8	IE	IE	-
2	TNBS, 20/0.8 per rat	50	8	IE	IE	-
3	TNBS, 10/0.8 per rat	50	8	IE	IE	-
4	TNBS, 5/0.8 per rat	50	8	IE	IE/PE	PE/EE
5	TNBS, 5/0.8 per rat	50	4	IE	IE/PE	PE
6	TNBS, 5/0.8 per rat	25	8	IE	IE/PE	PE
7	Saline, 0.8/rat	-	8	IE	IE/PE	PE/EE

TNBS: Trinitrobenzene sulfonic acid; IE: Inflammation evaluation; PE: Pain evaluation; EE: Enterochromaffin cell evaluation.

all purchased from Sigma-Aldrich (St. Louis, MO, United States). Chloral hydrate was purchased from Kou Hing Hong Scientific Supplies (Hong Kong, China).

Animals
Male Sprague-Dawley rats (aged 6 wk with body weight around 220 g) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. Rats were housed 5 per cage and maintained at 25 ℃ under 12 h-12 h alternating light-dark cycle with free access to food and water. Rats were maintained in laboratory conditions for 1 wk to adapt to the environment before each experiment. All animal studies were carried out in accordance with the guidelines of the Committee on Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University.

Experimental design
The rats were randomly divided into 7 groups. TNBS (20 mg/0.4 mL per rat) was given to rats in group 1 (*n* = 10). The rats in group 2 (*n* = 10), group 3 (*n* = 10), and group 4 (*n* = 14) were intracolonically administered with TNBS at a dose of 20, 10 and 5 mg per rat in 0.8 mL ethanol, respectively. In the above 4 groups, TNBS in 50% ethanol saline solution was administered at a depth of 8 cm from the anus. Rats in group 5 (*n* = 14) were given TNBS (5 mg/0.8 mL per rat in 50% ethanol) at a depth of 4 cm from the anus, while rats in group 6 (*n* = 14) were given TNBS (5 mg/0.8 mL per rat in 25% ethanol) at 8 cm from the anus. Group 7 (*n* = 14) was set as a control; rats in this group were given saline 0.8 mL at a depth of 8 cm from the anus (Table 1).

Five days after TNBS or saline administration, 4 rats in each group were selected randomly, and the colon tissues from these rats were harvested for inflammation evaluation. The remaining rats were allowed to recover until the pain threshold pressure was measured. At 4 wk after TNBS administration, five rats in each group were selected randomly for pain threshold pressure evaluation; then the colon tissues were collected for inflammation recovery examination. At 8 wk after TNBS administration, the rest of the rats in each group went through pain threshold pressure evaluation to test the stability of visceral hyperalgesia. In order to further investigate

if the TNBS-induced PI-IBS model had EC cell hyperplasia in the colon tissue, a part of proximal and distal colon tissues were also collected for EC cell number counting and serotonin [5-hydroxytryptamine (5-HT)] content determination.

Induction of colitis
Colitis was induced according to previous reports, with little modification[16]. Briefly, rats were fasted for 24 h before experiments, and then deeply anesthetized with chloral hydrate (350 mg/kg, i.p.). A fine plastic catheter (external diameter = 0.96 mm) was gently inserted into the descending colon at a depth of 4 cm or 8 cm from anus. The rats were kept in a head-down vertical position, and then TNBS was instilled slowly into the colon lumen within 1 min. After TNBS instillation, the catheter was left in place for 1 min and then slowly removed. The TNBS-treated rats were left on a warm mound of bedding in head-down position to prevent drug leakage until they regained consciousness. The control rats were similarly administered with 0.8 mL saline instead of TNBS.

Tissue preparation
Rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.); then an approximately 6 cm long piece of colon tissue with drug administration position in the middle was removed. After the photos were taken, 3 cm of the distal part (proximal to the anus) was fixed in 4% paraformaldehyde for histological evaluation., the remaining 3 cm (distal to the anus) was placed in liquid nitrogen and stored in a freezer at -80 ℃ for myeloperoxidase activity assay. In addition, approximate 3 cm of proximal colon (1-2 cm from cecum) and 3 cm of distal colon (1-2 cm from anus) tissue were also harvested from the PI-IBS model rats; the proximal part (about 1 cm) was fixed in 4% paraformaldehyde for EC cell number counting; the rest was placed in liquid nitrogen, and stored at -80 ℃ for 5-HT content determination.

Histological evaluation
The colon sections (5 μm thick) collected at 5 d and 4 wk after TNBS administration were all stained with hematoxylin and eosin (H and E). Masson trichrome staining[21] was performed on sections collected at 4 wk after TNBS administration for fibrosis evaluation. All sections were examined under a Nikon light microscope (Nikon Inc., Japan). The severity of the acute inflammation and the degree of inflammation resolution was graded using the macroscopic and histological scoring criteria (Tables 2 and 3), which were modified based on previous reports[22,23] according to the pathologist's suggestion. Five random fields were selected in each slide; images were captured with 100 × magnifications and analyzed using Image J NIH software. For fibroplasia evaluation, the area stained blue by Masson trichrome staining was measured and adjusted to reflect the total area of the colon tissue[21].

Myeloperoxidase activity assay
Myeloperoxidase (MPO) is an enzyme released by neu-

Table 2 Criteria for macroscopic scoring of colonic ulceration and inflammation

Score	Appearance
0	Normal appearance
1	Ulceration with inflammation at 1 or 2 sites
2	More sites of ulceration and inflammation
3	Major sites of damage extending > 1.5 cm along length of colon
4	Major sites of damage extending > 3 cm along length of colon

trophils in tissue under inflammatory conditions, and the level of MPO activity correlates directly with severity of inflammation[17]. In this study, MPO activity was measured by the modified method described by Krawisz *et al*[24] and Diop *et al*[18]. Briefly, the colon tissues were cut into small pieces and homogenized in 0.5% hexadecyltrimethylammonium bromide 1 mL per 100 mg of colon tissue. The homogenates were centrifuged at 19 000 *g* at 4 ℃ for 15 min. Aliquots of 80 mL supernatant were mixed with 120 μL potassium phosphate buffer (50 mmol, pH 6.0) with 0.0005% o-dianisidine dihydrochloride and 0.1% hydrogen peroxide. MPO activity was calculated from the rate of absorbance change during 1 min at 460 nm; one unit of MPO activity is equal to 1.13×10^{-2} changes in absorbance at 25 ℃. The results were normalized to the wet weight of colon tissue and expressed as MPO units/mg tissue.

Abdominal withdrawal reflex

Abdominal withdrawal reflex (AWR) test was performed as previously described to detect the pain threshold pressure[25]. Briefly, rats were lightly anesthetized with ether in order to place a 6 cm long flexible latex balloon into the descending colon and rectum through the anus. The end of the balloon was secured at least 1 cm proximal to the anal verge. Rats were then allowed to recover for at least 30 min. The tube of the balloon was connected *via* a Y-connector to a sphygmomanometer and colorectal distension was applied in increments of 5 mmHg until a visible contraction of the abdominal wall was observed by an investigator blinded to the treatment. The pain threshold pressure was defined as the intensity of colorectal distension that elicited an observable AWR, i.e., a sudden and persistent abdominal muscle contraction with abdomen lift off the platform (Score 3). The pain threshold pressure of all groups was recorded and repeated five times with intervals of at least 5 min for recovery.

Enterochromaffin cell counting

Tissue sections (5 μm thick) were deparaffinized in xylene, and rehydrated with graded ethanol for silver staining according to a method previously described, with little modification[26]. Sections were incubated with 5% ammoniacal silver solution for 4 h at room temperature, then 2 h in 56 ℃ and subsequently 12 h at room temperature in a dark humidified chamber. After rinsing

with water, 5% sodium hyposulfite was added and sections were incubated for 5 min at room temperature. The brown to black silver precipitate in the cytoplasm of EC cells was considered as a positive reaction. Five random fields at 200× magnifications for each section were captured and saved in the same size and resolution by a researcher blinded to treatment. After calibration, the mucosal fields were circled, and the areas of mucosa were calculated using Image J NIH software. EC cell density was calculated and expressed as the number of EC cells per mm^2 of mucosal area.

Serotonin content assessment

5-HT content in the colonic tissue was assayed following the previously reported procedure[27]. Briefly, the colon segment was homogenized in 15% iced trichloroacetic acid; the supernatant of each sample was filtered using 0.22 μm filters and extracted with diethyl ether, then the prepared samples were added to derivatization solution and analyzed by capillary electrophoresis with laser-induced fluorescence detection.

Statistical analysis

Data are presented as mean ± SE. Differences between two groups were analyzed by Student *t* test. When multiple groups were compared, data were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

RESULTS

Effect of trinitrobenzene sulfonic acid concentration on body weight and mortality rate

Based on the findings from our previous systematic review, TNBS (20 mg/rat, in 50% ethanol) was selected and given to rats at 8 cm from anus in 0.4 or 0.8 mL volume. As shown in Table 4, compared to the control group, the body weight of TNBS-treated rats all decreased markedly with loose and bloody stools at 5 d post-TNBS ($P < 0.01$). Moreover, the body weight in high-concentration TNBS (50 mg/mL)-treated rats was significantly lowered when compared to that treated with low-concentration TNBS (25 mg/mL) ($P < 0.05$), and 3 rats in the high-concentration TNBS (50 mg/mL)-treated group were found dead. At 4 wk after TNBS administration, the average body weight of high-concentration TNBS-treated rats was still significantly decreased compared to that of rats treated with low-concentration TNBS and saline ($P < 0.05$); no significant difference was found in the body weight between low-concentration TNBS-treated rats and the saline-treated ones.

Effect of trinitrobenzene sulfonic acid dosage on the severity of acute inflammation and inflammation resolution

Five days after TNBS administration, the results from MPO activity assay and histopathological evaluation showed that, TNBS, when administered at the doses of

Table 3 Criteria of histological scoring in colon tissue

Variables	Severity and scoring			
	0	1	2	3
Ulceration	No ulcer	Ulcerations not exceeding lamina muscularis mucosae	Ulcerations not exceeding submucosa	Ulcerations exceeding submucosa
Edema	Normal thickness	Submucosal expansion < 30%	Submucosal expansion 30%-100%	Submucosal expansion > 100%
Inflammatory cells	No infiltration	Few scattered cells	Distributed but not dense	Dense
Fibroplasia	Normal collagen	Increase < 30%	Increase 30%-50%	Increase > 50%

Table 4 Effects of trinitrobenzene sulfonic acid concentration on body weight and mortality rate

Treatment	TNBS concentration	Body weight (g)		Mortality rate (%)
		5 d	4 wk	
Saline	-	249.6 ± 26.7	343.0 ± 28.9	0
TNBS 20 mg/0.8 mL per rat	25 mg/mL	214.5 ± 16.8[b]	324.5 ± 24.8	0
TNBS 20 mg/0.4 mL per rat	50 mg/mL	179.2 ± 21.4[d]	291.8 ± 42.6[a]	33

[a]$P < 0.05$, [b]$P < 0.01$ *vs* saline-treated group; [d]$P < 0.01$ *vs* TNBS (20 mg/0.8 mL)-treated group. TNBS: Trinitrobenzene sulfonic acid.

20, 10, and 5 mg/0.8 mL per rat in 50% ethanol, dose-dependently induced acute inflammation and damage in the colon tissue of rats (Figure 1). The results indicate that the higher the dose TNBS used, the more severe the inflammation and damage induced. As shown in macroscopic appearance and H and E sections (Figure 1A and B), high dose TNBS (20 mg/rat) induced multiple larger and deeper ulcerations, and dense inflammatory cell infiltration when compared to that treated with low dose TNBS.

As shown in Figure 2, four weeks after TNBS (5-20 mg/rat) administration, there was no significant difference in MPO activity between TNBS-treated rats and the control groups, suggesting no acute inflammation was left at this period. However, results from pathological evaluation showed that the histological scores in high dose (20 mg/rat) and median dose (10 mg/rat) TNBS-treated groups were still significantly higher compared to that of the control ($P < 0.05$), but no significant difference was found between low dose TNBS-treated rats and the control, suggesting low dose TNBS-treated rats had completely recovered from the initial inflammation at 4 wk post TNBS. As shown in Figure 2A and B, the colons from high and medium dose TNBS treated rats lost their normal appearance, and more collagen was found in the submucosa and smooth muscle in Masson staining sections, suggesting the occurrence of fibroplasia.

Effect of trinitrobenzene sulfonic acid administration position, and ethanol percentage on the severity of acute inflammation and inflammation resolution

Knowing that low dose TNBS (5 mg/0.8 mL per rat in 50% ethanol)-treated rats can completely recover from initial inflammation at 4 wk after TNBS administration, further studies were based on this dosage selection. As shown in Figure 3, compared to the control, MPO activ-

ity assay and histopathological evaluation all demonstrated that low dose TNBS (5 mg/0.8 mL per rat, in 50% ethanol), when given to rats at 8 cm or 4 cm from anus, induced marked acute inflammation and ulcers at 5 d post TNBS administration ($P < 0.01$), no difference was found between these two groups. However, the same dose TNBS (5 mg/0.8 mL per rat, 8 cm depth) in 25% ethanol induced significant mild non-ulcer inflammation compared to that treated with TNBS in 50% ethanol ($P < 0.01$). Four weeks after TNBS administration, there were no significant differences in MPO activity and histopathological score among all TNBS-treated groups and the control, suggesting that the rats treated with low dose TNBS (5 mg/0.8 mL per rat) had all completely recovered at 4 wk after TNBS administration.

Effect of trinitrobenzene sulfonic acid administration position, and ethanol percentage on the visceral hyperalgesia

To investigate whether the low dose TNBS (5 mg/0.8 mL per rat)-treated rats acquired long-lasting visceral hyperalgesia after inflammation resolution, AWR test was applied to all these TNBS-treated rats at 4 and 8 wk after TNBS administration. As shown in Figure 4, at 4 wk after TNBS administration, the pain threshold pressure in all TNBS-treated rats was decreased significantly compared to the control ($P < 0.05$), but no significant difference was found among these TNBS-treated rats. At 8 wk after TNBS administration, the pain threshold pressure in rats treated with TNBS at the depth of 8 cm was still significantly decreased compared to that of the controls ($P < 0.05$), but no significant difference was found in rats treated with TNBS at 4 cm from anus or in 25% ethanol.

Enterochromaffin cell hyperplasia in trinitrobenzene sulfonic acid-induced post-infectious irritable bowel syndrome rat model

To investigate whether the rats treated with low dose TNBS (5 mg/0.8 mL per rat, in 50% ethanol) at 8 cm from anus also acquired other features of PI-IBS, the EC cell number and 5-HT content in the proximal and distal colon tissues were further tested at 4 and 8 wk after TNBS administration. As shown in Figure 5, compared to the control, 5-HT content in the proximal colon, but not the distal colon, were significantly increased, with 32% ($P < 0.01$) and 23% ($P < 0.05$) increased at 4 and 8 wk after TNBS administration, respectively. A

Figure 1 Acute colonic inflammation and damage induced by different doses of trinitrobenzene sulfonic acid at 5 d after trinitrobenzene sulfonic acid administration. Panel A depicts the appearance of colon tissue in saline (A1), high dose trinitrobenzene sulfonic acid (TNBS) (20 mg/rat, A2), medium dose TNBS (10 mg/rat, A3), and low dose TNBS (5 mg/rat, A4)-treated groups; Panel B depicts the representative histological changes of colon tissue in saline (B1), high dose TNBS (B2), median dose TNBS (B3), and low dose TNBS (B4)-treated groups (hematoxylin and eosin staining, 100×); Statistical analysis of myeloperoxidase (MPO) activity is shown in panel (C), and pathological score in panel (D). Data are shown as mean ± SE, *n* = 4 per group. [a]*P* < 0.05, [b]*P* < 0.01 *vs* saline-treated group; [c]*P* < 0.05 *vs* high dose TNBS-treated group (one-way analysis of variance, Student-Newman-Keuls).

similar trend can also be found in the EC cell number; i.e., there were 68% and 60% increases at 4 and 8 wk after TNBS administration, respectively (*P* < 0.01), suggesting this TNBS-induced PI-IBS model also presents EC cell hyperplasia in the colon tissue.

DISCUSSION

TNBS is an agent commonly used in inducing post-inflammatory IBS model currently. Concerning the variations in developing a protocol of the TNBS-induced PI-IBS rat model and the three necessary features of PI-IBS animal model (i.e., initial inflammation/infection, inflammation/infection resolution, and acquired symptoms of IBS), the present study investigated the effects of TNBS dosage and concentration, intracolonic TNBS administration position, and ethanol percentage on the development of the TNBS-induced PI-IBS rat model. Our results showed that: (1) TNBS induced dose- and concentration-dependent acute inflammation and inflammation resolution at the dose range of 5-20 mg/rat; (2) Intracolonic TNBS administration position affected the persistence of later acquired visceral hyperalgesia, but not the initial inflammation severity; (3) Ethanol percentage affected the TNBS-induced inflammation severity and later acquired visceral hyperalgesia, and low ethanol percentage reduced the degree of TNBS-induced acute inflammation and shortened the persistence of acquired visceral hyperalgesia; and (4) The protocol with TNBS at 5 mg/0.8 mL per rat, dissolved in 50% ethanol, intracolonically administered 8 cm from anus may be a proper protocol in which rats can recover completely from the

Figure 2 Effect of trinitrobenzene sulfonic acid dosage on severity of acute inflammation and inflammation resolution. The colon tissue was collected at 4 wk after trinitrobenzene sulfonic acid (TNBS) administration. Panel A depicts the appearance of colon tissue in saline (A1), high dose TNBS (20 mg/rat, A2), medium dose TNBS (10 mg/rat, A3), and low dose TNBS (5 mg/rat, A4)-treated rats; Panel B depicts the representative histological changes of colon tissue in saline (B1), high dose TNBS (B2), medium dose TNBS (B3), and low dose TNBS (B4)-treated rats (Masson trichrome staining, 100×); Statistical analysis of myeloperoxidase (MPO) activity is shown in panel (C), and pathological score in panel (D). Data are shown as mean ± SE, n = 4 per group. [b]$P < 0.01$ *vs* saline-treated group (one-way analysis of variance, Student-Newman-Keuls).

initial inflammation but acquire persistent visceral hyperalgesia and EC cell hyperplasia at 4 wk after TNBS administration.

As shown in our results, in the dose range of 5-20

Figure 3 Effects of trinitrobenzene sulfonic acid administration position and ethanol percentage on severity of acute inflammation and inflammation resolution. Panel A depicts the appearance of colon tissue in rats treated with trinitrobenzene sulfonic acid (TNBS) at 4 cm or 8 cm from anus or in 25% ethanol. The colon tissues were collected at 5 d or 4 wk after TNBS administration, respectively; Statistical analysis of pathological score is shown in panel (B), and myeloperoxidase (MPO) activity in panel (C). Data are shown as mean ± SE, n = 4-5 per group. [a]P < 0.05 *vs* saline-treated rats, [c]P < 0.05 *vs* rats treated with TNBS at 8 cm from the anus in 50% ethanol (one-way analysis of variance, Student-Newman-Keuls).

Figure 4 Effects of low dose trinitrobenzene sulfonic acid administration position and ethanol percentage on later acquired visceral hyperalgesia. Statistical analysis of pain threshold pressure at 4 wk and 8 wk after trinitrobenzene sulfonic acid (TNBS) administration are shown in panel (A) and (B), respectively. Data are shown as mean ± SE, n = 5 per group. [b]P < 0.01 *vs* saline-treated rats (one-way analysis of variance, Student-Newman-Keuls). AWR: Abdominal withdrawal reflex.

mg per rat, the higher the dose of TNBS, the more severe the acute inflammation and mucosa damage. This finding is consistent with previous reports that the severity and duration in TNBS-induced inflammation is dose-dependent[13,14,17]. It is notable that the same dose of TNBS (20 mg/rat) when given to rats in different vehicle volumes induced different degrees of damage; a higher TNBS concentration (50 mg/mL) induced

Figure 5 Persistent increases of enterochromaffin cell number and serotonin content in the colon tissue of trinitrobenzene sulfonic acid-induced post-infectious irritable bowel syndrome rat model. The colon tissue was collected at 4 wk and 8 wk after trinitrobenzene sulfonic acid (TNBS) administration. Panel A depicts the representative enterochromaffin (EC) cell staining in colon mucosa, the samples were from rats treated with saline (A1), or TNBS (A2) at 4 wk post TNBS (Scale bar, 100 μm). Statistical analysis of serotonin content is shown in panel (B), and EC cell number in panel (C). Data are shown as mean ± SE, *n* = 5 per group. [a]*P* < 0.05, [b]*P* < 0.01 *vs* saline-treated rats. 5-HT: 5-hydroxytryptamine.

more severe damage, even causing animal death in the acute phase. Our pathological results also showed that high-concentration TNBS-treated rats presented localized more severe inflammation and deeper ulcerations around the position of TNBS administration, which may be responsible for the occurrence of colitis complications, i.e., intestinal obstruction, intra-abdominal infection, and animal death. Concerning the fact that inflammation severity and duration have close correlation with TNBS dosage, the effects of TNBS dosage on inflammation resolution was assessed at 4 wk after TNBS administration. According to the pathologist's suggestion, fibroplasia evaluation index was added into the pathological evaluation criteria for the first time, so as to exactly evaluate inflammation resolution. Our results showed that the animals receiving low dose TNBS (5 mg/rat) recovered completely at 4 wk after TNBS administration, but those receiving high (20 mg/rat) and medium (10 mg/rat) dose were not recovered. This result seems to somewhat conflict with the finding from a previous study in which the rats receiving TNBS (5-20 mg/rat, in 25% ethanol) all recovered after 4 wk[14]. We speculate that this discrepancy may come from the protocol differences between these two studies, such as ethanol percentage, animal strain, or evaluation criteria. Consistent with our findings, the previous study showed that colonic inflammation induced by TNBS (30 mg/rat, in 50% ethanol) lasted for at least 8 wk[17], suggesting the

inflammation resolution is also dose-dependent. Based on previous findings and our results, TNBS dosage plays a critical role in TNBS-induced inflammation severity and resolution, and low dose TNBS (5 mg/rat)-induced inflammation can recover completely at 4 wk after TNBS administration.

Currently, 25% and 50% ethanol are both used as the carrier of TNBS in establishing TNBS-induced PI-IBS model and TNBS is commonly given to rats at the depth of either 4 cm or 8 cm from the anus. Based on low dose TNBS (5 mg/0.8 mL per rat) selection, we further investigated the effects of ethanol percentage and intracolonic administration position on the severity and resolution of TNBS-induced inflammation. Our results showed that rats treated with low dose TNBS at a depth of 4 cm or 8 cm presented similar inflammation severity and resolution, while the rats treated with TNBS in 25% ethanol developed milder non-ulcer inflammation compared to those treated with TNBS in 50% ethanol. As the carrier of TNBS, ethanol is known as a breaker to the mucosal barrier, and 30%-50% ethanol alone has been proved to induce acute inflammation and hyperemia[18,28]. Moreover, previous study have shown that 30% ethanol alone induced small areas of hyperemia, while TNBS combined with 30% ethanol induced larger area of necrosis and hyperemia in the colon[18]. These findings indicate that ethanol alone can induce concentration-dependent inflammation, and can be considered

Table 5 Detailed protocols in developing trinitrobenzene sulfonic acid induced post-inflammatory irritable bowel syndrome model in Sprague-Dawley rats

Procedures	Cautions
1. 7-wk-old male Sprague-Dawley rats, fast 24 h before TNBS administration	
2. Mix 1 volume of 12.5 mg/mL TNBS-saline solution with 1 volume of absolute ethanol	Fresh preparation
3. Weight and anesthetized rats with chloral hydrate (350 mg/kg, i.p.)	Anesthetization may last for 3-4 h
4. Insert a catheter into the colon at 8 cm from anus	Proceed carefully to avoid damage the colon wall
5. Keep the rat with head-down vertical position, instill 0.8 mL TNBS solution slowly into the colon lumen within 1 min	Handle slowly to avoid TNBS leakage
6. Keep the rat in head-down vertical position for 1 min before gently removing the catheter	
7. Put the rats in a mound of bedding in head-down position until consciousness recovery	Ensure TNBS solution remains completely in the colon; Keep the rats warm
8. On day 3, 7, 14, 21 and 28, observe and weight the rats	On day 3, body weight decreased by 10%-20% with unformed bloody stool; On day 7, body weight regained and reached the original level, the stool become formed but still soft; From day 7 to day 28, body weight increased smoothly, and finally reached the control level

TNBS: Trinitrobenzene sulfonic acid.

as an invasive helper in enhancing the effects of TNBS. Therefore, it seems reasonable that milder inflammation was induced in rats treated with equal dose of TNBS (5 mg/rat) but in lower ethanol percentage (25%). These results can also explain why high dose or high concentration TNBS have also been used by some researchers in establishing a PI-IBS model: low concentration ethanol may ameliorate the effects of TNBS[13,14].

Visceral hyperalgesia, the major feature of IBS, was further tested in low dose TNBS (5 mg/0.8 mL per rat) treated rats. From 4 wk to 8 wk post TNBS intracolonic administration at a depth of 8 cm, significant and persistent decrease in visceral pain threshold pressure was found, while the intracolonic TNBS administration at a depth of 4 cm or in 25% ethanol presented short-term acquired visceral hyperalgesia. Nowadays, the duration and severity of inflammation are considered as important risk factors in the development of PI-IBS[29], and severe inflammation which results in deep impairment of the underlining nerve fibers has been proposed to play an important role in the pathogenesis of IBS[30]. Based on the above evidence, we believe the unstable visceral hyperalgesia (i.e., lasting for less than 4 wk) in rats treated with TNBS in 25% ethanol may have close correlation with the mild non-ulcer inflammation observed at the acute phase of inflammation. These results also provide us with the information that mild inflammation, even without ulceration, can induce later short-term visceral hyperalgesia. However, it seems interesting that the same dose of TNBS when given at the different positions of colon (transverse *vs* descending) induced different features of acquired visceral hyperalgesia (persistent *vs* short-term), even though the TNBS-induced acute inflammation and damage was similar at first. Considering the unclear and complex pathogenesis of visceral hypersensitivity in PI-IBS, it is not easy to provide a clear explanation for the current findings. However,

during the acute phase of TNBS-induced inflammation, obvious colon dilation and a large amount of retained feces were found in the inflamed transverse colon, but not in the inflamed distal colon. We speculate that the persistent colonic inflammation and dilation may play important roles in enteric nerve system plasticity, and thus influence the feature of acquired visceral hypersensitivity. Currently, peripheral and central neuroplastic changes are considered to be associated with post-inflammatory persistent visceral hypersensitivity[1,31]. It is also well known that transient colorectal distension in the neonatal period can result in chronic visceral hypersensitivity and motility dysfunction in adulthood[25,32], and the underlying mechanism is thought to be associated with alterations in peripheral and central nerve systems[33]. Moreover, considering the different structure and functions of the transverse and descending colons, the regional differences in gut flora[34], mast cells[35] and EC cells[36] may also contribute to the different feature of acquired visceral hypersensitivity observed in our study. Further studies are needed to clarify these issues.

EC cell hyperplasia has been found in the colonic specimens of IBS and PI-IBS patients[37-39], and EC cell hyperplasia has been reported to play important role in the development of visceral hypersensitivity in IBS patients[40,41]. To identify whether visceral hyperalgesia is also accompanied with EC cell hyperplasia in the TNBS-induced PI-IBS rat model, EC cell number and 5-HT content were further investigated in the proximal and distal colon of PI-IBS rats. As shown in our study, the EC cell number and 5-HT content in the proximal colon, but not the distal colon, were significantly and persistently increased in this TNBS model, suggesting the occurrence of EC cell hyperplasia. However, this result differed from the finding from IBS patients, as EC cell hyperplasia in rectal mucosa was commonly reported[3]. This may be explained by the different distribution of

EC cells in the gut mucosa between human beings and rats: the vast majority of EC cells in humans mainly resides in the small intestine and rectum[42] while the major source of EC cells in rats largely locates in the cecum with a decline trend from the proximal to the distal colon[36]. Consistent with our findings, previous studies also showed that 5-HT content in the rat proximal colon tissue was significantly higher (about 5 fold) than that in the distal colon[43,44]. Therefore, it seems that EC cell hyperplasia in the proximal colon can be regarded as one of the features of the TNBS-induced PI-IBS rat model.

This present study, for the first time, showed that TNBS dosage and concentration, position of intracolonic TNBS administration, and ethanol percentage play important roles in developing the TNBS-induced PI-IBS model in Sprague-Dawley rats, suggesting more attention should be paid to these factors when developing a PI-IBS model with TNBS. Concerning the gene differences among animal strains, the protocol set up here may need some modulation when other rat strains except Sprague-Dawley rats, are used. Though this TNBS model presented persistent visceral hyperalgesia and EC cell hyperplasia for as long as 4 wk, more studies are still needed to observe the long-lasting visceral hyperalgesia and identify other features of PI-IBS in this model.

In the present study, we found out that the protocol with TNBS at 5 mg/0.8 mL per rat, dissolved in 50% ethanol, intracolonically administered at 8 cm from the anus resulted in persistent visceral hyperalgesia and colonic EC cell hyperplasia after complete recovery from the initial inflammation in rats. With this protocol, low-dose TNBS-induced mild mucosa damage and colonic inflammation is reproducible without any animal loss; visceral hyperalgesia and EC cell hyperplasia which last for at least 4 wk occur as early as 4 wk after TNBS administration. The details of this protocol are presented in Table 5. Ensuring the results from different laboratories would be comparable, we believe that widespread adoption of a recommended protocol will save a great quantity of time and resources.

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COMMENTS

Background
Post-infectious irritable bowel syndrome (PI-IBS) is a subgroup of IBS in which the patients developed their symptoms after recovery from an acute gastrointestinal infection. Previous systematic review showed that the trinitrobenzene sulfonic acid (TNBS)-induced model is the most commonly used PI-IBS model, but there are large variations in the protocol of model development. These variations make comparison between studies to be very difficult, if not impossible.

Research frontiers
This study aimed to investigate the effects of TNBS dosage, concentration, intracolonic administration position, and ethanol percentage on TNBS-induced acute inflammation, inflammation resolution, and later acquired features of IBS

(i.e., visceral hyperalgesia and enterochromaffin cell hyperplasia).

Innovations and breakthroughs
Recent studies have highlighted the important status of PI-IBS in functional diseases, as the clear onset and well defined pathophysiological changes of PI-IBS will help people understand not only the PI-IBS but also other subtypes of IBS. Moreover, the validated animal models, which aim to mimic one or more features of human disease, play important roles in the studies of mechanisms and potential therapeutic agents for diseases. This is the first study aimed to investigate the effects of key factors on developing TNBS-induced PI-IBS model in rats. The results and recommended protocol presented in this study will save a great deal of time and resources and ensure the results from different laboratories be comparable.

Applications
This study provides direct information about the effects of key factors on TNBS-induced acute inflammation, inflammation resolution, and later acquired features of IBS. More attention should be paid to these key factors when developing PI-IBS rat model with TNBS.

Peer review
This is a good descriptive study in which authors investigate the effects of TNBS dosage and concentration, intracolonic administration position, and ethanol percentage on the developing TNBS-induced PI-IBS rat model. The topic is interesting and useful for the study of pathophysiology of PI-IBS and the development of therapeutic agents.

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ORIGINAL ARTICLE

Cost-benefit analysis of esophageal cancer endoscopic screening in high-risk areas of China

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Abstract

AIM: To estimate the cost-benefit of endoscopic screening strategies of esophageal cancer (EC) in high-risk areas of China.

METHODS: Markov model-based analyses were conducted to compare the net present values (NPVs) and the benefit-cost ratios (BCRs) of 12 EC endoscopic screening strategies. Strategies varied according to the targeted screening age, screening frequencies, and follow-up intervals. Model parameters were collected from population-based studies in China, published literatures, and surveillance data.

RESULTS: Compared with non-screening outcomes, all strategies with hypothetical 100 000 subjects saved life years. Among five dominant strategies determined by the incremental cost-effectiveness analysis, screening once at age 50 years incurred the lowest NPV (international dollar-I$55 million) and BCR (2.52). Screening six times between 40-70 years at a 5-year interval [i.e., six times(40)f-strategy] yielded the highest NPV (I$99 million) and BCR (3.06). Compared with six times(40)f-strategy, screening thrice between 40-70 years at a 10-year interval resulted in relatively lower NPV, but the same BCR.

CONCLUSION: EC endoscopic screening is cost-beneficial in high-risk areas of China. Policy-makers should consider the cost-benefit, population acceptance, and local economic status when choosing suitable screening strategies.

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INTRODUCTION

Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer death worldwide[1]. Although the mortality of EC has

sharply reduced over the last three decades, EC remains the fourth leading cause of cancer death in China with a mortality of 15.21/100 000[2]. According to the "Third National Retrospective Sampling Survey of Death Causes Report in 2004-2005 of China", EC continues to be the major public health burden in some high-risk areas, where the mortality of EC was three times higher than the average of the country. EC is a fatal disease, with a 5-year survival rate of less than 20% even in developed countries[3,4].

To explore suitable control measures in high-risk areas of China, a great number of EC screening studies using endoscopic examinations (i.e., endoscopy with mucosal iodine staining and index biopsy as a screening technology, combined with pathological examination for confirming and staging the disease) have been conducted for several decades[5-10]. Through early detection and subsequent treatment, the 5-year survival rate of EC increased to 86%[10]. Furthermore, obvious reductions in incidence and mortality rates of EC were observed under endoscopic screening[11].

A national screening program for EC in high-risk areas has become available in 73 sites of 27 provinces of China based on evidence from previous studies. Nevertheless, due to lacking comprehensive health economic evaluations on such programs, two key public health questions remain to be answered: is the endoscopic screening cost-beneficial in the long run? Should we use the same screening strategy in both developed and developing high-risk areas of China?

The objective of this paper is to explore appropriate screening strategies for EC in high-risk areas of China from the health economic perspective by comparing the long-term cost-benefits of 12 endoscopic screening alternatives. It will provide valuable data for policy makers to make decisions on the current screening program.

MATERIALS AND METHODS

Decision analysis model

A Markov model was constructed to evaluate the cost-benefit of different screening strategies for EC. In each strategy simulation, a hypothetical cohort with 100 000 participants entered the model at age 40 years and were followed up until the age of 70 years. Costs and benefits were all discounted at an annual rate of 3%[12]. TreeAge Pro 2009 Suite by TreeAge Software Inc. was used for all analysis.

The natural history of EC was categorized as the following health status: normal, mild dysplasia (mD), moderate dysplasia (MD), severe dysplasia/carcinoma *in situ* (SD/CIS), intramucosal carcinoma (IC), submucosal carcinoma ($T_1N_0M_0$) (SC), invasive carcinoma (INC), and death. Figure 1 depicts the detailed transition processes of EC in the Markov model. Each rectangle represents a health state. During a Markov cycle (1 year), one could transit from his/her current health state to another (indicated by arrows between different states) or remain in the same state (indicated by half-circle arrows on the rectangles). Prior to the development of IC, the condi-

Figure 1 Bubble diagram representing the health states of esophageal cancer and transitions in natural-history Markov model.

tion could spontaneously regress. Once IC developed, no regression could occur. We used the model to evaluate all screening alternatives and non-screening outcomes.

Screening strategies

In the context of lacking guidelines for EC screening worldwide, we explored 12 screening strategies using endoscopic examinations. These strategies were performed at varying starting age for screening (40, 45 or 50 years), screening frequency (once, twice, thrice, or six times in the lifetime), and intervals of follow-up for mD and MD cases (e.g., 5 or 3 years). The strategies were listed as "t(y)nf/t(y)f", where t denotes the screening frequency, y represents the starting age of screening, nf means we do not follow up the mD and MD cases diagnosed by screening, and f means the mD and MD cases are followed up every 5 years and 3 years, respectively. For twice and thrice screening strategies in the lifetime, the screening intervals were 10 years; for six times screening strategy, the screening intervals were 5 years.

Screening, diagnosis, and treatment procedures for the strategies were all based on the current practice manuals. The participants were screened using endoscopy with iodine staining. If endoscopy revealed a suspected lesion (mD or worse), index biopsy combined with pathological examination were performed consecutively. The detailed procedures of endoscopy were the same with those in the literature[13]. For SD/CIS and IC cases detected by screening, Endoscopic Mucosal Resection and/or Argon Plasma Coagulation served as the standard treatment. For detected patients with SC or worse, therapies included esophagectomy, radiotherapy, and other routine treatments. Subjects who were not screened would be diagnosed and treated if they presented with symptomatic EC. All patients were followed up once by endoscopy in the first year after treatment.

Model parameters

The data used in the model were compiled from a variety of sources: (1) the results of our prospective cohort study based on the EC chemoprevention trial of selenomethionine and celecoxib in "early detection of EC" (EDEC) program; (2) the results of our population-based

Table 1 Non-age-specific parameters

Parameters	Value	Parameters	Value	Parameters	Value[1]
Initial probability[2]		Transition probability-continued		Compliance of screening[2]	67% (30%-100%)
Normal	0.8895	SD/CIS	see Table 2	Sensitivity of endoscopy[6]	96% (92%-99%)
mD	0.0820	IC	see Table 2	Specificity of endoscopy[6]	90% (59%-100%)
MD	0.0180	INC		Screening cost (I$ per capita)[2]	
SD/CIS	0.0090	Recovering to post-INC	0.7696	Direct cost	61.50 (37.00-119.00)
IC	0.0008	Relapsing to INC after treatment	0.2304	Indirect cost	8.31 (8.09-8.53)
SC	0.0005	After treatment[4]		Treatment cost (I$ per capita)[24][2]	
INC	0.0002	Post-SD/CIS		Direct cost	
Transition probability[9,16-21][2,3]		Recovering to normal	0.9950	SD/CIS	1292 (1114-1565)
Normal		Relapsing to SD/CIS	0.0050	IC	1292 (1114-1565)
Remaining normal	0.9760	Post-IC		SC	1818 (1519-2799)
Progression to mD	0.0240	Remaining post-IC	0.9450	INC-screening group	2767 (2332-4031)
mD		Relapsing to IC	0.0500	INC-control group	4888 (4333-6396)
Regression to normal	0.0500	Relapsing and progression to SC	0.0050	Indirect cost	
Remaining mD	0.9000	Post-SC		SD/CIS	1654 (1341-1968)
Progression to MD	0.0500	Remaining post-SC	0.8500	IC	1654 (1341-1968)
MD		Relapsing to SC	0.0500	SC	3369 (2872-3866)
Regression to mD	0.0800	Relapsing and progression to INC	0.1000	INC-screening group	3369 (2872-3866)
Remaining MD	0.8000	Post-INC (Same with "INC")		INC-control group	5526 (4584-6466)
Progression to SD/CIS	0.1200	Death probability	see Table 2	Discount rate[12][5]	3% (0%-6%)

mD: Mild dysplasia; MD: Moderate dysplasia; SD/CIS: Severe dysplasia/carcinoma *in situ*; IC: Intramucosal carcinoma; SC: Submucosal carcinoma (T₁N₀M₀); INC: Invasive carcinoma; I$: International dollar. [1]For compliance, sensitivity and specificity of endoscopy, screening and treatment costs, and discount rate, the numerals before and in the parenthesis denote the base case and ranges used in sensitivity analysis; [2]Data were calculated in terms of the project called "Early Detection and Early Treatment of Esophageal Cancer in Demonstration Centers in China"; [3]Data were collected from the death registry report of Linzhou County during 2004-2006; [4]Post-SD/CIS, post-IC, post-SC and post-INC represent the "health" condition after treatment of SD/CIS, IC, SC and INC; [5]Costs and life years were discounted during the Markov cycles.

screening project "Early Detection and Early Treatment of EC in Demonstration Centers in China" (EDETEC); (3) surveillance data; (4) published literatures; and (5) unpublished data.

In the chemoprevention trial of EDEC program, 2213 asymptomatic adults from Linzhou County, Henan Province of China, underwent an endoscopic screening in 1999[14]. Among them, 2189 participants who had histological diagnoses at the baseline evaluations were surveilled until 2007. The primary end-point was the occurrence of EC, confirmed by village doctors through checking the histological diagnoses in medical records. The project EDETEC covering 11 high-risk areas of EC was launched by the Chinese central government in 2005. The purpose was to increase the early detection and treatment rate as well as the 5-year survival rate of EC, and to improve the screening, early detection and treatment program and so forth[15].

Probabilities

At the initial model cycle, a hypothetical cohort of 100 000 participants was distributed among various health states based on the proportion of each pathologic stage of EC in the 40-44 years age group. The proportions were calculated in terms of the screening results of Linzhou County in the project EDETEC between 2005 and 2008. Among the 8267 asymptomatic participants aged 40-69 years, 8.2% cases were identified as mD in the 40-44 years age group. Full details are presented in Tables 1 and 2.

In each cycle of a Markov process, the transitions among health states occurred with annual transition prob-

abilities. They were estimated in terms of: (1) published literatures[9,16-21]; and (2) the results of both EDEC and EDETEC projects in Linzhou County (Tables 1 and 2).

It is believed that persons with SD/CIS or lesser abnormality may not die from EC and that patients with IC or SC may die from all causes including EC. In patients with INC, we assumed that they may mainly die from EC. Therefore, in our model, the corresponding death probabilities for three different populations above were converted from non-esophageal-cancer mortality, all-cause mortality, and case fatality rate of EC, respectively. All age-specific mortality rates were obtained from the death registry reports of Linzhou County between 2004 and 2006. And they were converted to probabilities by the formula: Probability = 1 - Exp (-rt), where "r" represents the rate and 't' denotes the time (Tables 1 and 2).

Screening compliance

The compliance of EC screening in different settings varied from 33.4% to 77.1%[22]. In the EDETEC program, the screening compliance of EC in Linzhou County during 2005-2008 was 67% (8267/12 294), which was used as a baseline in this analysis (Table 1).

Screening and treatment cost

In our model, both screening and treatment costs included direct and indirect costs, which were calculated from a societal perspective. Direct costs referred to those associated with drugs, disposable supplies, equipment and facilities, staff, *etc*. In this study, we used costs rather than charges. And they were collected using Micro-costing methods in

Table 2 Age-specific parameters

Parameters	Value					
	40-yr	45-yr	50-yr	55-yr	60-yr	65-69-yr
Transition probability						
SD/CIS						
Regression to MD	0.17	0.15	0.14	0.12	0.11	0.09
Remaining SD/CIS	0.75	0.75	0.74	0.74	0.73	0.73
Progression to IC	0.08	0.10	0.12	0.14	0.16	0.18
IC						
Remaining IC	0.60	0.50	0.40	0.20	0.15	0.13
Progression to SC	0.40	0.50	0.60	0.80	0.85	0.87
SC						
Remaining SC	0.80	0.70	0.55	0.20	0.17	0.15
Progression to INC	0.20	0.30	0.45	0.80	0.83	0.85
Death probability						
Non-esophageal-cancer mortality	0.002270	0.003073	0.007054	0.017061	0.019744	0.024105
All-cause mortality	0.002438	0.003383	0.007967	0.019559	0.021985	0.027370
Case fatality rate of esophageal cancer	0.581700	0.581700	0.581700	0.581700	0.581700	0.581700

MD: Moderate dysplasia; SD/CIS: Severe dysplasia/carcinoma *in situ*; IC: Intramucosal carcinoma; SC: Submucosal carcinoma (T₁N₀M₀); INC: Invasive carcinoma.

the EDETEC program[23]. Indirect cost was also estimated from our EDETEC program, including those related to transportation, accommodation, and the productivity losses of both patients and their caregivers[24,25]. Considering differences in purchasing power, costs were presented in 2008 international dollars (I$).

Screening cost per capita using endoscopic examination was I$69.81. In screening group, the treatment costs for patients with SD/CIS or worse ranged from I$2964 to I$6136. In control group, the treatment cost for INC cases was I$10 414, much higher than that in screening group (Table 1).

Other variables and assumptions
According to a previous study in Linzhou County, the sensitivity and specificity of endoscopic examination were 96% and 90%, respectively[6] (Table 1). For individuals diagnosed as having precancerous lesions or EC (i.e., SD/CIS or worse), we assumed that they would complete the entire treatment procedures.

Health economic evaluation
The basic outcomes of the model were total costs (including screening costs and treatment costs) and expected life years. Then the net present values (NPVs) and the benefit-cost ratios (BCRs) were calculated under each of the strategies (for a hypothetical cohort of 100 000 subjects followed up from 40 years to 70 years of age).

For each screening cohort, the benefit consisted of the treatment cost averted and productivity gains from screening programs[25], and counted by the formula: benefit = GDP per capita of Linzhou in 2008 (I$6542) × (life years of screening cohort - life years of "non-screening" group) + treatment cost of "non-screening" group. The NPV was the benefit minus the total cost of the screening group; the BCR equaled to the benefit divided by the

total cost. The strategies with a NPV > 0 and a BCR > 1 were considered cost-beneficial.

In addition, the screening alternatives were compared using an incremental cost-effectiveness analysis. The strategies that were more expensive and gained fewer life years (dominated), or less costly and less cost-effective (extended dominated) than an alternative were excluded.

Sensitivity analysis
Given the uncertainty about some parameters, univariate sensitivity analyses were used to assess the robustness of the model results by varying the values of screening compliance, discount rate, screening cost, treatment cost, sensitivity and specificity of endoscopy within reasonable ranges (Table 1).

Model validation
Based on the established natural-history model, the validity of the Markov model was assessed by comparing the model-predicted age-specific incidence and the age-specific proportion of each stage of EC with the observed data in real-world conditions.

RESULTS
Baseline results
Compared with non-screening outcomes, the screening strategies could save life years of 2539-15 384 for a hypothetical population of 100 000, with NPVs of I$24 million-I$99 million and BCRs of 1.61-3.06. Strategies with higher screening frequencies were more cost-beneficial than those with lower screening frequencies (Table 3).

When compared with each other, it indicated that the once(50)f-, twice(40)f-, twice(45)f-, thrice(40)f-, and six times(40)f-strategies were cost-effective, dominating or extended dominating others. In other words, other

Table 3 Estimated epidemiological and economic effects for each strategy with 100 000 people under baseline assumptions

Screening strategies t(y)nf/ t(y)f[1]	Life years	Life years saved (LYS)	Costs (I$)	ICER (I$/LYS)	Benefit (I$)	NPV (I$)	BCR
Non-screening	1 811 125	-	46 354 958	D	-	-	-
Once(40)nf	1 813 664	2539	39 133 890	D	62 964 854	23 830 964	1.61
Once(45)nf	1 814 180	3055	38 213 022	D	66 340 477	28 127 455	1.74
Once(50)nf	1 814 634	3509	36 989 316	D	69 310 502	32 321 186	1.87
Once(40)f	1 817 922	6797	38 007 700	D	90 820 285	52 812 585	2.39
Once(45)f	1 818 783	7658	36 792 906	ED	96 452 865	59 659 959	2.62
Once(50)f	1 817 966	6841	36 117 125	/	91 108 128	54 991 003	2.52
Twice(40)f	1 822 516	11 391	39 532 080	940	120 873 795	81 341 715	3.06
Twice(45)f	1 821 595	10 470	38 665 956	702	114 848 701	76 182 745	2.97
Twice(50)f	1 819 124	7999	38 261 433	ED	98 683 654	60 422 221	2.58
Thrice(40)f	1 823 528	12 403	41 665 346	2108	127 494 203	85 828 857	3.06
Thrice(45)f	1 821 827	10 702	40 775 616	D	116 366 423	75 590 807	2.85
Six times(40)f	1 826 509	15 384	48 042 566	2139	146 995 621	98 953 055	3.06

I$: International dollar. ICER means incremental cost-effectiveness ratio, which is defined as the additional cost of a specific strategy divided by its additional life years, as compared with the next-less-expensive strategy. D means dominated, e.g., the screening strategy is more expensive and less effective than another strategy. ED means extended dominated, e.g., if a screening strategy has a higher ICER than the next more costly, more effective strategy, it is extendedly dominated by that more cost-effective strategy. NPV: Net present value; BCR: Benefit-cost ratio. [1]t(y)nf/t(y)f: t denotes the frequencies of screening, y represents the starting age of screening, nf means we do not follow up the mild dysplasia and moderate dysplasia cases diagnosed by screening, and f means the mild dysplasia and moderate dysplasia cases diagnosed by screening are followed up every five and three years, respectively. For twice and thrice screening strategies in the lifetime, the screening intervals were 10 years; for the six times screening strategy in the lifetime, the screening intervals were five years. "-": Life years saved, benefits, NPVs and BCRs of screening strategies were all calculated by comparing with non-screening group, accordingly, those are null for non-screening group; "/": As the cheapest strategy, the ICER is null for the once(50)f-strategy.

strategies cost more and saved fewer lives, and were excluded. Among the cost-effective screening alternatives, the once(50)f-strategy saved the lowest life years of 6841, and resulted in the fewest NPV of I$55 million and BCR of 2.52. The highest life years saved were observed in the six times(40)f-strategy, with the maximum NPV of I$99 million and BCR of 3.06. Compared with six times(40)f-strategy, the thrice(40)f-strategy saved fewer life years and yielded lower NPV, but had the same BCR.

Sensitivity analysis

When the sensitivity and specificity of endoscopy, screening and treatment costs, discount rate, and screening compliance were changed once at a time (Table 1), once(50)f-, twice(40)f-, twice(45)f-, thrice(40)f-, and six times(40)f-strategies kept dominant. Uncertainty in those parameters had little effect on the choice of cost-effective strategies.

NPVs and BCRs changed obviously with screening cost, compliance, and discount rate under all cost-effective strategies. Both NPVs and BCRs were relatively less affected by the treatment cost, sensitivity and specificity of endoscopic examination. No matter how these parameters varied within the ranges, the results showed that screening was cost-beneficial with positive NPVs and BCRs > 1. In general, our results were robust (Figure 2).

Model validation

Comparison of incidence: The cancer registry report in Linzhou County during 2004-2006 showed that the age-specific incidence rates of EC were 47.44 per 100 000, 247.77 per 100 000, and 398.00 per 100 000 for the age groups of 40-49, 50-59, and 60-69 years, respectively. The corresponding model-predicted rates were

46.19/100 000, 248.14/100 000, and 424.78/100 000, respectively. The modeled estimates were about 94%-103% of the observed rates.

Comparison of proportions: First and most important, in any age group, the proportion of each histological grade of EC predicted by model was quite close to the screening results of the EDETEC program in Linzhou County during 2005-2008. And the estimated proportions were within the 99% confidence intervals of the observed data. Secondly, in each age group, the proportions decreased with the severity of the disease. And the proportions of mD ranked first. Last but not the least, for each pathologic grade of EC, the proportions increased with age, and reached the top in the 65-69 year-old group. Such tendency fit the characteristics of natural history of EC, and was also in agreement with the previous reports in other high-risk areas of China[26,27]. In summary, the validity of the model was satisfactory (Figure 3).

DISCUSSION

It was the first comprehensive cost-benefit assessment for the EC screening using endoscopic examination in China. Compared with no screening, all 12 screening strategies covering a hypothetical population of 100 000 resulted in substantial NPVs and high BCRs. However, when compared with each other, only five strategies were cost-effective based on the incremental cost-effectiveness analysis. Among all cost-effective strategies, screening once at age 50 yielded the lowest NPV (I$55 million) and BCR (2.52). Screening six times for those between 40-70 years of age at a 5-year interval yielded the highest NPV (I$99 million) and BCR (3.06). Compared with the six times(40)f-

Figure 2 One-way sensitivity analyses for each cost-effective screening strategy. Strategies are expressed as t(y)f: t denotes the frequencies of screening; y denotes the starting age of screening; f means the mild dysplasia and moderate dysplasia diagnosed by screening were followed up every five and three years. For twice and thrice screening strategies in the lifetime, the screening intervals were 10 years; for the six times screening strategy in the lifetime, the screening intervals were 5 years. A: Treatment costs for invasive carcinoma of "non-screening" group; B: Treatment costs for invasive carcinoma of screening group; C: Treatment costs for submucosal carcinoma ($T_1N_0M_0$); D: Treatment costs for severe dysplasia/carcinoma *in situ*/intramucosal carcinoma; E: Screening costs; F: Specificity; G: Sensitivity; H: Screening compliance; I: Discount rate. Solid vertical lines represent the base cases of net present value (NPV)s/benefit-cost ratio (BCR)s. For B, C, D, E and I, the left of each bar, the lowest bound of NPVs/BCRs range, was counted on the basis of the maximum values of related parameters; and the right of each bar, the highest bound of NPVs/BCRs range, was counted according to the minimum values of related parameters. For other parameters, the left/right of each bar was calculated based on their minimum/maximum values.

Figure 3 Comparison of modeled age-specific proportion of each pathologic stage of esophageal cancer with screening results. EC: Esophageal cancer; mD: Mild dysplasia; MD: Moderate dysplasia; SD/CIS: Severe dysplasia/carcinoma *in situ*; IC: Intramucosal carcinoma; SC: Submucosal carcinoma ($T_1N_0M_0$); INC: Invasive carcinoma.

strategy, screening thrice between 40-70 years of age at a 10-year interval saved fewer life years and produced lower NPV, but had the same BCR. Under these strategies, the mD and MD cases diagnosed by screening were followed up every 5 years and 3 years, respectively; all patients with SD/CIS or worse found by screening were treated, and followed up once by endoscopy in the first year after treatment. The validation assessment and the sensitivity analysis showed that our results were reliable.

Previously, two similar investigations presented BCRs of 4 and 4-12 for EC screening in China, which were higher than those in our analysis[25,28]. Explanations of the discrepancy from our estimates were: (1) Liu *et al*[28] and Wei *et al*[25] investigated 40-69 year old asymptomatic persons using cross sectional analyses, while we conducted a hypothetic birth cohort analysis, and followed up the cohort from 40 years to 70 years of age. Previous studies did not consider that some "normal/mild/moderate" cases defined by screening would progress and suffer from EC in the following life years[9,19-20,29]. A prospective study found that 23.7% mD and 50% MD cases developed EC during the 13-year follow-up[19]. The treatment costs may be very high for these EC patients. Neglecting them would overestimate BCRs; (2) Compared with non-screening, most of the EC patients in the screening group were diagnosed at earlier stages (87% *vs* 8%)[24]. As a result, the treatment cost per capita for EC patients in the screening group was lower than that in "non-screening" group. According to the formula of BCR, we found that BCR was positively associated with the difference of treatment cost per person between screening and control groups. Unlike Wei *et al*[25], we estimated the costs from the perspective of resource expenditure other than hospital charges. The difference of treatment costs between the groups in our study was much smaller than that in prior studies. That could account for the difference of BCRs to some extent;

and (3) The costs and benefits were not discounted in previous studies[25,28]. Our sensitivity analysis showed that the discount rates were inversely associated with BCRs. And the BCRs of almost all strategies increased to nearly 4 when the discount rate declined to zero.

As the most widely used summary measures in health economic evaluations, the NPV and BCR are used to determine the return on any investment. Our study demonstrated that an investment of I$ 36 117 125 would result in a return of I$ 54 991 003 under once(50)f-strategy. These economic benefits resulted from a reduction in the incidence and mortality of EC, and the productivity gains of the prolonged life years through early detection and subsequent treatment. Our results revealed that the return increased with the screening frequency, and the six times 40)f-strategy resulted in the highest NPV. Although thrice(40)f-strategy yielded lower benefits, it was much less costly than the six times(40)f-strategy. It means that the thrice(40)f-strategy was a suitable alternative for the six times(40)f-strategy if there was an emphasis on capital constraints.

In addition to cost-benefit outcomes, some other factors should be considered when choosing reasonable screening strategy in different settings. First of all, endoscopy is an invasive examination. Concerns related to the high frequency of screening (e.g., six times in the lifetime) can lead to the great deduction of the compliance if it is not appropriately addressed, especially in the areas with low compliance at the time of initial screening, such as some villages of Ci County (33.7%)[22]. Moreover, the total costs of the screening strategy, life years saved, local economic level, and health resource status should also be weighed and balanced by policy makers. In summary, we recommended that once(50)f-strategy which was the cheapest would be suitable in underdeveloped settings with inadequate health resources, and that thrice(40)f-

strategy which could save more life years would be preferable in developed settings with adequate health resources.

One issue needed to be emphasized in our analysis was that most data used in our model were calculated from specific epidemiological data of Linzhou, the highest incidence area of EC worldwide. A great number of endoscopic screenings in this area have been performed since the 1980s, and systematic cancer incidence and death registration have been established. Therefore, the related data from Linzhou County were available and reliable. Our sensitivity analyses displayed that variation in some important parameters within wide ranges did not have a significant effect on our results. This further confirmed that our evaluation results mainly based on data from Linzhou were objective and applicable for other similar high-risk areas in China.

It is known that the cost-benefit of screening for EC (or any other cancer) is highly dependent on the incidence (and subsequent mortality) of that particular cancer. Based on our model prediction and area-specific incidence of EC in Cancer Registry Annual Report of China in 2004, we preliminarily and roughly estimated the cost-benefit of screening program in moderate-(around the national average level of EC incidence, 15.22/100 000), and low-risk areas (less than half of the national average level for EC incidence, 7.61/100 000). In moderate-risk areas, the BCRs ranged from 1.09-1.59, and screening once at age 50 incurred the highest BCR. In low-risk areas, only the strategy of screening once at age 50 remained cost-beneficial (with a highest BCR of 1.09). The results revealed that in moderate- or low-risk areas, screening program was not so cost-beneficial as that in high-risk areas. The screening once at age 50 was relatively preferable. Therefore, our results should be prudential to be used in moderate- or low-risk areas. However, more researches are needed in the future.

Our analysis had several limitations. First, the screening and treatment costs did not include program costs, which might account for a large part of the total costs[12]. The underestimation of costs may result in overrating the benefits of screening strategies, whereas the one-way sensitivity analysis of costs found that even when the costs were increased by over 20%, the screening was still considered as cost-beneficial. Second, in this study, the transition probabilities of all health states should change with age. However, those of normal, mD and MD states were fixed due to the unavailability of the data, which could affect the models' results to some extent. Hence, further studies on the natural history of EC appear warranted. Finally, although we performed one-way sensitivity analyses to evaluate the impact of each uncertainty on the results, we could not quantify the total impact of combinations of the parameter values. We did not conduct a multivariate probabilistic sensitivity analysis, since data on the probability distributions of variables were unavailable. This may more or less influence the outcomes of the sensitivity analysis.

In conclusion, EC endoscopic screening is cost-beneficial in high-risk areas of China. The strategy with once screening at age 50 years in the lifetime is the cheapest but saves fewer life years. If decision makers wish to save more life years and get more benefit, the strategy of thrice screening from 40 years of age at an interval of 10 years would be preferable. In different high-risk areas of EC, policy makers should consider the cost-benefit of screening, acceptability in the population, local health resources and economic level when choosing appropriate screening strategies.

ACKNOWLEDGMENTS

COMMENTS

Background

Esophageal cancer (EC) remains the fourth-leading cause of cancer death in China, and continues to be the major public health burden in some high-risk areas. Previous studies found that EC screening program using endoscopic examination (*i.e.*, endoscopy with mucosal iodine staining and index biopsy as a screening technology, combined with pathological examination for confirming and staging the disease) could increase the 5-year survival rate, decrease the incidence and mortality of EC. A national screening program for EC in high-risk areas has become available in 73 sites of 27 provinces of China. Nevertheless, the health economic effects in the long run on such programs remain unknown. And whether screening strategy is suitable in regions with different health resources and economic level is not clear.

Research frontiers

To assess the cost-benefit of screening program in the long run, large-population-based perspective studies are difficult and expensive to conduct, and results would be obtained in decades. Instead, in the area of health economic evaluation for secondary prevention of cancer, the research hotspot is to use Markov model to explore suitable strategies which are cost-effective and cost-beneficial.

Innovations and breakthroughs

Previous researches with regard to cost-benefit analyses of EC screening program in China were cross-sectional studies without follow-up, and only evaluated the health economic effects of one screening strategy which is used currently. The authors conducted a hypothetic birth cohort analysis and followed up the cohort from 40 years to 70 years of age on the basis of Markov model, and compared 12 hypothetic screening strategies (different at starting age of screening, screening intervals, *etc*.) so as to explore preferable screening strategies in different areas.

Applications

The study results suggest that EC endoscopic screening is cost-beneficial in high-risk areas of China. The strategy, screening once at age 50 years in the lifetime, is the cheapest but saves fewer life years. If decision makers wish to save more life years and get more benefit, the strategy, screening thrice from 40 years of age at an interval of 10 years, would be preferable. The results will provide policy makers important information on updating such screening program in high-risk areas.

Terminology

Markov model: Markov model is considered as a powerful tool for simulating the development process of chronic diseases. In Markov models, health states passed through by patients are defined separately; and then through modeling on the basis of a system of transitional probability among states within a cycle (usually 1 year), the development of diseases and the medical resources used in population could be estimated; Cost-benefit analysis: Cost-benefit analysis is a systematic process for calculating and comparing benefits and costs of a project to see whether the benefits outweigh the costs for two purposes: (1) to determine if it is a sound investment; and (2) to see how it compares with alternate projects.

Peer review

The authors present the results of a decision analysis of endoscopic screening for esophageal squamous cell cancer for a high-risk region in China. They conclude that endoscopic screening, compared to no screening, is cost-effective, with several different screening schedules that could be used. Overall, this is a nicely done study and is well-written.

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BRIEF ARTICLE

Cervical inlet patch-optical coherence tomography imaging and clinical significance

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Abstract

AIM: To demonstrate the feasibility of optical coherence tomography (OCT) imaging in differentiating cervical inlet patch (CIP) from normal esophagus, Barrett's esophagus (BE), normal stomach and duodenum.

METHODS: This study was conducted at the Veterans Affairs Boston Healthcare System (VABHS). Patients undergoing standard esophagogastroduodenoscopy at VABHS, including one patient with CIP, one representative patient with BE and three representative normal subjects were included. White light video endoscopy was performed and endoscopic 3D-OCT images were obtained in each patient using a prototype OCT system. The OCT imaging probe passes through the working channel of the endoscope to enable simultaneous video endoscopy and 3D-OCT examination of the human gastrointestinal (GI) tract. Standard hematoxylin and eosin (H and E) histology was performed on biopsy or endoscopic mucosal resection specimens in order to compare and validate the 3D-OCT data.

RESULTS: CIP was observed from a 68-year old male with gastroesophageal reflux disease. The CIP region appeared as a pink circular lesion in the upper esophagus under white light endoscopy. OCT imaging over the CIP region showed columnar epithelium structure, which clearly contrasted the squamous epithelium structure from adjacent normal esophagus. 3D-OCT images obtained from other representative patients demonstrated distinctive patterns of the normal esophagus, BE, normal stomach, and normal duodenum bulb. Microstructures, such as squamous epithelium, lamina propria, muscularis mucosa, muscularis propria, esophageal glands, Barrett's glands, gastric mucosa, gastric glands, and intestinal mucosal villi were clearly observed with OCT and matched with H and E histology. These results demonstrated the feasibility of using OCT to evaluate GI tissue morphology *in situ* and in real-time.

CONCLUSION: We demonstrate *in situ* evaluation of CIP microstructures using 3D-OCT, which may be a useful tool for future diagnosis and follow-up of patients with CIP.

INTRODUCTION

Cervical inlet patch (CIP) is characterized by the presence of heterotopic columnar gastric mucosa in the upper esophagus, most commonly located just below the upper esophageal sphincter (UES). Other sites for heterotopic gastric mucosa have been reported in the duodenum, jejunum, cystic duct, ampulla of vater, gallbladder, rectum and the anus[1-7], but their etiology and pathological significance remain unclear. The incidence of CIP has been reported from as low as 1%, to as much as 10% of endoscopic cases in different adult studies[8,9]. A large autopsy series of 1000 children demonstrated a prevalence of 4.5%[10]. During esophagogastroduodenoscopy (EGD), the region just below the UES is often quickly traversed after overcoming the initial resistance. CIP is usually best seen at the end of an EGD exam while withdrawing back through the esophagus and specifically looking for the condition. One study found almost a 6-8 fold increase in the incidence, from 0.3% to 2.3%, depending upon the endoscopist's awareness of this entity and thoroughness of examination[11]. Although generally asymptomatic, CIP can present with dysphagia[12], stricture[13], ulcers[14], bleeding[15] or fistula[16]. It is unclear whether CIP is congenital or acquired. One postulate is that CIP originates from incomplete embryonic replacement of the stratified epithelium, which normally starts at the 4th month of gestation. The greater incidence of CIP seen in pediatric populations and in the upper esophageal pouch of children with tracheoesophageal fistula supports this hypothesis[10,17,18]. Also, immunohistochemical studies suggest an embryologic origin for CIP on account of differences in endocrine markers such as serotonin, glucagon, pancreatic polypeptide, somatostatin and neurotensin in histologic specimens of CIP and Barrett's esophagus (BE)[19].

A second postulate is that CIP, especially as noted in

adults, is an acquired metaplastic change occurring in the squamous mucosa of the esophagus and is associated with predisposing factors for gastroesophageal reflux disease (GERD), such as sliding hiatal hernia[20]. Its incidence is up to four-fold higher in patients having BE[21] and CIP was found in almost a third of patients having dysplastic BE or adenocarcinoma[22]. Thus, long-standing acid reflux is thought to lead to columnar metaplasia in the upper esophagus, similar to BE. Several reports suggest that CIP may progress to adenocarcinoma[23-26].

In this study, we evaluate whether optical coherence tomography (OCT) can assess epithelial differences in CIP compared to normal esophagus, BE, normal stomach and duodenum. OCT is an emerging medical imaging technology that enables micron-scale, cross-sectional, and three-dimensional (3D) imaging of biological tissues *in situ* and in real-time[27,28]. OCT is similar to ultrasound B-mode imaging, except that echoes of light, instead of sound, are used to achieve micron-scale image resolutions. *In vivo* endoscopic OCT imaging was first demonstrated in rabbit gastrointestinal (GI) and respiratory tracts in 1997[29], and was quickly adopted by multiple groups for investigations in the human GI tract[30-36]. A prospective study involving 121 patients demonstrated 97% sensitivity and 92% specificity for diagnosing BE[33]. Our group has developed a portable, catheter-based prototype OCT system, where the OCT probe can be passed through the accessory channel of a standard endoscope, and achieves imaging speeds of up to 100 000 axial scans per second with axial resolutions of 5 μm to 7 μm in tissues[37]. Real-time cross-sectional OCT image display and 3D capture capabilities were demonstrated in animals[37], and humans[36,38,39]. 3D-OCT volumetric imaging enables the synthesis of *en face* views (similar to magnification endoscopy images), the generation of virtual cross-sectional images with arbitrary orientation, the average of multiple frames to reduce speckle and improve contrast, and quantitative measurements of tissue morphology. To our knowledge, this is the first description of *in vivo* CIP microstructure using OCT.

MATERIALS AND METHODS

Imaging protocol

This study was conducted at the Veterans Affairs Boston Healthcare System (VABHS), in compliance with an approved protocol by the institutional review boards at VABHS, Harvard Medical School and Massachusetts Institute of Technology. Five male Caucasian patients undergoing regular EGD at VABHS from August 2009 to April 2011 were enrolled in this study. This includes one patient with CIP, one representative patient with BE and three representative normal subjects. The representative patient with BE and normal subjects were selected from a large cohort of subjects who were imaged using OCT for another study. White light video endoscopy was performed using the Evis Extra Ⅲ high definition system (Olympus America, Center Valley, PA), and endoscopic

Figure 1 Endoscopic view of cervical inlet patch.

3D-OCT images were obtained using the system described below.

Optical coherence tomography system

The 3D-OCT endomicroscopy system was developed in collaboration with LightLab Imaging - St Jude Medical, Inc. and is similar to the system previously described by our group[37]. A Fourier Domain Mode Locking swept laser with a center wavelength of 1310 nm and average output power of 42 mW at a sweep repetition rate of 59 kHz was used as the light source. The full-width-half-maximum bandwidth of the laser sweep was about 120 nm, which supports about 5 μm axial resolution in tissue. The system sensitivity was 103 dB with 13 mW of incident power. The imaging probe, with an outer diameter of 2.5 mm, was introduced through a standard working channel of a high-definition endoscope (Olympus GIF-Q180) to enable simultaneous video endoscopy and 3D-OCT imaging examination. The output beam from the probe was focused to a 15 μm spot and was emitted at an angle of about 80 ℃ from the probe axis by a prism. The internal optics in the probe was rotated rapidly for radial scanning at 60 (or 70) frames per second (fps). Each image frame had about 512 × 1000 pixels at 60 fps (or about 512 × 900 pixels at 70 fps). To acquire a spirally scanned, volumetric OCT data set of the GI tract, the probe was pulled back at 1.0 mm/s along the sheath, which corresponds to a frame-to-frame spacing of 14-17 μm. At this image acquisition speed, a 20 mm × 8 mm × 2 mm 3D-OCT data set was acquired in 20 s.

Individual 2D-OCT frames were displayed on screen for real-time preview. The volumetric data sets were acquired and streamed to a hard drive. During post-processing, each 2D radial frame was unwrapped to create a rectangular frame. A custom program was written to detect the surface of the plastic probe sheath in each frame, which is used to flatten the image. The flattened 3D-OCT data sets were then loaded into Amira (ResolveRT, Mercury Computer Systems) for 3D rendering and visualization in different orthogonal imaging planes.

Histology analysis

Standard hematoxylin and eosin (H and E) histology was performed by the pathology service at VABHS on biopsy or endoscopic mucosal resection (EMR) specimens in order to compare and validate the 3D-OCT data. Photomicrographs of the H and E slides were taken under a standard Olympus BX40 microscope using a 4× objective.

RESULTS

The endoscopic view of a CIP in a 68-year old patient referred for endoscopic treatment for long-segment BE is shown in Figure 1. During retraction of the endoscope, a pink circular lesion was observed under white light endoscopy in the upper esophagus (about 20 cm from the tooth). The histology of the biopsies taken from the lesion later confirmed the finding of CIP (Figure 2C). Endoscopic OCT imaging was performed over the CIP under direct simultaneous visualization with a white light endoscope. From cross-sectional OCT images shown in Figure 2A and B, regions with CIP and the adjacent squamous epithelium can be identified. In addition, the CIP region clearly shows shallower light penetration compared with the adjacent normal esophagus. This is similar to typical images from normal gastric mucosa of representative other subjects. Zoomed views shown in Figure 2D and E clearly demonstrate columnar and squamous epithelium in the CIP and the adjacent normal esophagus, respectively. The columnar features observed in the CIP are consistent with the corresponding H and E histology shown in Figure 2C.

For comparison, OCT images of a normal gastro-esophageal junction (GEJ) obtained from a representative patient with chronic heartburn symptom (Figure 3). The *en face* OCT projection image at 350 μm underneath the tissue surface clearly shows the GEJ. The OCT imaging probe scans a large field (20 mm × 8 mm) on the tissue, which is about 100× larger compared to the region sampled by a standard biopsy (1-2 mm²). Regions with gastric glandular mucosa (left) and esophageal squamous mucosa (right) exhibit clearly different patterns. Cross-sectional OCT images in Figure 3B-D show the GEJ and esophageal squamous epithelium along the probe pullback and rotation directions, respectively. The GEJ, squamous epithelium, lamina propria/muscularis mucosa, and esophageal glands underneath the squamous epithelium are clearly observed. Features observed in OCT images also match the representative histology of a normal GEJ shown in Figure 3E.

3D-OCT images from a representative patient with a long segment BE confirmed with histology (Figure 4). The *en face* projection OCT image at 200 μm underneath the tissue surface shows a similar angulated pattern compared with the *en face* image shown in the gastric mucosa (Figure 4A). Cross-sectional OCT images (Figure 4B and D) clearly show layered structures, where the original squamous mucosa in the esophagus is replaced by the columnar BE mucosa. Two hyper-scattering layers are observed underneath the BE mucosa, where the top layer corresponds to the newly formed muscularis mucosa layer which replaces the lamina propria, and the bottom

Figure 2 Endoscopic optical coherence tomography imaging of cervical inlet patch. A: Cross-sectional optical coherence tomography images of cervical inlet patch (CIP); B: Adjacent squamous epithelium, respectively; C: Corresponding hematoxylin and eosin histology obtained from a biopsy at the CIP site; D: 3× magnification of the CIP; E: Squamous epithelium (SE) region marked in (A). Scale bars: 1 mm.

Figure 3 Three-dimensional-optical coherence tomography images of a normal gastro-esophageal junction. A: *En face* projection optical coherence tomography (OCT) image at a depth of 350 μm; B: Regions with gastric mucosa and squamous mucosa show distinct features; Cross-sectional OCT image along the probe pullback direction showing the gastro-esophageal junction (GEJ) and normal squamous epithelium (SE) clearly; C, D: Cross-sectional images of the GEJ and SE, corresponding to the green and blue dashed lines marked in (A), respectively. Structures, such as SE, lamina propria (LP)/muscularis mucosa (MM), esophageal glands (arrows) (EG), and gastric mucosa, can be clearly identified; E: Representative histology at the GEJ. Scale bars: 1 mm.

layer corresponds to the muscularis propria. These OCT features are confirmed with corresponding histology of an EMR specimen obtained at the imaging area in the same patient.

Representative OCT images of normal stomach from a patient with chronic heart burn symptom (Figure 5).

Figure 4 Three-dimensional-optical coherence tomography images of a long segment Barrett's esophagus. A: *En face* projection optical coherence tomography (OCT) image at a depth of 200 μm; B: Cross-sectional OCT images of the long segment Barrett's esophagus (BE) along the probe pullback direction; C, D: Cross-sectional OCT images, corresponding to the green and blue dashed lines marked in (A). BE glands, the muscularis mucosa (MM), and the muscularis propria (MP) layers are clearly seen; D: Histology of an endoscopic mucosal resection specimen obtained from the same subject shows corresponding features observed in the OCT images. Scale bars: 1 mm.

The *en face* projection image (Figure 5A) at a depth of 250 μm under the tissue surface represents the typical angulated gastric glandular mucosa pattern. Cross-sectional OCT images (Figure 5B and C) clearly show the gastric glandular mucosa. Gastric pits and gastric glands can be observed from cross-sectional OCT images and the image features match the representative histology of gastric mucosa shown in Figure 5D. Light penetration in normal gastric tissues is also shallower compared with normal esophagus and BE.

Furthermore, 3D-OCT images of normal duodenum from a patient with chronic heart burn symptom are shown in Figure 6. Distinctive features of the intestinal mucosal villi are observed in the *en face* OCT projection image (Figure 6A), as well as in the cross-sectional OCT images (Figure 6B and C). The length of individual villi, measured to be around 300-600 μm, matches the corresponding histology shown in Figure 6E. These results demonstrate the feasibility of using OCT to evaluate GI tissue morphology *in situ* and in real-time.

DISCUSSION

CIP is an under-appreciated entity in general gastroenterologist's practice. In this study, we present imaging results from OCT, a relatively new imaging technology, to describe the gastric type of epithelial patterns in CIP, as clearly distinct from normal esophageal squamous epithelium, BE, or from normal duodenum. Under OCT, CIP exhibits similar columnar structures compared with normal gastric mucosa, and the imaging depth in both CIP and gastric tissues are low. In practice, obtaining biopsies from CIP in patients with troublesome supraesophageal or laryngeal symptoms may be difficult owing to poor view just below the UES. OCT may allow "optical biopsy" of the CIP epithelium without the need for obtaining tissue specimens, and may be used to assess changes suspicious for malignancy in the future. Given its small diameter (2.5 mm) and flexibility, the OCT probes may be introduced orally or nasally without an endoscope, and with better tolerance and potentially less motion artifacts. This may further negate the need for sedation, nursing, or use of the endoscopy unit which has implications beyond endoscopy costs.

There are a number of case reports of adenocarcinoma arising from heterotopic gastric mucosa in the upper esophagus[23-26,40]. To our knowledge, 31 cases have been reported in the literature where esophageal adenocarcinoma was found arising from an inlet patch[41-46] and two cases where laryngeal squamous cell carcinoma was found associated with or bordering inlet patches[47]. The pathogenetic link between BE and CIP raises concerns of dysplastic transformation in CIP. Using immunohistochemical markers to address potential cellular origins,

Figure 5 Three-dimensional-optical coherence tomography images of a normal stomach. A: *En face* projection optical coherence tomography (OCT) image at a depth of 250 μm; B: Cross-sectional OCT image along the probe pullback direction, corresponding to the red dashed line marked in (A); C: Cross-sectional images of the gastric mucosa, corresponding to the blue dashed line marked in (A). Gastric pits (GP) and gastric glands (arrows) (GG) can be identified; D: Representative histology of a gastric mucosa. Scale bars: 1 mm.

Figure 6 Three-dimensional-optical coherence tomography images of a normal duodenum. A: *En face* projection optical coherence tomography (OCT) image at a depth of 350 μm; B: Cross-sectional OCT images of the duodenum along the probe pullback direction; C: Cross-sectional OCT image, corresponding to the blue dashed line marked in (A); Mucosal villous structures (Vi) in the duodenum are clearly seen; D: Corresponding histology of the duodenum showing the villi. Scale bars:

Lauwers *et al*[48] demonstrated similar mechanisms of pathogenesis for CIP and BE on the basis of similarity between immunohistochemical staining patterns between the two entities. Furthermore, the similarity in the expression pattern for cytokeratins 7 and 20 and MUC6 mucin protein were not influenced by the presence or absence of GERD in these CIP patients[48-50]. Based on

these findings, Lauwers *et al*[48] suggested that CIP may arise as a metaplastic change occurring in the esophageal epithelium. In light of these findings and the pathogenetic similarity between CIP and BE based on the cytokeratin expression study by Lauwers, the suspicion that the CIP, at least in adults, arises from local stem cells within the esophagus at or near this "heterotopic" patch

is strong. However, unlike BE, a consensus guideline for surveillance of CIP has not been established on account of its relatively low incidence and lack of information on its natural history for dysplastic changes. In this study, we present an alternative approach to evaluate CIP based on OCT imaging. Recently, balloon based OCT probes have been developed in order to allow imaging over the entire esophageal lumen for screening purposes[35,51-53]. The balloon design also helps stabilize the imaging probe to minimize motion artifacts. The advantages of OCT, such as real-time imaging, large area of coverage, and depth resolved imaging, *etc*., suggest that it may be a useful tool for detection of various GI diseases, including CIP and Barrett's esophagus.

In addition, OCT enables visualization of the deeper esophageal glands underneath squamous mucosa, which may not be accessible with standard or jumbo biopsy forceps. As suggested by Lauwers *et al*[48] CIP may arise from submucosal esophageal mucous glands. If these glands are the origin of dysplasia or malignant transformation, 3D-OCT may be uniquely suited for identifying these early dysplastic changes and following them up. Recently, novel methods have been developed to perform OCT imaging with contrast agents, such as gold nanoparticles[54-57], and therefore enable molecular targeted imaging for early cancer detection. In the future, OCT may also be combined with biomarkers, e.g., the superficial expression of Lgr5 in BE. Localization with depth resolution of such markers may help with directed biopsies or targeted ablation.

Presently, there are no commercially available OCT systems for endoscopic applications. The OCT probe used in this study passes through the working channel of a standard white light endoscope, and the entire OCT system is portable and could fit in a standard endoscopy suite to provide complementary real-time information on tissue microstructures during endoscopy.

One limitation of the current study is the small sample size. Multiple normal subjects and patients with BE were imaged and representative results were shown from a large cohort, but only one subject with CIP was available. The objective of this pilot study is to demonstrate the feasibility of *in situ* imaging of CIP using 3D-OCT and identify characteristic imaging features of CIP compared to other organs in human upper GI tract. One set of representative OCT images from each organ was demonstrated. However, it is not possible to reach any statistical conclusion from this feasibility study.

In conclusion, we demonstrate *in situ* evaluation of CIP microstructure using endoscopic 3D-OCT. OCT imaging visualized columnar epithelial structures within the CIP region, which clearly contrasted with the squamous epithelium from adjacent normal esophagus, gastric mucosa in the stomach and villous structure in the duodenum. The microstructural features observed with OCT also matched those from H and E histological sections. These results demonstrated the feasibility of using OCT to evaluate GI tissue morphology *in situ* and

in real-time. Since OCT imaging can be performed with small diameter probes introduced orally or nasally, this emerging technology might be used to screen patients with troublesome upper esophageal symptoms for CIP, BE and other changes in the epithelium, even without endoscopy or the need for conscious sedation.

ACKNOWLEDGMENTS

COMMENTS

Background
Cervical inlet patch (CIP) is an under-appreciated entity encountered by gastroenterologists in general practice. Although rare, several reports suggest that CIP may progress to adenocarcinoma. Optical coherence tomography (OCT) is an emerging medical imaging technology that enables micron-scale, cross-sectional, and 3D imaging of biological tissues *in situ* and in real-time.

Research frontiers
This study evaluates whether OCT can optically assess epithelial differences in upper human gastrointestinal (GI) tract *in situ*.

Innovations and breakthroughs
OCT imaging over the CIP region showed columnar epithelium structure, which clearly contrasted the squamous epithelium structure from adjacent normal esophagus. OCT images obtained from other patients demonstrated distinctive patterns of the normal esophagus, Barrett's esophagus, normal stomach, and normal duodenum. Microstructures, such as squamous epithelium, lamina propria, muscularis mucosa, muscularis propria, esophageal glands, Barrett's glands, gastric mucosa, gastric glands, and intestinal mucosal villi were clearly observed with OCT and matched with hematoxylin and eosin histology. OCT may allow real-time "optical biopsy" of the CIP epithelium without the need for obtaining tissue specimens, and may be used to assess suspicious changes of malignancy in the future.

Applications
Given its small diameter and flexibility, the OCT probe may be introduced orally or nasally without an endoscope or need for moderate sedation, and with better tolerance and potentially less motion artifacts compared to endoscopy. In addition, the OCT imaging probe scans an approximately 100× larger field compared to biopsy, and therefore, might be useful in the future to screen for CIP.

Peer review
The paper is a very interesting piece of work exploring the utility of OCT primarily in characterizing cervical inlet patch but also in other esophageal disorders. It provides a reader with the future directions of and technological advances in upper GI endoscopy in an era when a number of non-white light endoscopic contrast techniques are competing for prime time use to more accurately delineate and diagnose pathology.

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BRIEF ARTICLE

Endomysial antibodies predict celiac disease irrespective of the titers or clinical presentation

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Abstract

AIM: To investigate the association between serum antibody levels and a subsequent celiac disease diagnosis in a large series of children and adults.

METHODS: Besides subjects with classical gastrointestinal presentation of celiac disease, the study cohort included a substantial number of individuals with extraintestinal symptoms and those found by screening in at-risk groups. Altogether 405 patients underwent clinical, serological and histological evaluations. After collection of data, the antibody values were further graded as low [endomysial (EmA) 1:5-200, transglutaminase 2 antibodies (TG2-ab) 5.0-30.0 U/L] and high (EmA 1: ≥ 500, TG2-ab ≥ 30.0 U/L), and the serological results were compared with the small intestinal mucosal histology and clinical presentation.

RESULTS: In total, 79% of the subjects with low and 94% of those with high serum EmA titers showed small-bowel mucosal villous atrophy. Furthermore, 96% of the 47 EmA positive subjects who had normal mucosal villi and remained on follow-up either subsequently developed mucosal atrophy while on a gluten-containing diet, or responded positively to a gluten-free diet.

CONCLUSION: Irrespective of the initial serum titers or clinical presentation, EmA positivity as such is a very strong predictor of a subsequent celiac disease diagnosis.

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INTRODUCTION

Recent serological screening studies have revealed that up to 1%-2% of the Western population might be affected by celiac disease[1,2]. However, due to its heterogeneous clinical picture the disease remains markedly underdiagnosed. Sensitive serum endomysial (EmA) and transglutaminase 2 antibodies (TG2-ab) are widely used as a method to select subjects for further investigations, but the diagnosis is based on the presence of small-bowel mucosal villous atrophy and crypt hyperplasia[3,4]. Unfortunately, the histological definition of the disease involves several problems. First, invasive studies are needed to acquire the mucosal specimens. In addition, biopsy samples may be of poor quality or wrongly orientated, increasing the risk of false positive or negative results[5]. The mucosal damage may be patchy and missed even if several samples are taken[6,7]. Finally, the histological lesion develops gradually and interpretation of borderline cases can be challenging. Since particularly EmA and high values of TG2-ab seem to predict celiac disease with a high specificity, it has been advocated that in seropositive subjects endoscopic studies might not always be needed to establish the diagnosis[8-15]. However, most studies so far have been carried out in tertiary centers with high-risk patients, and the results might not be applicable in everyday clinical practice.

In our local health-care district active celiac disease case-finding has been carried out since the 1980s. As a result, a substantial part of the patients are detected because of atypical symptoms or by active risk-group screening, and currently about 0.7% of the population have a biopsy-proven diagnosis[16]. Hence, we now sought to establish whether the serum antibodies could predict subsequent celiac disease also in subjects with mild or atypical clinical presentation. Because of the high specificity, EmA has traditionally been considered the gold standard for celiac disease serology, and was thus chosen as the primary inclusion criterion[17,18]. In addition, the results were compared to the widely used serum TG2-ab.

MATERIALS AND METHODS

The study cohort comprised consecutive EmA positive children and adults investigated at the Departments of Pediatrics and Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital. Primary care physicians were encouraged to refer individuals with celiac disease suspicion for further investigations applying a low index of suspicion. In addition, subjects who participated in population-based research studies were accepted. In the hospital demographic data, a family history of celiac disease and symptoms leading to the disease suspicion were recorded, and all subjects underwent extensive clinical, serological and histological evaluations. Thereafter, voluntary EmA positive children and adults continued in the trial. Participants who showed small-bowel mucosal villous atrophy and crypt hyperplasia (Marsh III) received a celiac disease diagnosis and were placed on a gluten-free diet. Subjects who had normal villi continued on a gluten-containing diet and were placed on regular serological and histological follow-up. In addition, the possibility to start an experimental trial with a gluten-free diet was offered to EmA positive individuals with normal villous structure (Marsh 0-II). Those who consented were re-evaluated after one year, and if a positive clinical, serological and histological response was observed, celiac disease diagnosis was established. Finally, serum TG2-ab were used for comparison in all from whom they were available.

Serum immunoglobulin A (IgA)-class EmA were measured by an indirect immunofluorescence method using human umbilical cord as antigen[19]. A dilution of 1:5 was considered positive, and positive sera were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000. The antibody titers were further graded as low (1:5-1:200) and high (1:500-1:4000). Serum IgA-class TG2-ab were measured by enzyme-linked immunosorbent assay (ELISA) (Celikey, Phadia GmbH, Freiburg, Germany) according to the manufacturers' instructions. Serum TG2-ab values $\geq$ 5.0 U were considered positive, and the values were further graded as low (5.0-29.9 U/L) and high ($\geq$ 30 U/L)[13]. Total IgA values were tested in all subjects negative to the IgA class serological tests. In case of IgA deficiency the corresponding antibodies were measured in immunoglobulin G (IgG) class.

Upon upper gastrointestinal endoscopy a minimum of three forceps specimens were taken from the distal duodenum, and small-bowel mucosal morphology was determined from several well-oriented biopsy sections as previously described[17]. The degree of mucosal damage was further graded according to the Marsh-Oberhuber classification, where Marsh 0 represents normal mucosa, Marsh I-II represents increased intraepithelial lymphocytosis without (I) or with (II) hyperplastic crypts and Marsh III partial (a), subtotal (b) or total (c) villous atrophy[20,21]. A patchy mucosal lesion was graded according to the most severe histological damage.

Genotyping of the participants for celiac disease-associated human leukocyte antigen (HLA)-DQB1*02 and DQB1*0302 alleles (DQ2 and DQ8) was performed using the DELFIA® Celiac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or the SSP™ DQB1 low resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) according to the

Table 1 Demographic data on the study participants and primary reason for celiac disease suspicion *n* (%)

Female	265 (67)
Age below 18 yr	92 (23)
Age (yr), median (range)	40 (1-79)
Main reason for disease suspicion	
Gastrointestinal symptoms[1]	166 (43)
Anemia or malabsorption	38 (10)
Extraintestinal symptoms[2]	50 (13)
Screening in at-risk groups[3]	97 (24)
Screening in the population[4]	39 (10)
Unknown	5 (1)

[1]Diarrhea, abdominal pain, flatulence, constipation, dyspepsia, and heartburn; [2]Osteoporosis, infertility, aphthous stomatitis, short stature, delayed puberty, arthralgia, ataxia, epilepsy, fatigue, and alopecia; [3]Family history of celiac disease, type 1 diabetes, thyroid disorders, Sjögren´s syndrome, and immunoglobulin A nephropathy; [4]Population-based research studies that included serological screening.

Table 2 Serum endomysial and transglutaminase 2 antibody values, divided according to the clinical presentation

	EmA, titer		TG2-ab, U/L	
	Low 1:5-200	High 1:≥ 500	Low 5.0-29.9	High ≥ 30
	n = 224, %	*n* = 154, %	*n* = 116, %	*n* = 166, %
Abdominal symptoms	45	40	45	45
Anemia or malabsorption	8	12	5	13
Extraintestinal symptoms	9	16	5	13
Screen-detected subjects	38	31	45	28
	P = 0.061		*P* = 0.002	

EmA: Endomysial; TG2-ab: Transglutaminase 2 antibody.

manufacturer's instructions.

χ^2 with cross-tabulation was used for statistical analysis. A *P* value less than 0.05 was considered statistically significant.

The study protocol was approved by the Ethics Committee of Tampere University Hospital. All subjects or their parents gave written informed consent.

RESULTS

In total, 405 EmA positive children and adults participated in the study. In 10 subjects the quality of the small-bowel biopsies was insufficient, in 14 EmA was determined as positive (1:5) without further dilution and in three subjects the clinical data were ambiguous. These cases were excluded from further statistical analyses. One patient had selective IgA deficiency and the corresponding antibodies were measured in the IgG class. Gastrointestinal symptoms remained the primary reason for celiac disease suspicion, but almost half of the patients were detected on the basis of extraintestinal symptoms or by screening of at-risk groups and the population (Table 1).

By definition, all participants were positive for EmA.

Table 3 Association between high and low serum endomysial and transglutaminase 2 antibody values and small-bowel mucosal morphology

	EmA (L)		TG2-ab(U/L)	
	Low 1:5-200	High 1:≥ 500	Low 5.0-29.9	High ≥ 30
	n = 227, %	*n* = 156, %	*n* = 146, %	*n* = 169, %
Marsh 0	5	1	4	1
Marsh I - II	16	5	16	5
Marsh IIIa	22	13	24	12
Marsh IIIb	28	29	31	28
Marsh IIIc	29	52	25	53
	P < 0.001		*P* < 0.001	

EmA: Endomysial; TG2-ab: Transglutaminase 2 antibody.

Table 4 Association between endomysial antibody titers and small-bowel mucosal damage

EmA titer	Subjects	Marsh 0- II, *n* (%)	Marsh III, *n* (%)
1: ≥ 5	372	57 (15)	315 (85)
1: ≥ 50	323	39 (12)	284 (88)
1: ≥ 100	282	26 (9)	256 (91)
1: ≥ 200	243	22 (9)	221 (91)
1: ≥ 500	155	9 (6)	146 (94)
1: ≥ 1000	96	3 (3)	93 (97)
1: ≥ 2000	48	2 (4)	46 (96)
1:4000	20	0 (0)	20 (100)

EmA: Endomysial.

Serum TG2-ab were measured in 316 EmA positive subjects and proved positive in 286 (91%) of them. Altogether 41% of the participants had high EmA and 54% high TG2-ab value defined at baseline. There was a significant association between serum TG2-ab level and clinical presentation, low antibody values being more common in the screen- than symptom-detected subjects (Table 2). A similar trend was observed with EmA, but the results were not statistically significant (*P* = 0.061).

Small-bowel mucosal villous atrophy and crypt hyperplasia (Marsh III) were found in altogether 85% of the EmA-positive subjects. There was a significant association between high antibody values and more severe small-bowel mucosal deterioration; in total 94% of those with high EmA titer evinced villous atrophy (Table 3). There was in this respect no significant difference between children and adults. The percentage of subjects evincing severe small-bowel mucosal damage increased progressively with higher EmA titers, but only the highest titer 1:4000 was 100% predictive of subsequent villous atrophy and crypt hyperplasia (Table 4).

In total, 40 patients had low and 17 high serum antibody values without simultaneous villous atrophy (Table 5). Irrespective of the baseline titers, 45 (79%) of these subjects (96% of those who remained on follow-up) either subsequently developed villous atrophy while on a gluten-containing diet, or experienced a positive clinical and serological response and disappearance of early mucosal changes on a gluten-free diet (Table 5). The pres-

Table 5 Baseline and follow-up data on subjects with positive endomysial antibodies but normal small-bowel mucosal villous structure

	Low[1] EmA and TG2-ab, *n* = 40	High EmA or TG2-ab, *n* = 17
Baseline		
Age, median (range), yr	39 (5-68)	39 (6-70)
Females, *n* (%)	30 (75)	11 (65)
Age below 18 yr	7 (18)	7 (41)
Gastrointestinal symptoms	28 (70)	12 (71)
Extraintestinal symptoms	2 (5)	4 (23)
Screen-detected subjects	10 (25)	1 (6)
EmA, median (range), titer	1:50 (1:5-1:200)	1:500 (1:5-1:2000)
TG2-ab, median (range), U/L	6.3 (0-24.8)	45.5 (13.9-> 100)
HLA DQ2 or DQ8, *n* (%)	33/33 (100)	16/16 (100)
Marsh 0	9 (23)	3 (18)
Marsh Ⅰ - Ⅱ	31 (77)	14 (82)
Follow-up		
Celiac disease diagnosis	29 (73)	16 (94)
Villous atrophy later[2]	12 (30)	8 (47)
Positive response to GFD	17 (43)	8 (47)
Gluten, no villous atrophy	2 (5)	0
Lost to follow-up	9 (22)	1 (6)

[1]EmA titer 1: < 500, TG2-ab value < 30.0 U/L; [2]Up to 10 yr of follow-up. EmA: Endomysial; TG2-ab: Transglutaminase 2 antibody; HLA: Human leukocyte antigen; GFD: Gluten-free diet.

ence of the celiac disease-associated HLA-DQ2 or DQ8 genotype was assessed in 299 EmA positive subjects and was found in all of them.

DISCUSSION

In our large series consisting of both children and adults, approximately half of the participants evinced high serum EmA levels, which was indicative of subsequent small-bowel mucosal villous damage in up to 94% of them. The results showed a high antibody titer to be an excellent predictor of villous atrophy and celiac disease also in high disease prevalence areas and in subjects with subtle or atypical symptoms. In the past few decades it has been observed that besides the classical gastrointestinal presentation, celiac disease patients may have a wide range of different extraintestinal symptoms. The patients may suffer for example from arthralgia or arthritis, osteoporosis, infertility and different neurological symptoms. In addition, screen-detected celiac patients may show only minor laboratory abnormalities or have no symptoms at all[3]. It was essential to investigate the performance of the celiac autoantibodies also in these atypical patients, as they are frequently seen in clinical practice, and may in fact represent the most common clinical presentation of celiac disease[16].

In patients with classical gastrointestinal celiac disease, Valdimarsson *et al*[8] observed a 100% positive predictive value of EmA for celiac disease in 19 adults, and suggested that histological confirmation might not be necessary in all such seropositive patients. Recently, similar results have been obtained with high values of TG2-ab. In a study by Barker *et al*[10], 48 out of 49 children having TG2-ab more than five times the upper limit of normal (ULN) had diagnostic small bowel mucosal damage. Likewise, Donaldson *et al*[11] observed a 100% positive predictive value for celiac disease by using the same cut-off level. In adults, Hill *et al*[13] suggested that TG2-ab levels more than ten times ULN would be exclusively indicative for celiac disease, and some other authors have presented comparable results[12].

Our findings thus largely accord with those in earlier studies carried out in specialized centers with high-risk patients having classical gastrointestinal presentation of celiac disease. Nevertheless, in the present study there was still a subpopulation of individuals in whom the current histological criteria were not fulfilled. Approximately 6% of the participants with high and up to 21% of those with low antibody values had normal small-bowel mucosal villous structure, and in total this was seen in 15% of the EmA-positive subjects. It could thus be argued that EmA are not sufficiently specific for a definite diagnosis of celiac disease as such. Interestingly, however, there is an increasing body of data showing that EmA positivity is a very strong predictor of forthcoming celiac disease also in subjects with initially normal villi[17,22-26]. In line with this conception, almost all of our EmA positive patients who had no structural villous damage either evinced a positive serological, clinical and histological response to a gluten-free diet, or subsequently developed villous atrophy while on a normal diet. The existence of a celiac-type disorder in these individuals was further supported by the presence of the relevant HLA type in all in whom it was measured. There is thus strong evidence that, irrespective of the initially normal villous morphology, these EmA positive subjects are truly suffering from celiac disease.

Our study is subject to some limitations. First, although a high percentage of the participants had mild or atypical clinical presentation, the number of those found by population-based serological screening was rather low. Consequently, the results cannot be generalized to this patient group, and further studies are needed[27]. Secondly, the mucosal biopsies were taken from the distal duodenum as previously recommended[28]. Judging from recent evidence, however, villous atrophy can occasionally be detected only in the bulb area of the small intestine, and in theory celiac disease might in such cases already have been confirmed at the time of the first biopsy[7]. Nevertheless, interpretation of bulb specimens may be biased on Brunner glands or peptic inflammation, and their role in the diagnostics remains controversial. In addition, a patchy small-bowel mucosal lesion is always possible in celiac disease, which further highlights the importance of serology in the diagnosis.

Although EmA shows excellent specificity for an untreated celiac disease, it has certain limitations. The immunofluorescence method is laborious, time-consuming and always somewhat subjective. Since TG2-abs can be easily measured using a practical ELISA method, it would be tempting to use it instead of EmA. Neverthe-

less, TG2-ab are measured by commercial tests which use different epitopes of TG2 as antigen, and thus the specificity figures for the method have been somewhat inconsistent. Consequently, the positive predictive value of TG2-ab has sometimes been rather low, particularly in low-risk populations[29]. TG2-ab can also be positive in some conditions such as in liver diseases[30]. For these reasons, we decided to use the more laborious and time-consuming but celiac disease-specific EmA as the primary inclusion criterion in our series. As a consequence, the results should not be applied to TG2-ab positive EmA negative subjects. Finally, since antibody-negative subjects were not included in our study, the overall sensitivity of the serological tests could not be obtained.

To conclude, EmA positivity as such is a very strong predictor of a subsequent celiac disease diagnosis also in patients with low serum antibody titers and subtle or atypical clinical presentation. Judging from the findings here, invasive endoscopic studies might not be obligatory in all such seropositive patients.

COMMENTS

Background
The diagnosis of celiac disease is based on the presence of small-bowel mucosal villous atrophy and crypt hyperplasia, but this histological definition involves several problems. Since particularly endomysial (EmA) and high values of transglutaminase 2 antibodies (TG2-ab) seem to predict celiac disease with high specificity, it has been advocated that in seropositive subjects with gastrointestinal symptoms endoscopic studies might not be obligatory to establish the diagnosis.

Research frontiers
New diagnostic criteria of celiac disease based to serological tests might make the burdensome and invasive endoscopic investigations unnecessary. The authors aimed to investigate the association between serum antibody levels and a subsequent celiac disease diagnosis in children and adults with a heterogeneous clinical presentation.

Innovations and breakthroughs
The results showed that high positive values of serological tests are excellent predictors of subsequent villous atrophy and celiac disease also in subjects with atypical or subtle clinical presentation. In addition, positivity of EmA is a very strong indicator of celiac disease diagnosis irrespective of the baseline titers.

Applications
These results indicate that gastrointestinal endoscopy might be omitted and celiac disease diagnosis established without further histological confirmation in children and adults with positive EmA.

Terminology
Celiac disease is a chronic autoimmune-based triggered by ingested gluten in genetically susceptible individuals. EmA and TG2-ab are serological test with high accuracy for an untreated celiac disease.

Peer review
This is a nice study that highlights the significance of positive EmA results in a large series of children and adults. The paper is well-written and adds pertinent information to the literature.

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BRIEF ARTICLE

Efficacy of mosapride citrate with polyethylene glycol solution for colonoscopy preparation

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Abstract

AIM: To evaluate the efficacy and safety of adjunctive mosapride citrate for bowel preparation before colonoscopy.

METHODS: We conducted a randomized, double-blind, placebo-controlled study with mosapride in addition to polyethylene glycol (PEG)-electrolyte solution. Of 250 patients undergoing colonoscopy, 124 were randomized to receive 2 L PEG plus 15 mg of mosapride citrate (mosapride group), and 126 received 2 L PEG plus placebo (placebo group). Patients completed a questionnaire reporting the acceptability and tolerability of the bowel preparation process. The efficacy of bowel preparation was assessed by colonoscopists using a 5-point scale based on Aronchick's criteria. The primary end point was optimal bowel preparation rates (scores of excellent/good/fair *vs* poor/inadequate).

RESULTS: A total of 249 patients were included in the analysis. In the mosapride group, optimal bowel preparation rates were significantly higher in the left colon compared with the placebo group (78.2% *vs* 65.6%, *P* < 0.05), but not in the right colon (76.5% *vs* 66.4%, *P* = 0.08). After excluding patients with severe constipation, there was a significant difference in bowel preparation in both the left and right colon (82.4% *vs* 66.7%, 80.8% *vs* 67.5%, *P* < 0.05, *P* < 0.01). The incidence of adverse events was similar in both groups. Among the subgroup who had previous colonoscopy experience, a significantly higher number of patients in the mosapride group felt that the current preparation was easier compared with patients in the placebo group (34/72 patients *vs* 24/74 patients, *P* < 0.05).

CONCLUSION: Mosapride citrate may be an effective and safe adjunct to PEG-electrolyte solution that leads to improved quality of bowel preparation, especially in patients without severe constipation.

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Key words: Mosapride citrate; Bowel preparation; Polyethylene glycol-electrolyte solution; Colonoscopy; Prokinetics

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INTRODUCTION

Polyethylene glycol (PEG)-electrolyte solution is used worldwide for bowel cleansing. Approximately 2 L of this oral solution, along with a laxative, is usually required for adequate bowel preparation in Japan[1]. However, the need to drink such large volumes of liquid with an unpalatable taste has a negative impact on patient compliance[2]. A thorough bowel preparation is required for safe and effective colonoscopy, and inadequate preparation not only decreases the sensitivity, but also increases the difficulty of the procedure[3-5]. Therefore, more effective bowel preparation regimens for colonoscopy are required to improve the acceptability and tolerability of the procedure. Prokinetics such as domperidone, metoclopramide, and cisapride have been used in combination with PEG-electrolyte solution to improve the quality of bowel preparation[6-12]. However, the addition of prokinetic agents to PEG-electrolyte solution has not been proven to improve patient tolerance or colonic cleansing[10-12] and is sometimes associated with serious adverse effects. For example, domperidone and metoclopramide may cause extrapyramidal symptoms with long-term use[13]. Cisapride was withdrawn from the market because of severe cardiac side effects, including QT-interval prolongation and ventricular arrhythmias[14]. Thus, safer and more effective prokinetic agents are needed.

Mosapride citrate (mosapride) is a selective 5-hydroxy-tryptamine-4 (5-HT4) receptor agonist. Mosapride enhances gastric emptying and motility by facilitating acetylcholine release from the enteric cholinergic neurons, without blocking dopaminergic D2 receptors[15]. It is known to be effective in gastroesophageal reflex disease[16], functional gastrointestinal disorders, such as functional dyspepsia[17], chronic gastritis with delayed gastric emptying, and diabetic gastroparesis[18]. As 5-HT4 receptors are also located in the human colon and rectum[19,20], mosapride is also expected to have a prokinetic effect on the colo-rectum. A few clinical studies have reported that mosapride in combination with PEG may enhance bowel cleansing and improve patient acceptability and tolerability[21,22]. However, the efficacy and tolerability of a PEG-electrolyte solution with or without mosapride has not been studied in a double-blind, randomized trial.

Figure 1 Steps of preparation for colonoscopy.

We conducted this study to evaluate the efficacy, acceptability, and tolerability of mosapride as an adjuvant to PEG-electrolyte solution for colonoscopy preparation.

MATERIALS AND METHODS

This was a prospective, double-blind, randomized, controlled study that included patients who underwent colonoscopy at Aichi Cancer Center Hospital (ACCH), Nagoya, from January 2009 to October 2009. This study was reviewed and approved by the ethics committee of ACCH.

Study population

All consecutive outpatients aged 20-80 years who were scheduled for colonoscopy at ACCH were evaluated for study inclusion. Patients with the following clinical features were excluded: presence of significant cardiac, renal, hepatic, or metabolic comorbidities; presence of ascites or bowel obstruction; known allergy to PEG-electrolyte solution; history of gastric stapling or bypass procedure; or a history of prior colonic or rectal surgery. A gastroenterologist assessed patient eligibility, and written informed consent was obtained from each patient prior to inclusion.

Randomization and blinding

Patients were randomly allocated to receive one of two bowel preparation regimens using a computer-generated random-number list. Patients were randomized in block sizes of two, with serially numbered, sealed, opaque envelopes. Concealed allocation was accomplished through non-research personnel who were not involved in this study. Comparisons between subjects who received 2 L PEG plus mosapride (mosapride group) and 2 L PEG plus placebo (placebo group) were made in a double-blind fashion.

Bowel preparation methods

The colonoscopy preparation steps used in this study are shown in Figure 1. The day before colonoscopy, all patients were instructed to eat a pre-packaged, low-residue diet (Enimaclin CS; Horii Pharmaceutical Ind., Ltd., Osaka, Japan) that consisted of a lunch, snack, and dinner, and were asked to drink more than 2 L of clear

liquid. On the day of the colonoscopy, all participants reported to the endoscopy room at 9:00 am, and received in-hospital bowel preparation. In-hospital preparation is important to ensure the uniformity of procedures within the study, and to remove any confounding caused by poor patient adherence. More than 10 toilet facilities were made available in the endoscopy unit for patient comfort. Six mosapride tablets (15 mg) (Gasmotin; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) or six identical-looking placebo tablets were administered orally with water at 09:30. The timing of administration of the mosapride tablets was based on its pharmacokinetics[23]. After 30 min, both groups were instructed to drink 0.25 L of PEG-electrolyte solution (Niflec; Ajinomoto Pharmaceticals Co., Ltd., Tokyo, Japan) every 15 min.

Evaluation of bowel preparation

The efficacy of bowel preparation was assessed using Aronchick's criteria[24], as follows: (1) Excellent (small volume of clear liquid, or greater than 95% of colonic surface seen); (2) Good (large volume of clear liquid covering 5% to 25% of colonic surface, but greater than 90% of surface seen); (3) Fair (some semisolid stool that could be suctioned or washed away, but greater than 90% of surface seen); (4) Poor (some semisolid stool that could not be suctioned or washed away, and less than 90% of surface seen); and (5) Inadequate (repeat preparation and colonoscopy needed). Participating endoscopists were trained to use Aronchick's criteria to achieve a good level of agreement. Investigators performed calibration exercises involving more than 20 colonoscopies prior to study commencement, based on their interpretation of scale anchors, to ensure that their findings agreed. The final assessment of bowel preparation was divided into two categories: optimal and non-optimal. Bowel preparations rated as fair, good, or excellent, based on Aronchick's criteria, were considered optimal; poor or inadequate ratings were considered non-optimal. After colonoscopy, two observers, including the endoscopist performing the procedure, determined the score by mutual agreement. They scored the quality of the preparation in the right colon (proximal to the splenic flexure), and the left colon (distal to the splenic flexure) and rectum separately. If the decision was discordant, a third expert reviewer graded and scored the recorded images, and this evaluation was used in the final analysis.

During or immediately following the colonoscopy, the investigator completed a physician questionnaire regarding the assessment of bowel preparation, amount of irrigation fluid used, time to reach the cecum, and ease of insertion into the cecum and visualization of the colonic lumen regardless of peristalses.

Patient tolerance and other measurements

The nursing staff recorded the time required to drink the indicated volume of lavage solution. They also recorded the time and number of motions from start of ingestion to the appearance of clear excretions. The nursing staff checked excretions until 1 h after patients finished the PEG + mosapride solution. If there was a solid stool with muddy excretions or no excretion at that time, we gave the patient an additional preparation, such as additional PEG or enema. A warm water enema of 500 mL volume was given until the excretions were clear. Patients who received an additional preparation were defined by Aronchick's criteria as inadequate. The patient questionnaire, which was administered before bowel cleansing, consisted of 20 questions pertaining to patient characteristics, tolerability, and acceptability of study medication. It also included questions about the following: age; height; body weight; average number of bowel movements per week for the last year; number of previous colonoscopies; compliance with ingestion of PEG-electrolyte solution; willingness to repeat the same preparation regimen again, if required; ease/difficulty of taking the preparation compared with previous experiences; and presence of subjective symptoms while drinking PEG-electrolyte solution, such as nausea, vomiting, fullness, abdominal pain, and circulatory reactions such as palpitations or chest discomfort. We defined patients who suffered from constipation (defined as < 2 bowel movements per week) for > 1 year as having severe constipation. Patients completed the questionnaire before undergoing the colonoscopy and submitted the form to the nursing staff.

Endpoints

The primary endpoint was the difference in optimal rate of colon cleansing in the mosapride and placebo groups. Secondary endpoints included differences in patients' acceptability and tolerance of solutions, time to first defecation, frequency of defecation, complete time for colonic preparation, time needed to reach the cecum, amount of irrigation fluid used, and subjective difficulty in colonoscopy insertion to the cecum and in observing the lumen of the colo-rectum because of peristalses.

Statistical analysis

The study was designed to detect an inter-group difference of 11% in the percentage of patients with optimal bowel preparation, with an α error of 5% and a power of 80%. This difference was based on a previous study[21]. The number of patients needed to demonstrate an 11% difference was 125 per treatment group, assuming a dropout rate of 10%.

The primary efficacy analysis was based on an intent-to-treat analysis and included patients who were randomized and received any treatment. The preparation of patients in this group was considered optimal or non-optimal based on the colonoscopist's score regarding cleansing. Patients who did not undergo colonoscopy because of preparation-related adverse events, or preparation failure, or in whom the right colon could not be reached because of bowel obstruction or for technical reasons were excluded. The rates of optimal preparation were compared between the groups by the Chi-square test or Fisher's exact test for categorical variables.

Table 1 Baseline characteristics

Variable	Overall		Excluding patients with severe constipation		*P* value	
	A (mosapride)	B (placebo)	C (mosapride)	D (placebo)	A *vs* B	C *vs* D
No. of patients	124	125	108	120		
Age (yr, mean ± SD)	67.3 ± 8.6	67.8 ± 10.1	67.3 ± 8.5	67.5 ± 10.2	NS	NS
< 60	21	21	18	21		
60-69	42	44	37	42	NS	NS
≥ 70	61	60	53	57		
Male	69	83	64	80	NS	NS
Female	55	42	44	40		
Body mass index (kg/m², mean ± SD)	22.5 ± 2.9	22.6 ± 2.7	22.7 ± 2.9	22.7 ± 2.6	NS	NS
Bowel movements per week						
< 2	16	5	0	0	< 0.05	NS
≥ 2	108	120	108	120		
Previous colonoscopy						
None (first time)	38	27	32	26	NS	NS
≥ 2	86	98	76	94		

P value by the χ^2 test. NS: Not significant.

Figure 2 Patient disposition flow chart. PEG: Polyethylene glycol.

For secondary endpoints, the Mann-Whitney *U* test was used for comparison between continuous variables. Categorical variables were analyzed using the corrected χ^2 or two-sided Fisher's exact tests, where appropriate. The criterion for statistical significance was $P < 0.05$.

All statistical analyses were performed using Statistical Analysis Software (SPSS, version 12.0J for the PC, SPSS Japan, Inc., Tokyo, Japan).

RESULTS

Patient characteristics

A total of 250 patients were randomized into two groups (Figure 2). Of those randomized to treatment, only one patient did not receive any treatment or undergo colonoscopy because he felt chilled and had a fever before treatment. Although 249 patients were analyzed, insertion of the colonoscope into the right colon failed in five (4%) patients in the mosapride group (advanced stenosing cancer in three and patient refusal in two), because of pain on colonoscopic advancement to the proximal colon. These five patients were excluded from the efficacy analysis of the right colon. Baseline characteristics of the patients are shown in Table 1. Differences in age, gender, body mass index, and the number of previous colonoscopies between the mosapride and placebo groups were not significant. However, significantly more patients suffering from severe constipation (defined as < 2 bowel

Table 2 Results of the preparation and endoscopic findings

Variable	Overall		Excluding patients with severe constipation		P value	
	A (mosapride)	B (placebo)	C (mosapride)	D (placebo)	A vs B	C vs D
No. of patients	124	125	108	120		
Time to first defecation (min, mean ± SD)	55.4 ± 27.3	71.2 ± 28.6	52.9 ± 26.2	70.4 ± 28.9	< 0.001	< 0.001
Frequency of defecation (times, median, quartile)	8.3 (4-18)	8.6 (4-18)	8.3 (4-18)	8.0 (4-18)	NS	NS
Time of bowel preparation (min, mean ± SD)	185.1 ± 63.8	198.0 ± 76.5	178.6 ± 58.2	198.0 ± 76.6	0.11	< 0.05
Elapsed time from last fluid intake to colonoscopy (min, mean ± SD)	154.6 ± 48.9	154.1 ± 48.2	157.1 ± 63.2	155.5 ± 62.1	NS	NS
Cecal intubation rate, *n* (%)	119 (96.0)	124 (99.2)	108 (100)	120 (100)	NS	NS
Insertion time[1] (min, median, quartiles)	7.8 (2-55)	8.5 (2-38)	7.4 (2-55)	8.5 (2-38)	NS	NS
Feel of peristalsis, *n* (%)	20 (16.1)	22 (17.6)	14 (13.0)	22 (18.3)	NS	NS
Amount of irrigation fluid						
None	67	65	57	62		
< 50 mL	40	47	33	46	NS	NS
50-100 mL	15	11	12	11		
> 100 mL	2	1	2	1		
Endoscopic findings						
Cancer	5	5	3	5		
Polyps	70	82	61	79	NS	NS
Diverticulosis	32	39	29	38		

P value by the Mann-Whitney *U* test. [1]Insertion time was based on patients in whom the cecal portion of the colon examined. NS: Not significant.

Table 3 Efficacy of overall colon-cleansing

Variable	Right colon		Left colon and rectum		P value	
	Mosapride	Placebo	Mosapride	Placebo	Right	Left
No. of patients	119	125	124	125		
Overall score						
Excellent	39	24	48	33	< 0.05	< 0.05
Good	34	38	37	39	NS	NS
Fair	18	21	12	10	NS	NS
Poor	3	3	2	4	NS	NS
Inadequate	25	39	25	39	0.07	0.06
Optimal ratings, *n* (%)	91 (76.5)	83 (66.4)	97 (78.2)	82 (65.6)	0.08	< 0.05

P value by the χ^2 test. NS: Not significant.

movements per week for > 1 year) were included in the mosapride group compared with the placebo group (*P* < 0.05). Therefore, we compared the efficacy, acceptability, and tolerability of the bowel preparation solution in subgroups of patients with or without severe constipation.

Bowel cleansing efficacy

As shown in Table 2, time to first defecation was significantly shorter in the mosapride group compared with the placebo group (*P* < 0.001). After excluding patients with severe constipation, the completion time for bowel preparation was significantly shorter in the mosapride group compared with the placebo group (*P* < 0.05). There were no differences in frequency of defecation, the elapsed time from last fluid intake to colonoscopy, time needed to reach the cecum, amount of irrigation fluid used, subjective difficulties in insertion to the cecum, and in observing the lumen of the colo-rectum between groups, or in the frequency of positive endoscopic findings.

The efficacy of bowel preparation is shown in Table 3.

Twenty-five (20.2%) patients required additional preparation in the mosapride group (mean 0.6 L additional PEG in 22 patients, 0.5 L enema in one patient, and both in two patients). Thirty-eight (30.4%) patients required additional preparation in the placebo group (mean 0.75 L PEG in 35 patients, 0.5 L enema in one patient, and both in two patients).

In the right colon, the number of bowel preparations rated as excellent was significantly higher in the mosapride group than in the placebo group (*P* < 0.05). However, the rate of optimal preparations did not differ significantly between groups (*P* = 0.08) (Table 3). After excluding patients with severe constipation, there were significant differences in the number of bowel preparations rated as excellent and the rate of optimal preparation in the mosapride group (*P* < 0.01 and *P* < 0.05 in the right colon and *P* < 0.05 and *P* < 0.01 in the left colon, respectively) (Table 4). In the left colon and rectum, the number of bowel preparations rated as excellent and the rate of optimal preparation were significantly higher

Table 4 Results of colon-cleansing efficacy excluding patients with severe constipation

Variable	Right colon		Left colon and rectum		P value	
	Mosapride	Placebo	Mosapride	Placebo	Right	Left
No. of patients	104	120	108	120		
Overall score						
Excellent	37	24	46	33	< 0.01	< 0.05
Good	31	37	33	38	NS	NS
Fair	16	20	10	9	NS	NS
Poor	2	3	1	4	NS	NS
Inadequate	18	36	18	36	< 0.05	< 0.05
Optimal ratings, *n* (%)	84 (80.8)	81 (67.5)	89 (82.4)	80 (66.7)	< 0.05	< 0.01

P value by the χ^2 test. NS: Not significant.

Table 5 Results of patient questionnaire *n* (%)

Variable	Overall		Excluding patients with severe constipation		P value	
	A (mosapride)	B (placebo)	C (mosapride)	D (placebo)	A *vs* B	C *vs* D
No. of patients	124	125	108	120		
Compliance > 80%	120 (96.8)	119 (95.2)	105 (97.2)	114 (95.0)	NS	NS
100% intake	112 (90.3)	115 (92.0)	97 (89.8)	110 (91.7)	NS	NS
Any symptom						
Nausea	5 (4.0)	6 (4.8)	3 (2.7)	4 (3.3)	NS	NS
Vomiting	0	1 (0.8)	0	0	NS	NS
Distension	40 (32.3)	31 (24.8)	31 (28.7)	28 (23.3)	NS	NS
Abdominal pain	4 (3.2)	2 (1.6)	3 (2.8)	2 (1.7)	NS	NS
Circulatory reactions	0	0	0	0	NS	NS
Willingness to repeat the same regimen	77/115 (66.9)	82/112 (73.2)	67/100 (67.0)	79/108 (73.1)	NS	NS
How easy/difficult to take preparation compared with previous one (easy/invariable/difficult)	34/42/10	24/67/7	32/37/8	23/66/5	< 0.05	< 0.05

P value by the χ^2 test. NS: Not significant.

in the mosapride group than in the placebo group ($P < 0.05$ and $P < 0.05$, respectively). These significant differences were maintained even after excluding patients with severe constipation ($P < 0.05$ and $P < 0.05$, respectively).

Patient tolerability and safety
There were no significant differences in compliance, as defined by > 80% and 100% intake of the PEG solution between the two groups (Table 5). Frequencies of symptoms such as nausea, vomiting, distension, abdominal pain, and circulatory reactions were similar in both groups. The proportion of patients who were willing to repeat the same preparation regimen was also similar in the two groups. However, among the subgroup of patients who had undergone a colonoscopy more than twice in the past, a significantly higher number of patients in the mosapride group felt that the current preparation was easier compared with patients in the placebo group ($P < 0.05$). This significant difference was maintained even after excluding patients with severe constipation ($P < 0.05$).

DISCUSSION

This is the first prospective, randomized, double-blind, placebo-controlled study to evaluate the efficacy, accept-

ability, and tolerance of mosapride as an adjuvant to PEG-electrolyte solution for colonoscopy preparation. Mosapride is a benzisoxazole derivative prokinetic drug that is used for the treatment of gastrointestinal symptoms associated with chronic gastritis and functional dyspepsia[16,17,25-28]. It facilitates acetylcholine release from the enteric cholinergic neurons by its selective 5-HT4 receptor agonistic action[26]. It is also active through its main metabolite M1, which is a 5-HT3 agonist. The action of mosapride resembles that of a previously used 5-HT4 agonist, cisapride, which had been reported to be useful for bowel preparation[29,30]. Cisapride had additional effects of blocking K channels and D2 dopaminergic receptors and was withdrawn after its K channel blocking properties led to reports of QT interval prolongation and cardiac arrhythmias. In contrast to cisapride, mosapride does not block K channels or D2 dopaminergic receptors and is believed to have less cardiac toxicity[31].

5-HT4 receptors are present in the myenteric plexus and the muscle of stomach and colon, and mosapride has high affinity for these receptors[20,29,32]. In human studies, mosapride has been found effective for slow transit constipation, outlet obstruction-type constipation, constipation in Parkinson's disease, and constipation associated with irritable bowel syndrome[33-35]. Recently, in guinea

pigs, it was reported that mosapride enhanced the colon cleansing action of PEG *via* an increase in colonic transit, reducing not only fecal residue but also excessive fluid in the colonic lumen[36]. However, it is unclear whether mosapride would have additive beneficial effects on bowel cleansing before colonoscopy in humans.

We found that the rate of optimal preparation was significantly higher in the mosapride group compared with the placebo group in the left colon and rectum, but not in the right colon. The number of patients with bowel preparations rated as excellent was significantly higher for the mosapride group compared with the placebo group, especially for the right colon. Kim *et al*[37] have also described this differential efficacy of mosapride between the right and left colon in guinea pigs, and ascribed this finding to the differential distribution of colonic 5-HT4 receptors. Although the rates of optimal preparation were not significantly different in the right colon, the number of bowel preparations rated as excellent was significantly higher with the use of mosapride. Furthermore, after excluding patients with severe constipation, the rate of optimal preparation was significantly higher in the mosapride group compared with the placebo group. These findings support the efficacy of mosapride for bowel preparation.

In this study, many patients required additional bowel preparation. One possible reason may be that the nursing staff checked excretions 1 h after finishing the preparation, which may have been too short an interval for the PEG solution to adequately cleanse the colon. However, the rate of inadequate cleansing was significantly lower in the mosapride group compared with the placebo group. Furthermore, the time to first defecation was also significantly shorter in the mosapride group. The beneficial effect of mosapride on gastric emptying was expected to ameliorate nausea, vomiting, and fullness of the abdomen during bowel preparation. Mishima *et al*[22] showed that administration of mosapride prior to PEG solution significantly decreased the incidence of uncomfortable abdominal symptoms. However, there were no significant differences in the frequencies of these symptoms between the mosapride group and the placebo group in this study, and more patients were willing to repeat the same preparation regimen in the placebo group. This finding may be due to more abdominal distension and pain in the mosapride group due to its prokinetic effects. According to a postmarketing surveillance study, the most common adverse events associated with mosapride are abdominal pain and loose stools (both 0.35%)[38]. On the other hand, a larger proportion of patients in the mosapride group than in the placebo group felt that the preparation was easier to complete. These findings may support the efficacy of mosapride in terms of patient tolerance and acceptability. It is possible that 2 L of PEG solution is so large that these symptoms are unavoidable; in addition, it is also possible that the dose of mosapride was not sufficient to alleviate these symptoms.

Co-administration of laxatives such as sennoside and bisacodyl with lavage solution has been shown to improve colonic cleansing during colonoscopy. Addition of these adjunctive therapies has also allowed for lower volume PEG solutions to be administered with equivalent or increased efficacy. However, the adjunctive therapies have to be taken the day before the procedure. This may lead to sleep disturbances and inconvenience due to frequent defecation. If it is possible to begin the bowel preparation using mosapride on the same day as the colonoscopy, patient tolerability may improve.

Recently, it has been suggested that co-administration of mosapride and PEG-electrolyte solution is useful in preparing the colon for barium enema examination as it allows good evacuation of remaining feces[39]. As a result, mosapride is now approved in Japan for preparation for a barium enema examination, and a total dose of 40 mg mosapride is used. Major side effects have not been reported in Japan with this dose. In the present study, we administered 15 mg of mosapride for colonoscopy preparation, which is the recommended usual daily dosage of mosapride for adult patients with chronic gastritis. However because the effects of mosapride are reported to be dose-dependent[35,40], additional studies that address optimal dosage and timing of administration are required to clarify the best regimen for colonoscopy.

One of the limitations of this study was that there was a significant difference in the number of patients with severe constipation between the two groups. However, even with the inclusion of patients with severe constipation, there was a non-significant trend for improved preparation in the mosapride group, and this difference became significant after the exclusion of this subgroup. It is possible that for patients with severe constipation, the dose of 15 mg mosapride may be insufficient. The second limitation of this study was its single center location. Finally, we did not evaluate laboratory abnormalities, as co-administration of mosapride and PEG-electrolyte solution is already common in Japan for preparation for barium enema examination[39]. No serious laboratory abnormalities have been reported in Japan with a 40-mg dose of mosapride.

In conclusion, we demonstrated that co-administration of mosapride with PEG-electrolyte solution improves the quality of bowel preparation for colonoscopy in the left colon. Mosapride may be an effective and safe adjunct to PEG that leads to improved quality of bowel preparation, especially in patients without severe constipation.

COMMENTS

Background
Although prokinetics have been used in combination with polyethylene glycol (PEG)-electrolyte solution to improve patient acceptability and tolerance, as well as improve bowel cleansing, the efficacy and safety of these agents remain unproven. Prokinetics such as domperidone, metoclopramide, and cisapride have been used in combination with PEG-electrolyte solution to improve the quality of bowel preparation. However, the addition of prokinetic agents to PEG-electrolyte solution has not been proven to improve patient tolerance or colonic cleansing and can be sometimes associated with serious adverse effects. Thus, safer and more effective prokinetic agents are needed.

Research frontiers

Mosapride citrate (mosapride) is a selective 5-hydroxytryptamine-4 (5-HT4) receptor agonist. Mosapride enhances gastric emptying and motility by facilitating acetylcholine release from the enteric cholinergic neurons, without blocking dopaminergic D2 receptors. It is known to be effective in gastroesophageal reflex disease, functional gastrointestinal disorders, such as functional dyspepsia, chronic gastritis with delayed gastric emptying, and diabetic gastroparesis. As 5-HT4 receptors are also located in the human colon and rectum, mosapride is also expected to have a prokinetic effect on the colo-rectum. A few clinical studies have reported that mosapride in combination with PEG may enhance bowel cleansing and improve patient acceptability and tolerability.

Innovations and breakthroughs

This is the first prospective, randomized, double-blind, placebo-controlled study to evaluate the efficacy, acceptability, and tolerance of mosapride as an adjuvant in PEG-electrolyte solution for colonoscopy preparation. This study demonstrated that co-administration of mosapride with PEG-electrolyte solution improves the quality of bowel preparation for colonoscopy in the left colon.

Applications

The study results suggest that mosapride may be an effective and safe adjunct to PEG, leading to an improved quality of bowel preparation, especially in patients without severe constipation. Additional studies that address optimal dosage and timing of administration are required to clarify the best bowel preparation method for colonoscopy.

Terminology

PEG-electrolyte solution: PEG-electrolyte solution is used worldwide for bowel cleansing. Approximately 2 L of this oral solution with some laxatives is usually required for adequate bowel preparation in Japan. However, the need to drink such large volumes of liquid with an unpalatable taste has a negative impact on patient compliance.

Peer review

This is an interesting and well written study. The methodology and evaluation of data is correct. The conclusion sounds good and useful for the general practice.

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BRIEF ARTICLE

Endoscopic ultrasound-guided biliary drainage with placement of a fully covered metal stent for malignant biliary obstruction

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Abstract

AIM: To determine the utility of endoscopic ultrasound-guided biliary drainage (EUS-BD) with a fully covered self-expandable metal stent for managing malignant biliary stricture.

METHODS: We collected data from 13 patients who presented with malignant biliary obstruction and underwent EUS-BD with a nitinol fully covered self-expandable metal stent when endoscopic retrograde cholangiopancreatography (ERCP) fails. EUS-guided choledochoduodenostomy (EUS-CD) and EUS-guided hepaticogastrostomy (EUS-HG) was performed in 9 patients and 4 patients, respectively.

RESULTS: The technical and functional success rate was 92.3% (12/13) and 91.7% (11/12), respectively. Using an intrahepatic approach (EUS-HG, $n = 4$), there was mild peritonitis ($n = 1$) and migration of the metal stent to the stomach ($n = 1$). With an extrahepatic approach (EUS-CD, $n = 10$), there was pneumoperitoneum ($n = 2$), migration ($n = 2$), and mild peritonitis ($n = 1$). All patients were managed conservatively with antibiotics. During follow-up (range, 1-12 mo), there was re-intervention (4/13 cases, 30.7%) necessitated by stent migration ($n = 2$) and stent occlusion ($n = 2$).

CONCLUSION: EUS-BD with a nitinol fully covered self-expandable metal stent may be a feasible and effective treatment option in patients with malignant biliary obstruction when ERCP fails.

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP)

is a well-established technique for providing biliary decompression in patients with bile duct obstruction. The success rate for effective biliary decompression with ERCP ranges from 90% to 95%[1]. However, there are patients in whom ERCP fails because of unsuccessful biliary cannulation, or inaccessible papilla due to duodenal stenosis caused by tumor invasion. In these cases, percutaneous biliary drainage (PTBD) is required. However, PTBD can lead to significant complications, including biliary peritonitis, hemobilia, pneumothorax, hematoma, liver abscesses, and patient discomfort related to the catheter[2].

Therefore, endoscopic ultrasound-guided biliary drainage (EUS-BD) using plastic stents has been introduced as an alternative to PTBD in cases of biliary obstruction when ERCP fails[3-6]. However, plastic stent malfunction due to stent clogging after EUS-BD is not uncommon[3,7,8]. Fully covered self-expandable metal stent (FC-SEMS) with a large bore diameter may have advantages over plastic stents. Recently, EUS-BD with a FCSEMS was introduced in a few cases of malignant biliary obstruction after a failed ERCP[9]. The current study was conducted to determine the feasibility, outcomes, and risks of EUS-guided hepaticogastrostomy (EUS-HG) and EUS-guided choledochoduodenostomy (EUS-CD) with an FCSEMS as a biliary diversion technique in patients with malignant biliary obstruction for whom interventional ERCP was unsuccessful.

MATERIALS AND METHODS

Study population
We collected data on all patients who presented with obstructive jaundice and who underwent EUS-BD after a failed ERCP during a 20-mo period from February 2009 to September 2010. Failed ERCP was defined as the inability to relieve jaundice or failed biliary cannulation. A total of 2209 ERCPs at Wonkwang University Hospital (*n* = 780) and Jeonbuk National University Hospital (*n* = 1429) were performed during the study period, and 366 required biliary decompression. Of the 22 (6%) patients who underwent alternative methods for biliary decompression as a result of failed ERCP, 13 had EUS-BD. Study eligibility was determined as follows: (1) Initial biliary cannulation or bile duct decompression by ERCP failed because of accompanying duodenal obstruction, periampullary tumor infiltration, and difficult cannulation (*n* = 11); (2) A high-grade left-sided hilar stricture as a result of segmental tumor progression with an occluded biliary metal stent was unable to be crossed by a guidewire (*n* = 2); (3) The patient refused PTBD (*n* = 13).

ERCP was performed by 3 experienced endoscopists (Kim TH, Kim SH and Lee SO). Each endoscopist performs 400 to 450 ERCPs annually. EUS-BD was performed by 2 experienced endoscopists (Kim TH and Kim SH) who perform more than 250 EUS procedures for pancreaticobiliary diseases annually.

In this study, stent occlusion was classified as predominantly tumor ingrowth (seen on fluoroscopy as a narrowing within the stent), tumor overgrowth (seen

radiographically or endoscopically as a new narrowing at the proximal or distal margin of the stent), or sludge/debris (demonstrated as echoendoscopic findings or multiple radiographic filling defects that disappeared after extraction with a biliary balloon along with endoscopic visualization of the extracted sludge)[10]. The Institutional Review Boards at Wonkwang University Hospital and Jeonbuk National University Hospital, South Korea approved this study. All patients provided written informed consent for participation in this study.

Techniques
Antibiotics were permitted in all cases for 3 d to 5 d before and after the intervention. ERCP was initially attempted in each patient by using a therapeutic duodenoscope (TJF-240; Olympus Optical Co, Tokyo, Japan). When the ERCP was unsuccessful, an EUS was performed using a GF-UCT 240 linear-array echoendoscope (Olympus Corp., Tokyo, Japan) in a second session (on the same or next day). These patients underwent an EUS-HG or EUS-CD. Using the transgastric approach, the echoendoscope was placed in the cardia or the lesser curvature of the stomach to view the dilated segment 3 of the liver (Figure 1). Using the transduodenal approach, the echoendoscope was placed in the duodenal bulb to image the extrahepatic bile duct (Figure 2). Color Doppler ultrasonography was used to identify the regional vasculature, and a bile duct puncture was performed with a 19-gauge needle (EUSN-19-T; Cook Endoscopy, Winston-Salem, NC, United States). To confirm successful biliary access, contrast medium was injected under fluoroscopy to demonstrate biliary opacification. A 0.035-inch guidewire was introduced through the EUS needle and advanced in an antegrade or retrograde fashion. Afterwards, 6F and 7F tapered, biliary, bougie catheters (catheter tip, 4F; Cook Endoscopy) were inserted and removed, over the guidewire to dilate the tract. If there was resistance when advancing the 6F bougie catheter, a needle-knife (Microtome; Boston Scientific, Natick, MA, United States) with a 7F shaft diameter or tapered MTW catheter (WTW, Endoscopie, Wesel, Germany) was inserted over the guidewire to dilate the tract. To accomplish this, the tip of a needle-knife (Microtome) with a pure current was gently inserted over the guidewire into the biliary system. The needle was then withdrawn, and the needle-knife was pushed in to dilate the tract. A commercially available, fully silicon-covered metal stent with an 8F deployment system and an olive tip (BONASTENT, a nitinol stent with a 10-mm diameter and 6-cm length; Standard Sci Tech Inc., Seoul, South Korea) was placed under echoendoscopic and fluoroscopic view.

Definition of events
Technical success was defined as the deployment of the metal stent across the stomach or duodenum, along with the flow of contrast medium and/or bile through the stent. Functional success was defined as a reduction in bilirubin to less than 50% of the pretreatment value

Figure 1 Endoscopic ultrasound-guided hepaticogastrostomy. A: In the intrahepatic approach, the linear array echoendoscope was placed in the lesser curvature of the stomach for viewing the left intrahepatic system. A 0.035-inch guidewire was introduced through the endoscopic ultrasonography-needle and advanced in an antegrade manner; B and C: A fully covered metal stent was placed under echoendoscopic and fluoroscopic view.

Figure 2 Endoscopic ultrasound-guided choledochoduodenostomy. A: The extrahepatic approach was carried out using an echoendoscope in the duodenal bulb, permitting imaging of the choledochus. Bile duct puncture was carried out with a 19-gauge needle; B: A 0.035-inch guidewire was introduced through the endoscopic ultrasonography-needle and advanced in a retrograde fashion; C and D: A fully covered metal stent was placed under echoendoscopic and fluoroscopic view.

within 2 wk[11]. An early complication was defined as any stent-related complication within 30 d, including complications of bile leakage, pneumoperitoneum, bleeding, and stent migration. A late complication was defined as any stent-related complication, such as stent migration and stent occlusion, occurring 30 d after stent placement. Biliary reintervention was defined as any type of endoscopic, percutaneous, or surgical procedure that was required to improve biliary drainage after placement of the stent. Stent occlusion was defined as the recurrence of jaundice and cholestasis and/or evidence of a dilated biliary system on ultrasound (US) or computed tomography (CT) with a direct view of the upper endoscope, which in all cases would require biliary intervention. Procedure time was defined as the time between puncture of the biliary tract with a 19-gauge needle and placement

of an FCSEMS.

Follow-up

Follow-up continued from stent insertion until the death of the patient or to the end of the study. Biochemical parameters and simple abdominal films were assessed at 2 d, 1 wk and 1 mo after stent placement and every 2 mo thereafter. Patient follow-up was based on outpatient examination findings. Imaging (US or CT and simple abdominal films) was routinely checked every 3 mo.

Statistical analysis

Procedure time (intrahepatic and extrahepatic approaches) and liver function test before and after the EUS-BD using a metal stent were compared using a 2-sample Wilcoxon signed-rank test. Statistical analysis was carried out using

Table 1 Patients' clinical characteristics and results of endoscopic ultrasound-guided biliary drainage ($n = 13$)

No. of patient	Age/sex	Diagnosis	Reason for failed ERCP	Biliary drainage route	Device for puncture/dilatation	Diameter length of stent (mm/cm)	Technical success	Functional success	Complication	Reintervention	Duration (mo) of 1st stent placement	Status
1	60/M	CBD cancer	Duodenal stenosis due to duodenal ulcer	Transduodenal	19G FN/cystostome	10/6	Success	Success	No	No	Whipple's operation	Alive
2	83/F	CBD cancer	Failed to access the bile duct	Transduodenal	19G FN/cystostome	10/6	Success	Success	Obstruction	Stent reinsertion	6	Dead
3	76/F	CBD cancer	Failed to access the bile duct	Transduodenal	19G FN/cystostome	10/4	Success	Success	Migration	Stent reinsertion	2	Dead
4	68/F	CBD cancer	Duodenal obstruction due to mass	Transduodenal	19G FN/cystostome	10/6	Success	Success	No	No	7	Alive
5	66/M	Pancreatic cancer	Periampullary tumor infiltration	Transduodenal	19G FN/tapered ERCP cannula and cystostome	10/4	Success	Success	Pneumoperitoneum/peritonitis	No	3	Dead
6	68/M	Pancreatic cancer	Periampullary tumor infiltration	Transduodenal	19G FN/bougie dilatator	10/6	Success	Success	No	No	1	Dead
7	67/F	Pancreatic cancer	Periampullary tumor infiltration	Transduodenal	19G FN/NK and bougie dilatator	10/6	Success	Success	No	No	3	Alive
8	59/M	Pancreatic cancer	Periampullary tumor infiltration	Transduodenal	19G FN/tapered ERCP cannula and cystostome	10/6	Success	Success	No	No	4	Alive
9	80/M	Pancreatic cancer	Periampullary tumor infiltration	Transduodenal	19G FN/tapered ERCP cannula and cystostome	10/6	Success	Success	No	No	3	Alive
10	81/M	Klatskin's tumor	Stricture could not be crossed (hilar)	Transgastric	19G FN/cystostome	10/6	Success	Fail	Migration/pneumoperitoneum/peritonitis	PTBD	5	Dead
11	55/M	Intrahepatic cholangiocarcinoma	Stricture could not be crossed (hilar)	Transgastric	19G FN/cystostome	10/6	Success	Success	No	No	3	Dead
12	62/M	Pancreatic cancer	Stricture could not be crossed (distal CBD)	Transgastric	19G FN	10/6	Failed					Dead
13	70/M	CBD cancer	Stricture could not be crossed (distal CBD)	Transgastric	19G FN/bougie dilatator	10/6	Success	Success	Migration	Stent reinsertion	2	Alive

F: Female; M: Male; CBD: Common bile duct; G: Gauge; NK: Needel knife; FN: Fine needle; PTBD: Percutaneous biliary drainage; ERCP: Endoscopic retrograde cholangiopancreatography.

SPSS 12.0 (SPSS, Chicago, IL, United States), and a 2-tailed P value of < 0.05 was considered statistically significant. Findings are expressed as the median and range.

RESULTS

Patient characteristics, procedural data, and follow-up results are presented in Table 1. A total of 13 patients (9 men and 4 women) with malignant biliary obstruction underwent EUS-BD with a fully covered metal stent after a failed ERCP. The etiology of biliary obstruction was pancreatic cancer in 5 patients, distal common bile duct cancer in 6 patients, Klatskin's tumor in 1 patient, and peripheral cholangiocarcinoma in 1 patient.

Reasons for failed ERCP were duodenal stenosis due to a previous duodenal ulcer ($n = 1$), tumor obstruction of the duodenum ($n = 1$), periampullary tumor infiltration ($n = 5$),

Table 2 Summary of published studies and current study on endoscopic ultrasound-guided biliary drainage with metal stent

Study	No. of EUS-BD-metal biliary stents	Technical success %	Clinical success %	Type of metal stent	Procedure-related complications (No. cases)
Will *et al*[6]	6	100	83.3	Partially covered (3), uncovered (3)	Cholangitis (1)
Artifon *et al*[27]	1	100	100	Partially covered	None
Bories *et al*[3]	11	91	100	Partially covered metal stent (3)	Biloma (1), cholangitis (1)
Park *et al*[9]	9	100	100	Fully covered	Pneumoperitoneum (2)
Park *et al*[28]	5	100	100	Fully covered	None
Siddiqui *et al*[29]	8	100	100	Fully covered	Duodenal perforation (1)
This study	13	92.3	91.7	Fully covered	Mild peritonitis (2)

EUS-BD: Endoscopic ultrasound-guided biliary drainage.

failure to access the common bile duct (n = 2), high-grade stricture of the distal common bile duct with an occluded biliary plastic stent (n = 2), and high-grade left-sided hilar stricture from segmental tumor progression with an occluded biliary metal stent (n = 2). Patients with obstruction of the duodenum received a concomitant duodenal stent at time of the procedure.

Technical and functional success

Technical success was 92.3% (12/13); there was 1 failure of guidewire insertion after puncture through the transgastric approach, because the diameter of intrahepatic bile duct was so small and had sharp angulation. During EUS-BD, no wire passage for rendezvous was attempted in any of the patients. For biliary access, a cystostome only was used in 6 of the 12 patients (4 extrahepatic approach and 2 intrahepatic approach), a tapered MTW catheter and a cystostome together were used 3 of 12 patients, and just bougie dilatators were used in 3 patients, respectively. Median procedure time was 19.5 min (range, 14-35 min). Nine patients were treated using an extrahepatic approach (all transduodenal). Four patients were treated using an intrahepatic approach (all transgastric). The median diameter of the left intrahepatic bile duct as determined by EUS was 7.3 mm (range, 5.3-9.4 mm).

Functional success was 91.7% (11/12). After the placement of a EUS-BD, the median bilirubin level decreased significantly from 11.6 mg/dL to 1.7 mg/dL (P = 0.001). The median alkaline phosphatase level also decreased significantly from 1629.0 IU/L to 113.0 IU/L (P = 0.001). Migration of a metal stent to stomach occurred in 1 patient treated with the transgastric approach.

Early complications after endoscopic ultrasound-guided biliary drainage

With the intrahepatic approach (n = 4), there was stent migration to the stomach with mild peritonitis (n = 1). With the extrahepatic approach (n = 9), there was pneumoperitoneum with mild peritonitis (n = 1). All patients with peritonitis were managed conservatively with antibiotics. Bleeding and cholangitis were not observed in any of the enrolled patients after the procedure. In addition, there was no significant pain after the procedure. Two patients with duodenal obstruction also received duodenal metal stents.

Follow-up

One patient with distal common bile duct (CBD) cancer

underwent curative resection (Whipple's operation), and this procedure did not disturb the operative procedure. None of the patients, except the patients that underwent the operation, were lost during the follow-up period (median, 5 mo; range, 1-12 mo). With regard to re-intervention, there was re-intervention in 4 of the 13 cases (30.7%), which was necessitated by stent migration (n = 3) and stent occlusion (n = 1). Stent migration occurred 2 d later (EUS-HG) and 2 mo later (EUS-CD and EUS-HG), respectively. In the patients with stent migration, the stents passed spontaneously without lodgment in the bowel. A second FCSEMS was placed through the previous choledochoduodenostomy site in 1 patient. In 2 patients with stent migration, PTBD was inserted into the obstructed left hepatic duct due to his severe general weakness. In 1 patient with stent occlusion by tumor ingrowth into the metal stent (choledochoduodenostomy), an additional FCSEMS was placed through the previous choledochoduodenostomy site.

DISCUSSION

EUS-BD may be an alternative method in some patients when endoscopic biliary drainage is unsuccessful because of failed biliary cannulation or tumor infiltration, which limits the endoscopic approach to the major papilla. At present, there are 2 approaches for EUS-BD, the transgastric route and the transduodenal route. A plastic or metal stent can be placed by this method. However, most papers reported EUS-BD with a plastic stent[4,5,7,8,12-14]. We thought that FCMEMS with a large bore diameter may have advantages over the plastic stent. There are a few papers describing EUS-BD with a metal stent[6,9,15] (Table 2), however, there are no reports regarding preoperative drainage of biliary obstruction by EUS-BD. The current study demonstrated that transduodenal EUS-BD using a nitinol FCSEMS may be safe and feasible after a failed ERCP. The reasons for failed ERCP were duodenal stenosis by a duodenal ulcer, periampullary tumor invasion, and difficult biliary cannulation. In one patient with distal CBD cancer who had duodenal stenosis due to a previous duodenal ulcer, initially EUS-BD was performed to resolve cholangitis and bile drainage, and Whipple's operation was performed. But EUS-guided metal insertion in this patient did not disturb the operation procedure.

A significant number of self-expandable metal stent

(SEMS) and plastic stents placed to palliate malignant obstructions will be occluded. Repeat transpapillary stent placement may be unsuccessful for an occluded biliary metal or plastic stent after the placement of a hilar metal stent or combined duodenal and biliary metal stent placement. In such circumstances, PTBD may be uncomfortable for patients and has a 10%-30% complication rate, with complications such as cholangitis, bile leak, peritonitis, and stent occlusion[16]. EUS-HG with a FCSEMS may be recommended in such circumstances. In our 2 patients with a hilar metal stent, dilation of the left main duct caused by segmental tumor progression was shown to be aggravated. In another 2 patients with periampullary cancer drained by a pigtail plastic stent, this stent was occluded by tumor ingrowth and bile plug. Because these patients had jaundice and cholangitis, EUS-HG with a FCSEMS was performed. Some experts have recommended the intrahepatic approach because it seems to be safer[7,15]. In the intrahepatic approach, one-step placement of a partially covered wall stent may have been limited in earlier studies because there was 1 bile peritonitis and 1 cholangitis. However, we found the transduodenal or extrahepatic approach to be safer and more effective and it is probably less technically difficult compared with the intrahepatic approach. The advantage is that the duodenum is very close to the extrahepatic bile duct and the duodenal wall is thin without major vascular structures.

There were complications with EUS-BD, including stent migration, pneumoperito-neum, and cholangitis[17,18]. In this study, during follow-up (median, 3 mo), there was re-intervention (4/12, 33.3%) necessitated by stent migration ($n = 3$) and stent occlusion ($n = 1$). The 3 stent migrations occurred 2 d later (EUS-HG), 2 mo later (EUS-CD), and 2 mo later (EUS-CD). The stent migration rate (3/12, 25.0%) was higher than that reported (7%) in another paper[9]. Although a flare at both ends and minimal shortening of this newly designed nitinol stent may contribute to the prevention of stent migration[19], a slippery covered metal stent with low axial force may lead to migration. In addition, marked CBD dilatation may have affected FCSEMS floating resulting in the minimization of the anchoring effect of the proximal flared end of the FCSEMS.

Studies with uncovered SEMS reported stent occlusion rates ranging between 18% and 46%[20,21] with the main cause of obstruction being tumor ingrowth. Although partially or fully covered SEMS were designed to prevent this complication, these stents can not completely protect tumor ingrowth in the biliary metal stent[22]. Occasional authors have reported no tumor ingrowth with covered SEMSs. However, these series often include few patients and frequently have a relatively high prevalence of tumor overgrowth or sludge formation as a cause of stent failure[23-25]. Our study shows tumor overgrowth was observed in one case with distal CBD cancer.

During the EUS-BD procedure, the leakage of bile into the peritoneum with peritonitis can occur because the gap between the stent and the fistula is likely to occur. The shortening of metal stent can lead to failure of correct stent placement, and stent migration and dislocation after placement. Experience with partially coverd SEMS is lim-

ited and associated with a higher complication rates due to stent shortening[3,6]. To prevent bile leakage, we placed a FCSEMS that is made of nitinol. This stent, with both ends flared, was designed to prevent distal or proximal migration. The nitinol stent has less shortening compared with a partially covered Wall stent, and a fully covered metal stent may prevent bile leakage[19]. Therefore, due to the characteristics of this stent, it may prevent bile peritonitis. Because the diameter of the working channel of the linear array echoendoscope is only 3.7 mm, it is possible to quickly perform one-step placement of FCSEMS with an 8F-diameter delivery device. In this study, although bile leakage occurred during the procedure, all patients had only mild peritonitis and were treated with conservative management, such as antibiotics and nothing by mouth.

If the gap between the bile duct and the GI tract grows farther after EUS guided biliary drainage with stent, early migration of stent into the abdominal cavity may occur, particularly in the case of a transgastric approach. A fatal complication due to sepsis after development of large fistula related to stent migration into the abdominal cavity has recently been reported[26]. However, bile leakage *via* the relatively large fistula can make bile peritonitis and the bile duct collapse. Therefore, it may be difficult or impossible to carry out EUS-BD in these patients. In such a case, PTBD or emergency surgery must also be considered. To prevent severe complications, an endoscopist must have considerable knowledge about periduodenal and hepatobiliary anatomy in linear array EUS. Also, the procedure must be performed quickly and efficiently with appropriately sized stents. When selecting a stent, stent structure, axial force and shortening of stent should be considered.

In conclusion, EUS-guided transgastric or transduodenal biliary drainage with one-step placement of an FCSEMS may be a promising alternative method to PTBD. It may be a reasonable, feasible, and promising minimally invasive endoscopic approach in selected patients as indicated as above. However, several complications can occur, and special attention of the potential complications such as peritonitis and migration are needed. Therefore, additional experience and the development of new comfortable accessories for this procedure are needed. Also, a large case series and prospective trials are needed to further assess this technique.

COMMENTS

Background
For palliation of patients with malignant biliary obstruction, percutaneous biliary drainage (PTBD) is a classic method in some patients when endoscopic biliary drainage is unsuccessful because of failed biliary cannulation or tumor infiltration, which limits the endoscopic approach to the major papilla. Recently endoscopic ultrasonography-guided biliary drainage (EUS-BD) with plastic stent or uncovered self-expandable metal stent (SEMS) has been introduced as an alternative to PTBD in these cases of biliary obstruction.

Research frontiers
The authors thought that a nitinol fully covered SEMS (FCSEMS) with a large bore diameter may have advantages over the plastic stent and other types of metal stent.

Innovations and breakthroughs
Technical and functional success with FCSEMS for palliation of patients with malignant biliary obstruction was respectively 92.3% (12/13) and 91.7% (11/12). Although there was early mild complication such as one case of immediate

stent migration and 2 cases of bile peritonitis, all complications improved with conservative treatment.

Applications

EUS-guided transgastric or transduodenal biliary drainage with one-step placement of a nitinol FCSEMS may be a promising alternative method to PTBD. Additional experience and the development of new comfortable accessories for this procedure are needed. Also, a large case series and prospective trials are needed to further assess this technique.

Terminology

There are 2 approaches for EUS-BD, the transgastric route and the transduodenal route. EUS-guided hepaticogastrostomy was performed through the transgastric route, and EUS-guided choledochoduodenostomy through the transduodenal route.

Peer review

The authors reported high success rate with this challenging procedure. They clearly demonstrated that extrahepatic approach is easier and safer than intrahepatic approach, Although technical expertise is important, the authors demonstrated more than 90% technical success with low major complication rates. This paper motivates talented endoscopists to solve biliary obstruction *via* EUS guided technics.

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BRIEF ARTICLE

Concomitant lung metastasis in patients with advanced hepatocellular carcinoma

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Abstract

AIM: To investigate the clinical features and prognostic factors of advanced hepatocellular carcinoma (HCC) patients presenting with lung metastasis at initial diagnosis.

METHODS: Between 2001 and 2010, we recruited 76 consecutive HCC patients initially presenting with lung metastasis, without co-existing metastasis from other sites. These patients were divided into three groups: untreated group (n = 22), single treatment group (n = 19), and combined treatment group (n = 35).

RESULTS: Metastasis of bilateral lung lobes was common and noted in 35 patients (46.1%), and most of patients (59/76, 77.6%) presented with multiple lung metastatic nodules. Nineteen patients (25.0%) received single-method treatment, including hepatectomy in 4, transcatheter arterial chemoembolization in 6, radiotherapy in 5, and oral sorafenib in 4. Thirty-five patients (46.1%) received combined treatment modalities. The overall median survival of the all patients was 8.7 ± 0.6 mo; 4.1 ± 0.3, 6.3 ± 2.5 and 18.6 ± 3.9 mo, respectively in the untreated group, single treatment group and combined treatment group, respectively, with a significant difference (log-rank test, $P < 0.001$). Multivariate analysis revealed that Child-Pugh score, the absence or presence of portal vein tumor thrombus, and treatment modality were three independent prognostic factors affecting survival of patients with advanced HCC and concomitant lung metastasis.

CONCLUSION: Combined treatment modalities tend to result in a better survival as compared with the conservative treatment or single treatment modality for HCC patients initially presenting with lung metastasis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. The number of new cases is estimated to be 500 000-1 000 000 per year[1]. Overall, 80% of HCC are attributed to chronic hepatitis B and C infection. Surgical resection with complete extirpation of tumor gives the best chance of a cure for patients with HCC[2]. However, a majority of HCC patients are considered as advanced or even at end-stage at their first hospital visit, with extensive tumor status, for example, macroscopic vascular invasion, extrahepatic metastasis, *etc*. Of various metastatic sites, the most common site is lung, followed by lymph node, bone and brain[3].

As one type of advanced HCC, HCC presenting with lung metastasis is not unusual, and its prognosis is very poor[4]. Nowadays, there are various treatment modalities for both intrahepatic tumor and extrahepatic metastatic foci of HCC, including surgical resection, transcatheter arterial chemoembolization (TACE), radiotherapy, chemotherapeutics, and recent molecular targeted therapeutic drugs[2]. However, the prognosis and treatment outcomes of advanced HCC presenting with lung metastasis remain poorly evaluated. We investigated the prognosis, treatment outcomes, and independent prognostic factors affecting the survival of a series of HCC patients presenting with lung metastasis at initial diagnosis.

MATERIALS AND METHODS

Patients

From January 2001 to December 2010, 103 consecutive patients were diagnosed as having lung metastasis at the first time of HCC diagnosis in the 5th Department of Hepatobiliary Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. To evaluate the efficacy of anti-tumor treatment modality for patients with HCC and concomitant isolated lung metastasis, we excluded those patients who showed evidence of severe organ failure (renal, respiratory, or cardiologic problems), poor liver function (Child-Pugh class C) that could affect survival, or co-existing metastasis from other sites. Therefore, 76 of the 103 patients (59 males and 17 females) were recruited for the study. The last follow-up date was January 30, 2011. This study protocol was approved by the Institutional Review Board of the Eastern Hepatobiliary Hospital.

We used a prospectively maintained database and conducted a retrospective study among these patients. We focused on the prognosis and prognostic factors affecting the survival of HCC patients presenting with lung metastasis. To investigate whether local regional or systemic therapy could affect survival, we stratified these 76 patients into three groups: untreated group (*n* = 22), single treatment group (*n* = 19), and combined treatment group (*n* = 35). All of the patients in the untreated group refused any anti-tumor invasive treatment other than conservative treatment, including the support of liver function, after being informed of the expenses and the possible side effects of any anti-tumor invasive treatment. The single treatment method for these patients included hepatectomy, TACE, radiotherapy, chemotherapeutics, and oral sorafenib, while combined treatment modalities were defined as more than one of the above treatment methods or pulmonary metastasectomy.

Laboratory tests

Laboratory blood tests including hepatitis B virus (HBV) markers, anti-hepatitis C virus, serum α-fetoprotein (AFP), carcinoembryonic antigen, platelet, serum albumin, serum total bilirubin, alanine transaminase, aspartate aminotransferase, and prothrombin time were performed.

Diagnosis of HCC and lung metastasis

The diagnosis of HCC was based on concordance between two imaging examinations [ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI)] showing arterial hypervascularity in a focal lesion $\geqslant$ 2 cm or with the combined criteria of an imaging examination and a serum AFP level greater than 400 ng/mL, according to the criteria of the Conference of the European Association for Study of the Liver[5]. In the present study, the diagnosis of HCC was also confirmed by pathohistology in 20 patients.

Lung metastases were diagnosed in all the 76 patients using imaging techniques, including chest CT (*n* = 62), positron emission tomography-CT (*n* = 11), and chest MRI (*n* = 3). In addition, one patient was further confirmed by pathohistology after a biopsy, and four patients after pulmonary metastasectomy.

Statistical analysis

Continuous data were expressed as mean $\pm$ SD or median (range). Categorical variables were compared by the χ^2 test or Fisher exact test, and continuous variables were compared by the Student *t* test or one-way analysis of variance. The survival rate was calculated using the Kaplan-Meier method, and the log-rank test was used to compare survival rates among three groups. Cox's proportional hazards model was used for multivariate analysis. All statistical analysis in this study were done using software package SPSS11.0 (SPSS Inc., Chicago, IL). A *P* value < 0.05 was defined to be statistically significant.

RESULTS

Patient characteristics

The baseline characteristics of these 76 patients with advanced HCC initially presenting with lung metastasis are summarized in Table 1. The mean age of patients was 52.4 $\pm$ 10.7 years. The most common etiology of the liver disease was HBV infection, which occurred in 63 patients (82.9%). There was no statistical difference in the baseline characteristics among the untreated, single treatment and combined treatment groups, including age, etiology of the liver disease, the presence of cirrhosis or ascites, AFP level, Eastern Cooperative Oncology Group (ECOG) scale, Child-Pugh score, and liver function.

Table 1 Baseline characteristics of hepatocellular carcinoma patients presenting with lung metastasis (mean ± SD) *n* (%)

	Total (*n* = 76)	Untreated group (*n* = 22)	Single treated group (*n* = 19)	Combined treated group (*n* = 35)	*P* value
Sex					
Male	59 (77.6)	16 (72.7)	15 (78.9)	28 (80.0)	0.804
Female	17 (22.4)	6 (27.3)	4 (21.1)	7 (20.0)	
Age (yr)	52.4 ± 10.7	52.8 ± 9.3	52.5 ± 11.6	52.1 ± 11.4	0.971
Etiology					
HBV	63 (82.9)	18 (82.9)	16 (84.2)	29 (82.9)	0.989
HCV	3 (3.9)	1 (4.5)	1 (5.3)	1 (2.9)	
Alcohol	5 (6.6)	1 (4.5)	1 (5.3)	3 (8.6)	
Non-B and non-C	5 (6.6)	2 (9.1)	1 (5.3)	2 (5.7)	
Cirrhosis	65 (85.5)	20 (90.9)	17 (89.5)	28 (80.0)	0.443
Platelet ($^{-3}$)	185.8 ± 75.5	180.9 ± 95.1	174.3 ± 74.0	195.1 ± 62.6	0.593
Albumin (g/L)	36.5 ± 4.3	36.3 ± 5.4	35.3 ± 3.5	37.3 ± 3.7	0.276
ALT (IU/L), median (range)	35 (10-352)	35 (15-352)	37 (15-124)	34 (10-210)	0.456
AST (IU/L), median (range)	35 (16-453)	41 (16-453)	44 (20-173)	33 (17-223)	0.364
Total bilirubin (μmol/L)	23.7 ± 13.3	28.1 ± 17.4	23.3 ± 8.1	21.2 ± 12.2	0.162
Prothrombin time (s)	12.9 ± 1.7	13.3 ± 2.3	13.0 ± 1.3	12.5 ± 1.3	0.241
AFP (ng/mL)					
< 400	36 (47.4)	10 (45.5)	7 (36.8)	19 (54.3)	0.461
≥ 400	40 (52.6)	12 (54.5)	12 (63.2)	16 (52.6)	
ECOG score					
0	17 (22.4)	4 (18.2)	4 (21.1)	9 (25.7)	0.672
1	54 (71.1)	16 (72.7)	15 (78.9)	23 (65.7)	
2	5 (6.6)	2 (9.1)	0 (0)	3 (8.6)	
Ascites					
None	67 (88.2)	18 (81.8)	16 (84.2)	33 (94.3)	0.303
Mild-moderate	9 (11.8)	4 (18.2)	3 (15.8)	2 (5.7)	
Child-Pugh score					
A	61 (80.3)	16 (72.7)	14 (73.7)	31 (88.6)	0.243
B	15 (19.7)	6 (27.3)	5 (26.3)	4 (11.4)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; ALT: Alanine transaminase; AST: Aspartate aminotransferase; AFP: Alpha-fetoprotein; ECOG: Eastern cooperative oncology group.

Characteristics of intrahepatic tumor and lung metastasis

For intrahepatic tumor status, there was no significant difference in tumor number, tumor location, and the probability of portal vein tumor thrombus or hepatic vein tumor thrombus among the three treatment groups, except for maximum tumor diameter (*P* = 0.007) (Table 2). As such, there is also no significant difference in number, location, and maximum diameter of lung metastatic nodules among these three groups (Table 2).

Treatment modalities

Among all the 76 patients, 22 patients did not receive any anti-tumor treatment but conservative treatment. We divided the remaining 54 patients into two groups according to their treatment schemes. Nineteen patients (25.0%) received single treatment modality (locoregional or systemic therapy), including hepatectomy in 4 patients, TACE in 6, radiotherapy for intrahepatic tumor and lung metastatic nodules in 5 (including γ knife radiosurgery in 3 and X-ray radiotherapy in 2), and oral sorafenib in 4. Thirty-five patients (46.1%) received combined treatment modalities (Table 3). The common treatment modality in the combined treatment group was radiotherapy (30/35, 85.7%), followed by hepatectomy (23/35, 65.7%), TACE (18/35, 51.4%), and oral sorafenib therapy (11/35, 31.4%).

In the combined treatment group, radiotherapy (γ knife radiosurgery in 24 and X-ray radiotherapy in 4) was mainly used for lung metastatic nodules (27/28) rather than intrahepatic tumor nodules (8/28). In addition, pulmonary metastasectomies were carried out in 4 patients who underwent hepatectomy during the same operation.

Overall survival

After a median follow-up of 8.7 ± 0.6 mo (range, 1.1-68.7 mo), 59 patients died and 17 patients remained alive. The causes of mortality were disease progression, including hepatic failure in 31 patients (52.5%); cachexia in 12 (20.3%); upper gastrointestinal bleeding in four (6.8%); pneumonia in one (1.7%); and pulmonary thromboembolism in one (1.7%). The cause of mortality of 10 patients was not confirmed. Among 17 surviving patients, only 4 patients were disease-free, including 3 patients who underwent hepatectomy and pulmonary metastasectomy, and one patient who received hepatectomy and subsequent γ-knife radiosurgery. Among 13 patients who were not disease-free but alive, 6 patients still took medicine of sorafenib, and achieved complete or partial response on radiographic examination (Figure 1A and B). The 6-mo, 1-year and 2-year cumulative survival rates of all these patients were 64.5%, 40.1% and 18.5%, respectively.

Table 2 Characteristics of intrahepatic tumor and lung metastasis of hepatocellular carcinoma patients presenting with lung metastasis (mean ± SD) *n* (%)

	Total (*n* = 76)	Untreated group (*n* = 22)	Single treated group (*n* = 19)	Combined treated group (*n* = 35)	*P* value
Intrahepatic tumor					
Maximum tumor diameter (cm)	9.3 ± 3.0	10.7 ± 3.2	9.7 ± 2.8	8.2 ± 2.5	0.007
Tumor number					
Solitary	29 (38.2)	9 (40.9)	6 (31.6)	14 (40.0)	0.791
Multiple/diffuse	47 (61.8)	13 (59.1)	13 (68.4)	21 (60.0)	
Tumor location					
Right	39 (51.3)	13 (59.1)	8 (42.1)	18 (51.4)	0.834
Left	15 (19.7)	4 (18.2)	4 (21.1)	7 (20.0)	
Both	22 (28.9)	5 (22.7)	7 (36.8)	10 (28.6)	
Portal vein tumor thrombus					
Absence	54 (71.1)	14 (63.6)	12 (63.2)	28 (80.0)	0.283
Presence	22 (28.9)	8 (36.4)	7 (36.8)	7 (20.0)	
Hepatic vein tumor thrombus					
Absence	67 (88.2)	19 (86.4)	15 (78.9)	33 (94.2)	0.238
Presence	9 (11.8)	3 (13.6)	4 (21.1)	2 (5.7)	
Lung metastasis					
Number of metastasis					
Solitary	17 (22.4)	4 (18.2)	3 (15.8)	10 (28.6)	0.479
Multiple	59 (77.6)	18 (81.8)	16 (84.2)	25 (71.4)	
Location of metastasis					
Right lung lobe	28 (36.8)	6 (27.3)	8 (42.1)	14 (40.0)	0.721
Left lung lobe	13 (17.1)	4 (18.2)	2 (10.5)	7 (20.0)	
Bialteral lung lobes	35 (46.1)	12 (54.5)	9 (47.4)	14 (40.0)	
Maximum metastasis diameter (cm)	2.5 ± 0.9	2.6 ± 0.9	2.2 ± 1.2	2.5 ± 0.8	0.445

Table 3 Treatment modalities for intrahepatic tumor and/or metastatic lung nodule(s)

Treatment modalities	*n* (%)
Single treatment modality in treated patients (*n* = 19)	
Hepatectomy	4 (21.1)
Pulmonary metastasectomy	0 (0)
Transcatheter arterial chemoembolization	6 (31.6)
Radiotherapy	5 (26.3)
Oral sorafenib	4 (21.1)
Combined treatment modalities in treated patients (*n* = 35)	
Hepatectomy + pulmonary metastasectomy	3 (8.6)
Hepatectomy + pulmonary metastasectomy + oral sorafenib	1 (2.9)
Hepatectomy + transcatheter arterial chemoembolization + radiotherapy	6 (17.1)
Hepatectomy + radiotherapy	9 (25.7)
Hepatectomy + transcatheter arterial chemoembolization + radiotherapy + oral sorafenib	2 (5.7)
Hepatectomy + radiotherapy + oral sorafenib	2 (5.7)
Transcatheter arterial chemoembolization + radiotherapy	6 (17.1)
Transcatheter arterial chemoembolization + radiotherapy + oral sorafenib	3 (8.6)
Transcatheter arterial chemoembolization + oral sorafenib	1 (2.9)
Radiotherapy + oral sorafenib	2 (5.7)

Figure 1 Overall survival. A: Computed tomography (CT) scan showing multiple metastatic nodules in both lung lobes before anti-tumor treatment in a 54-year-old male patient with lung metastasis due to hepatocellular carcinoma; B: CT scan showing the disappearance of previous lung metastatic nodules in 5 mo after γ-knife radiosurgery and oral sorafenib.

Comparison of survival among three treatment groups

The median survival time of combined treatment group was 18.6 ± 3.9 mo, which was longer than that of single treatment group (6.3 ± 2.5 mo) and untreatment group (4.1 ± 0.3 mo), and there was significant statistical differences among these groups (log-rank test, *P* < 0.001) (Figure 2). The 6-mo cumulative survival rate of combined treatment, single treatment and untreated group was 88.6%, 57.9% and 31.8%, respectively; and the 1-year survival rate of the three groups was 68.5%, 21.1% and 8.0%, respectively.

Table 4 Univariate and multivariate analyses to identify prognostic factors of overall survival for hepatocellular carcinoma patients presenting with lung metastasis		
Variables	***P* value**	
	Univariate analysis	**Multivariate analysis**
Sex		
Male/female	0.988	–
Age (yr)		
< 50/≥ 50	0.423	–
Etiology		
Non-hepatitis/hepatitis	0.111	–
Cirrhosis		
Absence/presence	0.36	–
ECOG score		
2000/1/2	0.012	0.3
Child-Pugh score		
A/B	< 0.001	< 0.001
Ascites		
No/yes	0.031	0.879
Platelet (^{3})		
< 100/≥ 100	0.198	–
Albumin (g/L)		
< 37.7/≥ 37.7	0.134	–
ALT (IU/L)		
< 40/≥ 40	0.734	–
AST (IU/L)		
< 40/≥ 40	0.356	–
Total bilirubin (μmol/L)		
< 17.1/≥ 17.1	0.319	–
Prothrombin time (s)		
< 14.0/≥ 14.0	0.251	–
AFP (ng/mL)		
< 400/≥ 400	0.03	0.384
Maximum intrahepatic tumor diameter (cm)		
< 8.0/≥ 8.0	0.039	0.214
Intrahepatic tumor number		
Solitary/multiple or diffuse	0.279	–
Intrahepatic tumor location		
Single lobe/both lobes	0.435	–
Portal vein tumor thrombus		
No/yes	< 0.001	< 0.001
Hepatic vein tumor thrombus		
No/yes	0.031	0.194
Lung metastatic tumor number		
Solitary/multiple	0.018	0.321
Lung metastatic tumor location		
Single lobe/both lobes	0.156	–
Maximum metastasis diameter (cm)		
< 2.5/≥ 2.5	0.129	–
Treatment modality		
No/yes	< 0.001	< 0.001

The cutoffs of continuous variable were set according to median value. ECOG: Eastern cooperative oncology group; ALT: Alanine transaminase; AST: Aspartate aminotransferase; AFP: α-fetoprotein.

Univariate and multivariate analyses

In investigation of prognostic factors of survival of HCC presenting with lung metastasis at initial diagnosis, univariate analysis showed that ECOG score, Child-Pugh score, ascites, AFP level, maximum intrahepatic tumor diameter, the absence or presence of portal vein tumor thrombus, the absence or presence of hepatic vein tumor thrombus, lung metastatic tumor number, and treatment modality had prognostic significance (Table 4).

Patients at risk	Total	6 mo	12 mo	18 mo	24 mo	30 mo	36 mo	42 mo	48 mo	54 mo	60 mo
Combined treated	35	31	20	15	8	4	2	2	2	2	1
Single treated	19	11	3	0	0	0	0	0	0	0	0
Untreated	22	7	1	0	0	0	0	0	0	0	0

Figure 2 Comparison of survival among three treatment groups. Survival curves of untreated group (*n* = 22), single treatment group (*n* = 19), and combined treatment group (*n* = 35).

In the Cox proportional hazard model, Child-Pugh score, the absence or presence of portal vein tumor thrombus at the initial presentation, and treatment modality were three independent prognostic factors that affected the survival of HCC patients that presented with lung metastasis at initial diagnosis (Table 4).

DISCUSSION

To the best of our knowledge, this is the first study that investigates in detail the prognosis and prognostic factors of advanced HCC presenting with lung metastasis at initial diagnosis. In our study, the variables of tumor status and liver function status at initial diagnosis were almost comparable. However, the survival times from date of diagnosis until death or last visit among the three groups were significantly different, and patients underwent specific combined treatment modalities had longer survival than those treated with single method or those without anti-tumor treatment. However, it must be noted that there are many therapeutic modalities for tumor nodules, whether regional or systemic, which have their own indications and contraindications, and could not be applied altogether on a specific individual.

There are many case reports and clinical studies of patients with advanced HCC and lung metastasis who underwent radical hepatectomy and pulmonary metastasectomy[6-12]. In the present study, four patients also underwent combined surgical resection, three of whom were still alive at the end of follow-up. We think that the combined radical surgery should be positively considered if intrahepatic tumor and lung metastatic lesion were completely resectable, if the remaining volume of the liver was adequate, and the lung metastatic lesion was single[13]. In theory, removing all existing tumor lesions,

including primary and second lesions, is the only possible therapeutic approach till now.

Recently, with development in radiotherapy techniques, radiotherapy has been shown to play a potential role in a wide spectrum of HCC, therefore it is necessary to evaluate the effect of radiotherapy[14-16]. In our center, stereotactic radiotherapy, particularly γ knife radiosurgery, has become the major therapeutic mordality for lung and brain metastases of HCC. In the present study, there were 35 patients who underwent radiotherapy for intrahepatic HCC tumors and/or lung metastatic nodules, accounting for more than 50%, whether or not they are combined with other anti-tumor treatment modalities. Despite advances in radiotherapy delivery, liver toxicity following radiotherapy remains a dose-limiting factor, and investigations to better understand the pathophysiology of radiotherapy-induced liver toxicity are warranted. There is a particular interest in combining radiotherapy with anti-vascular endothelial growth factor targeting agents for their independent activity in HCC as well as their radiation sensitization properties[17].

Sorafenib is a multikinase inhibitor with effects against tumor proliferation and angiogenesis, and was recently approved for the treatment of advanced HCC[18,19]. Maintenance sorafenib would probably prevent or delay the intrahepatic and extrahepatic spread of HCC after radiotherapy, which provides the rationale for the combination of these treatment modalities[20]. In the present study, 15 patients received oral sorafenib. Although we did not find its significant efficacy in HCC presenting with lung metastasis due to few cases and less strict design, we believed that sorafenib could be used as one part of combined treatment modalities for HCC patients with lung metastasis. However, a large-scale randomized controlled trial is needed to confirm it. Combining surgical resection with sorafenib would be considered as the most optimal treatment modality for HCC patients with lung metastasis.. However, it remains to be confirmed by a randomized controlled clinical trial in the future.

Till now, we have not obtained enough evidences to confirm the role of locoregional hepatectomy in HCC patients with lung metastasis. In our opinion, hepatectomy can be performed when primary intrahepatic HCCs are completely resected, the number of lung metastases is less than 3, and the diameter of individual lung metastasis is less than 3 cm. In addition, any of treatment modalities, such as radiotherapy or TACE, should be used together for lung metastasis.

In the univariate and subsequent multivariate analyses, Child-Pugh score was an independent prognostic factor for HCC patients with lung metastasis. This indicates that although invasive treatment other than conservative treatment can prolong the survival in well-selected patients, one must be careful before applying invasive treatment to all patients.

This study has several limitations. First, it was designed retrospectively. Although the patients' status, including ECOG scale, Child-Pugh score, and liver function, was reviewed in the medical records and did not differ statistically among the three treatment groups, the clinical circumstances at the initial presentation might differ. Therefore, the decision for the treatment mordalities might be biased, and the subsequent patient stratification into various treatment groups might also be biased. Second, treatment modalities, i.e., radiotherapy, TACE, varied, and the number of patients treated was too small to confirm the effectiveness of each treatment modality. Third, the indications of specific treatment modality varied, and selective modalities were given to selective patients with advanced HCC and lung metastasis. It is very hard to build up a standard therapeutic regime for all patients, regardless of primary and secondary tumor site, size and number, liver functional reserve, and patients' general condition.

In conclusion, the present study showed that the prognosis of advanced HCC with concomitant lung metastasis at initial diagnosis is very poor, and combined comprehensive treatment modalities tended to significantly prolong the survival of the patients compared with conservative treatment or single treatment modality. Furthermore, further randomized trials might be required to investigate the optimal treatment modality in the near future.

COMMENTS

Background
The prognosis and treatment outcomes of advanced hepatocellular carcinoma (HCC) presenting with lung metastasis remain poorly evaluated.

Research frontiers
There are various treatment modalities for advanced HCC, including surgical resection, transcatheter arterial chemoembolization, radiotherapy, chemotherapeutics, and administration of molecular targeted therapeutic drugs. This study investigated the prognosis, treatment outcomes, and independent prognostic factors affecting the survival of a series of HCC patients presenting with lung metastasis at initial diagnosis.

Innovations and breakthroughs
In this study, the survival times from date of diagnosis until death or last visit between untreated group, single treatment group, and combined treatment group were significantly different, and patients underwent specific combined treatment modalities had longer survival than those treated with single method or those without anti-tumor treatment. Multivariate analysis revealed that Child-Pugh score, the absence or presence of portal vein tumor thrombus, and treatment modality were three independent prognostic factors affecting survival of patients with advanced HCC and concomitant lung metastasis.

Applications
As one type of advanced HCC, HCC presenting with lung metastasis is not unusual, and its prognosis is very poor. The findings in this study may contribute to its prognosis, and combined treatment modalities tend to result in a better survival for patients with advanced HCC initially presenting with lung metastasis.

Peer review
The authors firstly investigated that combined treatment modalities tended to yield better survival prolongation compared with conservative treatment or single treatment modality for HCC patients initially presenting with lung metastasis, which may be contribute to its prognosis.

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BRIEF ARTICLE

Detection of eukaryotic translation initiation factor 4E and its clinical significance in hepatocellular carcinoma

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Abstract

AIM: To study the expression of eukaryotic translation initiation factor 4E (eIF4E), which is closely correlated with malignant tumors, and its relationship to prognosis in hepatocellular carcinoma.

METHODS: Western blotting was performed to quantify the eIF4E protein expression in the normal human liver cell line L02 and the hepatoma cell lines Hep3B, HepG2, and Huh7. Forty-six hepatocellular carcinoma samples with complete clinical data were obtained from Changzheng Hospital during the period of December 2008 to July 2009. The expression of eIF4E in the tumor samples and their adjacent tissues were detected by immunohistochemistry. The relationship between the test results and hepatocellular carcinoma (HCC) prognosis was statistically analysed by using a COX proportional hazard model.

RESULTS: Western blotting analysis showed that there were distinct eIF4E protein bands in all three of the hepatoma cell lines. In particular, the HepG2 cell line had the highest level of eIF4E protein expression. The L02 cell group had a low eIF4E expression. Immunohistochemical assay showed that there were 32 cases in which the tumour tissue expression was higher than their adjacent tissues, accounting for 69.57%. There were also 14 cases in which the tumour tissue expression was lower or no significant difference was found, accounting for 30.43%. COX proportional hazards model analysis showed that HCC prognosis was related to the depth of invasion, the overexpression of eIF4E and p53, possibly as independent HCC prognostic predictors.

CONCLUSION: In summary, eIF4E expression is associated with liver cancer, and patients with high eIF4E expression levels have a higher risk of recurrence.

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Key words: Hepatocellular carcinoma; Eukaryotic translation initiation factor 4E; Western blotting; Immunohistochemistry; Prognosis

INTRODUCTION

Eukaryotic translation initiation factor 4E (eIF4E) is a member of the eIF family. It can specifically bind to the cap structure located at the 5' end of mRNAs named the "m7GpppN cap", which is necessary for mRNA translation initiation, and affects mRNA metabolism, processing, transportation and translation[1]. It plays an important role

in regulating the initial stage protein synthesis[2,3]. eIF4E is highly expressed in a variety of human malignancies[4-7], which has been confirmed to be relevant to the occurrence, invasion and metastasis of carcinomas such as head and neck squamous cell carcinoma[8], laryngeal cancer, non-small cell lung cancer[9-11], breast cancer[12-18], thyroid cancer, oesophageal cancer, stomach cancer, cholangiocarcinoma, colon cancer[19], non-Hodgkin's lymphoma, acute or chronic myeloid leukaemia[20], and lymphoma[21,22]. Experiments have also confirmed that eIF4E is closely related to the prognosis of many carcinomas. However, eIF4E-related studies in the context of hepatocellular carcinoma (HCC) are still rare.

In this study, we separately compared the eIF4E expression levels in normal liver cells with liver cancer cell lines and liver cancer tissues with precancerous tissues. Additionally, we investigated the influence of eIF4E expression on the prognosis of liver cancer. This research may provide an experimental basis for exploring new ways to treat liver cancer.

MATERIALS AND METHODS

Study objects

We selected 46 patients with pathological evidence of HCC and complete clinical data from Shanghai Changzheng Hospital who had liver surgery from January 2007 to January 2009. In these 46 cases, there were 40 males and 6 females who ranged in age from 31 years to 77 years (median age: 52.26 years). With regards to histological grade, there were 42 cases of moderately differentiated HCC, and 4 cases were poorly differentiated. A total of 33 patients had a cancer embolus in the intrahepatic bile duct or vein or had an infiltrated peplos, and 13 patients had no cancer tissue in cutting edge and gallbladder and no infiltrated peplos. p53 pathological testing was positive in 39 patients and negative in 7 cases. None of the patients received preoperative radiotherapy or chemotherapy. The follow-up time was 24 mo, and no case was lost.

Major materials and reagents

The cell lines used for Western blotting were the human liver cancer cell lines Hep3B, HepG2, Huh7, and the normal human liver cell line L02, which was provided by Shanghai Cell Biology Institution of Academia Sinica.

eIF4E (P-2) is a mouse anti-human monoclonal antibody raised against full-length eIF4E (Santa Cruz Biotechnology, Inc.). It is recommended for the detection of eIF4E by Western blotting (dilution: 1:200; dilution range: 1:100-1:1000) and immunohistochemistry (including paraffin-embedded sections; dilution: 1:50; dilution range: 1:50-1:500). The streptavidin-perosidase (SP) kit was provided by Fuzhou Maixin Biotechnology, Inc.

Detection methods

Western blotting analysis: We tested the *eIF4E* gene expression level in normal liver cells and different liver cancer cell lines. The four of cell lines (*i.e.*, HepG2,

Hep3B, Huh7, and L02) were incubated with high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37.0 °C with 5% CO_2 until the cell concentration reached 5×10^6 cells/mL. Then, we sequentially performed the protein extraction, bicinchoninic acid protein quantification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis electrophoresis, protein transfer, membrane closure, antibody incubation, and Bio-Rad chemiluminescence.

Immunohistochemistry: We detected the eIF4E protein expression levels in HCC and their adjacent tissues (SP, a particular type of immunohistochemistry). The tumour and adjacent tissues from the same patient were fixed, dehydrated, sectioned, and made into paraffin biopsies. We made 46 paraffin sections. The steps of the SP kit included heating on a baking sheet, incubation, washing, sealing, staining, drying, dehydration, and mounting. To analyse the results, we used two scoring methods. The samples were placed in an electron microscope and were scored for staining intensity as follows. 0: No colour; 1: A yellow colour; 2: A claybank colour; and 3: A brown colour. We then graded the samples for the positivity rate as follows. 0: No positive tumour cell staining; 1: ≤ 10% positive cells; 2: 11% to 50% positive cells; 3: 51% to 75% positive cells; and 4: > 75% positive cells. Finally, we added the two scores together, and the sum represented the immunohistochemical score as follows. -: 0; +: 1 to 4; ++: 5 to 8; and + + +: 9 to 12. Each cancer tissue section was compared with its adjacent tissue.

Follow up: We analysed the number of cases that had HCC recurrence and metastasis during the post-operative 24 mo. The liver cancer recurrence risk was measured using COX proportional hazards model for statistical analysis. The patient age, gender, histological grade, depth of invasion, eIF4E, p53 status and other prognostic indicators were used for the COX proportional hazards model analysis.

Statistical analysis

We used the SPSS 17.0 statistical software for the statistical analysis. A COX proportional hazards model testing level of $\alpha = 0.05$ and a $P < 0.05$ was considered statistically significant.

RESULTS

eIF4E protein expression in liver cancer cell lines by western blotting

We tested the eIF4E protein expression level in the liver cell line L02 and the liver cancer cell lines Huh7, HepG2, Hep3B. The eIF4E protein bands are shown in Figure 1. The bands were detected by Bio-Rad chemiluminescence to obtain the data shown in Table 1.

The liver cancer cell lines HepG2, Huh7, Hep3B significantly expressed the eIF4E protein, and in particular, the HepG2 cell line had the highest level of eIF4E protein expression. The normal liver cell L02 also expressed

Figure 1 Eukaryotic translation initiation factor 4E protein and glyceraldehyde-3-phosphate dehydrogenase protein bands in a normal human liver cell line and three hepatoma carcinoma cell lines. A: Eukaryotic translation initiation factor 4E protein bands; B: Glyceraldehyde-3-phosphate dehydrogenase protein bands.

Table 1 Eukaryotic translation initiation factor 4E protein expression in the liver cells at different levels

	Huh7	HepG2	L02	Hep3B
GAPDH	827.165	884.682	885.437	848.552
eIF4E	3161.861	5651.885	775.440	4496.191

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; eIF4E: Eukaryotic translation initiation factor 4E.

eIF4E; however, its expression level was low. Glyceraldehyde-3-phosphate dehydrogenase, which was used as the internal reference had bands in each cell line, and no obvious differences were observed with this protein.

eIF4E protein expression in liver cancer and adjacent tissues by immunohistochemistry

We next detected the eIF4E protein expression level in HCC and adjacent tissues. There were 46 pathological tissue paraffin blocks in which 32 HCC tissue cases had higher eIF4E protein expression than their adjacent tissues, accounting for 69.57%. A total of 14 HCC tissue cases had lower expression or no significant difference compared with their adjacent tissues, accounting for 30.43%. The scores were weighted 154:97 = 1.59:1, meaning that, in general, HCC tissues had a higher eIF4E protein expression level than the adjacent tissues.

Figure 2 show that HCC tissues stained significantly stronger than adjacent tissues, indicating that tumour tissues had a higher eIF4E protein expression level. The lightly stained central area in Figure 2A represents necrotic tissue.

eIF4E may be an independent risk factor for liver cancer prognosis

Follow-up statistics showed recurrence in 33 cases and

Figure 2 Photo of hepatocellular carcinoma tissue and adjacent tissue by electron microscopy. A: Hepatocellular carcinoma tissue and adjacent interstitial tissue; B: Adjacent tissue. (Hematoxylin and eosin stain, ×100).

death in 12 cases. The patient age, gender, histological grade, depth of invasion, eIF4E overexpression, p53 positive status and other prognostic indicators were used for COX proportional hazards model for screening analysis. Ultimately, the depth of invasion, eIF4E, and p53 were included in the model with a Sig < 0.05 as shown in Table 2. The statistical significance suggests that these three factors are independent risk factors for liver cancer prognosis.

DISCUSSION

In eukaryotic cells, translational regulation plays an important role in gene expression. eIF4E is involved in the regulation of the mRNA translation process. It can enhance the translation of some important growth factors and cell growth regulators and affect protein synthesis, the cell cycle, cancer gene activation, and apoptosis; it also plays an important role in malignant transformation and metastasis.

eIF4E regulates the translation of cancer-related mRNAs (i.e., it is involved in the activation of proto-oncogenes, angiogenesis, apoptosis, invasion and metastasis) that are involved in tumour occurrence and development. Normal tissues have a low eIF4E expression level. eIF4E was overexpressed, in a variety of malignant tumours including head and neck squamous cell carcinoma, laryngeal cancer, lung cancer, breast cancer, thyroid cancer and other cancer tissues[4,23]. Its high expression was correlated with tumour invasion and metastasis. However, studies of eIF4E in liver cancer are rare. At present, there are studies

Table 2 COX proportional hazards model analysis

	B	SE	Wald	df	Sig	Exp(B)	95% CI for Exp(B)	
							Lower	Upper
Eukaryotic translation initiation factor 4E	1.971	0.926	4.529	1	0.033	7.179	1.169	44.100
Depth of invasion	3.122	1.211	6.650	1	0.010	22.690	2.115	243.423
Histological grade	0.410	1.156	0.126	1	0.723	1.506	0.156	14.527
Gender	1.671	1.152	2.104	1	0.147	5.319	0.556	50.890
Age	-0.017	0.028	0.354	1	0.552	0.983	0.930	1.040
p53	-3.208	0.825	15.118	1	0.000	0.040	0.008	0.204

B: Coefficient of regression; SE.: Standard error; Wald: The index of regression effect; df: Degrees of freedom; Sig: *P* value; Exp(B): Odds ratio.

that involve the targeting eIF4E in head and neck squamous cell carcinoma[24,25], breast cancer[13-18], non-small cell lung cancer[26], blood malignancies[27-29] and other studies[6,30-32]. However, few studies have focused on targeting eIF4E in HCC.

In this study, we tested the expression of eIF4E protein in a normal human liver cell line and three different liver cancer cell lines. eIF4E protein expression was high in the three liver cancer cell lines and higher than in the normal liver cell L02. The HepG2 cell line had an especially high level of eIF4E protein expression. By comparing 46 cases of human liver cancer and adjacent tissues, we found that eIF4E protein expression was higher in most of the cancer tissues than in the adjacent tissues. COX proportional hazards model analysis showed that the depth of invasion, eIF4E, and p53 status were independent risk factors of liver cancer prognosis.

Based on these studies, we believe that eIF4E protein expression may be closely associated with the occurrence of human liver cancer development and prognosis. It has been confirmed *in vivo* and *in vitro* that sorafenib treatment can inhibit the RAF/MEK/ERK signal transduction pathway, reduce the eIF4E phosphorylation level, reduce Mcl-l protein, and induce hepatoma cell apoptosis[33,34]. Accordingly, we suggest that lower levels of *eIF4E* gene expression may inhibit liver cancer. Targeting and adjusting the eIF4E level and activity may inhibit cancer cell growth[6,30,31,35], which may become a new paradigm in the field of the biological treatment of liver cancer[36].

COMMENTS

Background

Hepatocellular carcinoma (HCC), which has a poor prognosis and a low five-year survival rate, is the most common malignant tumour in our country. At present, there are no effective therapies including radiotherapy, chemotherapy, and surgery. Eukaryotic translation initiation factor 4E (eIF4E) plays an important role in the translation initiation phase of a eukaryotic cell. It has been confirmed that eIF4E can specifically bind to the 5' mRNA cap (m7GpppN) and modulate its translation and expression. Its expression is closely associated with the generation, infiltration, and metastasis of many tumours such as head and neck, larynx, lung, mammary gland, thyroid gland, oesophagus, stomach, bile duct, colon.

Research frontiers

There are many researchers that are targeting eIF4E in head and neck squamous cell carcinoma, breast cancer, non-small cell lung cancer, blood malignancies and other carcinomas; however, studies that involve the targeting eIF4E in HCC are rare.

Innovations and breakthroughs

Research concerning the effects of eIF4E on HCC is limited. The authors tested the expression of the eIF4E protein in liver cancer cell lines and cancer tissues and used COX proportional hazards model analysis to show that eIF4E was an independent risk factor HCC prognosis.

Applications

The targeted regulation of the level and activity of eIF4E may inhibit cancer cell growth, which may become a new treatment paradigm in the liver cancer field.

Terminology

eIF4E is a member of the eIF family. It can specifically bind to the cap structure located at the 5' end of mRNAs named the "m7GpppN cap", which is necessary for mRNA translation initiation, and affects mRNA metabolism, processing, transportation and translation. It plays an important role in regulating the initial stage protein synthesis. eIF4E is highly expressed in a variety of human malignancies, which has been confirmed to be relevant to the occurrence, invasion and metastasis of carcinomas such as head and neck squamous cell carcinoma, laryngeal cancer, non-small cell lung cancer, breast, thyroid cancer, oesophageal cancer, stomach cancer, cholangiocarcinoma, colon cancer, non-Hodgkin's lymphoma, acute or chronic myeloid leukaemia, and lymphoma. Experiments have also confirmed that eIF4E is closely related to the prognosis of many carcinomas.

Peer review

This paper is interesting and worth being published if authors can satisfactorily address the concerns raised regarding immunohistochemical expression of eIF4E.

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BRIEF ARTICLE

Association between body mass index and erosive esophagitis: A meta-analysis

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Abstract

AIM: To conduct a meta-analysis to estimate the determinants of the association between erosive esophagitis (EE) and body mass index (BMI).

METHODS: We identified the studies using PubMed. Studies were selected for analysis based on certain inclusion and exclusion criteria. Data were extracted from each study on the basis of predefined items. Meta-analyses were performed to verify the risk factors, such as obesity and gender.

RESULTS: Twenty-one studies were included in this systematic review. These studies demonstrated an association between increasing BMI and the presence of EE [95% confidence interval (CI): 1.35-1.88, overweight, odds ratio (OR) = 1.60, P value homogeneity = 0.003, 95% CI: 1.65-2.55, obese, OR = 2.05, $P <$ 0.01]. The heterogeneity disappeared by stratifying for gender. No publication bias was observed in this meta-analysis by the Egger method.

CONCLUSION: This analysis demonstrates a positive association between BMI and the presence of EE, especially in males. The risk seems to progressively increase with increasing weight.

INTRODUCTION

The symptoms of gastroesophageal reflux disease (GERD) are common health problems in industrialized societies. It is a highly prevalent gastrointestinal disorder encountered in clinical practice[1,2]. Erosive esophagitis (EE) is one of the most common forms of GERD. It occurs when excessive reflux of acid and pepsin results in necrosis of surface layers of the esophageal mucosa, thus causing erosions and

ulcers[3]. The etiology of EE may be multifactorial. Esophageal mucosal resistance, gastroesophageal reflux, volume and composition of the gastric contents, contact time for the refluxed material, the degree of incompetence of the intrinsic lower esophageal sphincter, and the presence of a sliding hiatus hernia are likely important determinants[4]. It is a chronic disease that exhausts socioeconomic and medical resources and its symptoms may lower the quality of life of the patients. Additionally, patients with EE are at increasing risk of developing Barrett's esophagus and esophageal adenocarcinoma[5].

During the past several decades, obesity has emerged as a major health concern in the Western world[6]. Several studies have found an increased risk of esophagitis in overweight patients[7-9]. Excess adiposity is a known risk factor for morbidity, including several cancers[10]. Recently, a relationship between obesity and GERD has been reported[11]. One recent population-based case control study reported a strong association between body mass index (BMI) and esophagitis in females, but not in males[12]. Given these associations, it would seem logical that increasing BMI is associated with EE. However, studies on the association between BMI and reflux esophagitis have yielded inconsistent results[13-16], though a few have found a strong relationship between obesity and EE[17,18].

The aim of this study was to investigate the effect of BMI on risk for EE by performing a meta-analysis of all available literature published in PubMed up to April 2011. By performing a meta-analysis of the studies that met our selection criteria, we hoped to better characterize the association between increased BMI and EE.

MATERIALS AND METHODS

Search strategy
Two investigators independently performed a systematic search of all existing English-language literatures published up to April 2011 using PubMed, an electronic search engine for published manuscripts. Search terms included "obesity", "BMI", "overweight" or "BMI", combined with "reflux or EE". A total of 268 articles were identified after the preliminary search was reviewed in further details.

Study selection
Studies were included if they met all the following inclusion criteria: (1) Cross-sectional, case control, or cohort studies that permitted assessment of a causal relationship between BMI and EE; (2) Studies with documented and clearly-defined BMI in kg/m^2 for all participants; (3) Studies that reported a relative risk or odds ratio (OR) with confidence intervals or provided sufficient data to permit their calculation; and (4) Studies with EE diagnosed by upper endoscopy. The inclusion criteria were not otherwise restricted by study size or publication type. The followings were chosen as the exclusion criteria: (1)

Studies not limited to humans or not written in English; (2) Studies that did not report risk estimates or raw data to allow independent calculation of these estimates; and (3) Case reports, case series or studies that lacked a control group.

Data abstraction
The abstracted data included information on the source of the study population, study design (case control, cohort, or cross-sectional), length of the study period, primary aim of the study, exposure definitions (BMI definitions of normal, overweight or obese), exposure measurement method (self-reported *vs* measured BMI), outcome definitions (diagnosis of EE with endoscopy), total number of subjects with EE, case and control criteria, ORs or risk ratios with and without adjustment for potential confounders and potential confounders used for adjustment.

Exposure definition
We defined body mass categories using the following BMI [weight (in kilograms)/height (in meters)^2]: "normal" (BMI between 18.5 and 25 kg/m^2), "overweight" (BMI between 25 and 28 kg/m^2), and "obese" (BMI $\geqslant$ 28 kg/m^2). These groupings represented the divisions or quartiles most frequently reported in the literature even though they differed somewhat from BMI categories in common use (overweight, BMI 25-29.9 kg/m^2; obese, BMI $\geqslant$ 30 kg/m^2)[11]. We also created a category that included both overweight and obese (BMI $\geqslant$ 25 kg/m^2). For each study, we selected the BMI classification that most closely approximated each of these categories. We included more than one estimate from the studies (e.g., if a study reported an OR for persons with a BMI 25-28 kg/m^2 and an OR for persons with a BMI $\geqslant$ 28 kg/m^2, both ORs were included in the summary estimate as BMI $\geqslant$ 25 kg/m^2)[11]. We then compared the risk of EE among the BMI categories.

We used estimates adjusted for potential confounders whenever they were available; if no adjusted estimates were provided, unadjusted estimates were used or calculated from the data[11].

Outcome definition
An outcome was defined as EE diagnosed with endoscopy. The severity of EE was graded from A to D according to the LA classification[19] or modified Savary-Miller classification (grade I, single or multiple non-confluent erosions; grade II, confluent non-circumferential multiple erosion; grade III, circumferential erosions; and grade IV, ulcer and/or stricture)[20].

Statistical analysis
The BMI data were extracted from each study and analyzed with STATA 11.0 (StataCorp, College Station, TX, United States, www.stata.com). Summary OR estimates were calculated using either relative risks (for cohort stud-

Figure 1 Flow diagram. BMI: Body mass index.

ies) or OR (for case control studies). Summary OR estimates were calculated based on the assumption of fixed effects and heterogeneity was tested using the Mantel-Haenszel method[21]. We evaluated heterogeneity by comparing the results between the fixed effects model and a random effects model[21]. Heterogeneity among the studies was analyzed using χ^2 test and considered present if $P \leqslant 0.05$ or if there was more than a 20% difference in the summary estimates between the two models. To enhance the confidence of the results of the statistics when the number of combined studies was deficient, we used the I^2 metric, which describes the proportion of variability across studies that is due to score heterogeneity. If $I^2 = 0$, there is no heterogeneity. $I^2 > 50\%$ is considered to be indicative of heterogeneity. Larger values indicate greater heterogeneity. If these tests indicated heterogeneity, we explored possible causes[21-24]. Then, to exclude the excessive influence of any single study, we assessed whether exclusion of any single study substantially altered the magnitude or heterogeneity of the summary estimate. We also stratified analyses by several factors[25-31]. Funnel plots were produced and Egger's test[32] was conducted to examine publication bias.

RESULTS

We identified 268 published articles or abstracts (Figure 1). After review of titles and abstracts, 31 articles appeared to meet the initial inclusion criteria. The excluded studies were review articles, animal experiments, case series that lacked appropriate control groups and studies that did not report the subject of interest. These 31 studies underwent a complete data abstraction. Ten additional studies were excluded after data abstraction for the following reasons: BMI categories that were inconsistent with the proposed reference ranges[7,33-36], inconsistent outcome definition[37], lack of proper control group[38],

and lack of evaluable risk estimates within the proposed categories[39-41].

The remaining 21 studies[4,8,12,42-59] (i.e., four cross-sectional, three cohort, 14 case control studies) were included in the primary analysis (Tables 1 and 2). Twelve studies were conducted for the primary purpose of evaluating the relationship between BMI and EE[4,44,45,49-52,54,55,57-59], eight studies were conducted to identify the variety of risk factors for EE, including BMI[8,12,42-48,53,56], and one study described the clinical characteristics of EE and non-erosive reflux disease, including BMI[43]. In Table 1, controls and normal groups were composed of general population and healthy volunteers. Eighteen studies were included in Table 3 because of their stratification by gender.

The pooled OR of EE related to BMI of 25 kg/m^2 or higher was 1.64-fold greater than that of EE related to BMI less than 25 kg/m^2 (OR, 1.64, 95% CI: 1.45-1.85, test for homogeneity, $P = 0.000$, $I^2 = 65.7\%$) (Figure 2, Table 3).

Stratification by gender and BMI category showed a homogeneous positive association between increased BMI and EE, and the strength of the association with increased BMI (Table 3). The risk for overweight males (OR, 1.40, 95% CI: 1.11-1.75, $P = 0.285$) increased further for obese males (OR, 1.75, 95% CI: 1.02-2.96, $P = 0.099$) (Figure 3). The pooled OR in females and males for BMI greater than 25 kg/m^2 were 1.45 (95% CI: 1.26-1.66) and 1.52 (95% CI: 1.24-1.87), respectively. Therefore, we considered there was a strong positive association between increasing BMI and EE in males, but not in females.

Evaluation of heterogeneity

The initial summary estimates for EE were heterogeneous, as described above. Stratification by BMI category did not substantially resolve the heterogeneity; however, additional stratification by gender provided more homogeneity. Stratification of the entire population by exposure measurement (e.g., self-report *vs* measured), or study design (case control *vs* cohort) did not substantially influence the initial heterogeneity (Table 3).

Publication bias

The rank correlation test did not suggest the presence of publication bias for the main summary estimates for either the overweight ($P = 0.656$) or the obese and overweight ($P = 0.804$). A review of funnel plots did not demonstrate patterns strongly suggestive of publication bias (Figure 4).

DISCUSSION

Our pooled results of observational studies demonstrated a positive association between increased BMI and the risk of EE. The strength of the association increased with increasing BMI and there was a trend towards a stronger association in males than in females. Unlike other non-modifiable risk factors such as age, race and gender, BMI is potentially modifiable. Thus, identifying a relationship

Table 1 Study characteristics

Authors	Yr	Design	Region	Population size	Case population	Reference population	Confounders adjusted for
Ha *et al*[43]	2010	Case-control	South Korea	*n* = 292 (EE), *n* = 500 (NERD)	Single hospital	Hospital controls	G, E, T, J, OD, WHR, TG
Nam *et al*[44]	2010	Cohort	South Korea	*n* = 495 (EE), *n* = 3779 (normal)	General population	General population	WC , WHR , VAT , SAT
Wang *et al*[46]	2010	Case-control	China	*n* = 70 (EE), *n* = 502 (non-EE)	General population	General population	A, G, S, B, T, E, C, tea drinking, spicy food consumption, betel nut use
Koo *et al*[45]	2009	Case-control	South Korea	*n* = 42 (EE), *n* = 987 (control)	General population	General population	G, T, E, TG ,
Koo *et al*[45]	2009	Case-control	South Korea	*n* = 42 (EE), *n* = 1007 (control)	General population	General population	G, T, E, TG ,
Chua *et al*[47]	2009	Case-control	Taiwan, China	*n* = 427 (EE), *n* = 427 (control)	Single hospital	Hospital controls	TG, Glucose intolerance, HDL-C, SBP
Song *et al*[48]	2009	Case-control	South Korea	*n* = 639 (EE), *n* = 5443 (non-EE)	Single hospital	Hospital controls	A, G, T, E, H, TC, HDL-C, LDL-C, TG, BP, fasting glucose
Lien *et al*[49]	2009	Case-control	Taiwan, China	*n* = 102 (EE), *n* = 1942 (non-EE)	Single hospital	Hospital controls	A, G, J
Lien *et al*[49]	2009	Case-control	Taiwan, China	*n* = 240 (EE), *n* = 1662 (non-EE)	Single hospital	Hospital controls	A, G, J
Nam *et al*[50]	2009	Cohort	South Korea	*n* = 552 (EE), *n* = 8019 (non-EE)	General population	General population	A, WC, E, T
Lee *et al*[51]	2009	Case-control	South Korea	*n* = 100 (EE), *n* = 100 (control)	Single hospital	Hospital controls	WHR, T, J, VAT, SAT, VAT/SAT
Chung *et al*[52]	2008	Case-control	South Korea	*n* = 3539 (EE), *n* = 3539 (control)	Single hospital	Hospital controls	E, T, metabolic syndrome
Zagari *et al*[53]	2008	Cross-sectional	Italy	*n* = 122 (EE), *n* = 911 (non-EE)	General population	General population	A, G, E, T, H, J, C, medication use, peptic ulcer
Lee *et al*[54]	2008	Case-control	South Korea	*n* = 292 (EE), *n* = 2896 (control)	Medical center	Medical center	G, TC, TG, WHR, J, T, OD, PBF
Kim *et al*[42]	2008	Case-control	South Korea	*n* = 1810 (EE), *n* = 20154 (normal)	Multiple hospital	Multiple hospital	G, E, J, H, TC, TG, T, medications for liver/heart disease
Moki *et al*[56]	2007	Case-control	Japan	*n* = 191 (EE), *n* = 4968 (non-EE)	General population	General population	A, G, BP, TG, FBG
Kim *et al*[58]	2007	Case-control	South Korea	*n* = 1090 (EE), *n* = 26229 (non-EE)	Single hospital	Hospital controls	A, G, E, T
Nocon *et al*[55]	2007	Cohort	Germany	*n* = 5289 (EE), *n* = 926 (non-EE)	General population	General population	A, T, E,
Kang *et al*[57]	2007	Cross-sectional	South Korea	*n* = 161 (EE), *n* = 2281 (non-EE)	Single hospital	Hospital controls	A, G, J, T, B, hypertensive drugs, lifestyle choices, abdominal obesity
Labenz *et al*[8]	2004	Cross-sectional	Germany	*n* = 2455 (EE), *n* = 2834 (control)	Medical center	Medical center	A, G, R, S, T, E, B, H, concomitant disease, concomitant medicatons
Nilsson *et al*[12]	2002	Case-control	Sweden	*n* = 179 (EE), *n* = 179 (control)	Multiple hospital	Multiple hospital	T, cholecystectomy, I, drugs use
Wilson *et al*[59]	1999	Case-control	United States	*n* = 189 (EE), *n* = 1024 (control)	Single hospital	Single hospital	A, G, J, R
Stene-Larsen *et al*[4]	1988	Cross-sectional	Sweden	*n* = 195 (EE), *n* = 1029 (control)	Single hospital	Single hospital	None

A: Age; B: Aspirin or NSAID intake; C: Coffee; D: Meal size; E: Alcohol/ethanol; F: Family history; G: Gender; H: *Helicobacter pylori* infection; I: Asthma or asthma medication; J: Hiatal hernia; K: Hospital visit or hospitalization; M: Marital status; O: Symptom checklist-90 score; P: Physical activity; Q: Psychosomatic symptoms; R: Race; S: Socioeconomic status, education; T: Tobacco; W: Right handedness; V: Comorbidity; X: Case control status; Y: Birthplace; Z: Hormone replacement therapy; VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue; BP: Blood pressure; SBP: Systolic; DBP: Diastolic blood pressure; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TG: Triglyceride; HbAlc: Hemoglobin Alc; OD: Obesity degree; WHR: Waist-to-hip ratio; WC: Waist circumference; PBF: Percentage of body fat; FBG: Fasting blood glucose; EE: Erosive esophagitis; NERD: Non-erosive reflux disease; NSAID: Nonsteroidal antiinflammatory drugs.

between obesity and EE might have significant implications for counseling.

A recent meta-analysis of BMI and GERD complications found heterogeneous results and it was not able to identify strata with homogeneous results[60]. It was pos-sibly due to their methods of stratification, the utilization of estimates with markedly different measures of BMI association, the absence of studies included in the cur-rent analysis, and the inclusion of studies that did not set up a non-GERD control group. In contrast, in the cur-

Table 2 Exposure and outcome definitions

Authors	Yr	Exposure (source)	BMI reference (kg/m²)	Exposure (definitions)			Outcome (source)	Outcome (definitions)
				BMI overweight (kg/m²)	BMI obese (kg/m²)	BMI overweight + obese (kg/m²)		
Ha *et al*[43]	2010	Measured BMI	≤ 25			≥ 25	Endoscopy	Los Angeles classification
Nam *et al*[44]	2010	Measured BMI	< 20	25-29.9	≥ 30	≥ 25	Endoscopy	Los Angeles classification
Wang *et al*[46]	2010	Measured BMI	< 25	25-30	> 30	≥ 25	Endoscopy	Los Angeles classification
Koo *et al*[45]	2009	Measured BMI	< 23	23-24.9	≥ 25	≥ 23	Endoscopy	Los Angeles classification
Koo *et al*[45]	2009	Measured BMI	< 23	23-24.9	≥ 25	≥ 23	Endoscopy	Los Angeles classification
Chua *et al*[47]	2009	Self-report	< 25		≥ 25	≥ 25	Endoscopy	Los Angeles classification
Song *et al*[48]	2009	Measured BMI			≥ 30		Endoscopy	Los Angeles classification
Lien *et al*[49]	2009	Self-report	< 24	24-26.9	≥ 27	≥ 24	Endoscopy	Modified Savary-Miller endoscopic classification
Lien *et al*[49]	2009	Self-report	< 24	24-26.9	≥ 27	≥ 24	Endoscopy	Modified Savary-Miller endoscopic classification
Nam *et al*[50]	2009	Self-report	< 20	25-29.9	≥ 30	≥ 25	Endoscopy	Los Angeles classification
Lee *et al*[51]	2009	Measured BMI	20-25	25-30	≥ 30	≥ 25	Endoscopy	Los Angeles classification
Chung *et al*[52]	2008	Measured BMI	< 23	23-24.9	≥ 25	≥ 23	Endoscopy	Los Angeles classification
Zagari *et al*[53]	2008	Self-report	20-24.9	25-29.9	≥ 30	≥ 25	Endoscopy	Modified Savary-Miller endoscopic classification
Lee *et al*[54]	2008	Measured BMI	< 20	25-30	> 30	≥ 25	Endoscopy	Los Angeles classification
Kim *et al*[42]	2008	Measured BMI	< 23			≥ 25	Endoscopy	Los Angeles classification
Moki *et al*[56]	2007	Measured BMI	< 25		≥ 25		Endoscopy	Los Angeles classification
Kim *et al*[58]	2007	Measured BMI	18.9-24.5	25-29.9	≥ 30	≥ 25	Endoscopy	Los Angeles classification
Nocon *et al*[55]	2007	Measured BMI		25-30	> 30		Endoscopy	Los Angeles classification
Kang *et al*[57]	2006	Measured BMI	< 25	25-30	> 30	≥ 25	Endoscopy	Los Angeles classification
Labenz *et al*[8]	2004	Measured BMI	< 25	25-30	> 30		Endoscopy	Los Angeles classification
Nilsson *et al*[12]	2002	Self-report	< 25	25-30	> 30	≥ 25	Endoscopy	Modified Savary-Miller endoscopic classification
Wilson *et al*[59]	1999	Measured BMI	< 20	25-30	> 30	≥ 25	Endoscopy	NA
Stene-Larsen *et al*[4]	1988	Measured BMI	< 25	25-28	> 28		Endoscopy	NA

BMI: Body mass index; NA: Not available.

Figure 2 Erosive esophagitis and body mass index (overweight and obese) in males and females. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. RR: Relative risk; OR: Odds ratio.

Study ID	RR/OR (95% CI)	Weight (%)
Nilssom *et al*[12], 2002	1.06 (0.56-2.01)	8.74
Moki *et al*[56], 2007	1.80 (1.30-2.50)	23.38
Nocon *et al*[55], 2007	1.36 (1.22-1.52)	48.66
Wang *et al*[46], 2010	2.46 (1.29-4.70)	8.61
Nam *et al*[44], 2010	1.57 (0.89-2.78)	10.61
Overall (I^2 = 36.3%, *P* = 0.179)	1.52 (1.24-1.87)	100.00

NOTE: Weights are from random effects analysis

Figure 3 Erosive esophagitis and body mass index (overweight and obese) in males. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. RR: Relative risk; OR: Odds ratio.

Table 3 Meta-analysis results in association between body mass index and erosive esophagitis

BMI category	OR (95% CI)	$P_{homogeneity}$	I^2 (%)	No. of studies
Overall				
Overweight	1.60 (1.35-1.88)	0.003	59.8	12[4,8,12,44,45,50,51,53,54,57-59]
Obese	2.05 (1.65-2.55)	0.000	74.2	15[4,8,12,44-46,50-54,56-59]
Overweight + obese	1.64 (1.45-1.85)	0.000	65.7	18[4,8,12,43-47,49,50-54,56-59]
Females				
Overweight	1.47 (1.15-1.88)	0.011	7.4	3[12,44,55]
Obese	3.76 (0.92-15.28)	0.340	78.0	3[12,44,55]
Overweight + obese	1.45 (1.26-1.66)	0.579	0.0	4[12,44,55,56]
Males				
Overweight	1.40 (1.11-1.75)	0.285	20.8	4[12,44,46,55]
Obese	1.74 (1.02-2.96)	0.099	52.1	4[12,44,46,55]
Overweight + obese	1.52 (1.24-1.87)	0.179	36.3	5[12,44,46,55,56]

BMI: Body mass index; OR: Odds ratio.

Figure 4 Evaluation of publication bias using a funnel plot. No significant funnel asymmetry was observed which could indicate publication bias. The horizontal line in the funnel plot indicates the random effects summary estimate, while the sloping lines indicate the expected 95% CI for a given standard error, assuming no heterogeneity between studies. Each trial is represented by a circle, the area of which represents the trial's precision. Larger circles represent trials that offer more information.

rent study, after the creation of more categories of BMI among the studies, stratification by gender demonstrated a homogeneous increase in EE with increasing BMI. A study showed a positive correlation between BMI and EE in females, but not in males[12] and a study of reflux patients showed that obese females, but not obese males, had an increased risk of severe esophagitis[55]. The study by Nilsson[12] also found that the association between obesity and EE was further strengthened by the use of oestrogen replacement medication. The prevalence of GERD symptoms as determined in a study investigating a cohort from North America did not differ between males and females[61]. In contrast, in another study, EE was more common in males than in females from Asia[42]. However, in our study, we found a strong positive association between increasing BMI and EE in males, but not in females. This may be because the populations of the included studies were from Asia.

Several hypotheses have been proposed to explain how obesity can cause EE. Abdominal fat may cause reflux through an increase in intrabdominal pressure and subsequent esophageal acid exposure[62,63]. Also, there was a suggestion that hormonal factors related to adiposity are more important than mechanical factors[63]. Obesity is also associated with increased transient lower oesophageal sphincter relaxation[64]. Strengths of this analysis include the use of strict criteria for defining our outcome of interest and the consistency of the BMI-EE association within the males despite different patient populations and different study designs. All the included studies used endoscopy to confirm the diagnosis of EE, which eliminated the possibility of false positive EE cases. Also, we included stratification by study design, location, and source population.

There are potential limitations of this analysis. First, only observational studies were included; study results may be influenced by the presence of measured or unmeasured confounding factors, such as physical activity. Second, bias may also exist in the present study because unpublished data were not included, nor were conference abstracts or articles published in a language other than

English. Third, the exposure definitions (i.e., normal, obese or overweight) differed slightly among the studies. We addressed this, however, by creating more comparable and consistent categories, although few differences still remained. Also, the accuracy of the BMI measurement and its reliability as a measure of adiposity are known to be imperfect.

In summary, based on our extensive review and synthesis of the literature, there appears to be a statistically significant association between elevated BMI and EE. Considering the prevalence of obesity and increasing incidence rates of EE, it is important to pay more attention to further studies that evaluate the influence of gender, ethnicity or age on EE to examine this association. Several studies have found abdominal visceral obesity to be an independent risk factor for EE[44,57]. Nam *et al*[44] demonstrated that association between EE and abdominal visceral adipose tissue volume was consistent among males and females, unlike the association between EE and BMI. However, CT or MRI is needed to test abdominal visceral adipose, which are time consuming and costly. So, measuring BMI may be more feasible. It is also important to determine whether weight loss can decrease the incidence of EE. Further studies are needed to evaluate the relationship between obesity and EE.

COMMENTS

Background
Both obesity and erosive esophagitis (EE) have a high prevalence worldwide. The relationship between them remains controversial.

Research frontiers
Many studies have been performed to evaluate the body mass index (BMI) for gastroesophageal reflux disease risk. It has been found that there was a positive correlation between BMI and EE in females, but not in males.

Innovations and breakthroughs
Findings from this meta-analysis suggested the importance of BMI in EE, especially in males.

Applications
This study provided the potential measurement indicators to identify high-risk groups for EE in obesity population, especially in males.

Terminology
BMI: BMI is a heuristic proxy for human body fat based on an individual's weight and height. It is defined as the individual's body mass divided by the square of his or her height; EE: EE is a term used to indicate any inflammation, swelling, or irritation of the esophagus. The esophagus becomes inflamed (swollen, irritated and red).

Peer review
The meta-analysis presents the data on association between obesity and EE. The topic is interesting and the methodology of the meta-analysis is appropriate.

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BRIEF ARTICLE

Serum inter-cellular adhesion molecule 1 is an early marker of diagnosis and prediction of severe acute pancreatitis

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Abstract

AIM: To determine if serum inter-cellular adhesion molecule 1 (ICAM-1) is an early marker of the diagnosis and prediction of severe acute pancreatitis (SAP) within 24 h of onset of pain, and to compare the sensitivity, specificity and prognostic value of this test with those of acute physiology and chronic health evaluation (APACHE) II score and interleukin-6 (IL-6).

METHODS: Patients with acute pancreatitis (AP) were divided into two groups according to the Ranson's criteria: mild acute pancreatitis (MAP) group and SAP group. Serum ICAM-1, APACHE II and IL-6 levels were detected in all the patients. The sensitivity, specificity and prognostic value of the ICAM-1, APACHE II score and IL-6 were evaluated.

RESULTS: The ICAM-1 level in 36 patients with SAP within 24 h of onset of pain was increased and was significantly higher than that in the 50 patients with MAP and the 15 healthy volunteers ($P < 0.01$). The ICAM-1 level (25 ng/mL) was chosen as the optimum cutoff to distinguish SAP from MAP, and the sensitivity, specificity, positive predictive value, negative predictive value (NPV), positive likelihood ratio and negative likelihood ratio were 61.11%, 71.42%, 0.6111, 0.7142, 2.1382 and 0.5445, respectively. The area under the curve demonstrated that the prognostic accuracy of ICAM-1 (0.712) was similar to the APACHE-II scoring system (0.770) and superior to IL-6 (0.508) in distinguishing SAP from MAP.

CONCLUSION: ICAM-1 test is a simple, rapid and reliable method in clinical practice. It is an early marker of diagnosis and prediction of SAP within the first 24 h after onset of pain or on admission. As it has a relatively low NPV and does not allow it to be a stand-alone test for the diagnosis of AP, other conventional diagnostic tests are required.

INTRODUCTION

Most cases of acute pancreatitis (AP) are mild and self limiting, and recover spontaneously, but approximately 20% of attacks turn to severe acute pancreatitis (SAP) with a life-threatening morbidity and a mortality rate

of 20%-30%. Hence, early diagnosis and prediction of the severity of AP are of particular significance[1]. Early prediction of the severity of AP is still difficult in clinical practice. Ranson's score can only be evaluated 48 h after admission. The acute physiology and chronic health evaluation II (APACHE II) score can be used within a few hours after admission, but its complex and cumbersome performance limits its clinical use[2]. Several laboratory markers have been developed over the past decade for the early diagnosis and prediction of SAP. Since there is no correlation found between the degree of structural damage to pancreas and clinical manifestation of the disease, no ideal predictive system or biochemical marker has been available. Several reports showed that serum intercellular adhesion molecule-1 (ICAM-1) levels were elevated during the course of AP and correlated with the severity of the disease and patient outcome. ICAM-1 can be detected as an early marker in the diagnosis of lung injury[3,4]. However, the details of the clinical use of this test for early diagnosis and prediction of SAP remain obscure, especially within the first 24 h in the patients after admission. The aim of this prospective study was to evaluate the use of the ICAM-1 in early diagnosis and prediction of SAP.

MATERIALS AND METHODS

Study population
All patients with AP were included in the primary analysis according to the guidelines of diagnosis and treatment of AP established by Branch of Gastroenterology, Chinese Medical Association in 2003[5]. The diagnosis of AP was based on the following features: (1) Prolonged abdominal pain characteristic of AP; (2) Elevated serum amylase and/or lipase levels by at least 3-folds that of normal range; and (3) Characteristic findings of AP on abdominal ultrasonography and/or computed tomography (CT) scan. Patients who were admitted within the first 24 h of the onset of abdominal pain were not included in the study. Patients with an accompanying disease that might influence the outcome data were excluded, such as postoperative, post-traumatic, post-endoscopic retrograde cholangiopancreatographic pancreatitis. Other causes of acute abdominal pain were ruled out. Eighty-six patients with AP included in this pilot study were analyzed in a prospective 1-year investigation performed at a single institution.

Clinical assessment
The study was approved by the Committee of Research Ethics of our hospital, and informed consent was obtained from all the patients and healthy volunteers before enrollment. Demographics (gender, age, occupation, course and characteristics of symptom) and the cause of the pancreatitis (cholelithiasis, alcohol abuse, hyperlipidemia and others) were recorded. Routine clinical observation, laboratory test and treatment were performed. The APACHE-II score was determined within the first

24 h and 48 h of the onset of pain after admission[6]. Ultrasonography was performed every other day in all the patients and/or spiral CT with intravenous contrast was performed in some patients within 48-72 h after admission to assess the extent of inflammation and the degree of pancreas necrosis according to Balthazar's classification. After the examinations, patients' data were reviewed to determine the eligibility for inclusion into the study.

Clinical classification
Ranson's score was recorded in the first 24 h and 48 h after admission. Since Ranson *et al*[7] in 1974 identified 11 prognostic factors, considerable researches have been undertaken to find the ideal predictor(s) that allow rapid and correct assessment of the severity of AP to suit different clinical and regional settings. SAP was categorized based on the clinical and laboratory data using Ranson's score. Cases meeting less than three positive criteria were classified as mild acute pancreatitis (MAP) and those meeting three or more positive criteria were classified as SAP.

Sample collection and enzyme-linked immunosorbent assay for ICAM-1 and interleukin-6
To check the time kinetics of rise in plasma ICAM-1 and interleukin-6 (IL-6) during AP, its levels were quantified in two time-points. The potential of ICAM-1 and IL-6 to predict SAP within the first 24 h and 48 h after the onset of symptoms or on admission was examined. A 5-mL sample of peripheral venous blood was collected twice from the 85 patients with AP. Blood samples obtained from the 15 healthy volunteers served as controls. The plasma was separated by centrifugation at 3000 r/min for 10 min at 4 ℃ and stored at -20 ℃ until assayed. Plasma ICAM-1 and IL-6 were quantified with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Sen-Xiong Technology Limited Company, Shanghai, China). ELISA plates were read at 450 nm and data was collected. Measurement was performed according to the instructions of the manufacturer.

Presentation of data and statistical analysis
After the examinations, data from each patient were reviewed to ascertain the eligibility for inclusion into the study. The data of gender, etiology of SAP and MAP, and the categorical variables were compared using χ^2 test. Analysis of variance was performed in the continuous variables of age, ICAM-1, IL-6 levels and other relative laboratory tests for SAP and MAP using Student's *t* test. The differences were considered statistically significant if $P \leqslant 0.05$. In order to differentiate SAP from MAP within the first 24 h of the onset of symptoms, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and positive likelihood ratio (PLR) and negative likelihood ratio (NLR) at different serum levels of ICAM-1 compared with APACHE II and IL-6 levels were calculated, respectively. The level of higher

Table 1 Characteristics of 86 patients with acute pancreatitis

	SAP, $n = 36$	MAP, $n = 50$	P value
Age (mean ± SE, yr)	53.19 ± 14.9	49.4 ± 10.9	> 0.05
Gender (male/female)	18/18	30/20	> 0.05
Length of hospital stay (d)	18.3 ± 1.6	7.3 ± 1.4	< 0.001
Etiology, *n* (%)			
Gall stones	19 (52.7)	20 (40.8)	< 0.05
Alcohol	4 (11.1)	8 (16.3)	
Idiopathic	13 (36.1)	21 (42.8)	
Test at first 24 h			
Blood amylase (U/L)	456.8 ± 175.7	389 ± 125.7	< 0.05
Total bilirubin (mmol/mL)	39.10 ± 22.42	29.15 ± 25.88	> 0.05
Blood glucose (mmol/mL)	9.61 ± 4.77	6.87 ± 2.63	< 0.05
Blood calculus	2.41 ± 0.33	2.63 ± 2.16	> 0.05
Test at 48-72 h			
Pancreatic necrosis	16	2	< 0.01
Ranson's score	5.3 ± 0.5	1.5 ± 0.24	< 0.001

SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis.

Table 2 Acute physiology and chronic health evaluation Ⅱ score, serum intercellular adhesion molecule-1 and interleukin-6 levels in severe acute pancreatitis and mild acute pancreatitis patients within the first 24 h after admission

	SAP, $n = 36$	MAP, $n = 50$	Control	t	P value
APACHE-Ⅱ	14.47 ± 5.81	7.57 ± 1.44		7.9132	< 0.001
ICAM-1 (ng/mL)	29.68 ± 8.04	16.77 ± 4.37	8.12 ± 2.33	9.5028	< 0.001
IL-6 (ng/mL)	68.76 ± 28.62	35.95 ± 11.56	14.46 ± 3.53	7.2700	< 0.001

SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; APACHE-Ⅱ: Acute physiology and chronic health evaluation Ⅱ; ICAM-1: Intercellular adhesion molecule-1; IL-6: Interleukin-6.

Table 3 Changes of serum intercellular adhesion molecule-1 and interleukin-6 levels in severe acute pancreatitis patients after admission

	Type	< 24 h	48-72 h	t	P value
APACHE-Ⅱ	SAP	14.47 ± 5.81	16.6 ± 2.03	2.0776	< 0.05
	MAP	7.57 ± 1.44	9.51 ± 1.74	6.0216	< 0.001
ICAM-1 (ng/mL)	SAP	29.68 ± 8.04	44.76 ± 12.08	6.2353	< 0.001
	MAP	16.77 ± 4.37	24.10 ± 5.88	7.0038	< 0.001
IL-6 (ng/mL)	SAP	68.76 ± 28.62	42.19 ± 12.77	5.0868	< 0.001
	MAP	35.95 ± 11.56	21.76 ± 8.65	6.8798	< 0.001

SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; APACHE-Ⅱ: Acute physiology and chronic health evaluation Ⅱ; ICAM-1: Intercellular adhesion molecule-1; IL-6: Interleukin-6.

PLR and lower NLR was chosen as the optimum cutoff point. A receiver operating characteristic (ROC) curves were constructed to determine the reference cutoff point for ICAM-1, APACHE Ⅱ and IL-6 levels that could distinguish between SAP and MAP. An index of the goodness of the test of area under the curve (AUC) was used to measure the ability of distinguishing SAP from MAP. A perfect test had an area of 1.0, while a non-discriminating test had an area of 0.5. All statistical analyses were performed using SPSS v8.00 statistical analysis software (SPSS Inc., Cary, NC).

RESULTS

Clinical characteristics of AP
The prospective study population consisted of 86 consecutive patients with AP (48 men, 38 women with a mean age of 50.7 years; range, 17-79 years) and 15 healthy volunteers (10 men, 5 women with a mean age of 48.7 years; range, 28-58 years). Forty-nine (56.9%) patients were diagnosed as having MAP and 36 (41.8%) as having SAP according to the Ranson's criteria. The clinical characteristics of the patients with AP are summarized in Table 1. Gallstones were the most common cause of both SAP and MAP (*n* = 19, 52.7% and *n* = 20, 40.8%, respectively). There was no difference in the sex, race and etiology of the disease between the SAP and MAP patients. The mean serum amylase and glucose levels were significantly higher in SAP than in MAP (*P* < 0.05). There was no significant difference in the serum level of total bilirubin between the two groups at the first 24 h after the onset of pain.

Mean scores of APACHE-Ⅱ, serum ICAM-1 and IL-1 levels in patients with SAP and MAP
The mean scores of APACHE-Ⅱ in the SAP patients within the first 24 h after the onset of pain were also significantly higher than in the MAP patients *(P < 0.001)*. The mean serum level of ICAM-1 in the SAP patients

within the first 24 h after the onset of pain was significantly higher than in the MAP patients *(P < 0.001)* and the healthy controls *(P < 0.001)*. Significantly higher levels of IL-6 were found in the SAP patients as compared with the MAP patients at the 24 h and the healthy controls *(P < 0.001)*. The APACHE-Ⅱ scores within the first 24 h after admission to hospital were significantly higher in SAP than in MAP patients *(P < 0.001)* (Table 2). The mean serum level of ICAM-1 in SAP patients at 48-72 h after admission was obviously higher than in SAP patients within the first 24 h after admission *(P < 0.001)*. The level of IL-6 declined in the AP patients at 48-72 h after admission, but it was still obviously higher in the SAP patients than in the MAP patients *(P < 0.001)*. The mean scores of APACHE-Ⅱ in SAP and MAP at the 48-72 h after the onset of pain were slightly increased, but the scores in SAP were significantly higher than that in MAP in the first 24 h (Table 3).

Sensitivity, specificity, PPV, NPV, PLR and NLR of ICAM-1 in distinguishing SAP from MAP
The sensitivity, specificity, PPV, NPV, PLR and NLR at different serum ICAM-1 levels (10, 15, 25, 30, 35, 40 and 45 ng/mL) were determined respectively. The ICAM-1 level (25 ng/mL) with higher PLR and lower NLR was chosen as the optimum cutoff to distinguish SAP from MAP within first 24 h of the onset of symptoms, and the sensitivity, specificity, PPV, NPV, PLR and NLR were 61.11%, 71.42%, 0.6111, 0.7142, 2.1382

Table 4 Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio of acute physiology and chronic health evaluation Ⅱ, intercellular adhesion molecule-1 and interleukin-6 in distinguishing severe acute pancreatitis from mild acute pancreatitis

	Cutoffs	Sensitivity (%)	Specificity (%)	PPV	NPV	PLR	NLR
APACHE-Ⅱ	> 8	77.77	55.10	0.5600	0.7714	1.7320	0.2223
ICAM-1	25 ng/mL	61.11	71.42	0.6111	0.7142	2.1382	0.5445
IL-6	50 ng/mL	36.11	63.26	0.6190	0.5740	0.8826	1.1680

APACHE-Ⅱ: Acute physiology and chronic health evaluation Ⅱ; ICAM-1: Intercellular adhesion molecule-1; IL-6: Interleukin-6; PPV: Positive predictive value; NPV: Negative predictive value; PLR: Positive likelihood ratio; NLR: Negative likelihood ratio.

and 0.5445, respectively. The APACHE-Ⅱ scores (< 4, 4-8, 9-12, 13-16, 17-20 and > 20) were also calculated. The sensitivity, specificity, PPV, NPV, PLR and NLR at the optimum cutoffs of APACHE-Ⅱ score > 8 were 77.77%, 55.10%, 0.5600, 0.7714, 1.7320 and 0.2223, respectively. The different serum IL-6 levels (20, 30, 40, 50, 60, 70 and 80 ng/mL) were respectively calculated using the same method. The sensitivity, specificity, PPV, NPV, PLR and NLR at the optimum cutoffs of IL-6 50 ng/mL were 36.11%, 63.26%, 0.6169, 0.5740, 1.5869 and 0.9180, respectively (Table 4).

ROC curves and AUC of ICAM-1, APACHE-Ⅱ and IL-6 in distinguishing SAP from MAP

The optimum cutoffs of APACHE-Ⅱ score > 8, serum ICAM-1 (25 ng/mL) and IL-6 (50 ng/mL) which were used to distinguish SAP from MAP within the first 24 h of the onset of symptoms were analyzed by constructing a ROC curve. The AUC of ICAM-1, APACHE-Ⅱ and IL-6 in predicting SAP was 0.712, 0.770 and 0.508, respectively (Figure 1). The AUC demonstrated that the prognostic accuracy of ICAM-1 was similar to the APACHE-Ⅱ scoring system. The AUC of serum ICAM-1 level was obviously superior to that of IL-6. The serum IL-6 level was not capable of distinguishing SAP from MAP within the first 24 h of the onset of symptoms.

DISCUSSION

In China, the exact incidence of AP is still unknown, but it is increasing gradually in recent years. AP is a life-threatening illness with an annual incidence of 30-50 attacks per 100 000 inhabitants[8]. It is an inflammatory process that presents different severity degrees, ranging from a mild self-limiting disease with interstitial edema in the pancreas, to a severe disease with extensive necrosis[9]. It progresses to a severe illness with a prolonged course in about 15%-20% of the patients. These severely ill patients may develop organ failure and/or local complications such as pancreatic necrosis. Approximately, 75% of the cases are mild with a mortality below 1%. Eighty percent of the patients

Figure 1 Cutoffs of acute physiology and chronic health evaluation Ⅱ score > 8, serum intercellular adhesion molecule-1 (25 ng/mL) and interleukin-6 (50 pg/mL) levels that can distinguish severe acute pancreatitis from mild acute pancreatitis within 24 h of the onset of symptoms were analyzed by constructing a receiver operating characteristic curve. The area under the curve for intercellular adhesion molecule-1 (ICAM-1), acute physiology and chronic health evaluation Ⅱ (APACHE-Ⅱ) score > 8 and interleukin-6 (IL-6) in predicting severe acute pancreatitis was 0.712, 0.770 and 0.508, respectively.

could recover completely, while 20% had their disease worsened according to the Atlanta criteria. The mortality increases up to 20% if the disease progresses into a severe necrotizing form[10] and the mortality can be as high as 30%-40%[11]. Current management strategies for patients with SAP include early admission to intensive care units, vigorous intravenous resuscitation, and urgent endoscopic retrograde cholangiopancreatography when cholangitis or biliary obstruction is present, antibiotic prophylaxis in patients with pancreatic necrosis, and close patient monitoring.

Thus, the management of AP is still challenging mainly due to the delay in admission to hospital after the onset of symptoms and the difficulty in discriminating MAP from SAP, especially within the first 24 h. There is an urgent need for an early and accurate prediction of SAP to ensure timely interventions in a specialized care setting[12]. The early recognition and diagnosis are an important goal in the optimal management of SAP. Severity assessment is indispensable to the selection of proper initial treatment in the management of AP.

Several prognostic scoring systems are being used in predicting SAP: Ranson's, Glasgow's, APACHE-Ⅱ, the bedside index for severity of AP (BISAP), and computed tomography severity index (CTSI). Papachristou *et al*[13] reported that the AUC for BISAP, Ranson's, APACHE-Ⅱ and CTSI in predicting SAP are 0.81, 0.94, 0.78 and 0.84, respectively in 185 patients with AP and found that all these scoring systems had a high accuracy in predicting SAP in the first 24 h after admission. The components of the BISAP score are clinically relevant and easy to obtain. Lujano-Nicolás *et al*[14] evaluated the severity of AP according to the Ranson's and APACHE-Ⅱ scores on admission in 28 patients with AP and correlated these scales with the local pancreatic complications according to the Balthazar classification. They found that

these scales have discrepancies when compared with to-mographic Balthazar and these scales were not correlated well with the tomographic Balthazar degrees. The Ranson's prognostic signs and the Glasgow's score can only be applied 48 h after admission. The APACHE-Ⅱ score has the invaluable advantage of being useful within a few hours after admission, and it can be assessed serially. However, it is cumbersome, which limits its use in clinical practice. The current gold standard for staging AP combines the clinical criteria with CT, but because of the high cost, exposure to ionizing radiation, and lack of sensitivity and specificity in the early stage of the disease, it has limited availability.

Numerous efforts have been made in recent years to identify objective markers that can predict the severity of AP on admission. Various biochemical tests, such as C-reactive protein (CRP), tumor necrosis factor, IL-2 and IL 6, have been developed over the past decade for early diagnosis and prediction of severity of AP[15-17]. However, except for CRP, none of them can accurately predict the disease severity within 24 h of onset, and the outcome in triage to the intensive care unit has not been reported[18]. As there is no correlation between the degree of structural damage to pancreas and clinical manifestation of the disease, there has been no ideal predictive system or biochemical marker for this disease. Little is known about clinical predictors of early readmission for AP. The ideal predictors of the severity of AP are described as being simple, quick, highly sensitive, highly specific, safe, reproducible, and cheap. An immediate test with a high specificity, AUC and low NLR is required. Unfortunately, this ideal predictor is still not available[19].

Adhesion molecules are involved in the inflammatory response during AP. ICAM-1, a single-chain transmembrane glycoprotein with a molecular weight of 80-110 kDa, consists of five Ig-like domains, a hydrophobic transmembrane domain and a short cytoplasmic C-terminal domain. Its ligand includes lymphocyte function-associated antigen-1 and macrophage antigen-1. Under physiological conditions, ICAM-1 is expressed at a low level in endothelial cells and epithelial cells or constitutively on the surface of alveolar cells, providing the underlying molecular basis for cell recognition, activation, proliferation, differentiation and motility, thereby helping stabilize the internal environment of the body. ICAM-1 also plays a key role in pathological events, such as inflammatory reactions, including acute renal failure and acute pancreatitis[20].

One of the most common complications of AP is acute lung injury, during which ICAM-1 plays an important role by participating in leukocyte adhesion and activation as well as by inducing the "cascade effect" of inflammatory mediators, pulmonary microcirculation dysfunction and even acute respiratory distress syndrome, multiple organ failure or death. The upregulation of ICAM-1 expression in the lung during acute lung injury is one of main pathogeneses; the early detection of the ICAM-1 expression level may contribute to the prevention and treatment of acute lung injury[3,4]. Sun *et al*[20] in-vestigated the ICAM-1 in mediating the development of AP from a local disease to a systemic illness in rats and found that upregulation of ICAM-1 and subsequent leukocyte infiltration appear to be significant events of pancreatic and pulmonary injuries. Intracapillary leucocyte accumulation represents a novel protective and potentially lifesaving mechanism of hemostasis in acute pancreatitis. This process depends on the expression of lymphocyte function-associated antigen 1 and ICAM-1 and precedes the classical steps of the leucocyte recruitment cascade[21]. Chooklin *et al*[22] measured the serum levels of pro-inflammatory and anti-inflammatory cytokines in 51 AP patients who were diagnosed with pancreatitis-associated lung injury with and without the development of organ dysfunction and found that in the pathogenesis of respiratory complications in AP cytokines, chemokines and adhesion molecules, in particular ICAM-1, play major roles. High ICAM-1 concentration was found in plasma during AP, which was not reduced by Dx treatment. Dexamethasone down-regulates ICAM-1 expression, but it does not completely prevent leukocyte recruitment during sodium taurocholate-induced AP[23]. Pancreatic ICAM-1 expression was increased in single-nucleotide polymorphism as compared with the controls. After calcitonin gene-related peptide application, pancreatic ICAM-1 expression was attenuated[24]. Graft pancreatitis is induced by ischemia/reperfusion injury in which neutrophil infiltration is believed to be a crucial early event. The data suggested that ICAM-1 was already up-regulated during cold ischemia, possibly representing the mechanism of early neutrophil infiltration observed in human pancreatic ischemia/reperfusion injury[25].

Irrespective of the underlying etiology, the immune response is almost identical in severe cases of AP. While the triggering factors of AP are still poorly understood, cytokines are considered as important mediators in the pathophysiology of SAP. Perejaslov *et al*[26] reported that peak levels of sICAM-1 on admission in AP 87 patients had a subsequent decrease and these mediators are correlated with the disease severity, development of multiple organ dysfunction syndrome and necrosis and may be used as prognostic markers. Several reports showed that serum ICAM-1 levels are elevated during the course of AP and correlate with the severity of the disease and patient outcome. ICAM-1 can be detected as an early marker for the diagnosis of lung injury[20]. Although serum ICAM-1 level was increased in AP patients and closely related to the development of SAP in many clinical studies, the details of the use of ICAM-1 for early diagnosis and prediction of SAP remain obscure, especially in the first 24 h after the admission. The aim of our prospective study was to evaluate the use of the ICAM-1 in early diagnosis and prediction of SAP, and to compare the sensitivity, specificity and prognostic value of this test with those of APACHE-Ⅱ score and IL-6.

Among the 86 patients with AP in this study, 49 patients were diagnosed as having MAP and 36 as having SAP according to the Ranson's criteria. The mean serum

level of ICAM-1 in the SAP patients within the first 24 h of the onset of pain was significantly higher than in MAP patients and the healthy controls. The mean serum level of ICAM-1 in the SAP patients at 48-72 h after admission was obviously higher than in the SAP group. The result showed that ICAM-1 is a simple, rapid and reliable method for use within the first 24 h after admission. It has a higher specificity and a sensitivity for early diagnosis and prediction of SAP. The AUC value demonstrated that the prognostic accuracy of ICAM-1 is similar to the APACHE-Ⅱ scoring system and obviously superior to IL-6 in distinguishing SAP from MAP. Previous reports showed the usefulness of serum IL-6 level in determining the severity of acute pancreatitis[18], but our data indicated that the serum IL-6 level could not distinguish SAP from MAP within the first 24 h. The exact reason for the difference is still unknown. Although the APACHE-Ⅱ scoring system has a high prognostic accuracy, it is too complex and cumbersome, which limits its clinical use. However, ICAM-1 has a relatively low NPV and does not allow it to be a stand-alone tool for diagnosis of acute pancreatitis; and the use of other conventional diagnostic tools remains a requirement.

In conclusion, we found that serum ICAM-1 levels rose within the first 24 h after the onset of AP and that early measurement of serum ICAM-1 levels could distinguish SAP from MAP. It has a higher sensitivity, specificity and NPV. It could be used as a test in screening patients with AP and predicting the outcome in patients with SAP. The predictive accuracy of ICAM-1 is similar to APACHE-Ⅱ scoring system and obviously superior to IL-6. But its low NPV does not allow it to be a stand-alone tool for the diagnosis and prediction of AP. To identify a novel predictor of severity and outcome of AP is needed so as to improve the predictive accuracy.

COMMENTS

Background
Most cases of acute pancreatitis (AP) are mild and self limiting, and recover spontaneously, but approximately 20% of attacks turn to severe acute pancreatitis (SAP) with a life-threatening morbidity and a mortality rate of 20%-30%. There is no established biomarker for early diagnosis and prediction of severe pancreatitis. Early diagnosis and prediction of the severity of AP is of particular significance.

Research frontiers
Serum intercellular adhesion molecule-1 (ICAM-1) levels are elevated during the course of AP and correlate with the severity of the disease. ICAM-1 can be detected as an early marker for the diagnosis of lung injury. The details of the clinical use of ICAM-1 for early diagnosis and prediction of severity in AP remain obscure, especially in the first 24 h after the admission. This study evaluated the use of the ICAM-1 in early diagnosis and prediction of SAP, and compared the sensitivity, specificity and prognostic value of this test with those of acute physiology and chronic health evaluation (APACHE) Ⅱ score and interleukin-6 (IL-6).

Innovations and breakthroughs
Serum ICAM-1 is elevated within the first 24 h after the onset of symptoms. Elevated ICAM-1 levels are associated with the severity of AP with a higher sensitivity, specificity and negative predictive value for early diagnosis and prediction of severity in AP. The predictive accuracy of ICAM-1 is similar to the APACHE-Ⅱ scoring system and obviously superior to IL-6.

Applications
Serum ICAM-1 is an early marker for the diagnosis and prediction of SAP, and

the test is simple, rapid and reliable that can be used in clinic practice. The results obtained are important for both early diagnosis and treatment of pancreatitis.

Terminology
ICAM-1 is a single-chain transmembrane glycoprotein. Under physiological conditions, it is expressed at a low level in endothelial cells providing the underlying molecular basis for cell recognition, activation, proliferation, differentiation and motility. ICAM-1 also plays a key role in pathological events, such as inflammatory reactions, including acute renal failure and acute pancreatitis

Peer review
The manuscript reports that ICAM-1 is a useful marker for early diagnosis and a potential predictor of severe acute pancreatitis. These data are important for early treatment for pancreatitis patients.

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BRIEF ARTICLE

Prevalence of depressive and anxiety disorders in Chinese gastroenterological outpatients

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Abstract

AIM: To investigate the prevalence and physicians' detection rate of depressive and anxiety disorders in gastrointestinal (GI) outpatients across China.

METHODS: A hospital-based cross-sectional survey was conducted in the GI outpatient departments of 13 general hospitals. A total of 1995 GI outpatients were recruited and screened with the Hospital Anxiety and Depression Scale (HADS). The physicians of the GI departments performed routine clinical diagnosis and management without knowing the HADS score results. Subjects with HADS scores $\geq$ 8 were subsequently interviewed by psychiatrists using the Mini International Neuropsychiatric Interview (MINI) to make further diagnoses.

RESULTS: There were 1059 patients with HADS score $\geq$ 8 and 674 (63.64%) of them undertook the MINI interview by psychiatrists. Based on the criteria of Diagnostic and Statistical Manual of Mental Disorders (4th edition), the adjusted current prevalence for depressive disorders, anxiety disorders, and comorbidity of both disorders in the GI outpatients was 14.39%, 9.42% and 4.66%, respectively. Prevalence of depressive disorders with suicidal problems [suicide attempt or suicide-related ideation prior or current; module C (suicide) of MINI score $\geq$ 1] was 5.84% in women and 1.64% in men. The GI physicians' detection rate of depressive and anxiety disorders accounted for 4.14%.

CONCLUSION: While the prevalence of depressive and anxiety disorders is high in Chinese GI outpatients, the detection rate of depressive and anxiety disorders by physicians is low.

INTRODUCTION

Gastrointestinal (GI) disease is a serious illness, which

frequently affects a patient's physical and emotional wellbeing as well as being heavily affected by stress[1-3]. Meanwhile depression and anxiety have been identified as risk factors for some GI diseases[4-6].

Various studies using a variety of assessment methods have demonstrated that high levels of depression and anxiety exist in patients with GI symptoms[7-9]. It has also been shown that patients with comorbid anxiety and depressive disorders tend towards more severe symptoms, longer recovery times, poorer outcomes, and greater use of healthcare resources[10-12]. Despite the likelihood of GI patients to suffer from emotional distress, it has been reported that physicians in the GI department often fail to identify most cases of depression and/or anxiety, leading to under-treatment in 40%-90% of patients[13-16].

Patients with depressive and anxiety disorders often have one or more somatic symptoms (e.g., cardiopulmonary or gastrointestinal), which may be partly induced by emotional disorders[17-19]. On the other hand, many patients with depression or anxiety visit non-psychiatric departments, especially the GI department, for their physical complaints[20-22]. All these facts contribute to the low detection rate of emotional disorders among GI patients.

It is necessary to determine prevalence estimates of emotional disorders in GI patients to facilitate reasonable medical resources allocation. These have been assessed in a number of studies throughout America, Europe, and China, including the Hong Kong and Taiwan regions[15,23-25]. However, the economic status and cultural traditions of mainland China are unique, and likely to make the situation of mainland Chinese GI patients distinctive.

This is the first large-sample, multicenter study based on a mainland Chinese population to estimate the prevalence of depression and anxiety in adult GI outpatients. This cross-sectional study was carried out with GI outpatients from 13 tertiary general hospitals in Beijing, Shanghai, Guangzhou, Changsha and Chengdu. The purpose of this study was to characterize the prevalence of depressive and/or anxiety disorders among GI outpatients and to determine the non-psychiatric physician identification rate of these disorders in GI outpatients.

MATERIALS AND METHODS

Ethics
This survey was approved by the Shanghai Mental Health Center Ethics Committee. All patients provided informed written consent.

Subjects
A multi-center, cross-sectional study was carried out in the outpatient departments of 15 tertiary general hospitals in Beijing, Shanghai, Guangzhou, Changsha, and Chengdu to estimate the prevalence of depressive and anxiety disorders in adult outpatients from gastroenterology, gynecology, cardiovascular and neurology departments. However, only 13 hospitals provided complete data for the GI department (one lacked a GI department, and the other incomplete patient data). Consecutive patients visiting outpatient departments were recruited for the study. Patients were included if they were over 18 years, consented to study participation, and were able to complete the questionnaires. Exclusion criteria included being previously screened, serious physical or mental condition, language or hearing problem, incomplete records, or ongoing psychological treatment. About 140 consecutive GI outpatients were investigated in each hospital during 4-5 consecutive working days randomly selected from the 22 normal workdays in a month by using SAS (v9.0) software.

Research instruments
The somatic symptoms, as well as depression/anxiety, were assessed with the Patient Health Questionnaire 15-Item (PHQ-15)[26], Hospital Anxiety and Depression Scale (HADS)[27,28], and Mini International Neuropsychiatric Interview (MINI)[29]. PHQ-15 is a self-report questionnaire used to screen and assess somatic symptoms. It consists of 15 physical symptoms, scaled 0-2 points for each. The higher the score, the more severe the symptom. The 14-item HADS[27,28] questionnaire evaluates severity of anxiety and depression using 7 items for each affliction, and is widely used in general hospitals. Each item's severity is rated from 0 (none) to 3 (severe). Scores $\geq$ 8 indicate probable anxiety or depression with great reliability and validity[30], and were regarded as positive in the preliminary screening of this study. MINI[29], a structured diagnostic instrument, is used to make diagnoses according to the Diagnostic and Statistical Manual of Mental Health Disorders (4th edition, DSM-IV) and the International Statistical Classification of Disease-10. The MINI Chinese version has good reliability and validity[31-33].

Study design
This multi-center, cross-sectional study was carried out in five cities: Beijing, Shanghai, Guangzhou, Chengdu, and Changsha (representing north, east, south, west and central China, respectively). In the first stage, outpatients were screened with PHQ-15 and HADS scales, and then visited GI physicians for their original complaints. The coordinators calculated scores of both scales, kept physicians blind to results, and recorded physicians' diagnosis and treatment. In the second stage, subjects with HADS scores $\geq$ 8 were assessed and diagnosed by psychiatrists with MINI. The study design is shown in Figure 1.

Statistical analysis
Data analysis and management were performed using the Statistical Package for Social Sciences v17 (SPSS Inc., Chicago, IL, United States). Demographic data were described by frequency and percentage, and the lack of data in one item, such as sex or diagnosis, was treated as a missing value. Subjects who were positive in the preliminary screening stage but did not complete the psy-

Figure 1 Flowchart of the study on prevalence of depressive disorders and/or anxiety disorders among gastrointestinal outpatients from 13 general hospitals in China. PHQ-15: Patient Health Questionnaire 15-Item; HADS: Hospital Anxiety and Depression Scale; MINI: Mini International Neuropsychiatric Interview.

chiatrists' interview were excluded.

According to a previous publication[34], prevalence was described as the percentage of positive subjects among those who completed the trial, and adjusted prevalence was calculated according to the HADS score distribution among all eligible subjects. The 95% CI of adjusted prevalence were computed using the Gaussian approximation to the log-likelihood. Categorical data, such as differences of prevalence in sex and age, or differences between individuals with and without depressive and/or anxiety disorders, were compared using the χ^2 test at the < 0.05 significance level. Consecutive data with normal distribution, such as age, were expressed as mean $\pm$ SD and analyzed using the t test.

"Recognized" or "detected" indicated diagnosis of depressive or anxiety disorders according to a physician's clinical judgment, prescription of antidepressants or antianxiety drugs, or referral to psychiatry or psychology departments.

RESULTS

Demographic characteristics

The study comprised 1995 outpatients, aged 18-89 (45.2 $\pm$ 15.6) years and 54.19% were female. The patients' demographic characteristics are presented in the first two columns of Table 1. One thousand and fifty-nine subjects screened positive (HADS score $\geqslant$ 8), 580 (54.77%) of whom were female. Among these 1059, 674 completed the second stage screening of psychiatrists' interview with MINI. Among the missing subjects ($n = 385$), 173 (44.9%) did not complete the further interview and 212 (55.1%) refused to attend the interview (Figure 1). There were no significant differences between missed and followed-up cases in sex ($\chi^2 = 0.066$, $P = 0.797$) or age (43.72 $\pm$ 15.45 *vs* 42.89 $\pm$ 14.92, $t = -0.860$, $P = 0.390$).

Of the completed subjects, 371 (55.0%) were women

and 323 (47.9%) were diagnosed with one or more types of depressive disorders and/or anxiety disorders. Among the 323 confirmed subjects, 194 (60.1%) were women (other characteristics are described in the third column of Table 1). Subjects with depressive and/or anxiety disorders were more likely to be female and younger than those without such disorders (Table 1).

Prevalence of depressive and anxiety disorders in gastrointestinal outpatients

The adjusted prevalence of depressive and anxiety disorders are shown in Table 2. One hundred and eighty-one subjects had current depression disorders, 117 had current anxiety disorders, and 59 had current comorbidity. This indicates that 32.6% (59/181) of individuals with current depressive disorders had at least one type of anxiety disorder, and 50.4% (59/117) of subjects with current anxiety disorders were affected by depressive disorders as well.

The prevalence of all types of depressive disorders and anxiety disorders, according to DSM-IV criteria, are detailed in Table 2. Among the depressive disorders, depressive episode was the most common with an adjusted current prevalence of 11.23%, while substance-induced mood disorder had the lowest adjusted current prevalence (0.35%). Among 181 outpatients with depressive disorders, 51 (28.2%) had suicidal problems [suicide attempt or suicide-related ideation prior or current; module C (suicide) of MINI, score $\geqslant$ 1], indicating that over a quarter of individuals with depressive disorders were at suicide risk.

Sex differences among current prevalence of depressive disorders and/or anxiety disorders

The current adjusted prevalence of depressive disorders and/or anxiety disorders was significantly different between male and female outpatients (Table 3). The prevalence of depressive disorders, anxiety disorders, and either depressive or anxiety disorders was significantly ($P < 0.05$) higher in female GI outpatients. The adjusted current prevalence of depressive disorders with suicidal problems was statistically significantly higher in women (mean 5.84%; 95% CI: 4.44-7.24) than in men (mean 1.64%; 95% CI: 0.82-2.46) ($\chi^2 = 23.096$, $P = 0.00$), and the mean relative risk was 3.71 (95% CI: 2.10-6.56, $P < 0.01$).

Physicians' detection and treatment of depressive and anxiety disorders in gastrointestinal outpatients

Among 323 digestive outpatients who were diagnosed with depressive disorders and/or anxiety disorders by MINI, complete information of physicians' diagnoses and treatments was available for 290 cases ($n = 13$ missing diagnosis information, and $n = 21$ missing treatment information).

The detection rate by physicians was 4.14% (12/290). Among the 12 detected subjects, five were treated with psychotropic drugs, including amitriptyline or doxepin ($n = 2$). Another seven were referred to the psychiatry de-

Table 1 Baseline characteristics and comparison of subjects with and without depressive disorders and/or anxiety disorders *n* (%)

Characteristic	Screened subjects (*n* = 1995)	Frequency and percentage of subjects with and without depressive disorders and/or anxiety disorders according to the MINI (*n* = 674)			
		With depressive disorders and/or anxiety disorders (*n* = 323)	Without depressive disorders and/or anxiety disorders (*n* = 351)		
Sex				χ^2	*P* value
Male	914 (45.81)	29 (39.94)	173 (49.29)	6.116	0.013[a]
Female	1081 (54.19)	194 (60.06)	178 (50.71)		
Occupation				χ^2	*P* value
Laborer/attendant	282 (14.14)	52 (16.10)	46 (13.11)	5.595	0.588
Office worker	227 (11.38)	38 (11.76)	45 (12.82)		
Businessman	139 (6.97)	25 (7.74)	25 (7.12)		
Teacher	110 (5.51)	17 (5.26)	19 (5.41)		
Manager	244 (12.23)	36 (11.15)	48 (13.68)		
Farmer	264 (13.23)	43 (13.31)	40 (11.40)		
Soldier	9 (0.45)	0 (0)	3 (0.85)		
Other	720 (36.09)	112 (34.67)	124 (35.33)		
Age groups, yr		mean ± SD	mean ± SD	*t*	*P* value
		43.01 ± 14.80	46.12 ± 15.74	3.330	0.001[b]
18-29	377 (18.90)	69 (21.36)	85 (24.22)		
30-44	659 (33.03)	115 (35.60)	114 (32.48)		
45-59	562 (28.17)	93 (28.80)	95 (27.07)		
60-	397 (19.90)	46 (14.24)	57 (16.24)		

The last four columns present the characteristics and the comparison of the two groups (outpatients with and without depressive disorders and/or anxiety disorders). There are statistically significant differences between the two groups in sex ([a]*P* < 0.05 *vs* with depressive disorders group) and age ([b]*P* < 0.01 *vs* with depressive disorders group) by χ^2 test and *t* test, respectively. MINI: Mini International Neuropsychiatric Interview.

Table 2 Adjusted prevalence of depressive and anxiety disorders among gastrointestinal outpatients in 13 general hospitals in mainland China and Diagnostic and Statistical Manual of Mental Health Disorders (4th edition) by using the Mini International Neuropsychiatric Interview

Diagnosis	Frequency, adjusted prevalence (%) and 95% CI (%) based on results of the MINI			
	Current		Lifetime	
GI outpatients in 13 general hospitals				
Depressive disorders	181	14.39 (12.85-15.93)	228	18.35 (16.65-20.05)
Anxiety disorders	117	9.42 (8.14-10.70)	122	9.82 (8.51-11.13)
Comorbid depressive and anxiety disorders	59	4.66 (3.74-5.58)	69	5.46 (4.46-6.46)
Depressive disorders or anxiety disorders	239	19.20 (17.47-20.93)	281	22.71 (20.87-24.55)
DSM-Ⅳ by using the MINI interview				
Depressive episode	141	11.23 (9.84-12.62)	183	14.79 (13.23-16.35)
Depressive disorder with suicidal problems	51	3.91 (3.06-4.76)	58	4.46 (3.55-5.37)
Mood disorders due to physical disease	26	2.01 (1.39-2.63)	32	2.51 (1.87-3.15)
Dysthymia	16	1.25 (0.76-1.74)	34	2.66 (1.95-3.37)
Substance-induced mood disorders	4	0.35 (0.10-0.61)	4	0.35 (0.10-0.61)
General anxiety disorder	57	4.66 (3.74-5.58)	57	4.66 (3.74-5.58)
Specific phobia	20	1.65 (1.09-2.21)	20	1.65 (1.09-2.21)
Social phobia (social anxiety disorder)	20	1.60 (1.05-2.15)	20	1.60 (1.05-2.15)
Panic disorder	17	1.35 (0.84-1.86)	24	1.95 (1.34-2.56)
Agoraphobia	17	1.35 (0.84-1.86)	21	1.75 (1.17-2.33)
Obsessive-compulsive disorder	16	1.30 (0.80-1.80)	16	1.30 (0.80-1.80)

Depressive episode is the most common depressive disorder, while substance-induced mood disorder has the lowest adjusted current prevalence. The most common anxiety disorder is general anxiety disorder, followed by social anxiety disorder, panic disorder, agoraphobia, and obsessive-compulsive disorders. MINI: Mini International Neuropsychiatric Interview; GI: Gastrointestinal; DSM-Ⅳ: Diagnostic and Statistical Manual of Mental Health Disorders (4th edition).

partment. Meanwhile, three out of 67 (4.48%) subjects with suicide risk were identified and received psychiatric management, including psychiatry department referral (*n* = 1) and doxepin treatment (*n* = 2).

DISCUSSION

The current study evaluated the prevalence of depressive and anxiety disorders among mainland Chinese out- patients visiting GI clinics, regardless of confirmed GI diagnosis. The adjusted current prevalence of depressive disorders, anxiety disorders, and comorbid disorders was 14.39%, 9.42% and 4.66%, respectively.

It is well recognized that depressive and anxiety disorders impair life quality and cause a heavy disease burden[35-38]. Nevertheless, more than half of patients with depression or anxiety visit non-psychiatric depart- ments, especially the GI department, for somatic symp-

Table 3 Current adjusted prevalence of depressive disorders and/or anxiety disorders and comparison between men and women

Diagnosis based on MINI	Frequency, adjusted current prevalence (%) and 95% CI (%) based on results of MINI exam				χ^2	P value
	Men		Women			
Depressive disorders	70	12.06 (9.95-14.17)	111	16.40 (14.19-18.61)	7.555	0.006[b]
Anxiety disorders	46	8.00 (6.24-9.76)	116	10.75 (8.90-12.60)	4.339	0.037[a]
Depressive disorders and anxiety disorders	24	4.06 (2.78-5.34)	35	5.10 (3.79-6.41)	1.224	0.269
Depressive disorders or anxiety disorders	92	16.01 (13.63-18.39)	147	21.96 (19.49-24.43)	11.285	0.001[b]

There are significant differences between male and female patients in the current prevalence of depressive disorders ([b]$P < 0.01$), anxiety disorders ([a]$P < 0.05$) and either depressive or anxiety disorders ([b]$P < 0.01$), respectively. MINI: Mini International Neuropsychiatric Interview.

toms[20,21,39,40]. However, most general physicians are not appropriately trained in psychiatry and cannot diagnose or treat depressive and anxiety disorders. Thus, GI physicians tend towards a low detection rate[41-43]. It is meaningful to investigate overall prevalence of depressive and/or anxiety disorders in GI outpatients to understand the actual patient population involved and the importance of diagnosing such disorders.

According to our knowledge, this is the largest study investigating the prevalence of depressive and anxiety disorders in GI outpatients from tertiary general hospitals in mainland China. The reliability of the current prevalence figures was assured by the use of experienced psychiatrists administering a structured diagnostic instrument. The tertiary general hospitals enrolled in this study were distributed in north (Beijing), east (Shanghai), south (Guangzhou), west (Chengdu) and central (Changsha) China, and represent the majority of national tertiary general hospitals. In addition, the DSM-IV-based MINI was used by experienced psychiatrists to produce accurate and consistent diagnoses. Finally, the study was carried out in two stages, preliminary screening and diagnostic interview.

Prevalence of depressive disorders and/or anxiety disorders in general hospitals or primary care

The overall prevalence figures of depressive disorders and/or anxiety disorders in general medical care have been reported previously[15,44]. The current adjusted prevalence of depressive disorders in our study was 14.39%. However, this value was 19.5% in a meta-analysis of primary care patients in ten countries[41]. The current adjusted prevalence of anxiety disorders reported in our study was 9.42%, which was lower than the 19.0% prevalence reported among Belgian outpatients in 86 general practices[45] and the 19.5% prevalence reported in 15 United States general medical care centers[15]. These apparent discrepancies may be a result of subjects in the previous studies being from primary care and the Primary Care Evaluation of Mental Disorders being used for diagnosis.

Furthermore, other previous domestic investigations have reported varying prevalence of depressive disorders and anxiety disorders. Qin *et al*[46] reported prevalence of 11.01% for depressive disorders in internal medical outpatients from 23 general hospitals in Shenyang. The prevalence of depression was 12.5% in family practices in Taiwan[25], while the prevalence of anxiety disorders was 11.61% in six tertiary general hospitals in Shenyang[42]. Generally speaking, these different results were due to variances in subjects and investigation instruments. The prevalence of depressive disorders and/or anxiety disorders in our study and other domestic studies are lower than results from abroad, which may relate to differences in ethnicity or culture[47,48].

The 1.25% current prevalence of dysthymia, the third top depressive disorder in our study, was higher than the 0.6% prevalence in Shanghai subjects reported by the WHO[43] in 1990, but was similar to the 2.1% mean prevalence for all international sites that participated in the research and the 2.8% prevalence of dysthymia in the study of Qin *et al*[46]. It was lower than the 12.6% prevalence of dysthymia among outpatients from 86 general practices in Belgium[45].

It is well-known that comorbidity of depressive disorders and anxiety disorders can exacerbate symptoms, and co-occurrence of anxiety is an independent risk factor of suicide among depressive patients[35,49]. In the current study, anxiety disorders were comorbid in 32.6% of depressive individuals. This comorbid proportion in depressive patients was found to be 68.9% in a study conducted in 15 centers of China[50], and 50.6% in the United States[51]. It is a common phenomenon that depressive disorders and anxiety disorders are in comorbidity among outpatients in general medical care.

Detection rate by physicians in general hospitals or primary care

Detection rate in this study was 4.14%, similar to the 4% reported for Shenyang[42,46]. A United States-based study of outpatients with GI symptoms revealed that 52% of anxious patients and 26% of depressive patients were recognized by gastroenterologists[16]. Family practices surveyed in Taiwan[25] indicated that the recognition rate of depression disorders was 12.5%, and that of general anxiety disorder was 8.0%. Prevalence of depression disorders in internal medicine inpatients was 26.9% and only 40% of these patients received antidepressant treatment[52]. Another MINI-based study of internal medicine inpatients revealed that prevalence of depressive disorders was 26%, and 43.8% of them were treated with antidepressants[53]. A meta-analysis conducted by

Mitchell *et al*[41] indicated that correct diagnosis rate of clinicians was 47.3%-50.1%. The remarkable difference in detection rate between other investigations and ours suggests the urgent need to improve the diagnosing rates in mainland China.

Meanwhile, comorbid disorders deserve great attention due to their significant correlation to suicide risk. Current prevalence of depressive disorders with suicidal problems was 3.91% in our study, suggesting that over a quarter of patients with depressive disorders were at suicide risk, while only 4.48% of those patients were recognized. Carson *et al*[54] indicated that morbidity of major depression with suicide ideation was 29.9%, while its recognition rate by physicians was 58%. Moreover, prevalence of depression and/or anxiety disorders in our study was higher in females than in males, which is consistent with results in Qin's study[42,46], and reminds physicians to pay more attention to female outpatients with mood problems.

Discrepancies of prevalence and detection rate between our study and previous studies likely reflect the limitations of methodology, which require significant effort to be overcome in subsequent research.

These findings confirm the high prevalence of depressive and anxiety disorders and disappointing detection and treatment rate in the GI departments, and highlight the particular challenge posed by the contrasts between these two rates. Although all 13 tertiary hospitals represent the top general hospitals in China, low recognition and treatment rates raise significant concerns and indicate the need to improve the physician's abilities to diagnose and identify emotional disorders in GI patients.

Several potential explanations exist for the high prevalence of depressive and anxiety disorders and low detection rate in GI outpatients. Physicians are less specialized than psychiatrists in recognizing mental disorders correctly. Furthermore, culture may limit physicians' abilities in this regard. In the Chinese traditional culture, social and cognitive processes or mental status are closely related, which contributes to interpreting emotional distress and anxiety as social or ethical problems rather than mental disorders. Somatic symptoms can also serve as cultural idioms of depressive emotion[55-57]. Depressed or anxious people are inclined to experience physical symptoms, masking the underlying mental disorder[39]. In addition, there is a distorted cognition of mental disorders. It is common to consider depressive individuals as having no self-control and weak. Jorm *et al*[58] reported that around a quarter of Australian adults consider antidepressants as harmful to suicidal depressive patients, who are more likely to reject relevant treatments, including psychotherapy. Finally, the established stigma of mental disorders causes hiding of emotional problems and rationalization to resist therapy. Dramatic reports in the mainstream media of aggressive behavior by mental disorder sufferers prejudice both patients and physicians against the disorder[59-61].

Previous studies have proven that depressive and anxiety disorders influence prognosis of physical diseases, raise medical risk, and increase economic burden[62,63]. However, appropriate treatment does benefit recovery from physical disease and maintenance of social function[64-66]. Therefore, clinicians should improve their ability to diagnose depression and anxiety, especially in patients with complaints of unexplained GI symptoms.

Limitations
Several limitations exist in the current study. Firstly, excluding outpatients who could not complete the investigation due to severe physical or mental dysfunction may have biased the results since severity of physical symptoms is positively related to depression, anxiety or other mental problems[67,68]. Secondly, the 385 missing cases (due to busy schedules and denial of mental issues) from the diagnostic interview accounted for 19.3% of the total. There were no statistically significant differences between missing and follow-up cases in sex ($\chi^2 = 0.066$, $P = 0.797$) or age ($t = -0.860$, $P = 0.390$). Although statistical adjustment was performed, representation of the sample in the study may have been impacted.

COMMENTS

Background
Depressive disorders and anxiety disorders are common in general hospitals and represent significant risks to patients' quality-of-life. Patients visiting non-psychiatric departments may have at least one somatic symptom which is partly of emotional origin, challenging non-psychiatric physicians to detect emotional disorders.

Research frontiers
Emotional disorders in gastrointestinal (GI) patients have been assessed in a number of studies in America, Europe and China, including non-mainland regions of Hong Kong and Taiwan. However, the economic status and cultural traditions of mainland China are quite distinctive from foreign countries and even the non-mainland regions of China. It is important to understand the mental health situation of mainland Chinese GI patients.

Innovations and breakthroughs
The current study determined the prevalence of depressive and/or anxiety disorders and physicians' detection rates in tertiary care hospitals across mainland China. In particular, this is the first multi-center study from the mainland of China with a large number of patients to report the prevalence of depression and anxiety in adult GI outpatients. Furthermore, the diagnosis of depressive and anxiety disorders was made with the Mini International Neuropsychiatric Interview diagnostic instrument.

Applications
The results of this study suggest that clinicians should improve their abilities to detect emotional disorders. Furthermore, they serve to remind the government or medical institutions of the importance of promoting productive interactions between psychiatry and other departments.

Peer review
This study is well designed including group analysis and statistics. In particular, this is the first multi-center study from the mainland of China with a large number of patients to report the prevalence of depression and anxiety in adult GI outpatients.

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BRIEF ARTICLE

Evaluation of malignancy using Ki-67, p53, EGFR and COX-2 expressions in gastrointestinal stromal tumors

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Abstract

AIM: To investigate the role of expressions of Ki-67, p53, epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) in gastrointestinal stromal tumor (GIST) grading and prognosis.

METHODS: Tumor tissue was collected retrospectively from 96 patients with GIST. Antibodies against Ki-67, p53, EGFR and COX-2 were used for immunohistochemical staining. Tumor grading was designated according to a consensus system and the staining was quantified in 3 categories for each antibody in the statistical analysis.

RESULTS: The Ki-67 expression in GISTs was significantly associated with the size of the tumors, mitotic rate and the risk of malignancy (χ^2 = 15.51, P = 0.02; χ^2 = 22.27, P < 0.001; χ^2 = 20.05; P < 0.001). The p53 expression was also significantly correlated with mitotic rate and the risk of malignancy (χ^2 = 9.92, P = 0.04; χ^2 = 9.97; P = 0.04). Over-expression of Ki-67 was strongly correlated with poor survival (χ^2 = 10.44, P = 0.006), but no correlation was found between the expression of p53, EGFR or COX-2 and survival. Multivariate analysis further demonstrated that Ki-67 expression (relative risk = 15.78, 95% CI: 4.25-59.37) could be used as an independent prognostic value for GIST patients. Adjuvant imatinib therapy could improve clinical outcomes in the patients with high risk and intermediate risk of recurrence after complete tumor resections (median survival time: 52 mo *vs* 37 mo, χ^2 = 7.618, P = 0.006).

CONCLUSION: Our results indicated that the expression of Ki-67 could be used as an independent prognostic factor for GIST patients.

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is one of the most frequent mesenchymal neoplasms of the gastroin-

testinal tract. In the elderly, micro-GIST (the tumor size smaller than 1 cm) is detected in 20%-30% of individuals over 60 years old[1,2]. GIST occurs along the gastrointestinal tract and commonly invades in the stomach and small intestine. The tumors rarely arise from extragastrointestinal sites, such as omentum or mesentery[3]. Most GISTs express c-kit. Monoclonal antibodies against c-kit, DOG1 and protein kinase C theta have been developed as helpful diagnostic adjuncts in pathology[4-6].

GISTs have a wide clinical spectrum, ranging from virtually benign to highly aggressive tumors. Up to 30% of GISTs recur and progress to metastatic disease even after the complete excision of tumors. Despite a remarkable progress in the understanding of GISTs, it is still difficult to make a prognosis due to the variability of disease[7]. According to the National Institutes of Health (NIH) classification system, GISTs are classified into four categories: very low, low, intermediate and high risk[8]. The prognosis of patients is commonly stratified based on tumor size and mitotic counts in the NIH system. Previous studies have demonstrated that nuclear atypia and tumor necrosis all contribute to prognostic outcomes of GIST patients. Further, some studies showed that gastric GISTs had lower risks of recurrence than nongastric tumors with the same size and same mitotic count[9]. The four-point classification only distinguishes GISTs with high-risk from those with low-risk[10]. The system using multiple histopathological parameters for GIST prognosis is subjective and lacks reproducibility[11].

The proliferation marker Ki-67, tumor suppressor gene *p53*, cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) have been identified as prognostic biomarkers in tumors of epithelial origin. However, there has been no study analyzing these markers systematically in a large cohort of mesenchymal tumors, especially in GISTs[12,13]. In this study, Ki-67, p53, EGFR and COX-2 expressions were fully investigated in the GIST tumor specimens from 96 patients and the grade of the tumor was established based on the immunohistochemical staining of each protein. The grades were then compared with patients' clinical features and roles of prognostic values for GISTs were evaluated. The study indicated that determination of these tumorigenetic and cell proliferative proteins provides alternative measurements for follow-up and prognosis.

MATERIALS AND METHODS

Patients

From January 2005 through December 2009, 134 patients were initially diagnosed as having mesenchymal gastrointestinal tumors at Jilin University First Hospital. Thirty-three patients were excluded from the study due to recurred tumors or the tumors being partially resected. Thus, 101 patients underwent successful surgical operations for complete resection of tumors. Following the surgery, patients with high-risk and intermediate risk were treated with imatinib (Glivec®, Novartis Pharmaceuticals,

Table 1 Clinicopathological features of 96 patients with gastrointestinal stromal tumor

	n	% of 96 GISTs
Age (yr, median age = 55 yr)		
< 40	12	12.5
40-60	52	54.2
> 60	32	33.3
Sex		
Male	57	59.4
Female	39	40.6
Site		
Esophagus	3	3.1
Gastric	45	46.9
Intestine	37	38.5
EGIST	11	11.5
Tumor size (cm, median size = 7.0)		
⩽ 2	7	7.3
> 2 to ⩽ 5	29	30.2
> 5 to ⩽ 10	34	35.4
> 10	26	27.1
Mitotic rate (per 50 HPFs)		
⩽ 5	54	56.3
6 to 10	28	29.2
> 10	14	14.6
Risk of malignancy		
High risk	45	46.9
Intermediate risk	24	25.0
Low risk	24	25.0
Very low risk	3	3.1

GISTs: Gastrointestinal stromal tumors; EGIST: Extra GIST; HPFs: High-power fields.

Basel, Switzerland) at a dose of 400 mg/d for 3 years. No imatinib treatment was given before the surgery. Five cases were lost to follow-up. Ninety-six patients were retrospectively evaluated in the study. Informed consents were obtained from all patients and the study was approved by the local human ethical committee of Jilin University First Hospital. Original hematoxylin and eosin-stained sections were reviewed in each case by two pathologists (Jin MS and Wang YP) according to GIST characteristics described by Miettinen[14]. All tumors from 96 patients were confirmed to be GISTs based on a combination of histological evaluations (highly cellular spindles/epithelioids/mixed cell tumors), and c-kit, DOG1, CD34 positive staining. The clinical information regarding the patients is summarized in Table 1.

Immunohistochemical analysis

Histological sections (4 μm) of 10% formalin-fixed, paraffin-embedded material were used for immunohistochemical staining. Prior to a primary antibody staining, the slide was pretreated with citric acid or ethylenediaminetetraacetic acid buffer in a pressure cooker for antigen retrieval. Endogenous peroxidase activity was quenched by 3% H_2O_2 blocking reagent for 10 min. The slide was incubated with a primary antibody at 4 ℃ overnight, and then immunostained with the avidin-biotin peroxidase complex (DAKO, CA). Finally, the slide was stained with diaminobenzidine according to the manufacturer's protocol (DAKO, CA). The slide was

rinsed three times with phosphate buffered saline after each step of staining. The sections were stained with primary antibodies against c-kit (Clone: YR145, dilution: 1/50, Cell Marque Corporation, CA), CD34 (QBEnd/10, dilution: 1/100, Neomarkers, CA), Ki-67 (MIB-1, dilution: 1/100, DAKO, Carpinteria, CA), DOG1 (SP31, dilution: 1/100, Spring Bioscience, Pleasanton), SMA (IA4, dilution: 1/200, Cell Marque Corporation, CA), p27 (1B4, dilution: 1/20, Novocastra), p53 (SP5, dilution: 1/100, Zymed Laboratories, San Francisco), S-100 (6E6, dilution: 1/100, Neomarkers, CA), Desmin (D33, dilution:1/50, Cell Marque Corporation, CA), EGFR (EGFR.113, dilution: 1/200, Novocastra Laboratories Ltd, Newcastle, United Kingdom), and COX-2 (SP21, dilution: 1/50, Neomarkers, CA, United States), respectively. All primary antibodies used in the study were biotinylated monoclonal antibodies. The stained slides were evaluated quantitatively or semi-quantitatively by two independent pathologists who were blinded from clinical data. Percentages of positive cells stained with a special antibody observed by two pathologists were consistent and the mean values were determined.

The nuclear staining for Ki-67 and p53, and cytoplasmic immunostaining for EGFR and COX-2, were considered as positive cells of the reaction. According to previous studies[15,16], the following scoring assessments for Ki-67 and p53 were used. The score 0 was assigned for < 5%, 1 for > 5% and < 10%, 2 for > 10% of Ki-67 staining positive cells. The p53 scoring system was 0 assigned for < 5%, 1 for > 5% and < 25%, 2 for > 25% of p53 staining positive cells. EGFR scoring system was 0 assigned for < 10%, 1 for > 10% and < 60%, 2 for > 60% of EGFR positive cells based on the systems described by Nakagawa *et al*[17] and Gumurdulu *et al*[18]. The COX-2 scoring system was 0 assigned for no positive cells; 1 for < 25% and score 2 for > 25% of COX-2 staining positive cells according to Fux *et al*[19].

Statistical analysis

The Chi-square test and Fisher's exact test were used to analyze relationships between clinicopathological features and expression levels of biomarkers. Kaplan-Meier test was applied with a log-rank test to study associations between categorical variables and the mean values of survival among groups. Cox proportional hazards regression analysis was used to estimate a hazard risk for survival and 95% CI was applied. The SPSS program (version 18.0) was used for statistical analysis. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Clinicopathological findings and follow-up

Clinicopathological features of the patients are summarized in Table 1. The median age of 96 patients was 55 years (range, 26-82 years). Histomorphology showed that the neoplastic cells were predominantly spindle-shaped (83/96, 86.5%). Based on the modified NIH risk

consensus system, 45 (46.9%), 24 (25.0%), 24 (25.0%) and 3 (3.1%) cases were classified as high-risk, intermediate-risk, low risk and very low risk categories, respectively. Fifty-three cases (55.2%) had mild nuclear atypia; 32 cases (33.4%) showed severe nuclear atypia, but 11 patients (11.4%) had no nuclear atypia. Tumor necrosis was found in 39 cases of the patients (40.6%).

At the time of study, the mean or the median duration of the follow-up period was 31 mo or 29 mo, respectively. Medical charts were available for 96 of 101 patients (95%). Sixty-nine patients (54.2%) received the imatinib treatment at a dose of 400 mg/d for 13 mo to 36 mo (median, 26 mo). Thirty-seven patients (82%) from the high risk group and 15 patients (62.5%) from the intermediate group required the imatinib treatment. Disease specific 1, 2, 3 and 4 year survival probabilities were 0.97, 0.89, 0.79, and 0.77 (0.65-0.87), respectively. Of the 96 cases, 19 patients (19.8%) died from GISTs and 6 patients (6.3%) died from unrelated causes.

Immunohistochemical findings

Eighty-eight (91.3%) tumor specimens were stained positive for c-kit. The tumors isolated from 8 patients (8.7%) were negative for c-kit but positive for DOG1 and/or CD34 staining. Reactivity with Desmin was found in 3 (3.1%) cases. Positive SMA and S-100 staining were also noted in 46 (47.9%) and 12 (12.5%) cases, respectively. Based on the Ki-67 index, 53.1% of tumors (*n* = 51) scored 0; 34.4% (*n* = 33) scored 1; and 12.5% (*n* = 12) scored 2. 34.4% of tumor specimens (*n* = 33) were p53 staining positive in the nuclei of over 25% of the cells. EGFR staining was found in most cases. Forty-two (43.8%) cases scored 2, and 27 (28.1%) cases scored 1 for EGFR staining. COX-2 overexpressed in 36 (37.5%) cases (Table 2 and Figure 1).

Clinicopathological features and GIST grades categorized by the staining of Ki-67, p53, EGFR or COX-2 are established in Table 3. The expression of Ki-67 was significantly associated with tumor size (*P* = 0.02), mitotic rate (*P* < 0.001) and the risk of malignancy (*P* < 0.001). The p53 expression was also correlated with mitotic rate (*P* = 0.04), tumor site (*P* = 0.02) and the risk of malignancy (*P* = 0.04). The levels of COX-2 protein were significantly higher in gastric tumors and spindle cell-like tumors (*P* < 0.001 and *P* = 0.05, respectively). In contrast, no correlation was found between the EGFR expression and clinicopathological factors or the risk of malignancy.

Survival analysis

The 3-year survival rates for disease specific survival (DSS) were 100%, 89%, 79% and 67% for groups at very low-risk, low-risk, intermediate-risk and high risk by the modified NIH risk categories, respectively. Associations between DSS and different protein biomarkers were analyzed using a multivariate analysis (Table 3 and Figure 2). The survival rates were strongly associated with tumor size (*P* = 0.004), mitotic count (*P* = 0.001),

Table 2 Ki-67, P53, epidermal growth factor receptor, cyclooxygenase-2 expression related to clinicopathological features

Variable	Ki-67				P53				EGFR				COX-2			
	0+	1+	2+	P value	0+	1+	2+	P value	0+	1+	2+	P value	0+	1+	2+	P value
Tumor size (cm)																
≤ 2	6	1	0	0.02	3	3	1	0.47	1	3	3	0.41	2	2	3	0.07
> 2 to ≤ 5	21	7	1		8	14	7		6	12	11		17	7	5	
> 5 to ≤ 10	15	15	4		9	10	15		11	8	15		20	9	5	
> 10	9	10	7		5	11	10		9	4	13		21	1	4	
Total	51	33	12		25	38	33		27	27	42		60	19	17	
Mitotic rate (per 50 HPFs)																
≤ 5	39	12	3	< 0.001	17	20	17	0.04	16	16	22	0.77	38	8	8	0.41
> 5 to ≤ 10	10	14	4		8	13	7		7	6	15		14	8	6	
> 10	2	7	5		0	5	9		4	5	5		8	3	3	
Risk of malignancy																
Very low + low risk	23	4	0	< 0.001	9	11	7	0.04	6	11	10	0.56	17	6	4	0.50
Intermediate risk	13	9	2		9	11	4		7	5	12		12	5	7	
High risk	15	20	10		7	16	22		14	11	20		31	8	6	

EGFR: Epidermal growth factor receptor; COX-2: Cyclooxygenase-2; HPFs: High-power fields.

Figure 1 Images of gastrointestinal stromal tumor using hematoxylin-eosin staining and immunohistochemical staining. A: Hematoxylin-eosin stain; B: DOG1 stain; C: Ki-67 stain; D: P53 stain; E: Epidermal growth factor receptor stain; F: Cyclooxygenase-2 stain.

tumor location ($P = 0.018$), the NIH modified criteria ($P < 0.001$, Figure 2A), Ki-67 amplification ($P < 0.001$, Figure 2B), and adjuvant imatinib therapy (median survival period 52 mo *vs* 37 mo, $\chi^2 = 7.618$, $P = 0.006$, Figure 2C). No significance was found when comparing survival rates with the EGFR expression, or the COX-2 expression. In the high-risk group, Ki-67 overexpression was significantly associated with poor survival ($\chi^2 = 10.44$, $P = 0.006$), but no statistical significance was found between the p53 expression and survival ($\chi^2 = 4.744$, $P = 0.089$). Using a multivariate analysis, a poor survival was observed in the high risk category [relative risk (RR) = 12.23; 95% CI: 1.61-92.81] graded using the modified NIH risk consensus system or in category 2

scored by the Ki-67 expression (RR = 15.78; 95% CI: 4.25-59.37) (Table 3).

DISCUSSION

In the absence of reliable genetic and immunohistochemical biomarkers in GISTs, the tumor size and mitotic rate are often used to assess risk probabilities in GIST patients. Large retrospective cohort studies have shown that the NIH classification carries substantial prognostic value[20]. Using classical morphological parameters, our results were consistent with previous studies on the prognosis of GIST patients.

Ki-67, a nuclear protein associated with cell prolif-

Figure 2 Kaplan-Meier plots for predicting disease specific survival based on the modified National Institutes of Health consensus system (A), Ki-67 expression (B) and adjuvant imatinib therapy (C).

Table 3 Multivariate analyses for disease-specific survival

Variable	RR (95% CI)	*P* value
Risk of malignancy		
Very low/low risk	1.00 (reference)	< 0.001
Intermediate risk	1.32 (0.81-21.2)	
High risk	12.23 (1.61-92.81)	
Ki-67 expression		
0+	1.00 (reference)	< 0.001
1+	3.75 (0.97-14.54)	
2+	15.78 (4.25-59.37)	
P53 expression		
0+	1.00 (reference)	0.20
1+	2.11 (0.24-18.37)	
2+	4.49 (0.54-37.11)	
EGFR expression		
0+	1.00 (reference)	0.50
1+	1.97 (0.60-6.50)	
2+	1.17 (0.36-3.77)	
COX-2 expression		
0+	1.00 (reference)	0.19
1+	0.84 (0.42-4.36)	
2+	1.99 (0.55-7.21)	

EGFR: Epidermal growth factor receptor; COX-2: Cyclooxygenase-2; RR: Relative risk.

The tumor suppressor p53 plays an important role in the regulation of cell cycle, DNA repair and programmed cell death. The functional loss of p53 disrupts these pathways and results in the selection of tumor cells with growth advantage[24]. p53 has been reported as a prognostic marker in a wide variety of carcinomas, as well as in GISTs[25]. A study showed that impaired p53 expression was often found in advanced GISTs and a strong effect of p53 on the progression-free survival was also observed[18]. The accumulation of p53 protein was significantly associated with mitotic rate and the risk of malignancy in the present study.

The activation of EGFR is associated with cell growth and transformation. There are few reports analyzing the EGFR expression in GISTs. A study has suggested that a transforming growth factor alpha (TGF-α)/EGFR autocrine loop is present in GISTs, in which TGF-α promotes the proliferation of GIST tumor cells through an interaction of EGFR with HER-1[26]. Co-expressions of EGFR and several EGFR ligands were observed with the upregulation of ADAM17 in GISTs. The authors suggested that the EGFR activation was through shedding of EGFR ligands by ADAM17 and consequently resulted in GIST progression and growth[17]. To our knowledge, there has been no study assessing prognostic values of EGFR in a large cohort of GISTs. However, no significant association was found between the EGFR expression and prognostic analysis of GISTs in our study.

Increased COX-2 expression has been observed in colorectal adenoma and carcinoma[27]. The induction of COX-2 has been shown to promote cell growth, inhibit apoptosis and enhance cell motility and adhesion[28]. Over-expression of COX-2 has tumorigenic effects in animal models[29]. Expression levels of COX-2 and vascular endothelial growth factor were found to

eration, expresses in all cell cycle phases except for G0. A recent study demonstrated that the automated assessments of Ki-67 staining with computing image analysis can be used for prognostic assessments of patients with breast cancer[21]. However, the prognostic value of Ki-67 as a potential biomarker has not been fully investigated in GISTs[22,23]. The present study shows that the expression of Ki-67 or p53 is significantly associated with many clinicopathological features in GISTs; higher score for Ki-67 staining was directly correlated with poor survival; Ki-67 was superior to other protein markers tested in survival assessments, particularly in the high risk group, suggesting that Ki-67 immunostaining is a reliable and independent marker for the prediction of clinical outcomes in patients with GISTs.

be significantly higher in malignant GISTs than those in benign and intermediate GISTs[30]. A study reported a correlation between the COX-2 expression and tumor cell proliferation in GISTs, but no association was found between COX-2 expression and mortality, metastasis, tumor size, the risk of stages, the dose of tyrosine kinase inhibitors, or the rate of complete resection[31]. Our results demonstrated that levels of COX-2 expression were significantly different between gastric tumors and nongastric tumors ($P < 0.001$), but no significant relationship was found between the COX-2 expression and risk factors, or survival.

Imatinib therapy reduces rates of recurrence in GISTs. Nevertheless, it remains unclear how to screen patients who would be more likely to benefit from the adjuvant therapy. In our study, we found that imatinib treatment could significantly improve 3-year DSS rates in the intermediate and high risk categories of patients after a complete tumor resection.

In conclusion, Ki-67 expression is significantly associated with GIST malignancy and can be used as a putative prognostic marker in GISTs. p53 and COX-2 also provide additional valuable information in the evaluation of malignancy and types of GISTs.

COMMENTS

Background
Gastrointestinal stromal tumors (GISTs) are known to have a wide variability in malignancy and prognosis. Risk grading based on tumor size, location and mitotic counts has been proposed to predict adverse outcomes of GIST in the literature.

Research frontiers
Recent molecular studies have found that the deregulations of Ki-67, cyclin A, B1, D1, E, cdc2 and other cell-cycle regulators were significantly associated with a shorter disease-free survival in GISTs.

Innovations and breakthroughs
In this study, expressions of Ki-67, p53, epidermal growth factor receptor (EGFR) and COX-2 were investigated in a large cohort of GIST patients and their roles as prognostic values for GISTs were also evaluated. To our knowledge, this is the first assessment of the prognostic value of EGFR in patients with GISTs.

Applications
The immunohistochemical staining of these tumorigenetic and cell proliferative proteins provides an alternative approach for follow-up and clinical decisions regarding the treatment for GISTs.

Peer review
This paper describes a retrospective clinicopathological study of GIST.

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BRIEF ARTICLE

Effects of glycine on phagocytosis and secretion by Kupffer cells *in vitro*

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Abstract

AIM: To investigate the effects and mechanisms of action of glycine on phagocytosis and tumor necrosis factor (TNF)-α secretion by Kupffer cells *in vitro*.

METHODS: Kupffer cells were isolated from normal rats by collagenase digestion and Percoll density gradient differential centrifugation. After culture for 24 h, Kupffer cells were incubated in fresh Dulbecco's Modification of Eagle's Medium containing glycine (G1: 1 mmol/L, G2: 10 mmol/L, G3: 100 mmol/L and G4: 300 mmol/L) for 3 h, then used to measure phagocytosis by a bead test, TNF-α secretion after lipopolysaccharide stimulation by radioactive immunoassay, and microfilament and microtubule expression by staining with phalloidin-fluorescein isothiocyanate (FITC) or a monoclonal anti-α tubulin-FITC antibody, respectively, and evaluated under a ultraviolet fluorescence microscope.

RESULTS: Glycine decreased the phagocytosis of Kupffer cells at both 30 min and 60 min ($P < 0.01$, $P < 0.05$). The numbers of beads phagocytosed by Kupffer cells in 30 min were 16.9 ± 4.0 (control), 9.6 ± 4.1 (G1), 12.1 ± 5.7 (G2), 8.1 ± 3.2 (G3) and 7.5 ± 2.0 (G4), and were 22.5 ± 7.9 (control), 20.1 ± 5.8 (G1), 19.3 ± 4.8 (G2), 13.5 ± 4.7 (G3) and 9.2 ± 3.1 (G4) after 60 min. TNF-α secretion by Kupffer cells in G1 (0.19 ± 0.03), G2 (0.16 ± 0.04), G3 (0.14 ± 0.03) and G4 (0.13 ± 0.05) was significantly less than that in controls (0.26 ± 0.03, $P < 0.01$), and the decrease in secretion was dose-dependent ($P < 0.05$). Microfilaments of Kupffer cells in G2, G3 and G4 groups were arranged in a disorderly manner. The fluorescence densities of microtubules in G1 (53.4 ± 10.5), G2 (54.1 ± 14.6), G3 (64.9 ± 12.1) and G4 (52.1 ± 14.2) were all lower than those in the controls (102.2 ± 23.7, $P < 0.01$), but the decrease in microtubule fluorescence density was not dose-dependant.

CONCLUSION: Glycine can decrease the phagocytosis and secretion by Kupffer cells *in vitro*, which may be related to the changes in the expression of microfilaments and microtubules induced by Kupffer cells.

INTRODUCTION

Glycine has been well characterized in the spinal cord as an inhibitory neurotransmitter which activates expression of the glycine-gated chloride channel (GlyR) in postsyn-

aptic membranes. Kupffer cells contain a GlyR similar to that described previously in the central nervous system[1,2]. Many studies have shown that dietary or intravenous glycine has a protective effect in rat models against endotoxic shock, hemorrhagic shock, liver ischemia-reperfusion, liver transplantation, and alcohol-induced liver injury and is most likely to exert this effect by inactivating the Kupffer cells *via* this newly identified GlyR[3-10]. Our previous studies also indicated that glycine protected rats from thioacetamide-induced liver injury and intestinal endotoxemia[11,12]. The mechanism involved may be related to inhibition of the release of pro-inflammatory cytokines by Kupffer cells induced by glycine. *In vivo* and *in vitro* experiments have found that glycine inhibits the secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in Kupffer cells[13-15]. However, the impact of glycine on phagocytosis by Kupffer cells has not been reported, and the mechanisms underlying the effect of glycine on TNF-α secretion by Kupffer cells have not been fully understood. Our *in vitro* study showed that lipopolysaccharide (LPS) probably enhanced or inhibited the phagocytosis of Kupffer cells by acting through mechanisms involving microfilaments or microtubules[16]. This study aimed to investigate the effects of glycine on phagocytosis and the mechanisms underlying TNF-α secretion by Kupffer cells *in vitro*.

MATERIALS AND METHODS

Animals
Adult male Wistar rats weighing 300-330 g were obtained from the Experimental Animal Center of Shanxi Medical University (China). All animals were fed with standard laboratory chow and water was available *ad libitum*. The experimental protocols were approved by the Shanxi Animal Research Ethics Committee.

Reagents
Polystyrene beads (1.1 μm), monoclonal anti-α tubulin-fluorescein isothiocyanate (FITC) conjugate, LPS (*Escherichia coli* Serotype 0128:B12), collagenase IV, phalloidin-FITC, hydroxyethyl piperazine ethanesulfonic acid (HEPES) Percoll, and Dulbecco's Modification of Eagle's Medium (DMEM) were purchased from Sigma (St. Louis, United States); a radioimmunoassay kit for TNF-α measurement was purchased from the Radio-Immunity Institute of the Chinese Liberation Army Omni-hospital (Beijing, China); glycine, sodium pentobarbital, fetal bovine serum (FBS), penicillin G, streptaquaine, insulin, glutamine, trypan blue, and all other reagents not specifically mentioned elsewhere were prepared by Beijing Chemical Inc. (Beijing, China).

Isolation and culture of Kupffer cells
Kupffer cells from Wistar rats were isolated by collagenase digestion and differential centrifugation, using Percoll density gradients as described previously with slight modifications[17]. Briefly, the liver was perfused *in situ* through the portal vein with Ca^{2+} and Mg^{2+} free Hanks'

balanced salt solution (HBSS) containing 0.5 mmol/L ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N- tetraacetic acid (EGTA) at 37 ℃ for 5 min at a flow rate of 26 mL/min. Subsequently, perfusion was performed with HBSS containing 0.05% collagenase IV at 37 ℃ for 5 min. After the liver was digested, it was excised and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze, and the filtrate was centrifuged twice at $50 \times g$ at 4 ℃ for 3 min to remove parenchymal cells. The nonparenchymal cell fraction was washed with buffer and centrifuged on a density cushion of Percoll at $1000 \times g$ at 4 ℃ for 20 min to obtain the Kupffer cell fraction, and the cells obtained were washed with buffer again. The viability of isolated Kupffer cells was determined by trypan blue exclusion and routinely exceeded 90%. Cells were seeded onto 24-well culture plates (Corning, NY) or 25 mm × 25 mm glass coverslips at a density of 1×10^6 or 5×10^5 and cultured in DMEM supplemented with 10% FBS, antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin sulfate), 0.1 U/100 mL insulin and 15 mmol/L glutamine at 37 ℃ with 5% CO$_2$. Non-adherent cells were removed after 1 h by replacing the culture medium. All adherent cells phagocytosed latex beads and stained positive for catalase, confirming that they were Kupffer cells, and cells were cultured for 24 h before experiment.

Effects of glycine on Kupffer cells
Cells were seeded onto 24-well plates and 12 mm × 12 mm glass coverslips, and incubated with fresh medium containing glycine (G1: 1 mmol/L, G2: 10 mmol/L, G3: 100 mmol/L and G4: 300 mmol/L) at 37 ℃ with 5% CO$_2$ for 3 h. Phagocytosis and expression of microfilaments and microtubules by Kupffer cells were measured by the bead phagocytosis test, fluorescence staining and immunofluorescence staining, as described below.

Measurement of phagocytosis by Kupffer cells
Phagocytosis by Kupffer cells was evaluated by the Kupffer cell's ability to ingest polystyrene beads according to the modified method of Hirose *et al*[18]. Briefly, cells were seeded onto 12 mm × 12 mm glass coverslips or glass plates and incubated with fresh medium containing 0.05% polystyrene beads for 30 min or 60 min at 37 ℃ with 5% CO$_2$. Following vigorous pipetting to remove non-phagocytosed latex beads, the coverslips or glass plates were washed 3 times with PBS and fixed with 2% formaldehyde or methanol for 5 min. After staining by Giemsa's method for 15 min at room temperature and washing 3 times with PBS, the coverslips were inverted onto glass slides and observed under phase contrast microscope. The mean number of latex beads phagocytosed by each Kupffer cell was counted in at least 20 Kupffer cells per field at magnification of 200 times, 5 fields per coverslip in 6 coverslips.

Measurement of Kupffer cell secretion
Kupffer cells were seeded into 24-well plates at a density of 1×10^6/well and incubated with fresh DMEM

Figure 1 Effects of glycine on phagocytosis by Kupffer cells *in vitro*. A: Phagotosis by Kupffer cells in the control group 30 min after the addition of latex beads, 200×; B: Phagotosis by Kupffer cells in group G3 30 min after the addition of latex beads, 200×.

containing 100 ng/mL LPS for 60 min at 37 ℃ with 5% CO_2. At the end of this period, the medium was collected, centrifuged at $1000 \times g$ at 4 ℃ for 10 min, and the supernatant was stored at -80 ℃ until used for TNF-α assay. TNF-α in medium was measured using the radio-immunoassay kit. The levels of TNF-α in the wells represented the secretion of Kupffer cells.

Measurement of microfilament expression by Kupffer cells

Kupffer cells were stained with phalloidin-FITC according to the modified method of Wulf *et al*[19]. Briefly, Kupffer cells were seeded onto 12 mm × 12 mm glass coverslips at a density of 5×10^5 (1×10^4 to 2×10^4 cells/coverslip), fixed with 2% formaldehyde for 20 min and extracted with 0.5% Triton X-100 for 15 min. The fixed cells were then washed 3 times with PBS (10 mmol/L, pH 7.4) and stained with phalloidin-FITC for 45 min at room temperature in the dark. They were then washed for a further 3 times with PBS, the coverslips were inverted onto mounting medium applied to glass slides, and they were observed and photographed under a ultraviolet (UV) fluorescence microscope with a high magnification of 400 times. Mounted preparations could be stored in the dark at 2 ℃-8 ℃.

Measurement of microtubules in Kupffer cells

Microtubules in Kupffer cells were stained with a monoclonal anti-α tubulin-FITC antibody according to the method recommended by the producer. Briefly, Kupffer cells were seeded onto 12 mm × 12 mm glass coverslips at a density of 5×10^5 (1×10^4 to 2×10^4 cells/coverslip). They were then fixed with cold methanol for 10 min at -20 ℃ and rinsed twice with cold acetone (-20 ℃) for 10 s, then the cell layer was rehydrated in PBS (10 mmol/L, pH 7.4) for at least 30 min and stained with monoclonal anti-α tubulin-FITC (1:25 diluted with PBS containing 1% bovine serum albumin) in a dark-room for 60 min at room temperature. The stained cells were washed 3 times with PBS, the coverslips were inverted onto mounting medium applied to glass slides and observed and photographed under a UV fluorescence microscope. Mounted preparations could be stored in

Table 1 Effects of glycine on phagocytosis by Kupffer cells *in vitro* (mean ± SD)

Groups	Beads observed in Kupffer cells ($n = 6$)	
	30 min	60 min
Control	16.9 ± 4.0	22.5 ± 7.9
G1	9.6 ± 4.1[b]	20.1 ± 5.8
G2	12.1 ± 5.7[a]	19.3 ± 4.8
G3	8.1 ± 3.2[b]	13.5 ± 4.7[b,c]
G4	7.5 ± 2.0[b]	9.2 ± 3.1[b,d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control; [c]$P < 0.05$, [d]$P < 0.01$ *vs* G1.

the dark at 2-8 ℃. The fluorescence density was measured in 10 cells using the MIAS-300 picture analysis system from at least 5 fields in each picture at a high magnification of 400 times.

Statistical analysis

All results were expressed as mean ± SD. Statistical differences between means were analyzed by one-way analysis of variance or *t* test using the SPSS 12.0 statistical package. Statistical significance level was set at $P < 0.05$.

RESULTS

Effects of glycine on phagocytosis by Kupffer cells

When incubated in 5% CO_2 with fresh medium containing glycine at 37 ℃ for 30 min or 60 min, phagocytosis by Kupffer cells decreased significantly. The number of beads phagocytosed by Kupffer cells in groups G3 and G4 was less than that of group G1 in 60 min. There were no significant differences in the amount of beads phagocytosed by Kupffer cells among the G2, G3 and G4 groups (Table 1 and Figure 1).

Effects of glycine on TNF-α secretion by Kupffer cells

When incubated in 5% CO_2 with fresh medium containing glycine at 37 ℃ for 3 h, TNF-α secretion by Kupffer cells decreased significantly, and the decrease in secretion was dose dependent. TNF-α concentrations detected in the medium of groups G3 and G4 were significantly lower than in the medium of group G1 (Table 2).

Figure 2 Effects of glycine on expression of microfilaments by Kupffer cells *in vitro*. A: The expression of microfilaments by Kupffer cells in the control group, stained with Phalloidin-fluorescein isothiocyanate (FITC), 400×; B: The expression of microfilaments by Kupffer cells in group G3, stained with Phalloidin-FITC, 400×.

Figure 3 Effects of glycine on expression of microtubules by Kupffer cells *in vitro*. A: The expression of microtubules by Kupffer cells in the control group, stained with monoclonal anti-α tubulin-fluorescein isothiocyanate (FITC) conjugate, 400×; B: The expression of microtubules by Kupffer cells in group G3, stained with monoclonal anti-α tubulin-FITC conjugate, 400×.

Table 2 Effects of glycine on tumor necrosis factor-α secretion and microtubule density of Kupffer cells *in vitro* (n = 6) (mean $\pm$ SD)

Groups	TNF-α detected in medium (μg/mL)	Microtubule density of Kupffer cells
Control	0.26 ± 0.03	102.2 ± 23.7
G1	0.19 ± 0.03[d]	53.4 ± 10.5[d]
G2	0.16 ± 0.04[a,d]	54.1 ± 14.6[d]
G3	0.14 ± 0.03[a,d]	64.9 ± 12.1[d]
G4	0.13 ± 0.05[a,d]	52.1 ± 11.4[d]

[a]$P < 0.05$ *vs* G1; [d]$P < 0.01$ *vs* control. TNF: Tumor necrosis factor.

Effects of glycine on microfilaments of Kupffer cells

After 3 h incubation in 5% CO_2 with fresh medium containing glycine at 37 ℃, Kupffer cells stained with FITC-Phalloidin did not demonstrate organized microfilaments in groups G2, G3 or G4. There were no significant differences in the microfilament fluorescence densities among Kupffer cells in control, G1, G2, G3 and G4 groups (Figure 2).

Effects of glycine on microtubules of Kupffer cells

Following 3 h incubation in fresh medium containing glycine at 37 ℃ with 5% CO_2, Kupffer cells were stained with

monoclonal anti-α tubulin-FITC. A significant decrease in the fluorescence density of microtubules was observed in Kupffer cells incubated with glycine as compared with the controls. However, the fluorescence density of the microtubules did not show a dose-dependent decrease among G1, G2, G3 and G4 groups (Table 2 and Figure 3).

DISCUSSION

Kupffer cells are the main component of the host monocyte-macrophage system, and their two main functions are phagocytosis and secretion. There is much evidence indicating that activation of Kupffer cells and their production of pro-inflammatory cytokines contribute to the pathogenesis of different liver injuries, including alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD) and liver failure among others[20-22]. Tsujimoto *et al*[23] showed that phagocytic activity of Kupffer cells was decreased in a rat model of nonalcoholic steatohepatitis. Glycine is a nonessential amino acid and an inhibitory neurotransmitter in the central nervous system. Many studies have shown that dietary or intravenous glycine can protect against a variety of liver injuries[3-10]. In this study, we found that glycine decreases the phagocytosis and secretion of Kupffer cells *in vitro*.

Effects of glycine on phagocytosis by Kupffer cells

The mechanisms of Kupffer cell phagocytosis are still not completely understood. The ruffling of the cell membrane and formation of pseudopodia may play an important role and these effects are believed to be accomplished by the cytoskeleton. In the cytoskeleton, actin-myosin interaction through the calcium-calmodulin system plays a major role in this activity[24]. In this system, intracellular Ca^{2+} combines with calmodulin to form the active calcium-calmodulin complex, which activates an enzyme, myosin light chain kinase, to phosphorylate the light chain of myosin. Phosphorylated myosin, but not unphosphorylated myosin, can interact with actin to induce activity of the cell membrane and pseudopodia, leading to phagocytosis. This process is reversible, in that a phosphatase can catalyze dephosphorylation of myosin, restoring it to a form that can not be activated by actin.

Previous studies have shown that integrity of the cytoskeletal system is important for phagocytosis of Kupffer cells. Depolymerization of the cytoskeleton decreased phagocytosis by Kupffer cells[25-27]. However, the effects of glycine on phagocytosis by Kupffer cells have not been reported.

The present experiments show that glycine decreases phagocytosis by Kupffer cells *in vitro*, causes disordering of the microfilaments in Kupffer cells, and reduces their expression of microtubules. All these results show that glycine can decrease the phagocytosis of Kupffer cells by acting on the microfilaments and microtubules.

Effects of glycine on secretion by Kupffer cells

Some studies have shown that both CD_{14} and non-CD_{14} mechanisms are involved in the TNF-α secretion of monocytes and Kupffer cells, and that both endocytosis and Ca^{2+} are required for endotoxin-stimulated TNF-α release by Kupffer cells in rats[28-30]. Previous studies have shown that glycine can protect against many injuries and illnesses in rat models, most likely by inactivating Kupffer cells and decreasing TNF-α secretion[3-15]. An *in vitro* study has shown that glycine prevents the increases in $[Ca^{2+}]_i$ caused to LPS by activating chloride influx-reduced synthesis and release of toxic mediators by Kupffer cells[2]. Thus, glycine can activate the chloride influx, prevent the increases in $[Ca^{2+}]_i$ and reduce the TNF-α secretion of Kupffer cells.

Other studies have demonstrated the involvement of a microtubule-dependent mechanism in TNF-α secretion by monocytes. Taxol, a microtubule-stabilizing antineoplastic agent, induced expression of tumor TNF-α in macrophages[31]. Microtubule-disrupting agents such as colchicine had opposite effects on TNF-α production[32-34]. The present experiments showed that glycine significantly decreased TNF-α secretion and microtubule expression. Some of our results are consistent with previous reports[13-15], leading us to believe that glycine can prevent TNF-α secretion by Kupffer cells through disruption of microtubules.

In summary, glycine decreases both phagocytosis and secretion by Kupffer cells *in vitro*, which is probably related to glycine-induced changes in expression of microfilaments and microtubules in Kupffer cells.

COMMENTS

Background

Activated Kupffer cells are most likely involved in the pathogenesis of different liver injuries. Glycine generally is considered as a protective agent for liver injuries. The mechanism may be related to the fact that glycine inhibits the release of pro-inflammatory cytokines by Kupffer cells. So, it is very important to clarify the impact of glycine on the phagocytosis and secretion by Kupffer cells.

Research frontiers

It is believed that cytoskeleton plays a vital physiological role in phagocytosis by Kupffer cells, and depolymerization of cytoskeleton decreases the phagocytosis by Kupffer cells. Glycine protects against liver injuries by preventing the elevation of intracellular Ca^{2+} and reducing pro-inflammatory cytokines production by Kupffer cells. But the impact of glycine on phagocytosis by Kupffer cells is still unclear, and the mechanisms of glycine on tumor necrosis factor-α secretion by Kupffer cells have not been completely understood.

Innovations and breakthroughs

This is the first study to report that glycine decreases the phagocytosis of Kupffer cells by acting on the microfilaments and microtubules *in vitro*.

Applications

This study suggests that glycine may be an effective agent which could provide a future strategy for therapeutic intervention in the treatment of liver injuries induced by activated Kupffer cells.

Peer review

It is an interesting study with appropriate methodology and the results are clear and of great importance.

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CASE REPORT

Exceptionally rare cause of a total stomach resection

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Abstract

The first-ever case of a 54-year-old woman who overdosed on non-steroidal anti-inflammatory drugs in an attempt at suicide. Before that incident, she had not been treated for coexisting diseases such as rheumatoid arthritis or depression. At the time of admission to the General Surgery Department, the patient reported pains in the epigastric region with accompanying nausea and vomiting with mucous content as well as the inability to ingest food orally. Despite parenteral and enteral feeding, the patient exhibited a drop in body mass. The histopathologic examination of a sample taken from the stomach during gastroscopy showed some non-specific necrotic and inflammatory masses with granulation. Intraoperatively, a very small, infiltrated stomach with an initial section of duodenum was identified. A total stomach resection together with the reconstruction of digestive tract continuity was performed using the Roux-Y method. Histopathologic examination of the stomach revealed a deep, chronic and exacerbated inflammatory condition with an extensive ulceration over the entire length of the stomach, reaching up to the pylorus. Additionally, numerous lymphatic glands with inflammatory reaction changes were observed.

INTRODUCTION

Currently, total stomach resection is typically performed in adults with stomach cancer[1-9]. Other causes of total stomach resection include chemical burns of the digestive tract caused by the consumption of toxic, most often caustic, substances, which occurs accidentally in children and in adults who have attempted suicide[10]. Most recent studies describe burns in the upper part of the digestive tract occurring after the consumption of concentrated acids, pesticides and bases used as detergents, bleachers or rust-removers[11-13]. Drug poisonings usually cause symptoms in the central nervous system and the circulatory and

respiratory systems as well as bleeding because they most often involve the ingestion of soporific, psychotropic and cardiac medicines. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in treatments globally. In some countries, e.g., in the United States, NSAIDs are used protractedly and in large doses, particularly in elderly patients. The administration of NSAIDs involves the risk of complications occurring in the digestive tract. It has been shown that these medicines may damage mucous membranes in the stomach and induce complications such as ulcerations, hemorrhages, or perforations[14-17]. The aim of the article is to present a female patient who consumed a very large amount of NSAIDs and Tramal at the same time in a suicide attempt.

CASE REPORT

A 54-year-old female was admitted to the General Surgery Department of the University Hospital in Olsztyn from the Internal Medicine Department to undergo surgical treatment of a pylorus narrowing that was induced by the consumption of drugs in a suicide attempt. Approximately five months before that, the woman took 60 NSAID pills (including Ibuprom, Ketonal, Diklofenak, Aspirin) and also ingested 300 mg of Tramal. After that episode, she was treated at the intensive care unit and then at the department of internal diseases. During that time, the patient was fed parenterally and, later, also enterally. Prior to that incident, the woman had not been treated for coexisting diseases such as rheumatoid arthritis or depression. At the time of admission to the surgery department, the patient reported epigastric pain with nausea and vomiting with mucous content as well as the inability to consume food orally. Despite parenteral and enteral feeding, the patient exhibited a 16-kg drop in body mass. Upon admission to the surgery department, her weight was 59 kg. The patient also suffered from pain in the epigastric and umbilical regions. However, the belly was soft with no pathological resistance, and the liver was not enlarged. Biochemical examinations showed minimal anemia (hemoglobin 10.4 g/dL) and increased fibrinogen values (up to 479 mg%) at international normalized ratio 1.10. During gastroscopy, a very small stomach was noticeably covered by scars and numerous fibrin-covered ulcerations. The stomach lacked a mucous membrane and hemorrhaged when touched with the device. The length of the stomach in the upper region was 2-3 cm. The distal part of the stomach was excessively narrowed and inaccessible. The pylorus channel allowed only for the insertion of the Flokar Ch10/130 cm catheter, which was placed properly behind the ligament of Treitz, with resistance. The histopathologic examination of a sample taken from the stomach revealed the existence of necrotic and inflammatory masses with granulation. The patient was qualified to undergo surgery operational treatment. A very small, infiltrated stomach with an initial section of duodenum was identified intraoperatively. Those organs exhibited excessive inflammatory infiltration. There were also numerous enlarged lymphatic glands, which were removed. After preparation and total resection of the stomach (Figure 1) with the initial part of the

Figure 1 Pathological changes in atrophic gastritis after treatment. A: Tubular resected stomach as a whole, 9 cm long; B1 and B2: Cross-section of stomach with its 2 cm thick wall and its atrophic mucose membrane.

duodenum, a Roux-Y anastomosis was performed using a 25-mm circular stapler. The esophageal resection line was 10 mm and free from pathological changes; the esophagus was observed to have intact epithelium and stroma with dispersed inflammatory infiltration. The initial duodenum resection line was 12-mm long with a preserved margin of mucous membrane and chronic exacerbated inflammation in the mucous and submucosal membranes. Histopathologic examination of the stomach revealed a deep, chronic and exacerbated inflammatory condition with an extensive ulceration over the entire length, reaching up to the pylorus. Microscopic examination revealed numerous lymphatic glands with inflammatory changes (Figures 2 and 3). The post-operative period was complicated on the fifth day due to bile leakage from the part of the duodenum closed with a linear stapler. A relaparotomy revealed numerous adhesions, as well as an orifice, 2 mm in diameter, on the top of the closed, bloated duodenum

Figure 2 Representative photomicrographs of hematoxylin and eosin stained sections from the stomach's necrosis of submucous membrane. A: The inflammatory infiltration of the submucosal membrane of stomach (A1 magnitude 40× and A2 magnitude 100×); B: The ulceration-granulation in the stomach's submucosal membrane, magnitude 40×.

Figure 3 Representative photomicrographs sections from the stomach (magnitude 40×). A: The large, transmural inflammatory cell infiltration of submucosal membrane CD3 (+); B: Mixed inflammatory cell infiltrations CD3 (+).

stump, approximately 10 mm from the stitch line, out of which bile flowed heavily. Through the orifice, a drain of the same diameter was introduced into the duodenum as a duodenostomy. Four days after the surgery, the patient was treated with a respirator. On the following days, apart from some non-edemic and inflammatory changes in the lower segments of the lungs, the course of treatment was satisfactory. The patient was fed parenterally and enterally through a Flocare-type tube. When performing control examinations, the drain was removed from the duodenum stump (14th day). On the 16th day, the patient was administered an oral diet that was extended gradually. On the 21st day, she was discharged from the hospital, in good general condition, with body mass of 61.5 kg.

DISCUSSION

Benzodiazepines and other psychotropic drugs are the most common causes of suicide-related drug poisoning. Few studies have examined suicide attempts that involve NSAID overdoses. The long-term administration of NSAIDs, even in therapeutic doses, results in digestive tract complications in the form of inflammation, ulceration, mucous membrane bleeding and hemorrhage as well as perforations to the stomach and duodenum[14-17]. The consumption of NSAIDs in larger amounts causes damage to the stomach's mucous membrane, even resulting in total necrosis, as observed in the current case. During the six-month-long feeding treatment of the

patient and her endoscopic observation at the Department of Internal Medicine, preparations were made for surgery. The patient was in poor psychological condition and did not agree to undergo surgery. Within the borders of the stomach and duodenum, inflammatory infiltrate almost completely closed the pylorus with numerous adhesions to the surrounding tissues. The mucous membrane had slipped down completely, and its place was taken by necrotic and inflammatory tissues. It has already been proven that high doses of acetylsalicylic acid increase the risk of hemorrhage during the course of peptic ulcer disease[18]. In the current case, there was no information related to the adverse effects of NSAIDs administered to patients with osteoarthritis. When patients overdose for suicidal reasons, the duration of contact between the mucous membrane and the caustic substance is long, as in the case of our patient. The exposure time, type of drug (e.g., hydrophilic properties), concentration in the stomach, contents of the stomach before drug consumption, degree of pylorus contraction and operations performed on the digestive tract that impair its motor activity are of crucial importance with respect to the level of damage and the extent of changes to the stomach. The morphological changes to the stomach upon consumption of large amounts of NSAIDs may be accompanied by damage to the liver, kidneys and heart[17,19]. Nelson *et al*[20] described a case of liver failure requiring transplantation and simultaneous intestinal necrosis upon consumption of large amounts of acetaminophen and ibuprofen by their schizophrenic patient. The toxic activity of those drugs damages the mucous membrane and stops the production of prostaglandins in the intestines. A massive hemorrhage from a duodenum or stomach ulcer may often be observed among patients who have consumed NSAIDs even in therapeutic doses[21,22]. Morphological changes in the digestive tract are usually described in the literature as liquefactive or coagulative necrosis but refer to poisonings with concentrated bases or acids. Regardless of the type of poison consumed orally, chronic ulcerations of the stomach's mucous membrane, excessive inflammation, clots in the micro-circulation, bacterial colonization and increased fibroblastic activity may develop. The toxic activity of any drug depends not only on the consumed dose but also on other circumstances. Having a meal, drinking alcohol, and the ingestion of other medicines influences the patient's clinical condition. Most often, oral poisonings cause changes to the upper part of the digestive tract and lead to damage to the liver and kidneys. The current literature lacks reports on total resection of the stomach due to poisoning with NSAIDs and Tramal.

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CASE REPORT

Lamivudine treatment enabling right hepatectomy for hepatocellular carcinoma in decompensated cirrhosis

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Abstract

A 69-year-old man was admitted to our hospital in October 2003, for further examination of two liver tumors. He was diagnosed with hepatocellular carcinoma (HCC) arising from decompensated hepatitis B virus (HBV)-related cirrhosis. Long-term lamivudine administration improved liver function dramatically despite repeated treatment for HCC. His Child-Pugh score was 9 points at start of lamivudine treatment, improving to 5 points after 1 year. His indocyanine green at 15 min after injection test score was 48% before lamivudine treatment, improving to 22% after 2 years and to 5% after 4 years. Radiofrequency ablation controlled the HCC foci and maintained his liver function. In April 2009, abdominal computed tomography revealed a tumor thrombus in the right portal vein. Since his indocyanine green test results had improved to less than 10%, we performed a right hepatectomy, which was successful. To our knowledge, there have been no documented reports of patients undergoing successful right hepatectomy for HCC arising from decompensated cirrhosis. The findings observed in our patient indicate the importance of nucleoside analogs for treating HBV-related HCC.

INTRODUCTION

The hepatitis B virus (HBV) infects more than 400 million people worldwide[1] and is an important risk factor for the development of hepatocellular carcinoma (HCC). In Japan, about 1% of individuals in the general population are HBV carriers, accounting for about 14% of patients with liver cirrhosis[2] and 15%-20% of those with HCC[3,4]. The prognosis of patients with HCC arising from chronic liver disease is dependent not only on tumor factors but on hepatic functional reserve. Depending on patient age, liver transplantation may be a good therapeutic option in patients with poor functional reserve. Lamivudine treatment is beneficial in patients with HBV-related HCC because it contributes to improvement of remnant liver function. We describe here a patient with decompensated HBV-related cirrhosis who developed HCC. Lamivudine therapy improved liver function and enabled a right hepatectomy 5 years later.

Figure 1 Computed tomography in our patient. It shows a cirrhotic pattern of the liver and massive ascites at first admission (A); Dynamic computed tomography revealed two hepatocellular carcinomas, 4.5 cm (B) and 2.5 cm (C) in diameter, in the right lobe; These two lesions were treated by transcatheter arterial chemoembolization and radiofrequency ablation (D, E).

CASE REPORT

A 69-year old man was admitted to our hospital in October 2003 for examination of two liver tumors. He had been diagnosed with hepatitis B in 1994 and treated with glycyrrhizin. His liver function deteriorated gradually, with ascites appearing in May 2001. He was first admitted to our hospital for treatment of intractable ascites (Figure 1A). Laboratory tests showed that his serum albumin (alb) concentration was 2.7 g/dL, his total bilirubin (T-Bil) was 2.8 mg/dL, his aspartate aminotransferase (AST) was 54 IU/L, his alanine aminotransferase (ALT) was 43 IU/L, and his prothrombin time (PT) was 42%. Administration of diuretic drugs was not effective, but treatment with a preparation of albumin resulted in the disappearance of ascites 1 mo later. Afterward, the ascites was kept under control by administration of diuretics. In October 2003, a computed tomographic (CT) scan of the abdomen revealed two HCCs (4.5 and 2.5 cm in diameter) in the right hepatic lobe (Figure 1B, C). Laboratory tests showed alb 2.5 g/dL, T-Bil 2.4 mg/dL, AST 152 IU/L, ALT 98 IU/L, PT 47%, indocyanine green at 15 min after injection (ICGR15) 48%, alpha-fetoprotein 444 ng/mL, and protein induced by vitamine K absence or antagonist II <10 mAU/mL. He was positive for HBe antigen, negative for HBe antibody, and had an HBV-DNA viral load of 6.7 log copies/mL. Beginning in November 2003, he was treated with 100 mg/d lamivudine. The two HCCs were treated by transcatheter arterial chemoembolization (TACE) and radiofrequency ablation (RFA) (Figure 1D, E). Both tumors were treated successfully and the patient's liver function recovered gradually after initiation of lamivudine treatment. In September 2005, an abdominal CT scan revealed a recurrent HCC, located near one of the previously treated tumors; this lesion was treated successfully with TACE and RFA. At this time, laboratory tests showed alb 3.7 g/dL, T-Bil 0.7 mg/dL, AST 23 IU/L, ALT 22 IU/L, PT 84% and ICGR15 22%. All HCC treatments were based on clinical practice guidelines in Japan[5], with the patient providing informed consent.

In May 2006, two HCC recurrences were detected in the right liver lobe and treated with TACE and RFA. Laboratory tests showed good liver function, alb 3.9 g/dL, T-Bil 1.1 mg/dL, AST 22 IU/L, ALT 11 IU/L, PT 95% and ICGR15 25%. In June 2007, a recurrent HCC was treated with TACE and RFA. Liver function was also excellent at this time (alb 4.0 g/dL, T-Bil 0.7 mg/dL and PT 100%). In September 2007, his viral load had again increased, with breakthrough hepatitis, and the YMDD mutation was detected. Treatment with adefovir dipivoxil plus lamivudine resulted in a gradual reduction in viral load. In December 2007, abdominal CT revealed five HCCs in the right lobe; these were treated by TACE and RFA. The patient was then treated with low-dose cisplatin and 5-fluorouracil infused through the hepatic artery. Laboratory tests showed alb 4.1 g/dL, T-Bil 1.0 mg/dL, AST 38 IU/L, ALT 30 IU/L, PT 88% and ICGR15 5%. Due to the development of a pseudoaneurysm in his hepatic artery, infusion of chemotherapy was discontinued. In March 2009, two HCCs were detected in the right lobe and were treated by RFA. Laboratory tests showed alb 3.7 g/dL, T-Bil 0.7 mg/dL, AST 35 IU/L, ALT 31 IU/L, PT 77% and ICGR15 10%. In April 2009, abdominal CT and CT angiography revealed a tumor thrombus in the right portal vein, but no lesion could be detected in the left lobe (Figure 2). Although he was diagnosed with decompensated cirrhosis, of Child-Pugh C, when first hospitalized, lamivudine treatment improved his liver function sufficiently, with an improve-

Figure 2 Computed tomography during hepatic arteriography, showing a portal tumor thrombus (arrow) in the right portal vein. Hepatocellular carcinoma was not detected in the left lobe.

Figure 4 Abdominal computed tomography showing the recurrence of hepatocellular carcinoma (arrow) in the left lobe two months after right hepatectomy.

ment of ICGR15 results to < 10%, to allow the successful performance of a right hepatectomy in April 2009 (Figure 3A). After liver resection, his AST rose to 1220 IU/L, his T-Bil to 1.9 mg/dL, and his PT decreased to 54%, followed by gradual recovery of liver function. He recovered well and left the hospital 1 mo after surgery.

Histologic examination of the extracted specimen showed a moderately differentiated HCC with portal tumor thrombus (Figure 3B) and multiple intrahepatic metastases. Fibrosis of varying extent was observed in the cancer-free area, with some areas showing severe fibrosis with pseudolobules and others showing mild fibrosis (Figure 3C). Abdominal CT in June 2009 suggested the recurrence of HCC in the left lobe (Figure 4), and abdominal angiography revealed multiple HCCs. These tumors were treated by TACE, but this was not effective. The left lobe tumors subsequently enlarged and the patient's liver function deteriorated gradually. The patient died in December 2009 (Clinical course Figure 5).

DISCUSSION

Chronic hepatitis B is a progressive liver disease, leading to cirrhosis and HCC[6]. Before antiviral agents became

Figure 3 Macroscopic and microscopic appearance of the resected liver. A: A tumor thrombus was detected in the right portal vein (arrow); B: A moderately differentiated hepatocellular carcinoma with a trabecular pattern was seen in the right portal vein [hematoxylin and eosin (HE) stain, ×40]; C: Fibrosis of varying extent was observed in the cancer-free area. Some pseudolobules with severe fibrosis were present in the left part of the photograph (HE stain, ×40).

established as effective treatments for hepatitis B, the prognosis of patients with end-stage HBV infection was generally poor. The 5-year survival rates of patients with compensated and decompensated cirrhosis have been reported to be 55%-84%[7,8] and 14%[9], respectively. Lamivudine, an antiviral drug, is an oral nucleoside analog that inhibits DNA synthesis by terminating the nascent proviral DNA chain. It rapidly reduces both serum HBV-DNA and transaminase concentrations[10]. Prolonged viral suppression can result in histological improvement, including the regression of fibrosis[10-13]. Although a subgroup of individuals with extremely advanced disease require urgent transplantation, lamivudine treatment can achieve significant improvement in liver function and reduce the morbidity of many of patients

Figure 5 Clinical course of the patient. After lamivudine administration, his liver function gradually improved despite repeated treatments for hepatocellular carcinoma (HCC), and his indocyanine green at 15 min after injection (ICGR15) test score was 5% 4 years after initiation of lamivudine treatment. He underwent a successful right hepatectomy for HCC 5 years after beginning lamivudine treatment. alb: Albumin; PT: Prothrombin time; TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation; 5-FU: 5-Fluorouracil; CDDP: Cisplatin.

with decompensated cirrhosis[14-19]. At present entecavir is recommended as the primary oral agent for hepatitis B because of its strong antiviral effects and low resistance rate, as well as being effective in treating decompensated cirrhosis[20]. Long-term lamivudine monotherapy can induce the emergence of resistant viruses with an amino acid substitution in the YMDD motif of the viral DNA polymerase[21]. Particularly in patients with decompensated cirrhosis, breakthrough hepatitis resulting from such a mutation may lead to hepatic failure[22] if other antiviral drugs such as adefovir dipivoxil[23,24] are not administered. Long-term treatment with lamivudine has been reported to reduce the incidence of HCC[25,26]. In addition, lamivudine has been found to improve liver function[27,28] and survival[29] in patients with HBV-related HCC after initial treatment of HCC. We have described a patient with HCC arising from decompensated HBV-related cirrhosis. Long-term lamivudine treatment improved his remnant liver function dramatically, despite repeated TACE and RFA sessions for HCC. Although his Child-Pugh score at the start of lamivudine treatment was 9 points, it improved up to 5 points 1 year later. Moreover, he scored 48% on an ICGR15 test performed before his first treatment for HCC, but this score improved to 22% after 2 years and to 5% after 4 years, with the latter considered safe for the performance of a right hepatectomy[30].

Despite repeated RFA, the liver function of this patient was well maintained. Generally, RFA has been regarded as safe and effective for HCC, and has been found to maintain liver function[31-33]. In our patient, lamivudine and RFA were effective in maintaining liver function. A previous case report described a patient with decompensated HBV-related cirrhosis, who, following lamivudine treatment, underwent a hepatectomy for HCC after liver function had improved[34]. That patient, however, underwent a partial hepatectomy for a small HCC. To our knowledge, no prior report has described

a successful right hepatectomy for HCC arising from decompensated HBV-associated liver cirrhosis. The findings reported in this patient indicate the importance of nucleoside analogs for treating HBV-related HCC.

In conclusion, we found that lamivudine treatment was beneficial for our patient with decompensated HBV-related cirrhosis and HCC, increasing the likelihood of treatment for HCC.

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MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

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The International Liver Congress
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Internal Medicine 2012
New Orleans, LA 70166,
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April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

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Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
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Amman, Jordan

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Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
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Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
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May 19-22, 2012
2012-Digestive Disease Week
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June 2-6, 2012
American Society of Colon and
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San Antonio, TX 78249,
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Pancreatic Cancer: Progress and
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PancreasFest 2012
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OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

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The Viral Hepatitis Congress
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September 8-9, 2012
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1st World Congress on Controversies
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November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
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November 9-13, 2012
American Association for the Study
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Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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Instructions to authors

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ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3   **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4   **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462   PMCID:2516377   DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5   **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6   21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303   DOI:10.1136/bmj.325. 7357.184]

Volume with supplement

7   **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8   **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10   **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11   **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12   **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13   **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14   **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15   Morse SS. Factors in the emergence of infectious dis-

eases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16   **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

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Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
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Contents

Contents

Contents

ABOUT COVER   Editorial Board Member of *World Journal of Gastroenterology*, Hitoshi Asakura, Director, Emeritus Professor, International Medical Information Center, Shinanomachi Renga Bldg.35, Shinanomachi, Shinjukuku, Tokyo 160-0016, Japan

AIM AND SCOPE

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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

EDITORS FOR THIS ISSUE

Adjuvant treatment in biliary tract cancer: To treat or not to treat?

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Abstract

Biliary tract cancer is a rare malignant tumor. There is limited knowledge about biology and natural history of this disease and considerable uncertainty remains regarding its optimal diagnostic and therapeutic management. The role of adjuvant therapy is object of debate and controversy. Although resection is identified as the most effective and the only potentially curative treatment, there is no consensus on the impact of adjuvant chemotherapy and/or radiotherapy on the high incidence of disease recurrence and on survival. This is mainly due to the rarity of this disease and the consequent difficulty in performing randomized trials. The only two prospectively controlled trials concluded that adjuvant chemotherapy did not improve survival. Most of the retrospective trials, which had limited sample size and included heterogeneous patients population and non-standardized therapies, suggested a marginal benefit of chemoradiotherapy in reducing locoregional recurrence and an uncertain impact on survival. Well-designed multi-institutional randomized trials are necessary to clarify the role of adjuvant therapy. Two ongoing phase Ⅲ trials may provide relevant information.

INTRODUCTION

Biliary tract cancer (BTC) is a rare tumor accounting for approximately 4% of all malignant neoplasms of the gastrointestinal tract. Marked gender, ethnical and geographical variations exist and, in certain regions of the world, like Chile or North India, BTC is one of the most common causes of cancer mortality. The median age at presentation is the seventh decade of life with a male to female ratio of 1.5[1-5].

Surgical resection is the only potentially curative treatment for BTC. However, the resectability rate has been reported to range between 30% and 70%, with large variability based on tumor location (70% for ampullary cancer, 40%-50% for gallbladder, intrahepatic and distal extrahepatic cancer and 30% for hilar BTC)[6,7]. The type of resection and prognosis vary with anatomical location with a 5-year overall survival (OS) rate of 20%-40% for intrahepatic adenocarcinoma, 50%-70% for ampullary cancer, 25%-50% for distal cholangiocarcinoma and for gallbladder cancer and 15%-35% for hilar BTC[8].

Due to the rarity of this disease, in which patients with curatively resected tumors are in the minority, prospective trials have been rarely performed and, sometimes, eligibility criteria allowed the enrolment of both patients with pancreatic cancer and BTC, thus hampering

the interpretation of results. Accordingly, information with a high level of evidence is lacking and wide areas of debate and controversy on optimal adjuvant therapeutic management exist.

PROGNOSTIC FACTORS

The 5-year OS rate with surgery alone is disappointing, ranging from 15% to 70%[6]. Complete surgical resection with histologically negative (R0) surgical margins has been reported to be the most important prognostic factor[9]. Since prognosis varies also with anatomical location, the heterogeneity of patient population of the reported studies may affect the interpretation of the data[8]. Other prognostic factors such as tumor stage, nodal status, vascular and perineural invasion, elevated baseline CA 19-9 level and histologic grade have been identified in many reports[10-13]. Among those, the prognostic relevance of tumor stage, nodal status, histologic grade and resection margin is almost universally accepted and should be taken into account for the stratification of the patients in prospective trials and for the interpretation of the results in non-randomized series; while the prognostic role of elevated baseline CA 19-9 level and vascular and perineural invasion is still controversial[14-45].

THERAPEUTIC MANAGEMENT

Most patients with BTC are not suitable for curative surgery at diagnosis, and the rate of microscopically positive resection margins (R1) has been reported to be up to 74%[14]. In addition, locoregional failure occurs in more than half of the patients, even after R0 resection[9,10]. Isolated locoregional disease was reported in approximately 15% of patients with gallbladder cancer, 20% with periampullary cancer and 60% with hilar cholangiocarcinoma. In contrast, systemic disease, with or without concomitant locoregional recurrence, occurred in 85% of patients with gallbladder cancer, 75% with periampullary cancer and 41% with hilar cholangiocarcinoma[10,11]. Because of poor survival after curative resection due to frequent local relapse and distant metastases[9-11,36], the role of adjuvant therapy has been widely explored[16,32,38,41-56].

Chemoradiation

Previous studies, mainly focusing on adjuvant chemoradiation therapy (CRT) with a variety of regimens, led to conflicting results and the role of this therapeutic strategy remains controversial. No large randomized trial of adjuvant CRT has ever been performed. A small phase III European Organization for Research and Treatment of Cancer trial, including 92 eligible patients with periampullary adenocarcinoma, demonstrated no statistically significant difference in survival between adjuvant CRT following resection *vs* observation[37]. However, since this trial included a limited number of patients and an outdated chemoradiation in terms of imaging, techniques, planning, dose and concomitant radiosensitizing chemo-

Table 1 Survival outcome for adjuvant chemoradiation

Ref.	No. of patients	Site	Therapy		OS (median)	
			Yes	No	CRT	Obs
[38]	73	GB	25	48	58	50
[39]	96	PV	54	42	35.2	16.5
[41]	49	All	34	15	16.4	6.7[1]
[42]	48	EHBD	48		44%[2]	NA
[43]	84	All	34	50	42	29
[44]	125	PV	29	96	67	42
[45]	34	EHBD	34	0	35%[2]	NA

[1]Statistically significant; [2]5-year overall survival (OS). CRT: Chemoradiation; Obs: Observation; PV: Papilla of vater; GB: Gallbladder; EHBD: Extrahepatic bile duct; All: PV + GB + EHBD + intrahepatic bile duct; NA: Not applicable.

therapy, definitive conclusions on the role of modern chemoradiation are impossible to draw.

Conversely, a retrospective series of 73 patients with gallbladder cancer treated between 1985 and 2004 at Mayo Clinic[38] suggested that adjuvant CRT may obtain a statistically significant improvement in OS only for patients with lymph node involvement. Similarly, two retrospective series of fluoropyrimidine-based post-operative chemoradiation from MD Anderson Cancer Center[39] and from South Korea[40], including 96 patients affected by ampullary adenocarcinoma and 91 patients with extrahepatic bile duct cancer, respectively, suggested an improved OS only in patients with locally advanced tumor (T3/T4)[39] or with R1 resection[40]. A few other smaller retrospective series also reported a modest potential OS benefit with adjuvant CRT (Table 1)[38,39,41-45].

Apart from the controversial impact on OS, CRT may have a role in improving local control, especially in patients at higher risk of local failure, such as those with R1 margins and positive lymph nodes. In fact, 5-year local control rate raised from 40%-50% in patients with ampullary cancer treated with surgery alone to 65%-80% in those who received post-operative CRT[1,39-41,57].

Unfortunately, the retrospective nature of most of these studies, the small sample size, the lack of correction for multiple comparisons, patient selection bias; heterogeneity in terms of patients' characteristics, treatment regimens, tumor site and stage; long-lasting accrual periods, different surgical, radiotherapy and radiological techniques in different historical periods and other confounding factors do not allow to draw any firm conclusion on the role of CRT. In fact, younger and healthier patients were more likely to be offered adjuvant CRT. On the other hand, patients with high risk features were more likely to receive adjuvant therapy than those with favorable features.

Chemotherapy

A few studies evaluated the role of adjuvant chemotherapy in BTC. A retrospective single centre analysis on 42 patients suggested that postoperative gemcitabine-based chemotherapy may be a promising strategy to improve OS after surgical resection for hilar cholangiocarcinoma[55].

Consistently, the addition of fluorouracil-based chemotherapy to adjuvant CRT seemed to improve disease free survival (3-year DFS 45% *vs* 27%) and OS (3-year OS 63% *vs* 31%) compared to CRT alone in another retrospective series of 120 patients with radically resected extrahepatic BTC.

A phase III trial addressed the role of adjuvant chemotherapy with 5-fluorouracil and mitomycin-C in a series of 508 patients with surgically treated pancreaticobiliary malignancies including 335 patients with BTC[58]. OS was significantly increased when compared to observation arm only in the unplanned subset analysis of 61 resected patients with macroscopically positive resection margins (R2) affected by gallbladder cancer. Similarly, a more recent phase III trial exploring the role of single agent adjuvant chemotherapy with either gemcitabine or 5-fluorouracil, in 304 patients with ampullary adenocarcinoma submitted to curative resection did not demonstrate a survival benefit for any of the adjuvant therapy arms when compared to surgery alone[59].

GUIDELINES AND CURRENT CLINICAL PRACTICE

The National Comprehensive Cancer Network (NCCN) guidelines[60] recommend only observation or adjuvant CRT with concomitant fluoropyrimidine for patients with R0 margins or negative lymph-nodes and adjuvant therapy with concurrent 5-fluorouracil-based CRT followed or not by additional fluoropyrimidine- or gemcitabine-based regimens in patients with R1 margins or metastatic lymph nodes. The use of chemotherapy is recommended due to the high incidence of systemic relapse and to the results observed in the therapeutic management of advanced disease[61,62]. The European Clinical Practice guidelines[63] are more vague, only suggesting CRT as a possible therapeutic option after surgery for BTC. More restrictive indications derive from a modified and implemented version of NCCN guidelines proposed by a committee of specialists of the Middle East and North Africa Region, which recommend only observation or enrolment into a clinical trial after an R0 and/or a negative regional nodes resection, because of conflicting data regarding adjuvant CRT[64].

Given the lack of guidelines based on high level of evidence, it is not surprising that patients submitted to curative surgery for biliary tract tumors receive heterogeneous management around the world. A survey of therapeutic strategies recommended in the clinical practice in 2001-2002, reported that adjuvant CRT was widely adopted in the majority of American centers (71%), followed by Asian/Pacific centers (55%), but only by 29% of European institutions[65]. This scenario may have changed in more recent years with a trend towards possibly increasing use of adjuvant treatment due to the numerous positive experiences reported in the literature in the last decade[9-11,38-42,44,45,55,57-59,66,67]. However, eighty-eight percent of the interviewed physicians recognized the unmet need for achieving higher levels of evidence from large prospective trials to support the routine use of adjuvant treatment[65].

FUTURE DIRECTIONS

Altogether, the available data do not allow to answer the question whether patients submitted to curative resection for BTC should receive an adjuvant therapy and which treatment strategy may provide the largest benefit.

In fact, neither adjuvant CRT nor adjuvant chemotherapy with either single agent or a 5-fluorouracil-mitomycin doublet improved OS when compared to observation alone in phase III trials[14,58,59], while only a modest benefit in loco-regional control rather than on OS was suggested with CRT by retrospective series that, in any case, suffer from previously mentioned methodological limitations[1,38,41-45,65-68].

The causes of this disappointing scenario and of the lack of a convincing answer are manifold. First, when compared to trials on advanced disease, trials on adjuvant therapy are more demanding, also due to the involvement of different specialists (surgeon, radiologist, oncologist and radiotherapist); more resource- and time-consuming, due to the longer patient's life expectancy and to the inferior number of patients; and require a more selective choice of centers to be involved. Second, evidence-based information on the most active and effective chemotherapy regimen against unresectable or metastatic disease is limited as well. Accordingly, the selection of promising regimens that may have a relevant impact on disease natural history is challenging. Only recently, cisplatin and gemcitabine regimen became the new standard of treatment in advanced BTC[61] providing a rational for investigating the role of this combination in the adjuvant setting. Additionally, two ongoing phase III trials are currently exploring the role of capecitabine (NCT00363584) and of GEMOX regimen (NCT01313377) in the adjuvant setting and may provide further information in the next future. Third, the rarity of disease limits the interest of pharmaceutical companies while investigator initiated trials are hindered by the restricted availability of agents already registered and authorized for the treatment of the disease. Fourth, the choice of the most promising therapeutic strategy is crucial in this disease that has a very high rate of both local and systemic recurrence. Systemic chemotherapy and CRT, rather than being taken as alternative therapies, should be combined taken into account in the design of post-operative management. However, the role of sequential therapy with CRT followed by systemic chemotherapy or the inverse sequence was rarely addressed in prospective trials[55]. Fifth, the knowledge on tumor biology is limited and, at the moment, does not allow to identify new agents that may contribute to improve the outcome of the disease. Last but not least, the interpretation of trials result is often challenging due to the heterogeneity of enrolled patients population. Stratification based on tumor location, extent of resection, lymph node

status and resection margin status will be crucial to the success of future studies.

CONCLUSION

A multi-institutional worldwide effort to conduct well designed phase III trial and to expand biological knowledge of the disease is necessary to clarify the role of adjuvant therapy in BTC.

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FIELD OF VISION

Motor vehicle accidents: How should cirrhotic patients be managed?

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Abstract

Motor vehicle accidents (MVAs) are serious social issues worldwide and driver illness is an important cause of MVAs. Minimal hepatic encephalopathy (MHE) is a complex cognitive dysfunction with attention deficit, which frequently occurs in cirrhotic patients independent of severity of liver disease. Although MHE is known as a risk factor for MVAs, the impact of diagnosis and treatment of MHE on MVA-related societal costs is largely unknown. Recently, Bajaj *et al* demonstrated valuable findings that the diagnosis of MHE by rapid screening using the inhibitory control test (ICT), and subsequent treatment with lactulose could substantially reduce the societal costs by preventing MVAs. Besides the ICT and lactulose, there are various diagnostic tools and therapeutic strategies for MHE. In this commentary, we discussed a current issue of diagnostic tools for MHE, including neuropsychological tests. We also discussed the advantages of the other therapeutic strategies for MHE, such as intake of a regular breakfast and coffee, and supplementation with zinc and branched chain amino acids, on the MVA-related societal costs.

INVITED COMMENTARY ON HOT ARTICLES

We have read with great interest the recent article by Bajaj *et al*[1] describing the diagnosis and treatment of minimal hepatic encephalopathy (MHE) to prevent motor vehicle accidents (MVAs), and would strongly recommend it to the readers.

MVAs are serious social issues worldwide[2]. Various factors are intricately involved in the occurrence of MVAs, and driver illness is an important cause[2]. Besides acute myocardial infarction, epileptic seizure, and hypoglycemia related to the use of anti-diabetic agents, liver cirrhosis with MHE has been reported to increase the risk of MVAs[3-6]. Although MHE occurs in up to 80% of patients with chronic liver disease[3] and diagnostic tools and therapeutic strategies for MHE exist[7-11], little information is available about the management of patients with liver cirrhosis with regard to preventing MVAs and subsequently reducing the associated societal costs.

In their study, Bajaj et al[1] performed a cost-effectiveness analysis to identify management strategies for the diagnosis and treatment of MHE in patients with liver cirrhosis to reduce MVA-related societal costs. They found that the diagnosis of MHE by rapid screening using the inhibitory control test (ICT), and subsequent treatment with lactulose could substantially reduce societal costs by preventing MVAs[1]. This is a significant study, and we agree with the authors about the benefits of the use of the diagnostic test and therapeutic management. However, we suggest that the management strategy should be modified to some extent for use in general medical institutions to prevent MVAs on a larger scale.

The ICT is a computerized test of attention and response inhibition that has been used to characterize attention deficit disorders[12]. The ICT consists of the presentation of several letters at 0.5 s intervals, while the subject is instructed to respond or inhibit their response to the specific letter[13]. The ICT is considered reliable and sensitive for the diagnosis of MHE[5,13]. In addition, unlike standard neuropsychological tests, ICT results are significantly associated with the future occurrence of MVAs[3]. However, the test takes approximately 30 min to complete and patients need to be familiar with computer operation. Furthermore, validation and standardization are required for each population, and therefore, so far, the ICT is not universally accessible. Similarly, other diagnostic tools for MHE also require trained personnel and specialized equipment[11]. In fact, an American Association for the Study of Liver Disease (AASLD) survey showed that the majority of AASLD members are not able to test for MHE because of a lack of time, resources, and suitable personnel[14]. Along with Bajaj et al[14], we propose that rapid and simple tools for the diagnosis MHE should be developed urgently, such as biochemical tests or virtual reality driving simulations.

Lactulose has been used to treat hepatic encephalopathy since 1966[15]. Lactulose reduces blood ammonia levels and improves overt hepatic encephalopathy as well as MHE[7]. In their study, Bajaj et al[1] have demonstrated the benefits of lactulose therapy on the occurrence of MVAs and MVA-related societal costs. However, compliance with lactulose treatment is generally poor, primarily because of its side-effects such as abdominal discomfort[16,17]. Recently, other therapeutic strategies for MHE have been reported. First, as prolonged periods of fasting are linked to the development of MHE, having a regular breakfast improves the attention and executive functions of cirrhotic patients with MHE[18]. Second, coffee intake improves cognitive function in elderly people as well as in patients with type 2 diabetes mellitus[19,20]. Although the beneficial effects of coffee on cognitive function have never been investigated in cirrhotic patients, insulin resistance is frequently seen in patients with chronic liver disease[21-23]. In addition, coffee consumption is known to improve hepatic inflammation and fibrosis in patients with chronic liver disease[24]. Third, the blood ammonia level is regulated by the activity of ornithine transcarbamoylase and zinc is a coenzyme required for its up-regulation[25].

Figure 1 A proposed flow chart of drive permission for patients with chronic liver disease. MHE: Minimal hepatic encephalopathy; BCAA: Branched chain amino acids.

Oral zinc supplementation improves hyperammonemia as well as hepatic encephalopathy, as seen in a double-blind randomized controlled trial[26]. Finally, a decrease in serum branched chain amino acids (BCAA) levels is a feature of chronic liver disease[27]. BCAA is a source of glutamate, which detoxifies ammonia by glutamine synthesis in the skeletal muscle and brain[28]. Therefore, BCAA enhances the detoxification of blood ammonia by incorporating ammonia in the process of glutamine production and is currently used for treating patients with hepatic encephalopathy[29]. Thus, a therapeutic approach comprising the intake of a regular breakfast and coffee, and supplementation with zinc and BCAA may improve the cost-effectiveness of MVA-related events in cirrhotic patients with MHE (Figure 1).

Prevention of MVA by the diagnosis and treatment of MHE is an important component in the management of patients with liver cirrhosis. Collaborative researches among medical institutions, automobile companies, and governmental sectors may help further prevent MVAs and subsequently reduce MVA-related societal costs.

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TOPIC HIGHLIGHT

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Pregnancy related issues in inflammatory bowel disease: Evidence base and patients' perspective

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Abstract

Inflammatory bowel disease (IBD) affects women of childbearing age and can influence fertility, pregnancy and decisions regarding breastfeeding. Women with IBD need to consider the possible course of disease during pregnancy, the benefits and risks associated with medications required for disease management during pregnancy and breastfeeding and the effects of mode of delivery on their disease. When indicated, aminosalicylates and thiopurines can be safely used during pregnancy. Infliximab and Adalimumab are considered probably safe during the first two trimesters. During the third trimester the placenta can be crossed and caution should be applied. Methotrexate is associated with severe teratogenicity due to its folate antagonism and is strictly contraindicated. Women with IBD tend to deliver earlier than healthy women, but can have a vaginal delivery in most cases. Caesarean sections are generally recommended for women with active perianal disease or after ileo-anal pouch surgery. While the impact of disease activity and medication has been addressed in several studies, there are minimal studies evaluating patients' perspective on these issues. Women's attitudes may influence their decision to have children and can positively or negatively influence the chance of conceiving, and their beliefs regarding therapies may impact on the course of their disease during pregnancy and/or breastfeeding. This review article outlines the impact of IBD and its treatment on pregnancy, and examines the available data on patients' views on this subject.

INTRODUCTION

Inflammatory bowel disease (IBD) predominantly affects the younger-aged population and therefore is prominent in women of child-bearing age. It often requires medication for maintenance of remission[1]. Literature on pregnancy in IBD has mainly utilized tertiary hospital cohorts and focused on pregnancy outcomes. Current guidelines emphasize the importance of inducing and maintaining disease remission prior to conception and during pregnancy[1,2]. Studies of patients' perspective of the influence of IBD on fertility, pregnancy and breastfeeding

are highly relevant and beginning to emerge. This review article summarizes this evidence and examines the currently available data describing the patients' views.

LITERATURE SEARCH

A literature search was conducted using Pubmed from 1980 to 2010 with the search terms "IBD", "Crohn's disease (CD)", "ulcerative colitis (UC)", "pregnancy", "breast feeding" and "nursing". Further relevant articles were identified from the reference lists of identified articles.

Fertility and fecundity

Factors determining fecundity and fertility in IBD include disease systemic effects (e.g., fatigue and anaemia) as well as libido and sexual inactivity, which, in turn, may be influenced by body image issues and dyspareunia[3]. Furthermore, concerns over the influence of disease activity or medications on a successful pregnancy may also influence fecundability. In UC, overall fertility is comparable to that of the healthy population[3-5], but women with ileo-anal pouches (IPAA) have reduced fecundity[6,7]. Fertility in CD in remission is equivalent to that of healthy women[4,8,9] but may be reduced in patients with active disease[9-12]. As in UC, women with CD also have reduced fecundity following extensive surgery involving the pelvis[5-7,13-18].

Disease behaviour during and after pregnancy

Disease activity during pregnancy is similar to that in non-pregnant women as reported by two Danish cohort studies. In 97 women with UC, flare rates were 34% per year during pregnancy and 32% per year outside pregnancy[19], while in 68 women with CD there was also no increased risk of a flare during pregnancy[20]. The vast majority of women have quiescent disease during pregnancy as shown in a population based cohort of 461 women from the Northern California Kaiser Permanente population[21].

Women with IBD might experience a more benign course of disease in the post-partum period. A significantly reduced number of flares in comparison to pre-pregnancy in a small cohort of 18 CD and 19 UC patients was reported by Castiglione *et al*[22]. A larger pan-European study of 93 women with CD and 173 women with UC confirmed this finding[23]. The reason for this phenomenon is unknown, but it has been proposed that disparity between maternal and foetal human leukocyte antigen class Ⅱ antigens leads to a state of immuno-tolerance or suppression that in turn leads to a more benign course of IBD[24].

Effects of IBD and IBD disease activity on pregnancy

Active disease during pregnancy has been linked to adverse pregnancy outcomes of low birth weight, pre-term birth and foetal loss[19,25-27]. Population-based studies have, however, reported conflicting results. In 461 women (300 with UC) of the Kaiser Permanente population in North America, no correlation between adverse pregnancy outcomes and disease activity was found[21], while a study of 157 Danish women with CD revealed that active disease increased the risk of pre-term birth[28]. The higher proportion of patients with active disease in the Danish study (45% *vs* 20% in the Kaiser Permanente population) may explain this difference.

Some studies, including a meta-analysis by Cornish *et al*[29], reported an increase in pre-term births in IBD patients[21]. Other studies, however, that differentiated UC from CD found an increase in pre-term birth rates for patients with CD only[30,31]. CD women also delivered lower birth weight babies than healthy controls and UC women[30,31]. It is important to recognise that all studies examining pregnancy outcomes in IBD used healthy women controls rather than those with other chronic diseases.

Congenital abnormalities in offspring of mothers with IBD

Congenital abnormalities of variable severities occur in 3%-7% of babies of healthy mothers[32]. Studies of congenital malformations in the offspring of mothers with IBD have reported conflicting results. In a population based cohort of 262 women from Washington state, North America, an increased risk of congenital malformations was found in the offspring of UC patients (7.9% *vs* 1.7% in healthy controls; *P* < 0.001) but not in those of CD patients (3.4%; *P* = NS)[30]. The study did not control for medication use[30]. In contrast, the Hungarian Case Control Surveillance of Congenital Anomalies (HCCSCA) (1980-1996) found no increase in the risk for "any" malformation [odd ratio (OR): 1.2, 95% (confidence interval) CI: 0.9-1.8] in the offspring of 79 UC mothers compared to 95 control mothers after adjusting for parity, age and medication use[33]. Specific malformations of the limb (OR: 6.2, 95% CI: 2.9-13.1), urinary tract (OR: 3.3, 95% CI: 1.1-9.5) and multiple malformations (OR: 2.6, 95% CI: 1.3-5.4), however, were significantly increased[33].

An Italian case-control study reported a significantly increased risk of congenital anomalies in the offspring of 90 women with IBD (5.5% *vs* 0% in 240 healthy controls) and that CD and UC had equivalent risks[31]. The reported 0% incidence of congenital abnormality rate in healthy controls, however, was unusually low given this rate can approach 7%[32].

The risk of congenital malformations in the offspring of 461 women with IBD from the Northern California Kaiser Permanente population was not increased in comparison to 493 healthy controls[21]. Cornish *et al*[29] found in a meta-analysis of four studies an increased risk (OR: 2.37, 95% CI: 1.47-3.82) of reporting congenital abnormalities for UC, while for CD the risk increase was not statistically significant (OR: 2.14, 95% CI: 0.97-4.74; *P* = 0.06).

Data on congenital malformations may be contradictory due to differences in study design, the inclusion of cohort *vs* population subjects, and sample sizes. The majority of studies, however, report an increased risk for malformations in UC, but not in CD. Further prospective

Table 1   Food and Drug Administration categories of drug safety during pregnancy

FDA category	Definition
A	Controlled studies in animals and women have shown no risk in the first trimester, and possible foetal harm is remote
B	Either animal studies have not demonstrated a foetal risk but there are no controlled studies in pregnant women, or animal studies have shown an adverse effect that was not confirmed in controlled studies in women in the first trimester
C	No controlled studies in humans have been performed, and animal studies have shown adverse events, or studies in humans and animals not available; give if potential benefit outweighs the risk
D	Positive evidence of foetal risk is available, but the benefits may outweigh the risk if life-threatening or serious disease
X	Studies in animals or humans show foetal abnormalities; drug contraindicated

FDA: Food and Drug Administration.

studies adjusting for disease activity, medication and age of the mother are needed.

Medication during pregnancy

The risks and benefits of medication need to be considered on an individual patient level, since active disease poses a risk to the pregnancy[26-28]. The European Crohn's and Colitis Organisation (ECCO) guidelines state the risk of adverse pregnancy outcomes from active disease to be higher than the risk of using most IBD medications[2]. The United States Food and Drug Administration issued a categorisation of drugs safety in pregnancy (Table 1)[34].

Aminosalicylates: Sulfasalazine, mesalazine and balsalazide are category B medications, while olsalazine is category C. Initial case reports[35-37] suggesting sulphasalazine teratogenicity was refuted by a Danish cohort study demonstrating no adverse outcomes in 17 pregnant CD patients exposed to sulfasalazine[28]. Another study of 181 pregnant patients with IBD exposed to sulfasalazine reported lower rates of adverse outcomes than those expected in the general population[38]. Sulfasalazine impairs folate absorption, which is vital for neural tube development. Therefore folate supplementation is mandated[2].

Several studies have demonstrated the safety of mesalazine[39-41]. Robust data from a Danish population-based cohort and a prospective controlled trial of 165 pregnant women with mesalazine exposure reported no increased risk of congenital abnormalities[42,43]. It is unclear whether an excess of stillbirth and preterm birth in 88 pregnancies of women with CD on mesalazine reported in the population based study[43], occured as a result of IBD itself rather than the medication since this study incorporated 19 418 pregnancies of healthy women without any chronic illnesses and medication exposure as the control group. Thus, sulfasalazine and mesalazine are

considered safe in pregnancy.

Antibiotics: Antibiotics play an important role in the management of perianal CD[2]. Metronidazole is a category B medication. In a population based case-control study using HCCSCA data (1980-1991), metronidazole exposure during the second or third trimester was associated with cleft defects[44]. Other studies including two meta-analyses and a prospective controlled study, however, found no increased risk of congenital abnormalities relating to metronidazole use[45,46]. Quinolones can induce congenital abnormalities in animals due to their accumulation in bone and cartilage[47]. In 2 human studies totalling over 250 patients without IBD, no increased risk of adverse pregnancy outcomes or foetal malformations was found[48,49].

Short-term antibiotic use in pregnancy appears safe in largely non-IBD cohorts. In IBD, however, exposure to antibiotics can be prolonged and the associated risks may therefore be higher[2]. A small series of IBD patients found no adverse pregnancy outcomes in 27 patients exposed to metronidazole and 18 to ciprofloxacin[50].

Tetracyclines (retardation of foetal skeletal development) and sulphonamides (interferes with folic acid metabolism) should both be avoided in pregnancy[2].

Corticosteroids: Prednisone and budesonide have category C ratings. Corticosteroids cross the placenta and some adverse data exist in humans. A meta-analysis of ten cohort and case-control studies totalling 50 845 patients without IBD found no increase in overall risk of major congenital abnormalities, but there was a significant risk of oral clefts[51]. In contrast a prospective study of 262 women found that neither the overall risk for malformations nor the risk for clefts was increased[52]. No human data exist on orally administered budesonide, but studies looking at women exposed to inhaled or intra-nasal budesonide have found it to be safe during pregnancy[53,54]. Due to oral budesonide's high first pass hepatic metabolism, significant foetal exposure is less likely.

Thiopurines: Mercaptopurine and its pro-drug azathioprine are category D due to previous links with spontaneous abortions[55,56]. Animal studies, using doses of 1.5-2.5 mg/kg, have not reported any adverse outcomes apart from low birth weights[57,58]. Transplant and rheumatology cohorts demonstrated the safety of thiopurines[59-61]. Most studies in pregnant IBD patients report no adverse outcomes[62,63], but a Danish study reported a increased pre-term birth, low birth weight and foetal abnormalities in a cohort of only 10 patients[64]. That study compared pregnancy outcomes in thiopurine exposed patients to those of the general population rather than non-exposed IBD patients. It is therefore unclear whether the increased risk detected in the study was due to medication or IBD itself. In contrast, a prospective Austrian abstract of 33 women[65] and a French study on 86 women show no increase in adverse outcomes[66]. Paternal thiopurine exposure within 3 mo prior to conception led to a higher

rate of pregnancy related complications in one study[67], but another study reported no increased risk of adverse outcomes[68].

Thus, studies comparing outcomes of thiopurine exposed and unexposed IBD patients demonstrate no association with adverse outcomes. The single study showing a possibly increased risk is small and compares exposed patients to the general population[64]. Based on the overall evidence and despite the category D classification, the ECCO guidelines therefore consider thiopurines safe and well tolerated in pregnancy[2].

Methotrexate: Methotrexate is rated category X as it is clearly teratogenic and an abortifacient due its biological action as a folate antagonist. It is associated with numerous foetal abnormalities and high risk of foetal mortality and absolutely contraindicated for women attempting pregnancy[69,70].

Other immunosuppressants: Data on cyclosporine and tacrolimus mainly stems from the transplant and rheumatology literature and both are rated category C[2].

Biological agents: Infliximab has been classed as category B. It does not cross the placenta during the first trimester and hence there is no exposure in this critical phase of development[71]. Infliximab crosses the placenta in later stages of pregnancy and may be present in the newborn for several weeks[71]. Safety data for infliximab are provided by three large scale studies. The Crohn's Therapy Resource, Evaluation and Assessment Tool registry-a prospective, North American observational multi-centre study-enrolled infliximab-exposed and unexposed CD patients from 1999 to 2004[72]. No differences in miscarriages and neonatal complications were found between infliximab-exposed (117 pregnancies) and unexposed (49 pregnancies) women[72]. The retrospective Infliximab Safety Database (maintained by Centocor) found no differences in adverse outcomes between 96 pregnancies in women with infliximab exposure compared to the general population[73]. The Leuven group in Belgium treated 29 women treated with infliximab during 35 pregnancies and six women with adalimumab during seven pregnancies. In comparison to IBD patients without infliximab or adalimumab exposure, no increased risk of adverse events was found[74].

Adalimumab is also classed as a category B drug and expected to have the same placental transfer as infliximab. Apart from the Leuven experience[74] there are few reports on IBD patients exposed to adalimumab during pregnancy[75,76]. The organisation for teratology specialists' registry of women with rheumatoid arthritis compared 34 adalimumab-exposed pregnancies to 52 pregnancies of healthy women and found no increased risk of adverse pregnancy outcomes[77]. Certolizumab and natalizumab are category B and C drugs respectively, but there is currently little data on their effects in pregnancy[2].

Infliximab use in pregnancy resulted in the death of a 3 mo old child with disseminated Bacillus Calmette-Guérin (BCG) after receiving the live BCG vaccination[78]. Live vaccinations are contra-indicated for immuno-compromised patients. Newborns to mothers exposed to infliximab and adalimumab mothers should have their vaccination postponed[78].

Mode of delivery

Patients with IBD (especially CD) were more likely to have a caesarean section in Cornish's meta-analysis of six studies (OR: 1.5, 95% CI: 1.26-1.79; $P < 0.001$) compared to the general population[29], but there was no difference in caesarean rates between UC and controls[29]. Concerns regarding the preservation of the anal sphincter function and the development of perianal disease after traumatic injuries occurring during vaginal delivery may partially explain this phenomenon. However, the chance of developing perianal disease in women with CD without prior perianal involvement is low; in a population based cohort study from Mannitoba, Canada only one of 27 women without prior perianal disease developed perianal disease after vaginal delivery and episiotomy[79]. Conversely, in a self-report survey of 179 women without perianal disease, 18% reported perianal involvement after delivery[80], but it is possible that selection bias, that is, more women with than without perianal disease may have responded, and recall bias may have occurred[80].

A population based cohort study from Mannitoba of 11 patients with inactive perianal disease and two single case reports from France and United States, revealed that inactive perianal disease may tolerate a vaginal delivery with episiotomy if needed without risking a flare[79,81,82]. However, women with active perianal disease should be advised to have a caesarean section as a high risk of deterioration is anticipated. Delivery trauma can lead to poor-healing in the perineum. ECCO guidelines advise that elective caesarean section is indicated for all women with perianal involvement[2] even though there is little evidence suggesting harm from a vaginal delivery in cases of inactive disease.

ECCO guidelines recommend mandatory caesarean sections after IPAA[2]. Changes in anal sphincter function are temporary and long-term disturbances seem to be independent of the mode of delivery in patients with IPAA[14,16,83]. The largest study of 232 females with pregnancies after IPAA found no difference in pouch-related complications between women undergoing vaginal delivery or caesarean section[84]. In another survey, functional pouch outcomes of 85 women with vaginal deliveries after IPAA were no different to those of 343 age matched women who did not have children after IPAA[85]. A Finish survey study of 39 women with IPAA found no differences in pouch function after 19 vaginal deliveries in comparison to 21 caesarean sections and the rate of 5 tears after vaginal delivery was similar to a healthy control group[86]. In a cohort of women investigated four years after IPAA at the Cleveland clinic, United States no clinical differences were demonstrated between 20 women

Table 2 Medication recommendation for pregnancy and breast feeding

Class	Drugs	FDA category	Pregnancy advice	Detection in breast milk
Aminosalicylates	Sulfasalazine, balsalazide, mesalazine	B	Safe to use (folate supplementation for sulfasalazine)	Low levels detectable
Aminosalicylates	Olsalazine	C	Safe to use	
Antibiotics	Metronidazole	B	Safe to use	
Antibiotics	Ciprofloxacin		Limited data; probably safe	
Corticosteroids	Prednisolone, budesonide	C	Safe to use	Detectable
Thiopurines	Azathioprine, 6-mercaptopurine	D	Safe to use	Very low levels detectable
Folate antagonist	Methotrexate	X	Absolutely contraindicated	
Biological agent	Infliximab	B	Probably safe (avoid during 3rd trimester)	Not detectable
Biological agent	Adalimumab	B	Probably safe (avoid during 3rd trimester)	Very low levels detectable

FDA: Food and Drug Administration.

with at least one vaginal delivery compared to 62 women who only had caesarean sections[87]. Subclinical differences in anorectal physiology were however demonstrated as women with vaginal deliveries had significantly lower squeeze pressure on anorectal manometry and significantly more anal sphincter defects detected by anorectal sonography than those women with caesarean sections[87].

The ECCO guidelines state that women are at borderline incontinence after IPAA surgery and that any further disruption by a vaginal delivery might compromise this[2]. This theoretical concern is supported by sonographic and manometric evidence of sphincter dysfunction, but this does not translate to clinical differences in pouch function or quality of life. Precautionary caesarean sections are however recommended[2].

Thus, mandatory recommendation for caesarean sections in IBD is only for very specific indications and decisions should be made on a case by case basis.

Breast feeding
Breast milk provides ideal nutrition and has positive effects for the immune system of the newborn[88]. Data on the protective effect of breastfeeding against the development of IBD report are conflicting. Study design, recall bias, definition of breast feeding (especially duration) and the design bias often inherent in retrospective case-control studies may explain some of the differences[89]. A meta-analysis of 17 studies found reduced ORs of 0.67 (95% CI: 0.52-0.86) for CD and 0.77 (95% CI: 0.61-0.96) for UC. The relevance of a French case-control study reporting an increased risk (OR: 2.1, 95% CI: 1.3-3.4) of developing CD[90] remains unclear, in light of previous studies showing no effect or a protective effect[89,91].

Fewer patients with IBD breastfeed compared to the general population, and this may relate to fears about adverse effects of maternal medication[92]. Most maternal medications can be detected in breast milk, but this does not always lead to biological effects in the infant.

Based on two case reports of bloody diarrhoea in newborns breastfed by mothers taking sulfasalazine and 5-aminosalicylates respectively, the American Academy of Pediatrics advises against breastfeeding by mothers taking these medications[93-95]. In contrast, several studies demonstrate the safety of sulfasalazine and 5-aminosalicy-

lates while breastfeeding by detecting low levels of drug in breast milk or the infant's serum[39,96,97]. Based on above evidence mothers on sulfasalazine and 5-aminosalicylates should not be discouraged from breastfeeding unless the infant develops diarrhoea.

Since corticosteroids are found in human breast milk in low concentrations, women are advised to avoid feeding within 4 h of taking an oral dose to reduce exposure[2,98]. Thiopurine and associated metabolite levels are either at undetectable, or extremely low levels, in human breast milk[99-101], while infants' levels were undetectable[102,103]. Infliximab can not be detected in infants of breastfeeding mothers[104], but adalimumab has been found in miniscule concentrations in breast milk in a single case[105]. Methotrexate should be avoided as it is found in breast milk[106]. Sulfasalazine, 5-aminosalicylates, steroids and thiopurines are all considered advisable during breast feeding[2] (Table 2).

Women's beliefs and attitudes
Few studies have evaluated the perspectives of patients with IBD on fertility, pregnancy, breast feeding and pregnancy outcomes. Women with IBD may have fewer children as more stay "voluntarily childless"[107]. A survey of Crohn's and Colitis Foundation of America members reported that 18% of females with CD and 14% of females with UC decided to stay childless compared to 6% of the general population ($P = 0.001$ for CD, $P = 0.08$ for UC). Notably, the decision to stay childless amongst IBD patients contrasts to the much lower "involuntary" childlessness rates (inability to conceive) (5% in CD, 1.7% in UC and 2.5% in the general population, $P =$ NS)[107]. Concerns about the effect of pregnancy on their disease, about passing on IBD to their children and about their ability to look after children were given as the main reasons for voluntarily childlessness[107]. IBD patients have expressed a high interest in genetic testing to determine their future health or that of their family members[108], but there are no data whether women with IBD would base decisions to have children on the results of genetic testing should a reliable test become available.

Data from an Australian IBD cohort were recently described by Mountifield *et al*[109,110] in two studies. The first postal questionnaire study evaluated the experience and views regarding fertility in 255 women with IBD and

found live birth rates of 1.0 for CD, 1.2 for UC, which are considerably lower than those of the general population of 1.8[109]. This coincided with a fear of infertility in 42.7% of patients, which was particularly apparent in women with CD or previous surgery[109]. Unfortunately, the study did not report whether women fearing infertility were indeed experiencing problems conceiving. Conversely, women sought fertility advice only as often as the general population[109]. The study highlights a difference between the medical evidence and patients' perception of infertility as patients overestimate their risk of infertility largely. The second study examined the experiences and views of 219 women after pregnancy[110]. The patients' main concern related to potential harm towards their pregnancy from IBD medication (84%) rather than the need to control active disease[110]. "Free text" responses suggested that women would rather "put up with the disease than harm my baby with medications". The main concerns regarding side effects of IBD medication related to congenital abnormalities rather than more common adverse outcomes of pregnancy such as pre-mature delivery or low birth weight[110]. Of note, some patients considered steroid "rescue" therapy safer than continuation of IBD maintenance medication[110]. The authors suggested their findings reflected a lack of patients' knowledge in the field of IBD and pregnancy[110]. The study provides insight into patients' views: many opinions were contrary to current medical evidence, and women may make decisions based on incorrect perceptions.

Two population based Danish studies have examined adherence to IBD medication prior and during pregnancy in 58 women with CD and 63 women with UC. Adherence, measured by retrospective self-reporting, was relatively high in both CD (72%)[111] and UC (60%)[112] as adherence in the non-pregnant IBD population ranges from 55%-70%[113]. It is, however, difficult to interpret the study findings since there was no direct control group of non-pregnant patients included and selection bias (responders *vs* non-responders) and recall bias (the questionnaires were sent years after the pregnancies) may be likely. Furthermore, adherence was assessed using a simple question rather than a validated tool. Reasons for non-adherence were quiescent disease (59%) and a fear of negative effects on the unborn child (50%), while forgetfulness was uncommon (5%)[112]. These studies highlight that unwanted effects of medication play an important part in women's views and influence decision making.

CONCLUSION

IBD affects women of childbearing age and may have an effect on their offspring. Fertility is reduced in active CD and after surgery. The risk of active disease during pregnancy carries a significant risk to baby and mother. The limited data available on patients' beliefs and attitudes suggest that many women hold views contrary to medical evidence. Women with IBD are therefore at risk of making uninformed choices that could in turn lead to adverse outcomes. There is a need for further studies examining

women's views in more detail and to investigate whether these beliefs are driven by a lack of knowledge. In the meantime, women with IBD should receive advice and counselling by their physician prior and during pregnancy.

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Leaky gut and the liver: A role for bacterial translocation in nonalcoholic steatohepatitis

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Abstract

Gut flora and bacterial translocation (BT) play important roles in the pathogenesis of chronic liver disease, including cirrhosis and its complications. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen predispose patients to bacterial infections, major complications and also play a role in the pathogenesis of chronic liver disorders. Levels of bacterial lipopolysaccharide, a component of gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver disease. Impaired gut epithelial integrity due to alterations in tight junction proteins may be the pathological mechanism underlying bacterial translocation. Preclinical and clinical studies over the last decade have suggested a role for BT in the pathogenesis of nonalcoholic steatohepatitis (NASH). Bacterial overgrowth, immune dysfunction, alteration of the luminal factors, and altered intestinal permeability are all involved in the pathogenesis of NASH and its complications. A better understanding of the cell-specific recognition and intracellular signaling events involved in sensing gut-derived microbes will help in the development of means to achieve an optimal balance in the gut-liver axis and ameliorate liver diseases. These may suggest new targets for potential therapeutic interventions for the treatment of NASH. Here, we review some of the mechanisms connecting BT and NASH and potential therapeutic developments.

Key words: Nonalcoholic steatohepatitis; Bacterial translocation; Insulin resistance; Leaky gut; Lipopolysaccharide

INTRODUCTION

Bacterial translocation (BT) and the derangement of gut flora are of substantial clinical relevance to patients with chronic liver disease and cirrhosis[1,2]. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen predispose patients to bacterial infections and major complications[3,4]. Furthermore, levels of bacterial lipopolysaccharide (LPS), a component of gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver disease. Bauer *et al*[5-7] have demonstrated this phenomenon in cirrhosis. Impaired gut epithelial integrity due to alterations in tight junction proteins may be the pathological mechanism underlying bacterial trans-

location. Over the last decade, increased gut permeability and increased LPS levels have been described in patients with alcoholic and nonalcoholic steatohepatitis (NASH)[8,9]. Increased serum LPS levels and activation of proinflammatory signaling cascades have been suggested to be important for disease progression in these settings[10]. Some potential mechanisms to explain the association between BT and liver disease associated with lipid accumulation and the development of NASH are reviewed here. These mechanisms may suggest new targets for potential therapeutic interventions for the treatment of NASH.

BACTERIAL TRANSLOCATION, THE INNATE IMMUNE SYSTEM AND TOLL-LIKE RECEPTORS PLAY A ROLE IN THE PATHOGENESIS OF LIVER DAMAGE

Inflammation is involved in the pathogenesis of chronic liver diseases and plays a role in the development of progressive hepatic damage and fibrosis[11]. Liver inflammation and chronic damage are mediated by innate immune responses that are regulated by toll-like receptors (TLRs)[12]. Innate immune cells can both initiate and maintain inflammation in the liver. Bacteria translocated from the gut activate lymphocytes after interacting at the mesenteric lymph nodes (MLNs)[13].

Innate immune cells, particularly dendritic cells, play a pivotal role in sensing pathogens and initiating adaptive immune responses through the activation and regulation of T lymphocyte responses[11]. The immune system is abnormally activated in patients and experimental models with cirrhosis and ascites[14-16]. In an animal model of cirrhosis, systemic activation of the immune system occurs before ascites develop and is driven by the recirculation of cells activated in hepatic lymph nodes (HLNs)[13]. In compensated cirrhosis, bacterial DNA fragments reach the MLNs, where they elicit a local inflammatory response. Bacterial DNA fragments were present in the MLNs of 54% of rats with cirrhosis, indicating their potential role in systemic inflammation[13]. BT initiates a Th1 immune response in MLNs, leading to T-helper 1 (Th1) polarization and the production of tumor necrosis factor (TNF)-α by monocytes. The recirculation of these activated effector immune cells into the blood promotes systemic inflammation[16]. A systemic inflammatory state with increased circulating TNF-α has been linked to increased susceptibility to bacterial infections and hemodynamic dysfunction in patients with cirrhosis[15-18].

The liver provides a tolerogenic immune environment for antigen-specific T cells. The liver is a source for activated immune cells present in the blood. The activation of Kupffer cells, recruited macrophages, and inflammatory cells results in the production of cytokines and chemokines that lead to prolonged inflammation and hepatocyte damage[11]. A direct correlation between activated cells in the blood and HLNs, but not in MLNs, supports this concept, as does the fact that the changes in activated cells in the MLNs, but not in the blood or HLNs, can be reversed by gut decontamination with antibiotics[13]. Th1 cells and monocytes were expanded and activated to produce intracellular interferon (IFN)-γ and TNF-α in the MLNs of cirrhotic rats[18]. Abrogation of bacterial translocation by bowel decontamination reduced the number of activated Th1 cells and monocytes and normalized IFN-γ and TNF-α production by monocytes in the MLNs and blood[16,19].

TLRs and TLR ligands play roles in the pathophysiology of liver fibrosis[20,21] and cirrhosis, viral hepatitis, ALD[22,23], nonalcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma[24]. TLRs recognize pathogen-associated molecular patterns (PAMPs) to detect the presence of pathogens[24]. TLRs are expressed on immune cells, Kupffer cells, endothelial cells, dendritic cells, biliary epithelial cells, HSCs, and hepatocytes. TLR signaling induces potent innate immune responses in these cells[25]. The liver is constantly exposed to PAMPs, such as LPS and bacterial DNA, through bacterial translocation *via* the portal vein system connecting it to the intestine[25].

TLRs also play a role in the regulation of inflammation based on their ability to recognize endogenous TLR ligands, termed damage-associated molecular patterns (DAMPs)[24]. The liver not only represents a major target of bacterial PAMPs in many disease states but also upregulates several DAMPs following injury[11,24]. The activation of inflammatory cells, including Kupffer cells, is a crucial step in the activation of hepatic stellate cells (HSCs)[25]. Intestinal bacterial microflora and functional TLR4, but not TLR2, are required for hepatic fibrogenesis[20]. Crosstalk between TLR4 signaling and transforming growth factor beta (TGF-β) signaling in HSCs has been reported[25]. Quiescent HSCs have been shown to be the target through which TLR4 ligands promote fibrogenesis. TGF-β signaling through the TLR4-MyD88-NFkappaB axis provides a novel link between proinflammatory and profibrogenic signals[20].

The activation of innate immune responses involving TLR4 and complement play important roles in initiating alcoholic steatohepatitis and fibrosis[26]. Activation of the TLR4-mediated myeloid differentiation factor 88 (MyD88)-independent [TRIF/interferon regulatory factor (IRF)-3] signaling pathway in Kupffer cells contributes to alcoholic steatohepatitis, whereas activation of TLR4 signaling in HSCs promotes liver fibrosis[26].

Activation of the innate immune system and increased release of proinflammatory cytokines and other mediators play an important role in the development of alcoholic liver disease (ALD)[27,28]. Alcohol-induced hepatocellular damage may occur as a result of bacterial or endotoxin translocation due to a reduction in reticuloendothelial system function in ALD. The recognition of gut-derived endotoxin by TLR4 contributes to the development of ALD through the activation of TLR-induced intracellular signaling pathways, cytokine production, and ROS[29]. TLR-dependent, ethanol-induced oxidative stress is important for the regulation of NFkappaB activation and

cytokine production by Kupffer cells[28]. Kupffer cells are stimulated by gut-derived endotoxin *via* mechanisms dependent on increased gut permeability and alcohol-induced liver injury[28].

TLR9 and TLR2 mediate *Propionibacterium acnes*-induced sensitization to LPS-triggered acute liver injury in mice[12]. Ligand-specific activation of TLR2 and TLR9 is dependent on the common TLR adaptor MyD88. MyD88 in immune cells, but not in liver parenchymal cells, plays important roles in inflammatory cell recruitment and liver injury[12].

Activation of TLR9 induces type I interferons *via* IRF-7. Type I IFNs were upregulated during TLR9-associated liver injury in WT mice. Type I IFN signaling is therefore required for protection from immune-mediated liver injury[30]. Type I IFNs have anti-inflammatory effects mediated by endogenous interleukin (IL)-1ra, which regulates the extent of TLR9-induced liver damage[30,31]. These data support the notion that bacterial translocation, the innate immune system and TLRs play an important role in the pathogenesis of liver damage.

BACTERIAL TRANSLOCATION IS ASSOCIATED WITH FAT ACCUMULATION IN THE LIVER

Several mechanisms have been proposed to explain the association between fat accumulation in the liver and bacterial translocation. A link between inflammation and hepatic steatosis was shown both in alcoholic and non-ALD[32,33]. The consumption of refined carbohydrates in soft drinks has been postulated to be a key factor in the development of NAFLD.

Results of several studies have shown that an increased consumption of fructose may result in an increased lipid accumulation in the liver which was accompanied by insulin resistance and elevated plasma triglycerides (Ackerman, 2005 No. 260; Jurgens, 2005 No. 261; Faeh, 2005 No. 262; Lewis, 2004 No. 264). Consumption of high levels of fructose lead to liver damage through overfeeding and also may induce a proinflammatory response by increasing intestinal translocation of endotoxin[34]. In a mouse model, hepatic lipid accumulation was higher in mice consuming fructose; these mice also showed high endotoxin levels in portal blood, lipid peroxidation and TNF-α expression[34].

Macrophages facilitate the clearance of cholesterol from the body *via* reverse cholesterol transport[35]. LPS has been shown to suppress PPARgamma1 and its downstream target genes in macrophages, inducing foam cell formation. This was proposed as a mechanism underlying the development of bacterial infection-induced atherosclerosis[35]. LPS induces the expression of adipocyte enhancer-binding protein 1 (AEBP1) during monocyte differentiation. LPS-induced down-regulation of pivotal macrophage cholesterol efflux mediators, leading to foam cell formation, is mediated by AEBP1. AEBP1-independent pathways contribute to the delayed effects of LPS

on macrophage cholesterol efflux and the development of foam cells[35].

The published data support the hypothesis that bacterial translocation may underlie some of the mechanisms associated with fat accumulation in the liver.

BACTERIAL TRANSLOCATION IS ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction is a pathogenic feature of NASH[29,36] and there is evidence that mitochondrial damage contributes to apoptotic/necrotic cellular damage in NASH[37]. NASH is associated with an increase in reactive oxygen species (ROS) production in Kupffer cells and hepatocytes[38]. The greater the decrease in cytochrome and oxidase activity seen, the more significant is the increase in ROS production. Mitochondrial dysfunction and overproduction of ROS play key roles in the progression of chronic hepatitis C and ethanol-induced liver injury. Ethanol also causes bacterial translocation in the intestine, and the resulting LPS activates Kupffer cells to produce pro-inflammatory cytokines[38]. It has been suggested that NASH may also result from increased ROS production in Kupffer cells and hepatocytes that may be dependent on bacterial translocation[38]. Therefore, in addition to its effects which are directly or indirectly associated with fat delivery, BT may also be associated with mitochondrial dysfunction that further contributes to fat accumulation in NAFLD.

BACTERIAL TRANSLOCATION IS ASSOCIATED WITH THE DEVELOPMENT OF NONALCOHOLIC STEATOHEPATITIS

Evidence supporting a role for the liver-gut axis in the pathogenesis of NAFLD has been slowly accumulating over the past 7 years[39-41]. Both preclinical and clinical data suggest an association between BT, small intestinal bacterial overgrowth (SIBO) and NASH[42,43]. Recently, the presence of SIBO has been associated with the severity of liver steatosis[44]. Exposure to bacterial products of intestinal origin, most notably endotoxin, including LPS, leads to liver inflammation, hepatocyte injury and hepatic fibrosis[43].

TLR4[45] and its coreceptor, myeloid differentiation factor-2 (MD-2), recognize LPS and activate proinflammatory signaling pathways. TLR4 can specifically recognize LPS as a danger signal and induce activation of inflammation-associated genes[46,47]. A 4-wk high-fat diet increased plasma LPS concentration two to three times[48] and the LPS recognition complex (TLR4 and MD-2) activates NADPH in liver steatosis and induces fibrosis in a NASH model in mice. These data support the role of these receptors in the development of steatosis, inflammation and fibrosis in NASH[49].

Figure 1 Bacterial translocation is associated with the development of nonalcoholic steatohepatitis. NASH: Nonalcoholic steatohepatitis.

The suppression of inflammation and immune tolerance are known to occur in normal livers. Suppressed inflammation has been shown despite bacterial colonization in normal human livers maintaining liver immune homeostasis[50]. In spite of increased bacterial colonization of liver tissues, lower levels of TLR2/4 mRNA and TLR4 and pIKKalpha, a marker for nuclear factor-kappa B (NFkappaB) activation, proteins were found in liver tissues from healthy subjects compared with samples from patients with primary biliary cirrhosis and NASH. Although these data raise the question of whether BT initiates the inflammatory process in the liver or whether it is instead a result of the inflammatory process, these results further support a role for BT in the pathogenesis of the disease.

SIBO also plays a role in NASH *via* interactions with TLR-4 and the induction of IL-8[43]. SIBO has been reported to coexist with NASH[51-56]. Patients with NASH show higher levels of expression of TLR-4/MD-2 on CD14-positive cells[43]. Serum levels of the proinflammatory cytokine IL-8 were higher in samples from NASH patients than in those from control subjects and were correlated positively with TLR-4 expression.

Leptin is a proinflammatory cytokine associated with the progression of NASH. Leptin enhanced TNF-α production and caused a dose-dependent increase in MAPK activity in LPS-stimulated KCs[57]. KCs isolated from the leptin receptor-deficient Zucker rat (fa/fa) showed reduced production of TNF-α upon stimulation with LPS[57]. Furthermore, treatment of normal rats with leptin increased LPS-induced hepatic TNF-α production in vivo, and leptin receptor-deficient Zucker rats showed reduced hepatic TNF-α production upon addition of LPS *in vivo*[57].

Following BT, LPS activates inflammasomes[58-60]. Inflammasomes respond to endogenous and exogenous danger signals by inducing the processing of pre-IL-1ss

into secreted IL-1ss[36]. In the methionine-choline-deficient (MCD) or high fat diet-induced models, saturated fatty palmitic acid activates the inflammasome and sensitizes hepatocytes to LPS-induced IL-1ss release. Hepatocytes exposed to saturated fatty acid release danger signals that trigger inflammasome activation in immune cells[36]. LPS treatment significantly increased hepatic TNF-α production in MCD mice. LPS also induced a significant increase in TUNEL-positive cells[61]. This increase in apoptosis was inhibited by treatment with a neutralizing anti-mouse TNF receptor antibody or pentoxifylline.

In humans, dietary fructose intake has been associated with increased intestinal permeability and translocation of bacterial endotoxin; plasminogen activator inhibitor (PAI-1) may also contribute to the development of NAFLD in humans[62]. Plasma concentrations of endotoxin and PAI-1 and hepatic mRNA expression levels of TLR4 and PAI-1 were higher in NAFLD patients than in control subjects. Serum levels of LPS-binding protein (LBP) were increased in obese patients with NAFLD[63]. Plasma levels of LBP were further increased in patients with steatohepatitis when compared with patients with simple steatosis[63]. TNF-α mRNA expression in liver tissue was significantly higher in patients with NASH than in control subjects and was correlated with the increase in plasma levels of LBP.

BT is also involved in nitric oxide synthase (NOS) upregulation through the activation of both endothelial NOS and inducible NOS[64-66]. The prevention of intestinal gram-negative bacterial translocation by norfloxacin corrects circulatory changes by decreasing nitric oxide (NO) production in cirrhosis. Norfloxacin administration significantly decreased the incidence of gram-negative bacterial translocation and production of proinflammatory TNF-α, IFN-γ and IL-6[66,67].

The published data support the hypothesis that BT is associated with the development and maintenance of continued lipid accumulation, inflammation and fibrosis in patients with NASH (Figure 1).

BACTERIAL TRANSLOCATION IS CLINICALLY RELEVANT IN CHRONIC LIVER DISEASE

BT was shown to affect the development of chronic liver disease and the associated complications. It is also associated with an impaired prognosis[68]. Bacterial DNA is a marker of bacterial translocation and can be detected in uninfected patients with cirrhosis and ascites[69,70]. It is associated with a marked inflammatory response and with the activation of the inducible form of NOS and the release of NO. A similar effect is observed in patients with SBP[68].

The induction of cirrhosis in rats by CCl4 led to prolonged oxidative stress in the intestine, accompanied by increased sugar content in both the intestinal brush border and the surfactant layers[71]. This was accompanied by changes in bacterial flora in the gut, and these bacteria

showed increased hydrophobicity and adherence to the mucosa. These data support the notion that oxidative stress in the intestine during cirrhosis alters mucosal glycosylation and increases the hydrophobicity of the luminal bacteria, enabling increased bacterial adherence to epithelial cells, facilitating BT[71].

In a human trial, the presence of bacterial DNA was associated with aggravation of peripheral vasodilation and with a worsening of intrahepatic endothelial dysfunction[68]. Patients exposed to bacterial DNA had a significantly lower mean arterial pressure and systemic vascular resistance. In response to increased blood flow caused by postprandial hyperemia, these patients had greater increases in hepatic vein pressure gradient and impaired hepatic vasorelaxation[68]. In contrast, a prospective trial of 151 patients with cirrhosis and ascites found no evidence that the detection of bacterial DNA in the ascites of cirrhotics is of clinical or diagnostic relevance to the detection of SBP[72]. This discrepancy in the published data remains to be resolved.

Increased intestinal permeability and abnormal motility were frequently observed in cirrhotics without ascites, even in the absence of evidence of BT. It has been suggested that these factors facilitate BT and thus precede it[73]. Systemic reactivity to microbial components as measured by the development of antibodies was suggested to reflect the compromised mucosal immunity in cirrhotic patients[74]. The presence of bacterial DNA in blood and ascites correlates with BT and is frequent in patients with advanced cirrhosis without overt infection; BT can also precede the occurrence of overt bacterial infection in patients with cirrhosis[73]. Altered permeability of the mucosa and deficiencies in host immune defenses that allow bacterial translocation from the intestine due to intestinal bacterial overgrowth have been implicated in the development of SBP[71]. Altered intestinal permeability was observed in 45% of patients with cirrhosis and was associated with Child-Pugh status, with the presence of ascites, and with a history of SBP[75,76]. SIBO is much more frequent in patients with cirrhosis and was highly correlated with BT, especially in ascitic patients[77].

Higher levels of *Enterobacteriaceae* were identified in cirrhotic rats than in healthy rats, and *Bifidobacteria* treatment resulted in lower levels of *Enterobacteriaceae*[61]. These results suggest the existence of an imbalance in the gut flora in cirrhotic rats, which may further result in BT and altered liver function[61].

BT to MLNs in cirrhosis has been linked to impaired host defense in these patients[78]. BT and endotoxemia are contributing factors in the expansion of specific subsets of lymphocyte populations[79]. In a clinical trial of 40 cirrhotics, the percentage of activated monocytes and T lymphocytes was increased in patients, and the proportions of effector cells and of those expressing CD95+ were higher. LBP modulates the biologic activity of circulating endotoxin, and its levels have been shown to rise in response to LPS[80-82]. Patients with elevated levels of LBP showed higher frequencies of regulatory T cells

(CD4+CD25+FoxP3+) than those with normal levels of LBP[79]. In a rat model, BT was associated with an increase in the phagocytic capacity of polymorphonuclear leukocytes[78]. Both TNF-α and IL-6 were increased in patients with translocation of bacterial DNA from gram-positive microorganisms regardless of endotoxin and LBP levels[83].

These data suggest that BT is of clinical relevance in patients with chronic liver disease and may be a contributing factor in the development of liver disease and the degree of severity of the associated complications.

BACTERIAL TRANSLOCATION: IMPLICATIONS FOR THERAPY

Detoxification of gut-derived toxins and microbial products from gut-derived microbes is one function of the liver. Levels of bacterial LPS are increased in the portal and/or systemic circulation in several types of chronic liver diseases. Increased gut permeability and LPS also play roles in several liver disorders. NASH is associated with increased serum LPS levels and the activation of proinflammatory signaling[8,11], both of which suggest BT as a potential therapeutic target in these disorders.

Probiotics have been suggested as a treatment for different types of chronic liver damage because of their abilities to augment intestinal barrier function and to prevent BT[84,85]. The administration of probiotics reduced BT in a rat model[86]. Both viable and heat-killed yeast cells prevented BT. This effect was suggested to be the result of an immune modulatory effect and the maintenance of gut barrier integrity[87,88]. Oral treatment with viable or heat-killed *Saccharomyces cerevisiae* strain UFMG 905 prevented BT in a murine model of intestinal obstruction. Treatment with either viable or heat-killed yeast reduced intestinal permeability and increased IL-10 levels. Orally administered probiotics, nonpathogenic *Escherichia coli*, and gentamicin decreased BT and attenuated liver damage, decreasing levels of TNF-α, IL-6, IL-10 and IL-12[89]. An enteral diet supplemented with *Chlorella* sp. microalgae had significant protective effects on the intestinal mucosal barrier in a rat model of obstructive jaundice and reduced BT[90].

Oral treatment with resveratrol, curcumin or simvastatin ameliorated small intestinal inflammation by maintaining gut barrier function, preventing BT, and decreasing Th1-type immune responses[91]. Oral administration of these compounds increased regulatory T cell numbers and augmented intestinal epithelial cell regeneration in the ileum. Levels of the anti-inflammatory cytokine IL-10 in the ileum, MLNs and spleen were increased, whereas the proinflammatory cytokines IL-23p19, IFN-γ and TNF-α were decreased[91]. Treated animals displayed fewer proinflammatory enterobacteria and enterococci and higher anti-inflammatory lactobacilli and Bifidobacteria loads.

Glutamine decreased intestinal permeability and BT to physiologic levels in treated animals and preserved intesti-

nal barrier integrity[92]. Arginine supplementation reduced intestinal permeability and BT, leading to mucosal ileum preservation[93]. A specific nutritional combination rich in protein, L-leucine, fish oil and specific oligosaccharides resulted in reduced BT along with reduced production of proinflammatory cytokines[94], and supplementation with honey in the presence of obstructive jaundice ameliorated BT[95].

Treatment with an anti-TNF-α mAb in a model of CCl4-induced cirrhosis decreased the incidence of BT in a TNF-α- and TNF-α receptor-independent manner without increasing the risk of systemic infection[96].

Desferrioxamine attenuated mucosal injury from post-hepatectomy liver dysfunction, and this was associated with decreased BT in the portal circulation, decreased portal endotoxin levels, and decreased systemic endotoxin levels[97]. Low concentrations of histamine inhibited bacteria from entering epithelial cells and inhibited intestinal BT[98]. In histamine-treated rats, the average numbers of bacteria in the liver and lymph nodes were much lower than those in control rats.

The sympathetic nervous system is activated in advanced cirrhosis, particularly in the splanchnic circulation, and exerts potent immunosuppressive actions. Splanchnic sympathectomy reduced bacterial translocation to MLNs in cirrhotic rats[99].

TREATMENT OF BACTERIAL TRANS-LOCATION AS A MEANS OF TREATING NONALCOHOLIC STEATOHEPATITIS

In light of the potential role of BT in the development of steatosis, steatohepatitis and fibrosis, several studies have evaluated the potential effects of treatments aimed at BT.

Probiotics exhibit immunoregulatory and anti-inflammatory activity. Administration of the probiotic VSL#3 modulated liver fibrosis *via* the modulation of collagen expression and impairment of TGF-β signaling in a NASH model[100]; however, this treatment did not prevent inflammation and steatosis.

Oxidative stress contributes to the development of NASH, suggesting that antioxidants, which decrease oxidative stress, may ameliorate the disease. Increasing hepatic α- or γ-tocopherol protected against LPS-induced NASH by decreasing liver damage, lipid peroxidation, and inflammation without affecting body mass or hepatic steatosis[101]. Resveratrol decreased NAFLD severity in rats *via* TNF-α inhibition and antioxidant activity[102]. Specifically, it decreased fat deposition, increased levels of activity of superoxide dismutase, glutathione peroxidase and catalase and decreased NOS in the liver.

LPS-induced liver injury was prevented by 8A8, a synthetic triglyceride containing an arachidonic acid branch, through the inhibition of TNF-α and NO production by hepatic macrophages[103]. Stimulation of peripheral blood mononuclear cells from NASH patients with LPS resulted in a strong increase in TNF-α production. Pentoxifylline caused a dose-dependent suppression of TNF-α secretion, suggesting that it may be able to serve as a potential treatment for NASH[104].

In an open-label trial, subjects with biopsy-proven NASH and insulin resistance were orally treated for 30 days with an IgG-enhanced fraction of enterotoxigenic *Escherichia coli* colostrum[105] (Imm-124©, Immuron, Australia). An alleviation of insulin resistance was detected by a decrease in fasting glucose levels, an elevation in the early peak of insulin secretion following glucose administration, improved OGTT, improved insulin secretion during the OGTT, and improvements in the HOMA score and HBA1C levels. Treated patients showed a decrease in serum levels of triglycerides, total cholesterol, and LDL cholesterol. A decrease in liver enzymes was noted in most treated patients. These effects were associated with increased serum levels of GLP-1 and adiponectin. An increase in CD25+ and CD4+CD25+Foxp3+ regulatory T cells was also noted. An anti-LPS effect along with the promotion of regulatory T cells suggests that a combined mechanism is responsible for these effects.

CONCLUSION

Alterations to intestinal microbiota seem to play important roles in the induction and promotion of liver damage progression and in the development and severity of NASH. Bacterial overgrowth, immune dysfunction, alteration of the luminal factors, and altered intestinal permeability are all involved in the pathogenesis of NASH and its complications, including progression to cirrhosis, infections, hepatic encephalopathy, SBP and renal failure[84]. A better understanding of the cell-specific recognition and intracellular signaling events involved in sensing gut-derived microbes will help in the development of means to achieve an optimal balance in the gut-liver axis and ameliorate liver diseases[36]. The data described here support the notion that BT may serve as a new therapeutic target for NASH.

BT induces an immune imbalance leading to a state of chronic inflammation, fat accumulation in the liver, mitochondrial dysfunction and NASH.

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ORIGINAL ARTICLE

Juvenile ferric iron prevents microbiota dysbiosis and colitis in adult rodents

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Abstract

AIM: To assess whether juvenile chronic ferric iron ingestion limit colitis and dysbiosis at adulthood in rats and mice.

METHODS: Two sets of experiments were designed. In the first set, recently weaned mice were either orally administered ferrous (Fe^{2+}) iron salt or ferric (Fe^{3+}) microencapsulated iron for 6 wk. The last week of experiments trinitrobenzene sulfonic acid (TNBS) colitis was induced. In the second set, juvenile rats received the microencapsulated ferric iron for 6 wk and were also submitted to TNBS colitis during the last week of experiments. In both sets of experiments, animals were sacrificed 7 d after TNBS instillation. Severity of the inflammation was assessed by scoring macroscopic lesions and quantifying colonic myeloperoxidase (MPO) activity. Alteration of the microflora profile was estimated using quantitative polymerase chain reaction (qPCR) by measuring the evolution of total caecal microflora, Bacteroidetes, Firmicutes and enterobacteria.

RESULTS: Neither ferrous nor ferric iron daily exposures at the juvenile period result in any effect in control animals at adulthood although ferrous iron repeated administration in infancy limited weight gain. Ferrous iron was unable to limit the experimental colitis (1.71 ± 0.27 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in colitic mice). In contrast, ferric iron significantly prevented the increase of MPO activity (1.64 ± 0.14 MPO U/mg protein) in TNBS-induced colitis. Moreover, this positive effect was observed at both the doses of ferric iron used (75 and 150 mg/kg per day po - 6 wk). In the study we also compared, in both rats and mice, the consequences of chronic repeated low level exposure to ferric iron (75 mg/kg per day po - 6 wk) on TNBS-induced colitis and its related dysbiosis. We confirmed that ferric iron limited the TNBS-induced increase of MPO activity in both the rodent species. Furthermore, we assessed the ferric iron incidence on TNBS-induced intestinal microbiota dysbiosis. At first, we needed to optimize the isolation and quantify DNA copy numbers using standard curves to perform by qPCR this interspecies comparison. Using this approach, we determined that total microflora was similar in control rats and mice and was mainly composed of Firmicutes and Bacteroidetes at a ratio of 10/1. Ferric juvenile administration did not modify the microflora profile in control animals. Total microflora numbers remained unchanged whichever experimental conditions studied. Following TNBS-induced colitis, the Firmicutes/Bacteroidetes ratio was altered resulting in a decrease of the Firmicutes numbers and an increase of the Bacteroidetes numbers typical of a gut inflammatory reaction. In parallel, the subdominant population, the enterobacteria was also increased. However, ferric iron supplementation for the juvenile period prevented the increase of Bacteroidetes and of enterobacteria numbers consecutive to the colitis in both the studied species at adulthood.

CONCLUSION: Rats and mice juvenile chronic ferric iron ingestion prevents colitis and dysbiosis at adulthood as assessed by the first interspecies comparison.

INTRODUCTION

In humans neonates never carry iron deficiency at birth and needs remain low during the first trimester of life because of a slowdown of red blood cells production as well as physiological hemolysis[1]. From the fourth month of life, iron requirements increase and appropriate diet supply, generally corresponding to food diversification, becomes necessary. Western countries recommendations leaded to iron fortification of food for infants to prevent any risk of anemia. Classical forms of supplementation are ferrous (Fe^{2+}) salts but are associated with frequent gastrointestinal side effects leading to poor compliance. In addition to this, presence of ferrous iron in the colon could select gut microbiota for humans that are unfavorable to the host because iron is known to be essential for the growth and virulence of many pathogenic enterobacteria[2]. To limit the unfavorable effects linked to ferrous iron absorption, food industry manufacturers have the possibility of using ferric (Fe^{3+}) forms mainly as of pyrophosphate. However, this form displays not only a poor solubility in the food matrix but also a low bioavailability. To bypass these constraints, ferric pyrophosphate may be microencapsulated in lecithin beads (Lipofer®). The incidence of the daily ingestion of this form in juvenile individuals has not yet been investigated.

At the gut level, and particularly during the perinatal period, the crosstalk between bacteria, epithelium and lymphoid tissue is establishing. This crosstalk is involved in modelling the memory mechanisms of systemic immunity response[3], when those systems mature to reach the full functionality of the intestinal barrier. In humans, postnatal dominant colonisation by facultative anaerobic bacteria, among which we find enterobacteria, quickly gives way to dominant anaerobic bacteria development within a couple of weeks, in order to reach an equilibrium during the second year of life[4]. Intestinal microbiota is a large and extremely complex ecosystem, constituted by dynamic and diverse bacterial communities. The adult human tract environment harbours different bacterial phylotypes superior to 400 different species[5,6]. In permanent contact with the mucosal surface through symbiotic interactions, it takes a prominent part on the maintenance of health of the host[7]. A 10/1 Firmicutes/Bacteroidetes ratio is considered to be representative of an health status[8] which reflects a stable equilibrium between bacteria. However, some groups, that are generally subdominant, such as enterobacteria may become potentially pathogenic and can affect host homeostasis[9]. Several studies have described a higher level of Bacteroidetes and enterobacteria associated to a reduction of Firmicutes in inflammatory bowel disease (IBD) patients[9-14]. IBD are a group of chronic disorders of the gastrointestinal tract and include Crohn's disease (CD) and ulcerative colitis. The pathogenesis is not known but involves, at least in part a loss of tolerance towards the commensal colonic microbiota. Therefore, disruption of this crosstalk results in a deregulation of the immune response to the gut microbiota and may lead to development of chronic inflammation such as IBD[9].

Since ferrous oral iron may be at the origin of enterobacteria proliferation[2] and since this development may contribute to intestinal mucosal barrier function deregulation leading to intestinal inflammation, we hypothesized that ferric iron supplementation may modulate microbiota profile settlement when given to juvenile animals and could modify the course of an experimental colitis in young adults. However, as individuals vary in their resistance to pathogenic stimuli and as interspecies comparisons remain difficult to address, particularly when analysing microbiota profile, this study aimed at: (1) comparing the incidence of microencapsulated ferric iron with the ferrous iron on experimental colitis in mice; (2) evaluating the dose-response effect of ferric iron in this model of inflammation in mice; and (3) analyzing the consequences of repeated exposure to ferric iron on a moderate colitis and microbiota dysbiosis on two models of rodents (mice and rats) to allow interspecies comparisons.

MATERIALS AND METHODS

Chemicals

Ferric pyrophosphate microencapsulated in lecithin beads (Lipofer®) used in this study was kindly provided as a stable solution by Lipofoods SA (Barcelona, Spain). Anesthetics were obtained from Centravet (Nancy, France). All other chemical molecules were purchased from Sigma-Aldrich SA (St Quentin-Fallavier, France) except if specified.

Animals

Experiments were conducted using male BalbC mice (15-17 g) and male Wistar rats (100-125 g) obtained from HARLAN Laboratories, Ganat (France). All animals were housed in stainless steel cages under controlled temperature (21 ± 1 ℃) and a 12 h light-dark cycles. They had free access to food (A04, SAFE, Epinay sur Orge, France) and water throughout the study. This study was performed at the Animal House Unit of the Institut Polytechnique LaSalle Beauvais (policy agreement No. A60) and received prior approval from both the animal protocol review committee and of the Picardie Council veterinary office.

Treatments

Two sets of experiments have been designed. In the first series of experiments, 4 groups of 16 male BalbC mice (10-12 g) were used. Three groups received either a solution of ferrous iron (150 mg/kg per day po) or ferric iron (75 or 150 mg/kg per day po - Lipofer®) daily for 6 wk. The 4th group received water. 2,4,6 Trinitrobenzene sulfonic acid (TNBS) colitis was induced the last week of the experiment on half of each group of animals ($n = 8$/subgroup). The other half served as control (sham colitis). In the second series of experiments, 16 male Wistar rats (75-100 g) were used. The animals were separated in two groups receiving either ferric iron (Lipofer®) at the dose of 75 mg/kg per day po or water under the same conditions for 6 wk. Colitis was also induced during the last week of the experiment. Half of each group of animals were submitted to TNBS colitis ($n = 8$), the other half ($n = 8$) served as a control (sham colitis). Body weight was monitored throughout the experiments.

Experimental colitis

Among the chemically induced experimental colitis[15], TNBS in 50% ethanol is one of the classical models because this mix induces a barrier break resulting in severe colitis with penetrating ulcers, a reduced colon length and thickening of the colon wall as observed in IBD patients[16]. Mice were fasted overnight prior to induction of colitis but were allowed free access to water. They were anesthetized with a mixture (50% v/v) of ketamine and xylazine (100 mg/mL) diluted in saline (NaCl 0.9%-w/v) at a dose 1 mL/kg ip. TNBS (Sigma Aldrich, France) diluted in 50% of ethanol (v/v) was injected *via* a polyethylene catheter inserted at 4 cm from the anus at the dose of 100 mg/kg in 25 μL to induce an experimental colitis. Control mice were also anesthetized and received an equal volume of saline. Following instillation mice were maintained in a head-down position for 2 min and received 0.2 mL sc of saline to prevent dehydration. Their awakening was closely monitored. Mice were sacrificed 7 d later. Rats were anesthetized with the same mixture of anesthetics at the dose of 5 mL/kg ip and the solution of TNBS was administered at 7 cm from the anus at the dose of 40 mg/kg in 100 μL. Saline (0.5 mL sc) was administered to prevent dehydration. Rats were sacrificed 7 d later. At sacrifice, macroscopic lesions were evalu-

ated and pieces of proximal colon (1 cm from the caecocolonic junction) and caecal content were collected, snap frozen and stored at -80 ℃ until further evaluation in both rat and mice experiments.

Assessment of the inflammatory reaction

Macroscopic damage scores: After sacrifice, the colon was removed immediately and severity of colonic mucosal alteration was determined according to a modified scale of Wallace *et al*[17]. Briefly, determination of the inflammatory damage was based on the presence of mucosal hyperaemia and bowel wall thickening, presence and extent of ulceration and necrosis, and the event of adhesions and diarrhea. Final quotation was ranging from 0 (normal appearance) to 10 (severe damage).

Myeloperoxidase assay: Myeloperoxidase (MPO) activity, a marker of polynuclear neutrophils, was measured in pieces of colon adjacent to the instillation point as described previously[18]. Briefly, frozen pieces of the proximal colon were homogenised in a phosphate buffer (50 mmol/L, pH = 6) containing hexadecyl trimethyl ammonium bromide (0.5% w/v) with a tissue lyser Ⅱ (Qiagen, France). The homogenates were submitted to 3 cycles of freezing/thawing (Liquid N2, 1 min/37 ℃, 10 min) and then further disrupted with a sonicator (Bioblock scientific, France) and then centrifuged (6000 *g* at 4 ℃ for 15 min). Supernatants were collected for measuring MPO activity and total protein contents. Samples were diluted into a reaction buffer containing O- dianisidine dihydrochloride (1 mg/mL) and hydrogen peroxide (3×10^{-4} % v/v). Human MPO from purified neutrophils was used as a standard. The absorbance was measured after 10 min of incubation at 450 nm. Total protein content was assessed from the supernatants according to Lowry's method (Bio Rad DC Protein Assay, France).

Real time quantitative polymerase chain reaction for microflora quantification

As we planned to assess microflora DNA from caecal content as well as comparing two species to validate the repeatability, we aimed at optimizing the bacterial DNA extraction by improving the extraction procedure of Gram positive bacteria and by determining the appropriate amount of the initial sample to treat.

DNA extraction: Total DNA from caecal samples (25, 50, 100 and 200 mg content) was extracted using the Qiamp DNA stool Mini Kit (Qiagen, France). Before proceeding according to the manufacturer's recommendations, frozen samples were lysed in an ASL lysis buffer and incubated for 3 consecutive cycles of freezing/thawing (Liquid N2, 1 min/37 ℃, 10 min). The lysates were clarified by centrifugation (14 000 *g* at 4 ℃ for 3 min). Polymerase chain reaction (PCR) inhibitors and impurities were absorbed by action of an inhibitex tablet (Qiagen). The supernatants were collected following a second centrifugation and DNA was automatically purified in the Qiacube automat (Qiagen,

Table 1 Primers used for real-time polymerase chain reaction amplification of *16S rRNA* gene

PCR assay	Primers	Primers Sequences 5'→3'	Accession number (NCBI)	Linear regression curves with coefficient of correlation	Sources of reference
Total Bacteria	UnivF	TCCTACGGGAGGCAGCAGTG	-	Y = -2.941 x + 36.580	Watanabe *et al*[21], 2001
	UnivR	TTACCGCGGCTGCTGGCACG		r^2 = 0.9997	Nadkarni *et al*[22], 2002
Bacteroidetes	BacF	CCTWCGATGGATAGGGGTT	-	Y = -2.908 x + 35.839	Firmesse *et al*[23], 2008
	BactR	TCCCCAGGTGGAATACTTAACG		r^2 = 0.9995	
Enterobacteria	EntF	CATTGACGTTACCCGCAGAAGAA	AX110239/AX109631	Y = -3.192 x + 36.762	This study
	EntR	CGCTTGCACCCTCCGTATTA	AF293850/U26176	r^2 = 0.9747	
Firmicutes	FirmF	ACCCGCGTCTGATTAGCTAGTT	M59090/L34627	Y = -3.298 x + 39.136	This study
	FirmR	CCTCTCAGGCCGGCTACTG	Y10584/FJ345661	r^2 = 0.9924	

Primers sequences were designed using the following website: www.ncbi.nlm.nih.gov/BLAST/. PCR: Polymerase chain reaction; NCBI: National Center for Biotechnology Information.

France) using Qiamp Minispin columns. Concentrations were determined on a Hellma TrayCel using a Biophotometer (Eppendorf, France). Absorbance ratios at 260/280 and at 260/230 were determined to quantify and assess the purity of DNA samples.

Plasmids for standard curves: Competent DH10b cells were used for the cloning experiments described previously[19]. The recombinant plasmids containing specific *16S rRNA* gene inserts either from Firmicutes, or Bacteroidetes, or enterobacteria or a consensus sequence for total microflora, were purified using a HiSpeed Plasmid Maxi kit (Qiagen, France). DNA concentration was determined following measurement of optical densities both at 260 nm and 280 nm before converting it into *16S rRNA* gene copy numbers as described previously[19]. Standard curves were established from serial dilutions of recombinants plasmids performed using real-time quantitative PCR. Copy numbers of the plasmid were calculated following a previously established equation [Copy numbers = 6.02 × 10²³ (copy/mol) × DNA amount (g)/DNA length (dp) × 660 (g/mol per dp)][20].

Real time polymerase chain reaction: Two pairs of primers (enterobacteria and Firmicutes) corresponding to specific bacterial regions targets within *16S rRNA* gene were designed (Table 1). The two other pairs used (Total Flora and Bacteroidetes) had already been designed[21-23]. qPCR was performed using an ABI Prism 7300 sequence detector system (Applied Biosystems, France) on both plasmids and samples extracted DNA. Reactions were performed in duplicate using the Sybr Green PCR master mix (Qiagen, France) in a final volume of 25 μL with 0.3 μmol/L final concentration of each primer and appropriate dilutions of DNA samples. Amplification was initiated at 95 ℃ for 3 min to activate Taq plus DNA polymerase followed by 40 cycles at 95 ℃ for 3 s and 61 ℃ for 30 s. Two consecutive tenfold series of dilutions were realized to verify the linearity (slope of -3.32). A melting step was added and curves were analysed to look for any unspecific amplification. Standard curves were obtained following amplification under similar conditions of different samples containing different numbers of copies from the respective specific clones of the targeted

gene. PCR efficiency (E) was calculated according to the equation from the standard curve: E = 10⁻(1/ slope) -1 according to Ibekwe and Grieve[24]. qPCR was realised using the cycle number threshold (Ct) and was based on the calculated standard curves. Each qPCR assay systematically included control reactions performed in parallel to the samples.

Statistical analysis

Results were expressed in mean ± SD error to the mean. Macroscopic lesions scores were compared using the Wilcoxon test for non parametric data followed by the Dunn post-test. For all the others parameters, data were submitted to an ANOVA followed by the Tuckey post-test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Optimization of DNA isolation and quantification of microflora for realising interspecies comparison

Because of high inter- and intra-species variations, we aimed at improving the DNA extraction steps by determining the best amount of caecal content to use and by adding the preliminary step to improve the extraction of Gram positive bacteria. The best yield of DNA extraction was obtained when using 100 mg of caecal content and by adding a preliminary thermic lysis step (data not shown). Specificity of the *16S rRNA* gene targeted primers used for qPCR was tested both in silico and using pure DNA extracts from specific target strains (positive controls) and by confronting it to no target species (negative controls). Selected primers (Table 1) were run and the specificities of the amplification products were confirmed by the analysis of the dissociation curve in both caecal samples and DNA controls. We thus determined that a unique target gene was amplified for each species. Analysis of the regression curve for Ct values obtained from serial dilution of DNA samples showed a linear correlation for all target DNA regions; for all the microbiota species studied, the coefficient of correlation (r^2) was higher than 0.97 and amplification efficiencies were between 100 % and 120 % which involves curve slopes ranging from -3.29 to -2.9 (Table 1). All below mentioned analyses have been performed under those conditions.

Figure 1 Effect of a 6 wk juvenile ingestion of ferrous (150 mg/kg per day po) *vs* **ferric (150 mg/kg per day po) iron on the inflammatory response at adulthood to a trinitrobenzene sulfonic acid-induced colitis in mice.** A: Macroscopic damage scores in non-inflamed (sham-colitis) and inflamed (trinitrobenzene sulfonic acid, TNBS) mice; B: Colonic myeloperoxidase (MPO) activity in non-inflamed (sham-colitis) and inflamed (TNBS) mice. Data are expressed as mean ± SE (n = 8 per group). [a]$P < 0.05$ *vs* TNBS-treated group, [b]$P < 0.01$ *vs* Sham-colitis.

Figure 2 Effect of a 6 wk juvenile ferric iron administration (75 and 150 mg/kg per day po) on the inflammatory response at adulthood to a trinitrobenzene sulfonic acid-induced colitis in mice. A: Macroscopic damage scores in non-inflamed (sham-colitis) and inflamed trinitrobenzene sulfonic acid (TNBS) mice; B: Colonic myeloperoxidase (MPO) activity in non-inflamed (sham-colitis) and inflamed (TNBS) mice. Data are expressed as mean ± SE (n = 8 per group). [a]$P < 0.05$, [b]$P < 0.01$ *vs* TNBS-treated group, [d]$P < 0.01$ *vs* Sham-colitis.

Effects of ferric iron on TNBS colitis

Repeated daily ingestion of ferrous iron (150 mg/kg per day po - 6 wk) by juvenile mice did not prevent the induction of a TNBS-induced moderate colitis. In fact, juvenile ferrous iron daily exposure failed to limit macroscopic lesions (Figure 1A) in TNBS treated mice. In contrast, juvenile ferric iron prevented these lesions (Figure 1A) in inflamed mice. Furthermore, while TNBS enema resulted in an increased MPO activity in controls (2.47 ± 0.22 MPO U/mg protein *vs* 0.91 ± 0.09 MPO U/mg protein), we did not observe any significant reduction of MPO activity in mice exposed to a repeated ingestion of ferrous iron (1.71 ± 0.27 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in the TNBS group) (Figure 1B). In contrast, the daily exposure of juvenile mice to the same dose of ferric iron (150 mg/kg per day po - 6 wk) was able to limit the inflammatory response since MPO activity was significantly lower ($P < 0.05$) (1.64 ± 0.14 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in the TNBS group) (Figure 1B). Furthermore, we observed that before inducing TNBS colitis (5 wk of treatment), mice treated with ferrous iron put on significantly ($P < 0.05$) less weight than mice treated with ferric iron and controls (124.6% ± 1.12% and 136.6% ± 1.33

% *vs* 143.7% ± 2.29 % in control, respectively).

Ferric iron juvenile supplementation and dysbiosis

We also aimed at determining the dose-response effect of ferric iron supplementation on the same model of colitis in mice.

In control animals, the repeated administration of both 75 and 150 mg/kg per day po (6 wk) of ferric iron did not result in any alteration of growth, nor did it result in colonic inflammation or an alteration of the microflora profile. In fact, weight gain under iron treatment remained similar whichever species considered (142.7% ± 2.66% for 75 mg/kg per day and 136.6% ± 1.33 % for 150 mg/kg per day *vs* 143.7% ± 2.29 % in controls). Similarly, no macroscopic alterations of the gut mucosa have been observed on mice following either the low or the high chronic ferric supplementation (Figure 2A). Those results were correlated to low levels of MPO activity in the colonic mucosa following exposure to both doses of ferric iron (1.65 ± 0.21 MPO U/mg protein and 1.58 ± 0.11 MPO U/mg protein for respectively 75 and 150 mg/kg per day ferric iron po *vs* 0.91 ± 0.09 MPO U/mg protein in controls) (Figure 2B). Daily consumption of ferric iron by juvenile rodents did not modify the bacte-

Figure 3 **Effect of a juvenile ferric iron administration for 6 wk on the microflora profile of mice and its evolution consecutively to a trinitrobenzene sulfonic acid-induced colitis.** A: Total Flora; B: Firmicutes; C: Bacteroidetes; D: Enterobacteria. Iron75: Ferric iron 75 mg/kg/d po - 6 wk; Iron150: Ferric iron 150 mg/kg per day po - 6 wk. Data are expressed as mean ± SE (n = 8 per group). [a]P < 0.05, [b]P < 0.01 *vs* Sham-colitis; [d]P < 0.01 *vs* TNBS-treated group.

ria profile since the total flora number remained at circa 11.8 log copy number/g of caecal content (Figure 3A). The Firmicutes number was estimated to be around 11.4 log copy number/g of caecal content (Figure 3B); The Bacteroidetes number remained around 10.5 log copy number/g caecal content (Figure 3C); and enterobacteria levels at 8.7 ± 0.1 log copy number/g of caecal content (Figure 3D).

Daily administration of both the doses of ferric iron on juvenile rodents prevented the TNBS-induced colitis at adulthood. In fact, exposure to ferric iron supplementation before inducing an experimental colitis limited the onset of macroscopic lesions (1.42 ± 0.72 AU and 0.2 ± 0.2 AU *vs* 5.75 ± 0.75 AU in TNBS-treated mice) (Figure 2A) and the increase of colonic MPO activity (1.58 ± 0.15 MPO U/mg protein and 1.64 ± 0.14 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein in TNBS-treated mice) (Figure 2B). The limitation of the inflammatory lesions was correlated to the maintenance of a healthy microflora profile. This supplementation also prevented the decrease of the Firmicutes (Figure 3B) and increase of the Bacteroidetes and enterobacteria populations (Figure 3C and D). Since efficiency was observed with the dose of 75 mg/kg per day po for 6 wk, we chose to continue with this dose of ferric iron.

Juvenile exposure to ferric iron comparably prevents a moderate TNBS-induced colitis in both rats and mice

Good comparisons need to be performed under the same conditions necessitating the induction of a comparable inflammatory reaction that is representative of pathophysiological conditions observed in humans. Using the optimised technique of extraction and quantification of DNA, total flora in control rats and mice was estimated to be around 11.8 log copy number/g of caecal content in control animals (Figures 3A and 4A). We also noticed that it is mainly composed of Firmicutes (around 11.3 log copy number/g of caecal content in both species) (Figures 3B and 4B) and Bacteroidetes (around 10.5 ± log copy number/g caecal content) (Figures 3C and 4C) followed by an enterobacteria population ranging from 8.6 ± 0.1 log copy number/g of caecal content in mice to 9.4 ± 0.1 log copy number/g of caecal content in rats (Figures 3D and 4D).

Instillation of TNBS resulted in a significant (P < 0.05) increase of macroscopic damage scores in rats (8.33 ± 0.35 AU) and mice (5.75 ± 0.75 AU) (Figures 2A and 5A) as well as a significant (P < 0.05) increase of MPO activity in rats (773.7 ± 36.62 MPO U/mg protein *vs* 496.1 ± 63.94 MPO U/mg protein) and mice (2.47 ± 0.22 MPO U/mg protein *vs* 0.91 ± 0.09 MPO U/mg protein)

Figure 4 Effect of a 6 wk juvenile ferric iron administration (75 mg/kg per day po) on the microflora profile of rats and its evolution consecutively to a trinitrobenzene sulfonic acid colitis. A: Total Flora; B: Firmicutes; C: Bacteroidetes; D: Enterobacteria; Iron75: Ferric iron 75 mg/kg per day po - 6 wk; Iron150: Ferric iron 150 mg/kg per day po - 6wk. Data are expressed as mean ± SE (n = 8 per group). [a]$P < 0.05$, [b]$P < 0.01$ *vs* Sham-colitis.

Figure 5 Effect of a 6 wk juvenile ferric iron supplementation (75 mg/kg per day po) on the inflammatory response at adulthood of rats submitted or not (sham-colitis) to a trinitrobenzene sulfonic acid-induced colitis. A: Macroscopic damage scores (MDS); B: Myeloperoxidase (MPO) activity Data are expressed as mean ± SE (n = 8 per group). [a]$P < 0.05$ *vs* trinitrobenzene sulfonic acid (TNBS)-treated group, [b]$P < 0.01$ *vs* Sham-colitis.

(Figures 2B and 5B). However, we did not observe any significant modification of total microflora neither in rats nor in mice 7 d after TNBS instillation (circa 11.7 log copy number/g of caecal content in both rodent species) (Figures 3A and 4A). However, the balance between the two major phyla of the bacterial population observed

was altered. A significant reduction ($P < 0.05$) of Firmicutes was registered in both colitic rats (-0.4 Δ log copy number) and mice (-0.3 Δ log copy number) (Figures 3B and 4B) as compared to controls. Conversely, significant higher levels of Bacteroidetes ($P < 0.05$) were detected in both the colitic groups; it increased by 0.5 Δ log copy

number in rats and by 0.7 Δ log copy number in mice (Figures 3C and 4C). Enterobacteria numbers were also found to be significantly higher ($P < 0.05$) in colitic rats and mice with increases of respectively 0.4 and 2 Δ log copy number/g of caecal content as compared to controls (Figures 3D and 4D).

Juvenile ferric iron supplementation for 6 wk, before inducing an experimental colitis, significantly limited the onset of macroscopic lesions not only in TNBS-treated mice (1.42 ± 0.72 AU *vs* 5.75 ± 0.75 AU) but also in TNBS-treated rats (7.25 ± 0.49 AU *vs* 8.33 ± 0.35 AU). Juvenile iron administration also significantly ($P < 0.05$) prevented the increase of colonic MPO activity in TNBS-treated mice (1.59 ± 0.5 MPO U/mg protein *vs* 2.47 ± 0.22 MPO U/mg protein) as well as in TNBS-treated rats (572.5 ± 26.39 MPO U/mg protein *vs* 773.7 ± 36.63 MPO U/mg protein) (Figures 2B and 5B). This juvenile ferric administration (75 mg/kg per day po - 6 wk) did not modify total bacteria number even when inducing colitis (Figure 3A and 4A). Juvenile ferric iron repeated administration before inducing the colitis not only prevented the increase of the Bacteroidetes population (Figures 3B and 4B) but also the enterobacteria population (Figures 3D and 4D) in both mice and rats.

DISCUSSION

When using a microencapsulated ferric pyrophosphate form (Lipofer®), we were the first to evidence a beneficial effect of ferric iron oral supplementation in juvenile animals to prevent the induction of colitis at adulthood. Ferric pyrophosphate is a water insoluble compound, marketed as a food additive in Europe to fortify infant cereals and chocolate powder drinks[25]. Because of its chemical composition, it has been microencapsulated to improve both its bioavailability and its incorporation in food to be fortified[26], rendering it a good alternative to the ferrous forms without having any of the side effects.

First, we provided evidence of the benefits of ferric iron supplementation during the juvenile period. Iron oral administration is recommended in young infants to prevent anemia and its consecutive neurologic and developmental deficits. Iron also has benefits, as a trace element, in supporting immune function by strengthening not only epithelial barriers but also cellular and humoral immune responses. However, ferrous iron may be at the origin of deleterious effects such as lipid peroxydation[27] or enterobacteria growth facilitation[2]. Here, with ferric iron, we did not observe any negative effect of a repeated administration for 6 wk. In fact, in non-inflamed mice, juvenile ferric iron ingestion did not result in either an inflammatory reaction or did it modify the microflora profile since both total bacteria and enterobacteria numbers remained unchanged as compared to non supplemented sham-colitic mice. This absence of action on gut microbiota profile is in favour of the ferric form instead of the ferrous form for infant food fortification.

Secondly, we aimed at evaluating the dose response effect of ferric iron supplementation in the juvenile period

the induction of an experimental colitis at adulthood. Environmental factors such as smoking, luminal enteric bacteria and trace elements such as iron are involved in the pathogenesis of IBD in a genetically susceptible host[28]. In fact, anemia is often described during chronic gut inflammatory disorders such as IBD[29]. This anemia is caused by two factors, the first being impaired proliferation and differentiation of erythroid progenitor cells, and the second being consecutive to iron retention within monocytes and macrophages both activated under the inflammatory conditions. Classically, patients receive either oral or systemic iron supplementation generally as ferrous sulfate or fumarate. However, ferrous iron oral administration often aggravates the inflammatory reaction because of its accumulation in the intestinal lumen and its participation in the Haber-Weiss reaction[27]. Compared to non supplemented mice, ferric iron juvenile supplementation proved to be efficient against this experimental colitis, even at the lowest dose used since their MPO activity levels were similar to those of control mice. The lowest dose of iron used corresponded to an overall daily intake of 1.2 mg iron per day, in other words to 15 μg iron/g mouse/day which is two times lower than the dose used by Werner *et al*[30] but it is however efficient in our model too.

The positive effect of iron supplementation in mice was corroborated by the results obtained in rats. In our study, we were able to demonstrate that ferric iron oral administration did not induce any alteration but rather prevented the course of an experimental colitis in both rodent species. In fact, both rats and mice submitted to iron supplementation did not develop any sign of inflammation. In both those rodent species, we also induced an experimental inflammatory reaction of comparative intensity. In fact, in comparison to the literature[31], they developed a moderate but homogenous colitis characterised by tissular lesions, ulcerations of the distal colon and neutrophil infiltration. These results are in agreement with a previous report indicating that the TNBS-induced colitis model is associated to an increase of inflammatory response including granulomas and tissular MPO activity[31-33].

Finally, we aimed at evaluating the reproducibility of colonic microflora evaluation under control conditions and its alteration during a moderate experimental colitis in rats and mice. This study is the first to evidence a comparative alteration of gut microbiota during an experimental colitis in two rodent species. Following the qPCR optimisation process described above, we observed no difference in total microbiota numbers between control rats and mice groups. In this work, we also found that caecal microflora of rodents is composed predominantly of Firmicutes followed by Bacteroidetes which profile correlates to the observations realised on humans. In fact, human Firmicutes population is 10 times higher than their Bacteroidetes population; which is considered to be a good indicator of health status[34,35]. In this study, we observed the same ratio profile not only in mice but also in rats. In non supplemented animals and under

inflammatory conditions, we did not observe any alteration of total bacteria numbers in both the rodent species. This is in agreement with literature which describes no principal differences in the composition of the total mucosal flora in IBD patients compared to controls[36]. Furthermore, while tissular healing might have already started in the animals, in both murine models we observed a net increase of Bacteroidetes and a reduction of the Firmicutes population indicating a clear reversion of this profile. Similar trends were also observed in clinical studies, showing a lower representation of Firmicutes phyla during inflammatory acute phases[34,37]. As already evidenced, the diminution of the Firmicutes phylum promotes the development and the invasion of tissues by opportunistic bacteria species and the gut becomes very susceptible to invading pathogens among which enterobacteria[38,39]. We also noted, under colitic conditions, a higher level of enterobacteria which is concordant with literature reporting a higher incidence of *Escherichia coli* in IBD patient compared to healthy subjects[40,41]. We chose to work in comparing rat and mice microbiota alteration during this moderate experimental colitis to ensure that not only could we obtain comparative results to human dysbiosis but also to make sure that the results obtained were linked to the intensity of the inflammatory response rather that to species sensitivity. Such comparisons are of great interest in testing the reproducibility of the method set up. Overall, our results are correlated to clinical observations realised on IBD patients such as fever, diarrhea, weight loss, rectal bleeding[42], and severe alteration of the microbiota equilibrium, especially with Bacteroidetes and enterobacteria increases in opposition to Firmicutes reduction[9,10]. In both rats and mice receiving ferric iron in the juvenile period for 6 wk the microbiota profile was not altered. In addition, rats and mice submitted to an experimental colitis during the last week of iron treatment did not display any modification of either total number bacteria or enterobacteria numbers as compared to non supplemented animals. This is in favour of a positive role of ferric iron onto gut microbiota equilibrium which will limit the onset of the inflammatory reaction as compared to classical ferrous forms. It reinforces the idea that iron is one of the key markers for limiting the onset of the inflammatory response in genetically susceptible patients[30]. Furthermore, we may suggest that ferric iron in contrast to ferrous iron contributes to the settlement of an appropriate microflora during the post natal period and that this profile is less sensitive to inflammatory stimuli. One cannot ascertain the mechanisms producing such results, but we may suggest that the lower incidence of ferric iron on colitis might be linked to, either a lower susceptibility to stimulate oxidative stress reactions as compared to ferrous iron, or to its interactions with the immune system which is partly driving an appropriate microbiota implantation during the perinatal period. Rather than working with animals whose microflora profile had been modified because they display a modified immune pattern[43,44], we decided to work on two rodent species of the same age, gender and submitted to a similar level of an inflammatory stimulus Since this microbiota/immune system interaction is said to be species, gender and age specific.

In conclusion, this study shows comparative rodent dysbiosis to human IBD dysbiosis following a moderate TNBS colitis. It also shows the benefits of ferric iron oral ingestion during the juvenile period in the prevention of an experimental colitis induced at adulthood in healthy animals. These interesting results would necessitate checking how anemic juvenile animals would react to such a treatment and especially to an induced colitis at adulthood. To further understand this incidence of ferric iron on overall intestinal functionalisation, one point we did not address is the role of the immune system and particularly its fine orientation modulation by iron fortification during the perinatal period in regards to the observed effects. We may hypothesize that iron could participate in reinforcing the immune system orientation, thus contributing to the limitation of the inflammatory response due to TNBS. This question will be addressed in another set of experiments. If these results are confirmed, this form of microencapsulated ferric iron could thus be clinically assessed as an interesting alternative to iron sulfate in young individuals at risk of anemia but also in subjects at risk of chronic gut inflammation such as CD.

ACKNOWLEDGMENTS

COMMENTS

Background

Juvenile iron supplementation is now widely accepted to limit the risk of anemia. However, oral iron ingestion may have side effects especially at this period when the crosstalk between bacteria, epithelium and the immune system is being set up. In fact, an impaired settlement of this crosstalk may predispose to chronic diseases such as inflammatory bowel disease (IBD). No one has so far evaluated the consequences of food matrix composition during the postnatal period on the predisposition to develop chronic diseases at adulthood. This study was thus aimed at evaluating the consequences of juvenile chronic iron exposure on the onset of experimental colitis and its related dysbiosis in both rats and mice at adulthood.

Research frontiers

In these recent years, research on the role of commensal microbiota has evidenced its strong impact on digestive physiology and particularly its modulating role in IBD. Moreover, recent data evidenced that microbiota implantation in the perinatal period conditions, the maturation of the gut mucosa and the immune system renders the host tolerant to commensal bacteria. Since oral ferrous iron may promote potential pathogenic bacteria growth, which may condition the development of chronic gut inflammation, the authors evaluated in this study the modulation by repeated juvenile administration of ferric iron on the onset of an experimental colitis at adulthood in two rodent species.

Innovations and breakthroughs

Literature largely describes the necessity to maintain a good equilibrium of the microbiota to stay healthy. Food matrix also conditions the diversity and stability of the microbiota especially during the postnatal period crucial for these aspects. This study is the first to evidence the preventive effects of oral ferric iron administration at the juvenile period on the inflammatory response and dysbiosis related to an experimental colitis at adulthood in two rodent species.

Applications

This study proposes to evaluate the consequences of oral supplementation at

infancy on risk of developing gut inflammation at adulthood. These results necessitate being completed by the evaluation of the consequences of ferric iron on the immune system maturation and to be clinically proven but they could represent a good opportunity for families at risk of developing IBD.

Terminology

IBD comprises chronic inflammations of the gut and more especially Crohn's Diseases and Ulcerative Colitis. Microbiota designates the pool of microorganisms harboured in our gut under physiological conditions. They divide into dominant and subdominant and control each other's growth to reach dynamic equilibrium. Dysmicrobism is an alteration of the microbiota equilibrium. Iron is a metal. In aqueous solution, it exists in two oxidation states: ferrous (Fe^{2+}) and ferric (Fe^{3+}).

Peer review

The study aimed to study rodent dysbiosis and compare it to human IBD dysbiosis following a moderate trinitrobenzene sulfonic acid colitis. It shows the benefits of ferric iron oral ingestion during the juvenile period in the prevention of an experimental colitis induced at adulthood. The paper is well written and has potential application in food supplemental.

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ORIGINAL ARTICLE

High dose glargine alters the expression profiles of microRNAs in pancreatic cancer cells

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Abstract

AIM: To investigate the effect of high dose glargine on the expression profiles of microRNAs in human pancreatic cancer cells.

METHODS: Real-time polymerase chain reaction array (RT-PCR) was applied to investigate miRNAs differentially expressed in Sw1990 cells treated with or without 100 IU/L glargine. Stem-loop RT-PCR was used to confirm the results of the array assay in Sw1990 and Panc-1 cells. The effects of miR-95 on cell growth, apoptosis, invasion and migration abilities were respectively examined by CCK8 assay, apoptosis assay, Matrigel invasion and migration assay in Sw1990 and Panc-1 cells. Nude mice xenograft models with Sw1990 cells were built to investigate pancreatic cancer growth *in vivo* after transfection by the lentivirus pGLV3-GFP- miR-95.

RESULTS: Ten miRNAs were significantly up-regulated and 2 miRNAs down-regulated in glargine treated Sw1990 cells when compared with non-treated cells (2.48-fold changes on average, $P < 0.01$). miR-95, miR-134 and miR-34c-3p are the top three miRNAs regulated by glargine (3.65-fold, 2.67-fold and 2.60-fold changes respectively, $P < 0.01$) in Sw1990 cells. Stem-loop RT-PCR confirmed that high dose glargine up-regulated the expression of miR-95 and miR-134 in both Sw1990 and Panc-1 cells. The most obvious change is the apparent increase of miR-95. Forced expression of miR-95 significantly increased cell proliferation (Sw1990: 2.510 ± 0.129 *vs* 2.305 ± 0.187, $P < 0.05$; Panc-1: 2.439 ± 0.211 *vs* 2.264 ± 0.117, $P < 0.05$), invasion (Sw1990: 67.90 ± 12.33 *vs* 47.30 ± 5.89, $P < 0.01$; Panc-1: 37.80 ± 8.93 *vs* 30.20 ± 5.14, $P < 0.01$), migration (Sw1990: 101 ± 6.00 *vs* 51.20 ± 8.34, $P < 0.01$; Panc-1: 91.80 ± 9.22 *vs* 81.50 ± 7.47, $P < 0.01$) and inhibited cell apoptosis (Sw1990: 22.05% ± 1.92% *vs* 40.32% ± 1.93%, $P < 0.05$; Panc-1: 20.17% ± 0.85% *vs* 45.60% ± 1.43%, $P < 0.05$) when compared with paired negative controls, whereas knockdown of miR-95 obtained the opposite effect. Nude mice xenograft models confirmed that miR-95 promoted the growth of pancreatic cancer *in vivo* when compared with negative control (tumor volume: 373.82 ± 23.67 mL *vs* 219.69 ± 17.82 mL, $P < 0.05$).

CONCLUSION: These observations suggested that modulation of miRNA expression may be an important mechanism underlying the biological effects of glargine.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in Western countries and has the poorest survival rate (< 5%) among the common malignancies[1,2]. Recently, antidiabetic therapies have been shown to affect the risk of pancreatic cancer. Some observational studies in humans have linked glargine with a putative increased cancer risk, including pancreatic cancer[3,4].

Glargine (A21Gly, B31Arg, B32Arg human insulin) is a widely used insulin analog in which a 24-h action profile is achieved by altering the amino acid sequence of the alpha (α) and beta (β) chains of the C terminus[5]. It has been shown that glargine increases resistance to apoptosis in several tumor cell lines[6]. Given the increased affinity to the insulin-like growth factor- I receptor (IGF-IR) *in vitro*[7], glargine may increase the bioavailability of IGF- I by altering the levels of IGF-binding proteins[8,9]. IGF- I is a more potent growth factor than insulin, promoting proliferation and inhibiting apoptosis, and plays an important role in facilitating malignant cell survival and metastasis[10,11]. This may be the theoretical basis for the potential carcinogenicity of glargine. However, data regarding the effect of glargine on pancreatic cancer are inconsistent. Administration of glargine didn't alter proliferation of Colo-357 pancreatic carcinoma cells and survival of patients with pancreatic carcinoma[12]. Thus, the role of glargine in pancreatic carcinogenesis deserves further investigation.

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs, 19-25 nucleotides in length, which are now recognized as crucial post-transcriptional regulators of gene expression[13-15]. It has been demonstrated that miRNAs play important roles in biological processes that affect tumor progression including migration, invasion, epithelial to mesenchymal transition (EMT) and metastasis[16-18]. miRNAs are promising as early biomarkers, prognostic indicators and therapeutic targets for anticancer treatments[19-21]. Aberrant miRNA expression has also been frequently reported in pancreatic cancer[22]. However, very few compounds, not to mention glargine, which affect cell growth and/or development, have been shown to affect miRNA expression.

In this present study, we elucidated the miRNAs signature in response to glargine treatment in human pancreatic cancer cells. Our results indicated that glargine alters specific miRNA expression in human pancreatic cells, especially miR-95. The effect of miR-95 on apoptosis, proliferation, migration and invasion ability of pancreatic cancer cells were further investigated. Moreover, nude mice xenograft models were built to investigate pancreatic cancer growth *in vivo* after transfection by the lentivirus pGLV3-GFP-miR-95. It therefore appeared that miR-95-related changes were important effects of glargine.

MATERIALS AND METHODS

Cell lines and cultures

Pancreatic ductal cancer cell lines Sw1990 and Panc-1 were conserved in our own laboratory and were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% fetal bovine serum (FBS; GIBCO) in a humidified incubator at 37 ℃ with an atmosphere of 5% CO_2.

miRNAs real time polymerase chain reaction array

Sw1990 cells (3×10^5 per well) were plated on 6-well plates in DMEM with 10% FBS. After 24 h of incubation at 37 ℃, the cells were treated with or without 100 IU/L glargine. Glargine was replenished every 24 h. The cultures were incubated for 2 d, then the total RNA was isolated from cell samples using Trizol reagent (Invitrogen) following the manufacturer's protocol. Then, cDNA synthesis was performed using Universal cDNA synthesis kit (Exiqon). The expression levels of 372 human mature miRNAs were examined using the miRCURY LNA™ Universal real time microRNA polymerase chain reaction system, Ready-to-use human panel I (Exiqon, kangchen, China).

Briefly, total RNA containing miRNA was polyadenylated, and cDNA was synthesized using a poly (T) primer with a 3'degenerate anchor and a 5'universal tag. Then, cDNA served as a template for microRNA quantitative real-time polymerase chain reaction (qPCR) using miRCURY LNA Universal RT miRNA PCR kit (Exiqon). The miRNA Ready-to-use human panel I is a 384-well PCR plate containing dried down LNA™ primer sets for one real-time PCR reaction per well. Three small RNA (U6snRNA, SNORD38B, SNORD49A) and three miRNA (miR-103, miR-191 and miR-423-5p) reference genes are included on the panel. The amplification profile was denatured at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 10 s and 60 ℃ for 60 s. At the end of the PCR cycles, melting curve analyses were performed. All reactions were conducted three times. Expression levels of mature miRNAs were evaluated using comparative CT method ($2^{-\Delta CT}$).

Stem-loop real-time reverse transcription-PCR

The miRNAs (miR-95, miR-134 and miR-34c-3p) were quantitated by stem-loop real time reverse transcription (RT)-PCR to confirm the reliability of the miRNA array assay. In brief, Sw1990 and Panc-1 cells (3×10^5 per well) were seeded on 6-well plates in DMEM with 10% FBS. After 24 h of incubation, the cells were treated with different concentrations of glargine (0-150 IU/L) for 48 h or treated with 100 IU/L glargine for different periods (24-72 h). Glargine was replenished every 24 h. Then the total RNA was isolated. 0.2-0.5 μg of total RNA was reverse transcribed to cDNA using a target-specific stem-loop primer indicated in Table 1. cDNA in water was added to 5 μL of the 2 × SYBR green master mix (Applied Biosystems Inc, Foster City, United States), 400 nmol/L of gene-specific primer and water used to make the solution up to 10 μL. The reactions were amplified at 95 ℃ for 10 min followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 60 s. U6 small nuclear RNA (U6) served as the endogenous control. The relative amount of each miRNA

Table 1 Primers used for reverse transcription or polymerase chain reaction of microRNAs

Gene name		Primer sequences (5'-3')
miRNA-95	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGTGCTCAAT
	Sense	CGGGTATTTATTGAGCA
	Antisense	AACTGGTGTCGTGGAG
miRNA-134	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGCCCCTCTG
	Sense	TGTGACTGGTTGACCAGAG
	Antisense	AACTGGTGTCGTGGAG
miRNA-34c-3p	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCGG-CAATTCAGTTGAGCCTGGCCGTG
	Sense	AATCACTAACCACACGG
	Antisense	AACTGGTGTCGTGGAG
U6	Stem-loop primer	CTCAACTGGTGTCGTGGAGTCG-GCAATTCAGTTGAGAAAAATAT
	Sense	CAAGGATGACACGCAAAT
	Antisense	TGGTGTCGTGGAGTCG

to U6 was described using the formula $2^{-\Delta Ct}$ where $\Delta Ct = $ (Ct miRNA - CtU6). Each sample was run in triplicate.

Vector constructs and lentivirus production

The miR-95 sequence was constructed as follows: (forward) hsa-miR-95-*Bam*H I : GATCCGTTCAACGGGTATT-TATTGAGCATTCAAGAGATGCTCAATATACCC-GTTGAACTTTTTTG; (reverse) hsa-miR-95-*Eco*R I : AATTCAAAAAAGTTCAACGGGTATATTGAG-CATCTCTTGAATGCTCAATAAATACCCGTT-GAACG. The sequence was amplified and cloned into the pGLV3-GFP vector (GenePhama) to generate pGLV3-GFP- miR-95. The negative control was pGLV3-shRNA-NC. Virus packaging was performed in HEK 293T cells after the co-transfection of 20 mg pGLV3-GFP-miR-95 vector with 15 mg of the packaging plasmid pHelper 1.0 Vector and 10 mg of the envelope plasmid pHelper 2.0 Vector using Lipofectamine 2000 (Invitrogen).Viruses were harvested 48 h after transfection, and viral titers were determined.

Oligonucleotide construction

After glargine treatment, the expression of miR-95 was increased most obviously in Sw1990 cells and Panc-1 cells, so we further investigated the functional roles of miR-95 in pancreatic cancer cells. miR-95 mimics, miR-95 inhibitor and negative control siRNA oligonucleotides were chemosynthesized (Shanghai GenePhama Co. Ltd). The oligonucleotides used in these studies were has-miR-95 mimics: 5'-UUCAACGGGUAU UUAUUGAG-CA-3' and 5'-CUCAAUAAAUACCCGUUGAAUU-3'. Mimics negative control: 5'-UUCUCCGAACGUGU-CACGUTT-3' and 5'-ACGUGACACGUUCGGAGAA TT-3', hsa-miR-95 inhibitor: 5'-UGCUCAAUAAAU-ACCCGUUGAA-3'. MicroRNA inhibitor negative control: 5'-CAGUACUUUUGUGUAGUACA A-3'.

Cell transfection

Cells were cultured to 80% to 90% confluence after be-

ing seeded into 6-well plates and were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For transient transfection, Sw1990 or Panc-1 cells in each well of a 6-well plate were transfected with 12.5 μL miRNA inhibitor or 7.5 μL miRNA mimic oligonucleotides. Transfection efficiency was evaluated by FAM in control vector or real-time PCR. For stably transfected cells, cells were transfected with lentivirus at 80%-90% confluency. Sw1990 cells (1×10^5) were infected with recombinant lentivirus in the presence of 5 μg/mL polybrene (GenePhama).

Cell proliferation assay

A total of 10^4 Sw1990 or Panc-1 cells per well were plated in 96-well plates before transfection and cultured for 24 h in normal conditions. They were then transfected with hsa-miR-95 mimics or hsa-miR-95 inhibitor along with paired negative controls. The cells were incubated at 37 ℃ for 48 h. Cell proliferation was assessed using Cell Counting Kit 8 (Dojindo, Tokyo, Japan) according to manufacturer's protocol.

Apoptosis assay

At 72 h after transfection, apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (Biovision, United States). Results were calculated by the percentage of apoptotic cells in all cells counted.

Matrigel invasion assay

At 48 h after transfection, the invasive ability of the cells was assayed using Transwells (8 mm pore size, Corning Costar Corp). The Transwells were put into the 24-well plates. First, 0.1 mL Matrigel (50 mg/mL, BD Biosciences) was added onto the plate surface and incubated for 2 h, and then the supernatant was removed. Freshly trypsinized and washed Panc-1 or Sw1990 cells were suspended in DMEM containing 1% FBS. Then 0.1 mL of the cell suspension (1×10^5 cells) was added to the upper chamber of each insert that was coated with Matrigel. Next, 0.6 mL of DMEM containing 10% FBS was added into the lower compartment, and the cells were allowed to invade for 24 h at 37 ℃ in a 5% CO_2 humidified incubator. After incubation, the cells were fixed with 95% absolute alcohol and followed by crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of × 200. Each experiment was performed in triplicate.

Cell migration

At 48 h after transfection, the ability of Panc-1 or Sw1990 cells to migrate was detected using Transwells (8 mm pore size, Corning Costar Corp). The Transwells were put into the 24-well plates. Freshly trypsinized and washed cells were suspended in DMEM containing 1% FBS. 5×10^4 cells/well were placed in the top chamber of each insert (BD Biosciences, NJ), with the non-coated membrane. 0.6 mL of DMEM containing 10% FBS was added into the lower chambers. After incubating for 24 h at 37 ℃ in

a 5% CO_2 humidified incubator, the cells were fixed with 95% absolute alcohol and stained with crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of $\times$ 200. Each experiment was performed in triplicate.

Mice xenografts

The stable cell line Sw1990 was harvested from tissue culture flasks after transfection with the pGLV3-GFP-miR-95 and control pGLV3-GFP vector using trypsin and washed three times with PBS. About 1×10^7 cells were implanted into the right flanks of female nu/nu mice (five in each group). Tumor volume (V) was measured with an external caliper every 4 d and it was calculated as V = 0.52 (length $\times$ width^2). After four weeks, all the animals were sacrificed and tumors were removed.

Statistical analysis

Data were expressed as the mean $\pm$ SD unless otherwise noted. The differences between groups were analyzed using a two-tailed Student's *t*-test when only two groups were present and the null hypothesis was rejected at the 0.05 level.

RESULTS

Glargine treatment alters miRNAs expression profiles

To study the responses of miRNAs to glargine, miRNA real time PCR array analysis of miRNA expression was conducted with total RNAs extracted from Sw1990 pancreatic cells treated with or without 100 IU/L glargine. Differential expression between glargine-treated and non-treated cells was defined using a cut off value of 2-fold change. We observed that 10 miRNAs were significantly up-regulated and 2 miRNAs were significantly down-regulated (2.48-fold on average, $P < 0.01$) in glargine treated Sw1990 cells when compared with non-treated cells. miR-95, miR-134 and miR-34c-3p are the top three miRNAs regulated by glargine (3.65-fold, 2.67-fold and 2.60-fold changes respectively, $P < 0.01$) in Sw1990 cells (Figure 1A).

Confirmatory studies with differentially expressed miRNAs by stem-loop real-time PCR

After treatment with increasing concentrations of glargine (50, 100, 150 IU/L) for 48 h, miR-95 was up-regulated by 1.18 fold ($P > 0.05$), 3.41 fold ($P < 0.01$) and 2.92 fold ($P < 0.01$) on average respectively in Sw1990 cells and 1.45 fold ($P < 0.01$), 3.41 fold ($P < 0.01$) and 2.92 fold ($P < 0.01$) on average respectively in Panc-1 cells, when compared with non-treated cells (0 IU/L). No obvious dose dependent responses were observed; miR-134 was up-regulated in a dose dependent manner (Sw1990: 1.69, 2.10 and 2.93 fold on average respectively, $P < 0.01$; Panc-1: 1.56, 1.99 and 2.88 fold on average respectively, $P < 0.01$) in both Sw1990 and Panc-1 cells; miR-34c-3p showed no significant changes (Sw1990: 1.03, 1.05 and 1.06 fold on average respectively, $P > 0.05$; Panc-1: 1.25, 1.25 and 1.19

fold on average respectively, $P > 0.05$) in both Sw1990 and Panc-1 cells (Figure 1B-1,2).

After treatment with 100 IU/L glargine for different periods (24, 48 and 72 h), miR-95 was up-regulated by 2.50 fold ($P < 0.01$), 3.10 fold ($P < 0.01$) and 2.99 fold ($P < 0.01$) on average, respectively, in Sw1990 cells and 2.31 fold ($P < 0.01$), 2.46 fold ($P < 0.01$) and 2.16 fold ($P < 0.01$) on average, respectively, in Panc-1 cells, when compared with the cells at 0 h; miR-134 was up-regulated by 2.22 fold ($P < 0.01$), 2.37 fold ($P < 0.01$), 2.17 fold ($P < 0.01$) on average, respectively, in Sw1990 cells and 2.10 fold ($P < 0.01$), 2.31 fold ($P < 0.01$) and 2.37 fold ($P < 0.01$) on average, respectively, in Panc-1 cells; miR-34c-3p showed no significant changes (Sw1990: 1.16, 1.14 and 1.13 fold on average respectively, $P > 0.05$; Panc-1: 1.17,1.24 and 1.13 fold on average, respectively, $P > 0.05$) in both Sw1990 and Panc-1 cells (Figure 1B-3,4).

miR-95 increases cell proliferation and inhibits cell apoptosis

We investigated the potential oncogenic role of miR-95 in Sw1990 and Panc-1 cells. First, we tested miR-95 expression using stem-loop real-time PCR. It increased or decreased after transfected with miR-95 mimics or anti-miR-95 inhibitor. We observed a significant increase in proliferation (Sw1990: 2.51 $\pm$ 0.13 *vs* 2.31 $\pm$ 0.19, $P < 0.05$; Panc-1: 2.44 $\pm$ 0.21 *vs* 2.26 $\pm$ 0.12, $P < 0.05$) after transfection of miR-95 mimics (Figure 2A-1). In contrast, anti-miR-95 inhibitor significantly decreased cell proliferation (Sw1990: 2.11 $\pm$ 0.07 *vs* 2.23 $\pm$ 0.13, $P < 0.05$; Panc-1: 2.09 $\pm$ 0.09 *vs* 2.31 $\pm$ 0.13, $P < 0.05$) (Figure 2A-2). These data indicate that cell proliferation can be significantly promoted by increase of miR-95 expression.

We further investigated the effect of miR-95 on apoptosis and found that apoptosis decreased dramatically (Sw1990: 22.05% $\pm$ 1.92% *vs* 40.32% $\pm$ 1.93%, $P < 0.05$; Panc-1: 20.17% $\pm$ 0.85% *vs* 45.60% $\pm$ 1.43%, $P < 0.05$) in Sw1990 and Panc-1 cells 72 h after transfection with miR-95 mimics (Figure 2B). It suggested that miR-95 may function as a strong apoptotic suppressor in human pancreatic cancer cells.

miR-95 regulates pancreatic cancer cell invasion and migration in vitro

In the cell invasion and migration assay, we observed that depletion of miR-95 significantly impaired the ability of Sw1990 cells to migrate and invade through the matrigel-coated membranes or the non-matrigel-coated membranes towards serum-containing medium (invasion: 49.40 $\pm$ 6.59 *vs* 65.80 $\pm$ 5.09; migration: 52.30 $\pm$ 10.87 *vs* 88.90 $\pm$ 10.46, $P < 0.01$), when compared with a paired negative control (Figure 3B); Increased expression of miR-95 significantly promoted the ability of Sw1990 cells to migrate and invade through matrigel-coated membranes or non-matrigel-coated membranes towards serum-containing medium (invasion: 67.90 $\pm$ 12.33 *vs* 47.30 $\pm$ 5.89; migration: 101.00 $\pm$ 6.00 *vs* 51.20 $\pm$ 8.34, $P < 0.01$), when compared with the paired negative control (Figure 3A). Similar results were found in Panc-1 cells (depletion of miR-95,

Figure 1 Effect of insulin glargine on miRNAs expression in Sw1990 cell line (A), insulin glargine up-regulated the expression of miR-95 and miR-134, miR-34c-3p showed no obvious changes in Sw1990 and Panc-1 cells (B). Ten miRNAs were significantly up-regulated and two miRNAs were down-regulated after 100 IU/L insulin glargine treatment for 48 h compared with non-treated cells. The cut off line for miRNA expression change was 2-fold. Data were presented as mean ± SD. Sw1990 and Panc-1 cells were incubated with increasing concentrations of insulin glargine (50-150 IU/L) for 48 h or treated with 100 IU/L insulin glargine for different periods (24-72 h). Then the expression of the three miRNAs were detected by stem-loop real-time reverse transcription-polymerase chain reaction array. miR-95 was significantly up-regulated, but no dose or time dependent changes were observed; miR-134 was up-regulated in a dose-dependent manner. miR-181a[*]: The miRNA of lower abundance.

invasion: 57.90 ± 10.55 *vs* 73.80 ± 11.95, migration: 66.40 ± 10.1 *vs* 99.50 ± 8.85, $P < 0.01$; forced expression of miR-95, invasion: 37.80 ± 8.93 *vs* 30.20 ± 5.14; migration: 91.80 ± 9.22 *vs* 81.50 ± 7.47, $P < 0.01$) (Figure 4A and B). These results indicated that miR-95 may be important in the progression of pancreatic cancer through increasing cell invasion and migration.

miR-95 promotes the growth of Sw1990 xenografts

Sw1990 cells transfected with pGLV3-GFP-miR-95 or negative control pGLV3-GFP were injected into the right flank of nude mice. Four weeks later, the tumor volumes of xenografts were 373.82 ± 23.67 mm[3] in the miR-95

transfected group and 219.69 ± 17.82 mm[3] in the negative control group. The weight of xenografts was 0.40 ± 0.08 g in the miR-95 transfected group and 0.23 ± 0.05 g in the negative control group. miR-95 significantly increased the growth of the Sw1990 xenografts (Figure 5, $P < 0.05$).

DISCUSSION

In the present study we demonstrated that glargine altered specific miRNA expression in human pancreatic cells at 50-150 IU/L, which is equivalent to 300-900 nmol and is much higher than the physiological concentration of

Figure 2 miR-95 promotes cell proliferation (A) and enhancement of miR-95 inhibit apoptosis (B). A total of 10^4 Sw1990 or Panc-1 cells per well were plated in 96-well plates for 24 h and then were transfected with hsa-miR-95 mimics or hsa-miR-95 inhibitor along with paired negative controls. The cells were incubated at 37 ℃ for 48 h and then cell proliferation was assessed using Cell Counting Kit 8. Data were shown as mean ± SD. $^aP < 0.05$ *vs* pairing negative control.

insulin (0.1-1 nmol). Our miRNA real time PCR array showed that high dose glargine (100 IU/L) up-regulated the expression of 10 miRNAs and down-regulated 2 miRNAs in Sw1990 pancreatic cancer cells. The most obvious change was the apparent increase of miR-95. Stem-loop real-time PCR confirmed the aberrant changes of miR-95 after treatment of high dose glargine in Sw1990 and Panc-1 pancreatic cancer cells. Then miR-95 showed significant anti-apoptotic and growth-promoting effects *in vitro* and *in vivo*. Ectopic expression and siRNA knock-down of miR-95 confirmed its invasion-promoting activity *in vitro*. Therefore, these results highlighted the miR-95-related changes as important effects of glargine.

Recent studies linked the use of glargine with increased risk of cancer. Hemkens *et al*[4] published a registry study that demonstrated a significantly increased risk of cancer diagnosis associated with high dosages of glargine. However, the Scottish study found a non-significant increased risk for specifically breast cancers[23]. The UK study found no link between glargine and cancer[3]. In addition, although glargine has been shown to increase resistance to apoptosis in several tumor cell lines, administration of

glargine didn't alter proliferation of Colo-357 pancreatic carcinoma cells *in vitro*[12]. Therefore, all epidemiological and laboratory evidence remains inconclusive and new indicators are needed to determine the role of glargine in carcinogenesis.

miRNAs have been recognized as promising diagnostic and prognostic markers for cancer diagnosis or treatment. For example , miR-34a family members were found to be directly regulated by TP53 and act as tumor suppressors[24]; miR-217 inhibited pancreatic cancer cell growth through targeting KRAS[25]; miR-10b promoted pancreatic cancer invasiveness and correlates with a poor prognosis[26]; The miRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) were found to inhibit tumor invasion and metastasis by regulating EMT[27]; miR-126 can inhibit cell adhesion, migration and invasion through the suppression of CRK[28]. Our study confirmed, for the first time, that miR-95 and miR-134 were primary glargine-responsive miRNAs.

miR-95 has been shown to be involved in carcinogenesis. A highly characterized example is colorectal carcinoma, in which miR-95 can promote cell proliferation by

Figure 3 Effect of miR-95 on tumor cell migration, invasion of Sw1990 cells. A: Invasion and migration assay. Representative fields of invasion (down) or migration (up) cells on the membrane (left, magnification of × 200). Average invasion or migration cell number per field (right). The invasion or migration cell number of Sw1990 transfected with miR-95-mimics drastically increased than that transfected with pairing negative control; B: The invasion or migration cell number of Sw1990 cells transfected with miR-95-inhibitor dramatically decreased than that transfected with pairing negative control. [b]$P < 0.01$ *vs* Sw1990-control, n = 10.

regulating sorting Nexin 1[29]. In pancreatic cancer, miR-95 is significantly upregulated in most tissues and cell lines[30]. In HeLa cells, inhibition of miR-95 caused a decrease in cell growth[31]. *miR-134* gene is located at 14q32, and is involved in several physiological and pathological pro-

cesses. For example, miR-134 plays an important role in translation-dependent guidance of nerve growth cones[32]; miR-134 is regarded as a potential plasma biomarker for the diagnosis of acute pulmonary embolism[33]; plasma miR-134 in bipolar disorder serves as a potential periph-

Figure 4 Effect of miR-95 on tumor cell migration, invasion of Panc-1 cells. A: Invasion and migration assay. Representative fields of invasion (down) or migration (up) cells on the membrane (left, magnification of × 200). Average invasion or migration cell number per field (right). The invasion or migration cell number of Panc-1 transfected with miR-95-mimics drastically increased compared with that when transfected with the pairing negative control. B: The invasion or migration cell number of Panc-1 cells transfected with miR-26a-inhibitor dramatically decreased compared with that when transfected with the pairing negative control. [b]*P* < 0.01 *vs* Panc-1-control, *n* = 10.

eral marker that can respond to acute manic episodes and is associated with effective mood stabilizer treatment[34]. Interestingly, recent studies indicated that miR-134 may also be involved in carcinogenesis. p53/p63/p73, the tu-

mor suppressors, were believed to be regulators of the miR-134 processing complex[35]. Our study showed that high dose glargine can significantly upregulate the expression of miR-95 (no time or dose dependent manner) and

A

B

Figure 5 miR-95 promotes the growth of Sw1990 xenografts. A: miR-95 strikingly increased the growth of Sw1990 cells xenografted in nude mice; B: At the end of the experiment, all animals were sacrificed and the tumors were removed. The tumors were much heavier in miR-95 group than in the vector group. Data were shown as mean ± SD ([a]$P < 0.05$ *vs* vector).

miR-134 (dose dependent manner) *in vitro*, and for the first time investigated the role of miR-95 in pancreatic cancer.

In conclusion, our study demonstrated that alterations of specific miRNAs and miRNA-related changes were important effects of glargine, suggesting an important and novel new mechanism by which glargine mediates its potent effects on cell growth and apoptosis.

COMMENTS

Background
Glargine is widely used in the treatment of type 1 and type 2 diabetes mellitus. Recently, this insulin analogue has been suspected to be associated with an increased risk of cancer, including pancreatic cancer, but available evidence remains inconclusive.

Research frontiers
Anti-diabetic therapies have been shown to affect the risk of pancreatic cancer. Metformin use was associated with reduced risk, and insulin or insulin analogue use was suspected to be associated with increased risk of pancreatic cancer in diabetic patients. However, data regarding the effect of glargine, one of the long-acting insulin analogues, on pancreatic cancer are inconsistent. Several researchers

believed glargine to be a motigen, not a carcinogen. New biomarkers are needed to determine the role of glargine in the carcinogenesis of pancreatic cancer.

Innovations and breakthroughs
Recent reports have highlighted that miRNAs play important roles in biological processes that affect tumor progression and are promising biomarkers for cancer diagnosis or treatment. This is the first study to show the effects of high dose glargine on miRNA expression in pancreatic cancer cells. miR-95 was proved to be affected by high dose glargine and to be involoved in the carcinogenesis of pancreatic cancer.

Applications
By understanding the effects of glargine on pancreatic cancer cells, this study may help to clarify the role of glargine in the progress of pancreatic cancer.

Terminology
Glargine is a widely used insulin analog in which a 24 h action profile is achieved. MicroRNAs (miRNAs) are endogenous, non-coding small RNAs, 19-25 nucleotides in length. It has been demonstrated that miRNAs play important roles in biological processes that affect tumor progression including migration, invasion and metastasis.

Peer review
The authors examined the effects of high dose glargine on pancreatic cancer cells *in vitro*; miR-95 is up-regulated significantly by glargine. miR-95 can significantly increase pancreatic cancer cell proliferation, invasion and migration and inhibit cell apoptosis. Moreover, miR-95 is proved to inhibit pancreatic

cancer growth *in vivo*. The results are interesting and may represent the role of glargine in pancreatic carcinogenesis.

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ORIGINAL ARTICLE

Suppression of colorectal cancer metastasis by nigericin through inhibition of epithelial-mesenchymal transition

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Abstract

AIM: To evaluate the effect of nigericin on colorectal cancer and to explore its possible mechanism.

METHODS: The human colorectal cancer (CRC) cell lines HT29 and SW480 were treated with nigericin or oxaliplatin under the conditions specified. Cell viability assay and invasion and metastasis assay were performed to evaluate the effect of nigericin on CRC cells. Sphere-forming assay and soft agar colony-forming assay were implemented to assess the action of nigericin on the cancer stem cell properties of CRC cells undergone epithelial-mesenchymal transition (EMT).

RESULTS: Compared with oxaliplatin, nigericin showed more toxicity for the HT29 cell line (IC50, 12.92 ± 0.25 μmol *vs* 37.68 ± 0.34 μmol). A similar result was also obtained with the SW116 cell line (IC50, 15.86 ± 0.18 μmol *vs* 41.02 ± 0.23 μmol). A Boyden chamber assay indicated that a significant decrease in the number of HT29 cells migrating through polyvinylidene fluoride membrane was observed in the nigericin-treated group, relative to the vehicle-treated group [11 ± 2 cells per high-power field (HPF) *vs* 19.33 ± 1.52 cells per HPF, $P < 0.05$]. Compared to the control group, the numbers of HT29 cells invading through the Matrigel-coated membrane also decreased in the nigericin-treated group (6.66 ± 1.52 cells per HPF *vs* 14.66 ± 1.52 cells per HPF, $P < 0.05$). Nigericin also reduced the proportion of CD133$^+$ cells from 83.57% to 63.93%, relative to the control group ($P < 0.05$). Nigericin decreased the number of spheres relative to the control group (0.14 ± 0.01 *vs* 0.35 ± 0.01, $P < 0.05$), while oxaliplatin increased the number of spheres relative to the control group (0.75 ± 0.02 *vs* 0.35 ± 0.01; $P < 0.05$). Nigericin also showed a decreased ability to form colonies under anchorage-independent conditions in a standard soft agar assay after 14 d in culture, relative to the control group (1.66 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$), whereas the colony numbers were higher in the oxaliplatin group relative to the vehicle-treated controls (14.33 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$). We further detected the expression of E-cadherin and vimentin in cells treated with nigericin and oxaliplatin. The results showed that HT29 cells treated with nigericin induced an increase in E-cadherin expression and a decrease in the vimentin expression relative to vehicle controls. In contrast, oxaliplatin downregulated the expression of E-cadherin and upregulated the expression of vimentin in HT29 cells relative to vehicle controls.

CONCLUSION: This study demonstrated that nigericin could partly reverse the EMT process during cell invasion and metastasis.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second most commonly diagnosed cancer in women[1], with a 5-year survival rate < 10% for patients with metastatic disease[2]. Despite the use of active targeted drugs for treatment of metastatic CRC, the cure rate has remained low in the past decade. Activating invasion and metastasis is the hallmark of cancer[3,4], during which malignant cells spread from the primary tumor to distant organs.

The pathogenesis of metastasis involves a series of steps, often termed the invasion and metastasis cascade, which includes the following: local invasion of the host stroma by tumor cells; detachment and embolization of tumor cell aggregates; extravasation of the tumor embolus; survival of tumor cells that are transported through the circulation and stop in the capillary bed; extravasation of the tumor embolus; proliferation of the tumor cells within the organ parenchyma, resulting in a metastatic focus; and reinitiation of these processes for the development of metastases. The first and decisive step of this process is the local invasion through the epithelial basement membrane, because it requires alteration in cell-cell and cell-matrix interactions, reconstruction of the extracellular matrix, remodeling of the cytoskeleton, and enhancement of cell modulation. Great progress has been made on the capacity for invasion and metastasis over the past decade with powerful novel research tools and refined experimental models becoming available. On the other hand, many critical regulatory genes have been identified.

Epithelial-mesenchymal transition (EMT), a transdifferentiation characterized by decreased epithelial markers such as E-cadherin and increased mesenchymal markers such as fibronectin, has become prominently implicated as a means by which transformed epithelial tumor cells acquire the ability to invade, resist apoptosis, and propagate[5-9]. More importantly, EMT has been shown to result in cancer cells with stem-cell-like characteristics that have a propensity to invade surrounding tissue and display resistance to chemotherapeutic interventions[6,10,11]. Nigericin is a potassium ionophore, which has been reported to be toxic to breast stem cells passing through EMT[12].

Lu *et al*[13] have reported that nigericin, like salinomycin, selectively inhibits Wnt1-mediated signaling in HEK293 cells at nanomolar concentrations.

In this study, we aimed to ascertain the specific activities of nigericin on human CRC cell lines. We selected CD133 as the marker of stem cells of CRC.

MATERIALS AND METHODS

Tumor cell preparation and cell culture
Human CRC cell lines, HT29 and SW116 were used. HT29 cells were cultured in McCoy's 5A medium (Gibco, United States) with 10% fetal bovine serum (FBS). SW116 cells were cultured in RPMI 1640 medium with 10% FBS. The cells were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Drugs and antibodies
Oxaliplatin and nigericin were both purchased from Sigma-Aldrich (St. Louis, MO, United States). Antibodies used for immunofluorescence staining and Western blotting were as follows: mouse anti-E-cadherin (Abcam Inc., Cambridge, MA, United States), mouse anti-vimentin (Abcam), mouse anti-CD133 (Abcam; used for Western blotting and immunocytochemistry), allophycocyanin-conjugated CD133 antibody (Miltenyi Biotec, Auburn, CA, United States) used for fluorescence-activated cell sorting (FACS), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam).

Analysis of cell viability
For assessment of cell viability in HT29 and SW116 cell lines under different treatments, cells growing at the exponential stage were plated in triplicate, in 96-well plates at a density of 2000 cells/well in a final volume of 100 μL. After incubation for 24 h, oxaliplatin, nigericin and dimethylsulfoxide (DMSO) control were added to each well of the plates. Cell viability was detected after 24 h using Cell Counting Kit 8 (DOJINDO, Japan). Absorbance for each well was read at 570 nm using a microplate reader. Growth inhibition was calculated as a percentage of the untreated controls. Experiments were done three or more times, often in triplicate, for each cell line, and IC_{50} was determined using the four-parameter logistic model.

Cell migration and invasion
Analysis of cell migration was performed using Boyden chambers according to the manufacturer's protocol (Becton Dickinson Labware, Bedford, MA, United States). For cell invasion study, the inserts of the chamber were prepared by coating the upper surfaces with Matrigel (BD Matrigel Matrix, Phenol Red-free). HT29 cells (3×10^3) treated with DMSO control, oxaliplatin and nigericin in McCoy's 5A medium without FBS were plated to the upper chamber. McCoy's 5A with 20% FBS as chemoattractants were plated in the lower chamber of the 24-well pates. After 24 h, nonmigrating or noninvading cells were removed mechanically from the upper chamber using a cotton swab. Cells that migrated or invaded to the lower

surface of the Transwell membrane were fixed in methanol for 30 min at 37 ℃ and stained with 0.05% crystal violet for 1 h. Cells were quantified by counting the number of stained nuclei in five individual fields by fluorescence microscopy, in triplicate.

Immunoblotting
Cell lysates were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to hydrophobic polyvinylidene fluoride (PVDF) membrane. After blocking in 5% skimmed milk solution for 2 h, the membranes were incubated with the primary antibodies diluted with anti-CD133, anti-E-cadherin, and anti-vimentin. Primary antibodies were detected with mouse secondary antibodies directed against human IgG and visualized with Odyssey Infrared Imaging System.

Real-time polymerase chain reaction
mRNA expression was determined by real-time polymerase chain reaction. RNA was extracted by using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reverse transcription was performed using Superscript Ⅱ (Invitrogen) according to the manufacturer's instructions. TaqMan reactions were done utilizing an ABI 7500 real-time quantitative polymerase chain reaction (PCR) system. For data analysis, raw counts were normalized to housekeeping gene average for the same time point and condition (ΔCt). The following primers were used in this study: CD133 forward CATCCACAGATGCTCCTAAGGC and reverse GCTTTATGGGGAGTCTTGGTC; E-cadherin forward CGAGAGCTACACGTTCACGG and reverse GTGTCG AGGGAAAAATAGGCTG; vimentin forward CTCCTCCCCCTGTCACATAC and reverse TGATTG-GCATCAGGACCGTTG. GAPDH was used as an internal control. Analysis was performed with the $\Delta\Delta$Ct method.

Flow cytometric analysis
HT29 and SW116 cells, after different treatments, were washed with PBS. Single cell suspensions were incubated with allophycocyanin (APC)-conjugated CD133 antibody (Miltenyi Biotec) for 30 min at 4 ℃. Mouse IgG1-APC was selected as an isotype control body. 7-Aminoactinomycin was used to eliminate the dead cells. The labeled cells were detected by the BD FACSVantage Systems (Becton Dickinson) according to the manufacturer's protocols. Gating was implemented on the basis of negative control staining profiles.

Colonosphere assay
McCoy's 5A with B27 supplement (Invitrogen), 20 μg/mL epidermal growth factor (Invitrogen), 20 μg/mL fibroblast growth factor (Invitrogen), and penicillin-streptomycin served as the stem cell medium (SCM) for this experiment. HT29 cells, after the indicated treatments, were plated at a concentration of 200 cells/100 μL SCM in each of the 20 wells of a 96-well ultralow-attachment plate (Corning Life Sciences, CA, United States). Cells

were supplemented with 100 μL SCM after 7 d of incubation and analyzed on day 14, and MTT solution (40 μL) was added to each well, and a colorimetric assessment was done. The average absorbance measurement for each group was used as an index of sphere number.

Soft agar colony-forming unit assays
We mixed 1.2% agar with 2 × McCoy's 5A medium at a ratio of 1:1 to make a 0.6% agar growth medium solution. We pipetted 2 mL of the 0.6% growth medium mixture into each well of the six-well cell culture cluster (Corning Life Sciences). We avoided bubble formation and spread the mixture evenly by slowly rotating the plate. We allowed the 0.6% agar growth medium layer to harden for 30-40 min at room temperature in a sterile laminar flow hood. We determined the concentration of HT29 cells treated with DMSO control, oxaliplatin and salinomycin, and adjust the suspension to 5×10^3 cells/mL in 0.3% agar diluted with PBS. We transferred 2 mL of the cell suspension to the 0.6% agar growth medium plate and cultured at 37 ℃ in the presence of 5% CO_2 for 14-21 d. We counted the number of colonies using a microscope.

Immunocytochemistry
Cells were directly sorted onto a glass slide, fixed with 4% paraformaldehyde, and stained with anti-E-cadherin, anti-CD133 and anti-vimentin monoclonal antibodies. Nuclei were identified by staining with 4', 6-diamidino-2-phenylindole. Subcellular localizations were determined by using confocal microscopy. The fluorescence intensity of each region was analyzed by different people on three occasions.

Statistical analysis
All values were shown as mean ± SD. Statistical significance was calculated by *t* test unless otherwise stated (SPSS 17.0), considering $P < 0.05$ as statistically significant.

RESULTS
Nigericin inhibits tumor growth and invasion
We examined the *in vitro* effect of nigericin on tumor growth and metastasis. Compared with oxaliplatin, nigericin exhibited more toxicity for the HT29 cell line (IC$_{50}$, 12.92 ± 0.25 μmol *vs* 37.68 ± 0.34 μmol) (Figure 1A). We also obtained similar results with the SW116 cell line (IC$_{50}$, 15.86 ± 0.18 μmol *vs* 41.02 ± 0.23 μmol) (Figure 1B). We then checked whether nigericin had functional influence on the migratory and invasive capacity of CRC cells. After incubation for 24 h, nigericin induced a conspicuous reduction in the number of cells migrating through the PVDF membrane relative to the vehicle-treated controls [11 ± 2 cells per high-power field (HPF) *vs* 19.33 ± 1.52 cells per HPF, $P < 0.05$] (Figure 1C and D). It was surprising that oxaliplatin promoted the migration of CRC cells through PVDF membrane compared with the vehicle-treated controls (38 ± 2 cells per HPF *vs* 19.33 ± 1.52 cells per HPF, $P < 0.05$) (Figure 1C and D). Compared to

Figure 1 Nigericin inhibits tumor growth and metastasis. A: Dose-response curves of HT29 cells treated with nigericin and oxaliplatin. Bars denote SD (n = 5); B: Dose-response curves of SW116 cells treated with nigericin and oxaliplatin. Bars denote SD (n = 5); C: Boyden chamber assays were done to compare the migratory capacities of HT29 cells treated with oxaliplatin and nigericin. Bars denote SD (n = 5); D: Also shown are phase-contrast images of HT29 cells migrating through the collagen membrane; E: Numbers of cells invading through the Matrigel-coated hydrophobic polyvinylidene fluoride membrane after treatment with the indicated compounds. Bars denote SD (n = 5); F: Images of HT29 cells migrating through the collagen membrane are also shown. DMSO: Dimethylsulfoxide; HPF: High-power field.

the control group, the numbers of HT29 cells invading through the Matrigel-coated membrane also decreased in the nigericin-treated group (6.66 ± 1.52 cells per HPF *vs* 14.66 ± 1.52 cells per HPF, $P < 0.05$) (Figure 1E and F). Correspondingly, oxaliplatin treatment increased the number of HT29 cells invading through the Matrigel-coated membrane (28.66 ± 2.08 cells per HPF *vs* 14.66 ± 1.52 cells per HPF, $P < 0.05$) (Figure 1E and F).

Effects of nigericin and oxaliplatin on expression of cancer stem cell marker

In order to complete subsequent experiments logically, we treated the HT29 cells with nigericin, oxaliplatin, and DMSO vehicle control for 3 d, and then replaced the culture medium containing drugs with normal McCoy's 5A medium with 10% FBS for another 3 d incubation.

The stem cell marker prominin-1 (CD133), a pentas-

pan membrane protein, may not be the only marker, but it remains the most widely reported marker of cancer stem cells (CSCs) of CRC validated by different groups[14-18].

We further assessed the expression of CD133 on HT29 cells after treatment with nigericin and oxaliplatin using flow cytometry. The results demonstrated that nigericin reduced the positive rate of CD133 from 83.57% to 63.93%, relative to the control group ($P < 0.05$) (Figure 2A and B). In contrast, oxaliplatin treatment increased the expression of CD133 from 79.18% to 97.22%. In order to verify this result, we selected the SW116 cell line to repeat the experiment. Similarly, nigericin decreased the proportion of CD133+ cells from 4.55% to 0.31%; on the contrary, the expression rate of CD133 increased from 4.55% to 36.89% (Figure 2C and D). The data from real-time PCR, Western blotting, and immunocytochemistry indicated analogous results (Figure 2E-G).

Figure 2 Effects of nigericin and oxaliplatin on expression of cancer stem cell marker. A: Percentages of CD133^{+} cells after treatment of HT29 cells with nigericin and oxaliplatin. Bars denote SD; B: CD133 fluorescence-activated cell sorting (FACS) profiles are indicated for HT29 cell treatment with nigericin and oxaliplatin; C: Percentages of CD133^{+} cells after treatment of SW116 cells with nigericin and oxaliplatin. Bars denote SD; D: CD133 expression in SW116 cells after treatment was assayed with FACS; E: Immunofluorescence staining analysis of CD133 expression in HT29 cells after treatment; F: CD133 protein expression in HT29 cells after treatment was assayed with immunoblotting; G: Real-time polymerase chain reaction analysis of CD133 mRNA expression in HT29 cells. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DMSO: Dimethylsulfoxid.

Effect of nigericin and oxaliplatin on sphere- or colony-forming ability of CRC cells

To evaluate the ability to form colonies or spheres of HT29 cells treated with nigericin and oxaliplatin in the absence of serum and without attachment to culture plates[19]. We performed the sphere-forming assay and soft agar forming assay under serum-free conditions.

Differences between the nigericin and oxaliplatin groups

were quantitated by plating a limited number of cells in each well of a low-attachment 96-well plate and evaluating the ability of HT29 cells to form colonospheres. Nigericin decreased the number of spheres relative to the control group (0.14 ± 0.01 *vs* 0.35 ± 0.01, $P < 0.05$), while oxaliplatin increased the number of spheres relative to the control group (0.75 ± 0.02 *vs* 0.35 ± 0.01, $P < 0.05$) (Figure 3A). Nigericin also showed a decreased ability to

Figure 3 Effects of nigericin and oxaliplatin on sphere- or colony-forming ability of cancer stem cells. A: Mean OD of sphere-forming HT29 cells after different treatments was assayed by methyl thiazolyl tetrazolium assay. Bars denote SD ($n = 5$); B: Numbers of colonies formed by HT29 cells after treatment in independent experiments. Bars denote SD ($n = 5$); C: Phase-contrast images of colonies formed in soft agar assays after treatment. DMSO: Dimethylsulfoxide; HPF: High-power field.

form colonies under anchorage-independent conditions in a standard soft agar assay after 14 d in culture, relative to the control group (1.66 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$), whereas the colony numbers were higher in oxaliplatin group relative to the vehicle-treated controls (14.33 ± 0.57 *vs* 7 ± 1.15, $P < 0.05$) (Figure 3B and C).

Up-regulation of E-cadherin and downregulation of vimentin in CRC cells after nigericin treatment

E-cadherin, encoded by the *CDH1* gene, has dual functions in epithelial cells: as a cell-cell adhesion molecule and as a negative regulator of the canonical WNT signaling cascade; in particular, of its central mediator β-catenin. E-cadherin downregulation in mammalian cell systems is sufficient to trigger EMT[20]. Gupta *et al*[12] have reported that nigericin preferentially kills cells that have undergone EMT. In colorectal carcinomas, the embryonic EMT is activated during tumor invasion in disseminating cancer cells[21]. Characteristic of these cells is a loss of E-cadherin expression.

We detected the expression of epithelial marker (E-cadherin) and mesenchymal marker (vimentin) of cells treated with nigericin and oxaliplatin to ascertain the effects of diverse compounds on EMT.

As shown in Figure 4A and B, nigericin induced an increase in expression of E-cadherin and a decrease in expression of vimentin relative to vehicle controls. In contrast, the expression of E-cadherin in the cells treated with oxaliplatin was downregulated in contrast to vehicle

controls; correspondingly, oxaliplatin treatment upregulated the expression level of vimentin. The data from real-time PCR showed similar results to immunocytochemistry and Western blotting (Figure 4C).

DISCUSSION

Significant progress has been made in understanding the molecular pathogenesis, diagnosis (hereditary and sporadic), and treatment of CRC. Despite the use of active targeted drugs for treatment of metastatic CRC in the past decade, and improvement of overall survival to nearly 2 years for nonresectable disease, the cure rate remains low[22].

5-Fluorouracil and oxaliplatin formed the mainstay of chemotherapeutic regimens for metastatic CRC. Oxaliplatin covalently binds to DNA, forming platinum-DNA adducts that cause prolonged G2 arrest and inhibition of growth, which lead to apoptotic cell death[23].

There is a large body of evidence that tumor cells that are resistant to chemotherapy represent a subpopulation of cells from the primary tumor, which is molecularly and phenotypically distinct. These cells are referred to by several names, including tumor-initiating cells, tumor-promoting cells, or more commonly, CSCs[16]. EMT is a highly conserved cellular process during embryonic development and a pathogenic feature in tumorigenesis[10,24].

During the process of EMT, epithelial cells lose the expression of E-cadherin and other components of

Figure 4 Upregulation of E-cadherin and downregulation of vimentin in cancer stem cells after nigericin treatment. A: Immunofluorescence staining analysis of treated HT29 cells using epithelial E-cadherin and vimentin staining; B: E-cadherin and vimentin expression in HT29 cells after treatment, assayed by Western blotting; C: Real-time polymerase chain reaction analysis of E-cadherin and vimentin mRNA expression in HT29 cells after treatment with dimethylsulfoxide (DMSO) vehicle, oxaliplatin and nigericin.

epithelial cell junctions, adopt a mesenchymal cell phenotype, and acquire motility and invasive ability[25,26]. Furthermore, Mani *et al*[10] have induced EMT in nontumorigenic, immortalized human mammary epithelial cells (HMLEs) by ectopic expression of either the Twist or Snail transcription factors; these cells formed > 30-fold more mammospheres than did HMLEs infected with the corresponding control vector. They have concluded that the cells generated by EMT acquired yet another attribute of mammary stem cells. EMT, which enables cancer cell dissemination, also imparts a self-renewal capability to disseminating cancer cells.

There is no consensus as to the exact criteria that define a CSC, because markers might vary according to the tumor type. In our study, we suggested CD133 as a marker of tumor-initiating cells of CRC[14-18].

We evaluated the effect of nigericin and oxaliplatin on CRC cell lines, including invasion and metastasis, and growth on colon cancer spheres, or colonospheres. From the results of cell viability and flow cytometry assays, we could see that nigericin specifically targets CD133+ cell

subpopulations within CRC cell lines. Moreover, nigericin induced inhibition of invasion and metastasis in HT29 cells. These effects may have been due to the fact that nigericin upregulated the expression of E-cadherin, while E-cadherin played an important role in cancer progression and EMT induction[27,28].

In a variety of human cancers, E-cadherin loss was closely related to poor prognosis, tumor progression, and metastasis[29,30]. Therefore, E-cadherin also could be a sign of drug efficiency of nigericin therapy in the future. Through analysis of the expression level of E-cadherin and vimentin, we may conclude that nigericin partly reverses EMT to affect the ability of CRC cells to invade and metastasize.

We further evaluated the effects of nigericin treatment on the characteristics of CSC phenotype. The nigericin treatment group had a decreased number of spheres or colonies relative to the vehicle control group. Our data led us to hypothesize further that nigericin treatment suppresses EMT-generating cells with the properties of stem cells. This hypothesis needs further studies using animal

experiments and preclinical and clinical trials. Nigericin may prove to be the therapeutic strategy that is effective in patients with metastatic disease.

However, the molecular mechanisms involved in the effect of nigericin are poorly understood. Lu *et al*[13] have reported that nigericin, as a potassium ionophore, selectively inhibits Wnt1-mediated signaling in HEK293 cells.

The polyether ionophores like nigericin interfere with transmembrane potassium potential and promote mitochondrial and cell potassium efflux. We hypothesize that nigericin treatment antagonizes the Wnt signaling cascade, while Wnt signaling plays a crucial role in embryonic development and cancer[31-35]. Besides, certain other CSC markers and signaling pathways, including EZH2 and Hedgehog pathways may also play some important roles in the mechanism of nigericin treatment, and thus need further studies[36]. Further studies will focus on the relation between nigericin-induced EMT and Wnt signaling.

We showed for the first time that nigericin not only partly reversed the EMT process during cell invasion and metastasis, but also suppressed some of the CSC phenotypes generated by EMT. EMT plays a pivotal role in tumor invasion and metastasis; therefore, nigericin treatment may be of benefit in the future.

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COMMENTS

Background
Despite therapeutic innovations, metastatic colorectal cancer (CRC) often has a poor prognosis and high mortality.

Research frontiers
Epithelial-mesenchymal transition (EMT) provides a new basis for understanding the progression of carcinoma towards dedifferentiated and more malignant states. The EMT program, which involves dissolution of adherens and tight junctions and a loss of cell polarity, dissociates the cells with epithelial cell sheets into individual cells that exhibit multiple mesenchymal attributes, including heightened invasiveness. EMT also generates cells with properties of stem cells. Nigericin has been reported recently to act as a selective breast cancer stem cell inhibitor. However, the effect of nigericin on CRC is unknown. In this study, the authors evaluated the anticancer effect of nigericin and its possible mechanisms.

Innovations and breakthroughs
Recent reports have highlighted the important role of EMT during the invasion-metastasis cascade. In particular, EMT can be seen at the edges of colon carcinomas that are invading adjacent tissues. This is the first study to report that nigericin could suppress CRC metastasis. Furthermore, our studies indicated the possible mechanisms of action of nigericin.

Applications
Through understanding the effect of nigericin on CRC cells, this study may indicate a future promising therapeutic strategy in the treatment of patients with metastatic colorectal carcinoma.

Terminology
E-cadherin is a hallmark of epithelial cell protein expression; vimentin is an intermediate filament component of the mesenchymal cell cytoskeleton. CD133 protein, a pentaspan cell surface receptor, is a putative CRC stem cell marker.

Peer review
The study investigated the antitumor activity of nigericin on CRC stem cells. The authors found that nigericin selectively targeted cancer stem cells, and inhibited EMT. It is well designed and well presented.

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BRIEF ARTICLE

Association between autoimmune pancreatitis and systemic autoimmune diseases

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Abstract

AIM: To investigate the association between autoimmune pancreatitis (AIP) and systemic autoimmune diseases (SAIDs) by measurement of serum immunoglobulin G4 (IgG4).

METHODS: The serum level of IgG4 was measured in 61 patients with SAIDs of different types who had not yet participated in glucocorticosteroid treatment. Patients with an elevated IgG4 level were examined by abdominal ultrasonography (US) and, in some cases, by computer tomography (CT).

RESULTS: Elevated serum IgG4 levels (919 ± 996 mg/L) were detected in 17 (28%) of the 61 SAID patients. 10 patients had Sjögren's syndrome (SS) (IgG4: 590 ± 232 mg/L), 2 of them in association with Hashimoto's thyroiditis, and 7 patients (IgG4: 1388 ± 985.5 mg/L) had systemic lupus erythematosus (SLE). The IgG4 level in the SLE patients and that in patients with SS were not significantly different from that in AIP patients (783 ± 522 mg/L). Abdominal US and CT did not reveal any characteristic features of AIP among the SAID patients with an elevated IgG4 level.

CONCLUSION: The serum IgG4 level may be elevated in SAIDs without the presence of AIP. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is an increasingly recognized type of chronic pancreatitis that is clearly distinct from other types of chronic pancreatitis. It is characterized by its morphology, immunologic features, pathology and glucocorticosteroid responsiveness[1-4].

Immunological examinations in AIP patients have demonstrated high incidences of hypergammaglobulinemia (43%), increased serum levels of immunoglobulin G (IgG) (62%-80%) and IgG4 (68%-92%), and the presence of antinuclear antibodies (40%-64%) and rheumatoid factor (25%). Among all the serological diagnostic features, an elevated serum level of IgG4 has the highest individual diagnostic value; however, it is not disease specific. Furthermore, an elevated serum IgG4 level correlates with the activity of AIP[5,6]. Kamisawa *et al*[7] reported an association between serum IgG4 level and extrapancreatic lesions in patients with AIP. AIP patients with a serum IgG4 level ≥ 2200 mg/L frequently exhibit extrapancreatic lesions.

The immunologic and histologic features of AIP and the glucocorticosteroid responsiveness suggest an autoimmune mechanism for the development of the disease[8]. AIP is accompanied by other autoimmune diseases (sclerosing cholangitis, sclerosing sialadenitis, retroperitoneal fibrosis, enlarged celiac and hilar lymph nodes, chronic thyroiditis and interstitial nephritis, *etc*.) in 50%-63% of cases, suggesting that AIP may be a systemic disorder[1-4]. The occurrence of autoimmune diseases in association with AIP is well documented[9,10], but the incidence of such associations has not been reported.

The aim of the present study was to assess the presence of AIP in different systemic autoimmune diseases (SAIDs) through measurement of the serum IgG4 level and examination of the morphology of the pancreas.

MATERIALS AND METHODS

Patients and diagnosis of diseases
Serum samples were obtained from 61 patients with different SAIDs who had been admitted to our Department of Rheumatology and had not participated in glucocorticosteroid treatment during the past 2 years. One male and 60 females (mean age 54.5 years, range 29-82 years) were recruited.

Autoimmune diseases were diagnosed according to standard diagnostic criteria[11-14]. The diagnosis of AIP was based on the HISORt criteria[15]. The most frequent diagnosis was Sjögren's syndrome (SS), but systemic lupus erythematosus (SLE), Hashimoto thyroiditis, Raynaud's syndrome, polymyositis and systemic sclerosis also occurred (Table 1).

Serum samples were additionally obtained from 7 age- and sex-matched healthy subjects, and 6 patients with AIP. In one AIP patient, the AIP was accompanied by rheumatoid arthritis and ankylosing spondylitis.

All participants provided their written informed consent. The study protocol was approved by the ethics committee at the University of Szeged and was carried out in full accordance with the most recent revisions of the Helsinki Declaration.

IgG4 assay
After collection, serum samples were stored at -70 ℃ until analyzed. The IgG4 subclass was determined by the ra-

Table 1　Distribution of gender and age in groups of patients

	No. of patients	Male/ female	Age mean (range)
Sjögren's syndrome	35	1/34	56.7 (29-82)
Systemic lupus erythematosus	22	0/22	50.2 (31-68)
Systemic sclerosis	4	0/4	59.5 (45-80)
Normal subjects	7	4/3	68 (56-80)
Autoimmune pancreatitis	6	3/3	53.7 (27-75)

Figure 1　Serum immunoglobulin G4 levels in different systemic autoimmune diseases and autoimmune pancreatitis. Dotted line: Cutoff value (400 mg/L).

dial immunodiffusion (RID) method (The Binding Site Limited, Birmingham, United Kingdom). The diameters of precipitation rings were measured after 72 h. The results were read using the RID reference table. The lowest detection limit was 22.4 mg/L. The intra- and inter-assay coefficients of variation were 3.26 and 0.89 CV%, respectively, as stated by the manufacturer. A cutoff value of 400 mg/L was employed.

Patients with a serum IgG4 level of > 400 mg/L were examined by a gastroenterologist. The clinical and laboratory data were reviewed and abdominal ultrasonography (US) and computed tomography (CT) were performed.

Anti-SS-A/SS-B autoantibody determination
The presence of anti-SS-A/SS-B autoantibodies was determined by means of commercial enzyme-linked immunosorbent assays, conducted according to the protocols provided by the manufacturers.

Experimental data were evaluated statistically with the independent-samples *t* test. *P*-values < 0.05 were accepted as being statistically significant. Statistical data is expressed as mean ± SD.

RESULTS
An elevated serum IgG4 level (mean value 919 ± 996 mg/L) was detected in 17 (28%) of the 61 SAID patients (Figure 1). Ten of the 17 patients had SS (mean serum IgG4 590 ± 232 mg/L) (2 cases were associated with Hashimoto's thyroiditis), 7 (mean serum IgG4 1388 ± 985.5 mg/L) were diagnosed with SLE. Two SLE patients showed markedly elevated IgG4 levels (> 3000 mg/L). In

one case, SLE was associated with Raynaud's syndrome, while the other patient suffered from xerophtalmia and bronchial asthma. The serum IgG4 level was elevated (mean serum IgG4 783 ± 522 mg/L) in 5 (83%) of the 6 AIP patients. The patient with a normal level of IgG4 had typical pancreatic histology and his condition improved with steroid therapy. The IgG4 levels in these SLE and SS patients were not significantly different from that in the AIP patients.

US examination revealed a normal pancreas in 11 of the 17 SAID patients with elevated serum IgG4 levels, but raised the suspicion of AIP by demonstrating a gracile pancreas in 2 cases (both suffered from SS), and widening of the body or the tail of pancreas, each in a further one patient (both suffered from SLE). However, in none of these 4 cases was AIP confirmed by an abdominal CT scan. The US examinations indicated pancreatic steatosis in 2 additional cases. None of the SAID patients had pancreatic duct dilatation.

The presence of anti-SS-A/SS-B autoantibodies and the potential relation of this to an elevated IgG4 level were examined in the patients with SS. Both anti-SS-A-positivity and anti-SS-B-positivity was detected in 22 patients; 7 of them exhibited an elevated IgG4 level. The anti-SS-A was positive and the anti-SS-B was negative in 9 cases; 2 of these patients had a high IgG4 level. In 4 patients with SS, neither anti-SS-A-positivity, nor anti-SS-B-positivity was found; an elevated IgG4 level was detected in only one of these cases.

DISCUSSION

The present study has demonstrated that the serum IgG4 level may be elevated in SAIDs, without the presence of AIP.

AIP can be complicated by a variety of extrapancreatic lesions, which appear synchronously or metachronously with the pancreatic lesion, share the same pathological conditions, and show a favorable response to glucocorticosteroid therapy, characteristics indicative of a common pathophysiological background. Among the variety of extrapancreatic diseases, lachrymal and salivary gland lesions are some of the most frequent, found in 23%-39% of patients with AIP[16,17]. Extrapancreatic lesions may mimic or be misdiagnosed as primary lesions of the corresponding organs, e.g., lachrymal and salivary gland lesions for SS. It is therefore necessary to differentiate between IgG4-related diseases and inherent diseases of the corresponding organ. When the pancreatic lesion is obscured, it may be difficult to detect these presumably IgG4-related extrapancreatic lesions[4].

IgG4 is the rarest of the 4 IgG subclasses in humans, with an incidence of about 4%. IgG antibodies are predominantly involved in the secondary immune response; complement activation is possibly their most important biological function. The main role of IgG4 is presumably to protect against the biological effects of the complement-fixing IgG subclasses and to act in parasitic infestation or various forms of atopy[18-20]. Serum IgG4 levels

are frequently and significantly elevated in AIP patients[6] and an elevated level of serum IgG4 has been included among the laboratory criteria for the diagnosis of AIP[4,15]. AIP patients with 3 extrapancreatic lesions have been reported to have significantly higher IgG4 levels than those lacking such lesions[16]. The optimal cutoff value for discriminating AIP patients with extrapancreatic lesions from those without was demonstrated on the basis of receiver operator characteristic curves to be 2200 mg/L[7].

The serum IgG4 level was measured in 61 SAID patients in our study, 28% of whom proved to have an elevated serum level of IgG4. However, none of them could be diagnosed with AIP according to the HISORt criteria. What could be the reason for this?

One explanation is the composition of our patient cohort. In Japan AIP predominantly affects men, with a male:female ratio of 2.85:1[16]. Moreover, there was a male preponderance in the United Kingdom, European and US studies (100%, 66% and 65% male, respectively), similar to in reports from Japan[21-24]. In contrast, there was only one male in our patient population.

Lachrymal and salivary lesions associated with AIP were previously considered to be complications of SS. However, in contrast to those accompanying SS, the lachrymal and salivary gland lesions associated with AIP yield negative results for anti-SS-A/SS-B autoantibodies and show numerous IgG4-positive plasma cell infiltrations in the affected tissues. These lesions are currently thought to correspond to Mikulicz's disease[25]. The explanation for our negative results may be that there was only one patient with negative SS-A/SS-B autoantibodies in our study group.

Another point is that autoantibodies against FcεRIα are detected in the sera of patients with different autoimmune diseases (such as SLE, dermatomyositis, pemphigus and pemphigoid); these antibodies are from subclasses IgG2 and IgG4, but they are functionally inactive[26]. In our study, elevated IgG4 levels were found in 7 patients treated for SLE.

Moreover, our 17 SAID patients with elevated IgG4 levels included 6 who suffered from different concomitant diseases which could cause the increase in the serum level of IgG4. In one patient, nodular sclerosis Hodgkin lymphoma (HL) was diagnosed histologically. HL cells frequently express interleukin 13 (IL-13) and its receptor. Besides exerting several effects on B cells (e.g., promoting their survival and proliferation), IL-13 switches the Ig class to IgG4 and IgE[27]. In another patient, bullous pemphigoid was identified, which is among the most common blistering autoimmune skin lesions. One of the features of the disease is the presence of autoantibodies against hemidesmosomal antigens (i.e., bullous pemphigoid antigen 1 and 2) in the serum and in affected areas of the skin. The major types of these autoantibodies are IgG4 and IgE[28]. In a third patient, cutaneous lymphocytic vasculitis was diagnosed, which could also explain the serum IgG4 elevation[29]. In 2 patients, the underlying disease was accompanied by Hashimoto's thyroiditis, which can elevate the IgG4 level since thyroglobulin autoantibodies

are from subclasses IgG2 and IgG4[30]. There was also one patient with bronchial asthma, in which disease elevated titers of IgG4 can be found[31].

Finally, SS was diagnosed in the remaining 4 patients, one of whom was seronegative, while the others were seropositive. The elevated serum IgG4 level in patients with seronegative SS may possibly be explained by the presence of Mikulicz's disease[32]. Furthermore, an elevated serum IgG4 level has also been reported in SS[33].

However, not all AIP patients display elevated serum IgG and IgG4 levels. IgG4-negative AIP patients seem to occur more frequently in Europe[34]. Furthermore, some AIP cases improve spontaneously[4]. Hence, it cannot be ruled out that our SAID cohort included AIP patients who were not diagnosed by the measurement of serum IgG4 or in whom the morphology of the pancreas had already normalized by the time of our examination.

Overall, it can be concluded that the serum IgG4 level may be elevated in SAIDs, but as a consequence of the concomitant SAID rather than of AIP. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs.

COMMENTS

Background
Autoimmune pancreatitis (AIP) is frequently associated with some other autoimmune disease, suggesting that it may be a systemic disorder. The determination of serum immunoglobulin G4 (IgG4) is a sensitive marker to diagnose AIP and IgG4-related diseases.

Research frontiers
IgG4 is a sensitive marker in the diagnosis of AIP. The association of AIP and systemic autoimmune diseases (SAIDs), and the usefulness of the determination of serum IgG4 in the diagnosis of AIP in patients with SAIDs are not defined.

Innovations and breakthroughs
The authors revealed that the serum IgG4 level may be elevated in SAIDs without the presence of AIP. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs.

Applications
This study provides important data about the serum level of IgG4 in SAIDs. The determination of serum IgG4 does not seem to be suitable for the differentiation between IgG4-related diseases and SAIDs. The diagnosis of AIP in SAIDs should be made on the results of morphological and histological examination.

Terminology
AIP is an increasingly recognized distinct type of chronic pancreatitis with a presumed autoimmune etiology. Immunoglobulin G (IgG) has four subclasses (IgG1 through IgG4) and the IgG4 subclass accounts for 3%-6% of total serum IgG.

Peer review
This paper discusses the relationship between autoimmune pancreatitis and an elevated serum IgG4 level in autoimmune diseases, and presents interesting and potentially important information. The patients used in this study are few and biased (1 male and 60 female cases), but it is highly significant as a clinical pilot study. This study is well-designed and written, but there are some points to be clarified.

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BRIEF ARTICLE

Clinical course of sub-centimeter-sized nodules detected during surveillance for hepatocellular carcinoma

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Abstract

AIM: To evaluate the outcome of sub-centimeter-sized nodules (SCSNs) detected during surveillance for hepatocellular carcinoma (HCC) in patients at risk.

METHODS: We retrospectively analyzed a total of 142 patients with liver cirrhosis or chronic hepatitis B or C without a prior history of HCC in whom a SCSN was detected during HCC surveillance. We calculated the rate of HCC development from SCSNs in the study population and analyzed the differences in the baseline clinical characteristics and imaging features between the patients with SCSNs that eventually developed into HCC and patients with SCSNs that did not develop into HCC.

RESULTS: During 667 person-years of follow-up, HCC developed in 33 patients. The calculated HCC development rate was 4.9% per year. The cumulative one-, two-, three- and five-year HCC development rates were 5.6%, 10.6%, 14.1% and 20.4%, respectively. Upon baseline comparison, the HCC group was older (54.4 ± 8.3 years *vs* 48.9 ± 9.4 years; *P* = 0.003) and had lower albumin levels (3.56 ± 0.58 g/dL *vs* 3.84 ± 0.55 g/dL; *P* = 0.012) and higher baseline alpha-fetoprotein (AFP) levels (8.5 ng/mL *vs* 5.4 ng/mL; *P* = 0.035) compared to the non-HCC group. Nodule pattern and initial radiologic diagnosis also differed between the two groups. Multivariate analysis revealed that age [*P* = 0.012, odds ratio (OR) =1.075, 95% confidence interval (CI) =1.016-1.137], sex (*P* = 0.009, OR = 3.969, 95% CI: 1.403-11.226), and baseline AFP level (*P* = 0.024, OR = 1.039, 95% CI: 1.005-1.073) were independent risk factors for developing HCC.

CONCLUSION: The overall risk of HCC development in patients with SCSNs is similar to that in liver cirrhosis patients. Patients with these risk factors need to be closely monitored during follow-up.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death in the world, and the ninth leading cause of cancer deaths in the United States[1-7]. The number of deaths per year from HCC is virtually identical to the incidence throughout the world, underscoring the high fatality rate of this aggressive disease[8]. The sole approach to achieve long-term survival is to detect the tumor at an early stage, when effective therapy can be applied[9]. Accordingly, the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases recommend performing screening for HCC in patients at risk who would be treated if diagnosed with this condition[10-12]. Under these guidelines, imaging criteria for the diagnosis of HCC are established for lesions 1 cm or larger in patients at risk, but owing to a high false-positive rate, a wait-and-see policy is recommended for nodules smaller than 1 cm in diameter[11,12]. However, the possibility remains high that minute hepatic nodules detected during surveillance may become malignant over time[13,14]. In addition, a delay in the start of treatment of early-stage HCC may be associated with a poorer patient survival[15]. Nevertheless, clinicians have limited data on the clinical course of sub-centimeter-sized nodules (SCSNs) detected during surveillance.

A variety of important risk factors for the development of HCC have been identified. These include chronic hepatitis B and C virus infection and cirrhosis due to almost any cause[16-22]. Almost 80% of cases are due to underlying chronic hepatitis B and C virus infection[17]. Since patients with chronic hepatitis B who may not have fully developed cirrhosis or have regressed cirrhosis as well as patients with cirrhosis are at increased risk of developing HCC, an updated the American Association for the Study of Liver Diseases guidelines recommended surveillance in patients with chronic hepatitis B[12].

The purpose of our study was to evaluate the outcome of SCSNs detected during HCC surveillance in patients at risk and to determine the risk factors for development of those nodules into HCC.

MATERIALS AND METHODS

Patients

This retrospective study was conducted according to the principles of the Declaration of Helsinki. The study involved patients with liver cirrhosis of any etiology or chronic liver disease including chronic hepatitis B and C virus infection, without a prior history of HCC in whom a SCSN was detected during HCC surveillance with ultrasonography (US) or computed tomography (CT) of the liver at Samsung Medical Center, Seoul, South Korea between January 1, 2005 and April 30, 2005 (*n* = 198). At our institution, patients at risk for HCC were followed with alpha-fetoprotein (AFP) and US every 6 mo. In case of a difficult US, such as in obese individuals, CT and US were performed alternately for HCC surveillance.

Even when an SCSN was detected, patients were usually followed with AFP and US every three or six mo as appropriate. However, if any SCSN enlarged or its appearance was typical of HCC, 3 mo surveillance was used for a certain period or other image modalities such as CT or magnetic resonance imaging (MRI) were performed additionally. The medical records of all patients were reviewed thoroughly. Patients who met any of the following criteria were excluded: (1) less than 12 mo of follow-up, except subjects who were diagnosed with HCC within 12 mo of follow-up; (2) subjects who were lost to follow-up and diagnosed with HCC at an outside hospital; and (3) any history of cancer. Thus, a total of 56 patients were excluded from the study. Forty patients had less than 12 mo of follow-up, seven patients were excluded because HCC was diagnosed at the time of inclusion in the study, and three patients were lost to follow-up. Additionally, three patients had hepatic nodules 1 cm or larger in size at inclusion, two patients had other types of cancer, and the etiology of liver disease in one patient was unclear.

Data collection

The following clinical and laboratory information was collected from each patient: age, sex, etiology of liver disease, presence of liver cirrhosis, the Child-Pugh classification, aspartate aminotransferase (AST), alanine aminotransferase (ALT), prothrombin time (PT), serum total bilirubin, platelet count, serum albumin, and baseline and follow-up AFP levels.

Image interpretation

The initial radiologic diagnosis of SCSNs was based on the results of US or CT during surveillance. In addition, all radiologic images were reviewed by one radiologist who had 11 years of experience in liver imaging interpretation. He did not participate in the initial patient selection and was blinded to the final diagnoses and clinical information such as AFP levels. Each detected lesion was evaluated for the number, location, and echogenicity/attenuation of nodules. Lesions were categorized as follows: (1) hypoechoic/low-attenuation; (2) hyperechoic/high-attenuation; and (3) mixed echoic/attenuation (Figure 1). All lesions were included in one of these three categories.

The diagnosis of HCC was based either on biopsy or the clinical criteria of the Korean Liver Cancer Study Group and the National Cancer Center, South Korea[3]. Briefly, the diagnosis of HCC was made when the AFP level was ≥ 400 ng/mL and at least one of the dynamic enhancement CT or MRI showed a vascular pattern typical of HCC in patients at risk including patients with HBV or HCV infection, or liver cirrhosis. If the AFP level was < 400 ng/mL, at least two of the dynamic enhancement CT, MRI or transarterial angiography must show vascular patterns typical of HCC in order to make a diagnosis of HCC.

Statistical analysis

Statistical analyses were conducted using PASW Statistics

Figure 1 Representative cases according to ultrasonography and computed tomography findings. A: Ultrasonography shows scattered sub-centimeter-sized low echoic nodules; B: Arterial phase computed tomography (CT) scan shows a 5 mm sized low attenuated nodule in left lobe of liver; C: Ultrasonography shows a 7 mm sized hyperechoic nodule in right lobe of liver; D: Arterial phase CT scan shows a 5 mm sized high attenuated nodule in right lobe of liver.

18 for Windows (SPSS, Inc, Chicago, IL, United States). The statistical results are presented as the mean ± SD, median (range), or number (%) of patients. The differences in the baseline clinical characteristics and imaging features between the lesions that eventually developed into HCC and those that did not (non-HCC) were statistically analyzed to identify significant risk factors for the development of HCC from SCSNs detected during surveillance.

Continuous variables were compared parametrically using Student's *t*-test or non-parametrically using the Mann-Whitney *U*-test. Categorical variables were compared using the χ^2-test or Fisher's exact test as appropriate. Multiple logistic regression analysis was performed on variables that were different between the non-HCC and HCC groups in the univariate analysis ($P < 0.100$), in order to identify variables independently associated with the development of HCC. HCC development rates were calculated using the Kaplan-Meier method. A two-sided *P* value < 0.05 was considered statistically significant.

RESULTS

A total of 142 patients were included in this study. Their characteristics are summarized in Table 1. Eighty-four patients (59.2%) were male and the mean age was 50.2 ± 9.4 (SD) years. The etiology of liver disease was hepatitis B virus infection in 126 patients (88.7%), hepatitis C virus infection in 9 (6.3%), and alcoholic liver disease in 7 (5.0%). One hundred and eleven patients (78.2%) had cirrhotic liver. Ninety-eight patients (88.3%) were Child-Pugh class A, 10 (9.0%) were class B, and 3 (2.7%) were class C. A total of 33 patients had at least one SCSN: 23 patients were detected by US and 10 were detected by CT. There was one SCNS in 26 patients (18.3%), two SCSNs in 7 (5.0%), three in 3 (2.1%), four in 1 (0.7%), and more than four in 105 (73.9%). The SCSNs were hypoechoic/low-attenuation in 77 patients (54.3%), hyperechoic/high-attenuation in 31 (21.8%), and mixed echoic/attenuation in 34 (23.9%). Initial radiologic diagnosis of the hepatic nodules was regenerative nodule (RN)/dysplastic nodule (DN) in 119 patients (83.8%), hemangioma in 17 (12.0%),

indeterminate nodule in 5 (3.5%), and arterioportal shunt in 1 (0.7%).

During 667 person-years of follow-up (mean, 28.5 ± 20.0 mo), HCC developed in 33 patients (23.2%). The mean durations of follow-up were 32.6 ± 19.5 and 64.3 ± 17.6 in the HCC and non-HCC groups, respectively. The mean time to diagnosis of HCC after detection of SCSNs was 33.1 ± 18.9 mo. Except for one biopsy-proven case, most of the HCC cases were diagnosed according to the clinical criteria of the Korean Liver Cancer Study Group and the National Cancer Center, South Korea[3], which were not same as the international guidelines[10,11] at that time. However, when retrospectively reevaluated, all diagnoses of HCC were satisfied with the updated American Association for the Study of Liver Diseases guidelines[12]. Following diagnosis, twelve patients (36.4%) underwent radiofrequency ablation, 13 (39.4%) underwent transarterial chemoembolization, 5 (15.2%) underwent surgical resection, 1 (3.0%) underwent liver transplantation, and 2 (6.0%) did not receive any treatment.

The calculated HCC development rate was 4.9% per year. The cumulative one-, two-, three- and five-year HCC development rates were 5.6%, 10.6%, 14.1% and 20.4%, respectively.

Clinical features and initial radiologic results of patients in the HCC and non-HCC groups

Patients diagnosed with HCC were older (54.4 ± 8.3 years *vs* 48.9 ± 9.4 years; $P = 0.003$) and had lower albumin levels (3.56 ± 0.58 g/dL *vs* 3.84 ± 0.55 g/dL; $P = 0.012$) and elevated baseline AFP levels [8.5 (range: 3.2-211.6) ng/mL *vs* 5.4 (range: 1.0-55.9) ng/mL; $P = 0.035$] compared to patients with non-HCC nodules. In terms of nodule pattern, patients diagnosed with HCC had more hypoechoic/low-attenuation nodules and less hyperechoic/high-attenuation nodules than patients with non-HCC nodules [23 (69.7%) *vs* 54 (49.5%) and 1 (3.0%) *vs* 30 (27.5%), respectively, $P = 0.011$]. In the initial radiologic diagnosis of hepatic nodules, RN/DN accounted for 31 (93.9%) in patients diagnosed with HCC, while RN/DN and hemangioma accounted for 88 (80.7%) and 17 (15.6%), respectively, in patients with non-HCC nodules ($P = 0.036$). There were no significant differences in

Table 1 Baseline characteristics of high-risk patients who had sub-centimeter-sized nodules (*n* = 142)

Baseline characteristics	Number of patients
Age (yr)	50.2 ± 9.4
Male	84 (59.2)
Etiology of liver disease	
Hepatitis B infection	126 (88.7)
Hepatitis C infection	9 (6.3)
Alcohol liver cirrhosis	7 (5.0)
Liver cirrhosis	111 (78.2)
Child-Pugh A	98 (88.3)
Child-Pugh B	10 (9.0)
Child-Pugh C	3 (2.7)
AST (UI/L)	47.9 ± 26.8
ALT (UI/L)	53.2 ± 42.5
PT (INR)	1.19 ± 0.17
Bilirubin (mg/dL)	1.24 ± 0.97
Platelets (10^9/L)	125.3 ± 60.3
Albumin (g/dL)	3.77 ± 0.56
Baseline AFP (ng/mL, range)	5.7 (1.0-211.6)
Number of nodules	
One	26 (18.3)
Two	7 (5.0)
Three	3 (2.1)
Four	1 (0.7)
Over four	105 (73.9)
Nodule pattern	
Hypoechoic/low-attenuation	77 (54.3)
Hyperechoic/high-attenuation	31 (21.8)
Mixed	34 (23.9)
Initial radiologic diagnosis	
RN/DN	119 (83.8)
Hemangioma	17 (12.0)
Indeterminate nodule	5 (3.5)
Arterioportal shunt	1 (0.7)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PT: Prothrombin time; AFP: Alpha-fetoprotein; RN: Regenerative nodule; DN: Dysplastic nodule; INR: International normalized ratio. Data are shown as the mean ± SD, median (range) or *n* (%) of patients.

Table 2 Risk factors for the development of hepatocellular carcinoma from sub-centimeter-sized nodules

Variables	Diagnosis		*P* value
	HCC (*n* = 33)	Non-HCC (*n* = 109)	
Age (yr)	54.4 ± 8.3	48.9 ± 9.4	0.003
Male	24 (72.4)	60 (55.0)	0.070
Etiology of liver disease			0.364
Hepatitis B infection	27 (81.8)	99 (90.8)	
Hepatitis C infection	3 (9.1)	6 (5.5)	
Alcoholic liver cirrhosis	3 (9.1)	4 (3.7)	
Liver cirrhosis	29 (87.9)	82 (75.2)	0.123
AST (UI/L)	49.3 ± 20.4	47.5 ± 28.5	0.736
ALT (UI/L)	50.9 ± 36.5	53.9 ± 44.3	0.722
PT (INR)	1.23 ± 0.17	1.18 ± 0.17	0.088
Bilirubin (mg/dL)	1.27 ± 0.78	1.23 ± 1.02	0.831
Platelets (10^9/L)	110.1 ± 53.9	129.9 ± 61.6	0.099
Albumin (g/dL)	3.56 ± 0.58	3.84 ± 0.55	0.012
Number of nodules			0.390
One	4 (12.1)	22 (20.0)	
Two	1 (3.0)	6 (5.5)	
Three	0 (0.0)	3 (2.8)	
Four	0 (0.0)	1 (0.9)	
Over four	28 (84.8)	77 (70.6)	
Nodule pattern			0.011
Hypoechoic/low-attenuation	23 (69.7)	54 (49.5)	
Hyperechoic/high-attenuation	1 (3.0)	30 (27.5)	
Mixed	9 (27.3)	25 (22.9)	
Initial radiologic diagnosis			0.036
RN/DN	31 (93.9)	88 (80.7)	
Hemangioma	0 (0.0)	17 (15.6)	
Indeterminate nodule	2 (6.1)	3 (2.8)	
Arterioportal shunt	0 (0.0)	1 (0.9)	
Baseline AFP (ng/mL, range)	8.5 (3.2-211.6)	5.4 (1.0-55.9)	0.035

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PT: Prothrombin time; AFP: Alpha-fetoprotein; RN: Regenerative nodule; DN: Dysplastic nodule; INR: International normalized ratio; HCC: Hepatocellular carcinoma. Data are shown as the mean ± SD, median (range) or *n* (%) of patients.

sex, etiology of liver disease, presence of liver cirrhosis, Child-Pugh class, AST, ALT, PT, bilirubin, platelet count, or number of nodules between patients diagnosed with HCC and patients with non-HCC nodules (Table 2).

Multivariate analysis revealed that old age [*P* = 0.012, odds ratio (OR) = 1.075, 95% confidence interval (CI) = 1.016-1.137], male sex (*P* = 0.009, OR = 3.969, 95% CI: 1.403-11.226), and high baseline AFP level (*P* = 0.024, OR = 1.039, 95% CI: 1.005-1.073) were associated with an increased risk of developing HCC from SCSNs detected during surveillance (Table 3).

DISCUSSION

The purpose of our study was to evaluate the outcome of SCSNs detected during surveillance in patients at risk and to determine risk factors for developing HCC from those nodules. The current practice guidelines recommend follow-up of SCSNs every few months in order to detect growth suggestive of malignant transformation[10-12]. However, early diagnosis of HCC has a significant impact on survival because it enables the timely implementation of

Table 3 Multivariate analysis of risk factors for the development of hepatocellular carcinoma from sub-centimeter-sized hepatic nodules

Variables	*P* value	OR	95% CI
Age (yr)	0.012	1.075	1.016-1.137
Male	0.009	3.969	1.403-11.226
PT (INR)	0.877	0.698	0.007-66.718
Platelets (10^9/L)	0.917	0.999	0.990-1.009
Albumin (g/dL)	0.478	0.624	0.169-2.298
Nodule pattern	0.081		
Nodule pattern (1)[1]	1.000	0.812	0.233-2.827
Nodule pattern (2)[2]	0.054	0.075	0.006-1.026
Baseline AFP (ng/mL)	0.024	1.039	1.005-1.073

[1]Between hypoechoic/low-attenuation and mixed echoic/attenuation; [2]Between hyperechoic/high-attenuation and mixed echoic/attenuation. OR: Odds ratio; CI: Confidence interval; PT: Prothrombin time; AFP: Alpha-fetoprotein; INR: International normalized ratio. Data are shown as the mean ± SD, median (range) or number (%) of patients.

effective treatment strategies, including hepatic resection, loco-regional ablative therapy, and liver transplantation[23,24]. In addition, even in cases of HCC that are

detected early and can be treated with radiofrequency ablation, a delay (more than five weeks) in treatment may be associated with poorer patient survival[15]. Therefore, in the present study, we focused on the SCSNs, which have not been investigated so far, even though occasionally encountered in practice, and identified clinical risk factors for the development of HCC from SCSNs.

Several studies have reported an HCC yearly incidence in HBV or HCV infection, which is between 2%-8% per year depending on the study population[12,18,21,25-33]. In the present study, the annual HCC incidence from SCSNs was 4.9% per year, which is similar to above-mentioned HCC incidences of 2%-8%/year in chronic HBV or HCV infection. Thus, although the detection of SCSNs during surveillance is not infrequent and their management could be a major clinical challenge, it seems that the HCC incidence does not increase significantly in patients with SCSNs compared to patients without SCSNs.

There have been a few studies of sub-centimeter-sized HCC[34,35]. Park *et al*[34] reported that small (5-10 mm) arterially enhancing nodules at the hepatic arterial phase of CT in surveillance for HCC have a 29.5% probability of developing into HCC over a mean 35.7 mo of follow-up on a per-person basis. They also identified the presence of HCC treatment history, a larger size of small (5-10 mm) arterially enhancing nodules, presence of coexistent HCC, and absence of coexistent typical arterioportal shunts as independent risk factors for future development of HCC. In our study, SCSNs had a 23.2% probability of developing into HCC over a mean of 28.5 mo of follow-up. The unique feature of the present study that differentiates it from that of Park *et al*[34] is that our study population had no prior HCC history and included 77 (54.3%) patients who had hypoechoic/low-attenuation SCSNs. In addition, patients diagnosed with HCC had more hypoechoic/low-attenuation SCSNs than patients with non-HCC nodules (69.7% *vs* 49.5%; *P* = 0.011; Table 2), although the difference was not significant in the multivariate analysis (*P* = 0.081). This could be due to hemangiomas, which are mainly hyperechoic/high-attenuation and benign, because 17 patients with a hemangioma were included only in the non-HCC group (Table 2). Therefore, we selected patients who had RN/DN and performed a subgroup analysis. The proportion of patients with hypoechoic/low-attenuation SCSNs did not differ between the two groups (70.0% *vs* 56.5%, *P* = 0.134). According to our results, non-enhancing minute hepatic nodules also might have considerable malignant potential and should receive as much attention as enhancing nodules.

A study by Forner *et al*[36] evaluated the accuracy of contrast-enhanced US and dynamic MRI for the diagnosis of nodules 20 mm or smaller detected during US surveillance. The study included 89 patients with cirrhosis, of whom 13 patients (14.6%) had a SCSN. Among those with SCSNs, 2 (15.4%) were ultimately diagnosed with HCC. Significant differences were found in age, no-

dule size, and the presence of a halo between patients diagnosed with HCC and patients with non-HCC nodule in all subjects, although multivariate analysis was not performed. In our study, old age, male sex, and high baseline AFP levels were associated with an increased risk of developing HCC from SCSNs detected during surveillance. Among these variables, male sex was the strongest risk factor (*P* = 0.009, OR = 3.969, 95% CI: 1.403-11.226). Elevated baseline AFP levels may be affected by undiscovered HCC. Therefore, we excluded subjects who were diagnosed with HCC at the time of inclusion in the study. Additionally, we investigated the change in AFP levels and calculated the AFP ratio as the last AFP level divided by the baseline AFP level. The AFP ratios were also significantly elevated in the HCC group compared to the non-HCC group [1.0 (range: 0.2-74.3) *vs* 0.7 (range: 0.0-4.7); *P* = 0.040], even though baseline AFP levels were elevated. Thus, elevated AFP level at baseline could be considered a risk factor for developing HCC, and an increased AFP ratio during follow-up should be considered a critical warning sign for HCC development.

The present study had some limitations. First, the retrospective design likely introduced selection bias. Second, there was a lack of histological confirmation for the benign lesions, which were defined on the basis of radiologic images. However, it is unlikely that HCCs were incorrectly categorized as benign because our follow-up period was sufficiently long. Furthermore, pathological confirmation of these lesions would not be practical in clinical settings. Last, our assessments regarding the number, location, nodule pattern, and size of SCSNs had an element of subjectivity due to the small nodule sizes and sometimes ill-defined margins. To overcome this limitation, all radiologic images were reviewed by an experienced radiologist who was blinded to the final diagnoses.

In conclusion, the overall risk of HCC development in patients with SCSNs is similar to that in liver cirrhosis patients. However, since old age, male sex, and high baseline AFP level are associated with an increased risk of developing HCC from SCSNs, patients with these risk factors need to be closely monitored during follow-up.

COMMENTS

Background
During hepatocellular carcinoma (HCC) surveillance, the detection of sub-centimeter-sized nodules (SCSNs) is not infrequent and their management is a major clinical challenge. Owing to a high false-positive rate, a wait-and-see policy is recommended for those nodules. However, the possibility remains high that small nodules detected during surveillance may become malignant over time and a delay in the start of treatment of even early-stage HCC may be associated with a poorer patient survival.

Research frontiers
Clinicians have limited data on the clinical course of SCSNs. In this study, the authors investigated outcomes of SCSNs detected during HCC surveillance in patients at risk.

Innovations and breakthroughs
This is the first report to evaluate the outcome of SCSNs detected during surveillance in patients with cirrhosis or chronic liver disease and to determine

risk factors for developing HCC from those nodules. Therefore, the study could provide valuable information to clinicians managing patients with chronic liver disease.

Applications

The study results suggest that patients with risk factors such as old age, male sex and high baseline alpha-fetoprotein need to be closely monitored during follow-up.

Peer review

This study is very informative for clinicians because the detection of SCSNs during surveillance is frequently encountered in practice setting. In addition, their results have scientific relevance for understanding the epidemiology of the disease.

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BRIEF ARTICLE

Family history influences the early onset of hepatocellular carcinoma

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Abstract

AIM: To evaluate the relationship between a positive family history of primary liver cancer and hepatocellular carcinoma (HCC) development in Korean HCC patients.

METHODS: We studied a total of 2242 patients diagnosed with HCC between January 1990 and July 2008, whose family history of primary liver cancer was clearly described in the medical records.

RESULTS: Of the 2242 patients, 165 (7.4%) had a positive family history of HCC and 2077 (92.6%) did not. The male to female ratio was 3.6:1, and the major causes of HCC were chronic hepatitis B virus (HBV) infection in 75.1%, chronic hepatitis C virus infection in 13.2% and alcohol in 3.1%. The median ages at diagnosis in the positive- and negative-history groups were 52 years (range: 29-79 years) and 57 years (range: 18-89 years), respectively ($P < 0.0001$). Furthermore, among 1713 HCC patients with HBV infection, the number of patients under 45 years of age out of 136 patients with positive family history was 26 (19.1%), whereas those out of 1577 patients with negative family history was 197 (12.5%), suggesting that a positive family history may be associated with earlier development of HCC in the Korean population ($P = 0.0028$).

CONCLUSION: More intensive surveillance maybe recommended to those with a positive family history of HCC for earlier diagnosis and proper management especially when HBV infection is present.

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Key words: Liver cancer; Hepatocellular carcinoma; Family history; Epidemiology

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for up to 90% of primary liver cancers. It is the fifth most common can-

cer and the third most common cause of cancer-related death worldwide[1-3]. The major risk factors for the development of HCC include liver cirrhosis of any etiology, chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infection, heavy alcohol consumption and non-alcoholic steatohepatitis[2,4].

Familial clustering has been reported in many types of cancer including pancreas, colon, stomach, lung and breast cancers based on either meta-analyses or registry-based studies throughout the world[5-9]. However, no data on familial clustering for HCC is available in Korea to date although a few studies are reported in some other countries[10-15]. The development of HCC in Caucasian populations are reported to be less related to chronic HBV infection, but the clustering of HBV infection among family members was reported to be the major cause associated with family histories of HCC in Asia[3,12,14,16]. Still, the impact of family history of HCC in the development of HCC remains to be determined along with the possible confounding effects of important risk factors for HCC. Here, we report a large retrospective cohort study evaluating the effect of family history of HCC on its development among Korean patients with various risk factors.

MATERIALS AND METHODS

Study population
This study was a single-hospital-based study; cases were retrospectively evaluated. The data were recruited retrospectively from the medical records of 2242 patients who had first been diagnosed with and treated for HCC between January 1990 and July 2008. Before the analysis, the diagnosis of HCC was reconfirmed based on the American Association for the Study of Liver Diseases Practice Guidelines for Management of HCC[17], with either positive histopathology on liver biopsy and/or non-invasive criteria of hepatic imaging demonstrating one or more space-occupying lesions showing arterial hypervascularization on triphasic computed tomography and/or magnetic resonance imaging with or without an elevated alpha-fetoprotein (AFP) level.

All patients were screened for hepatitis virus infection when diagnosed with HCC, and if a patient had no evidence of chronic viral hepatitis, he or she underwent studies for autoimmune hepatitis and metabolic and/or genetic disorders such as Wilson's disease, hemochromatosis, primary biliary cirrhosis. HBV infection was diagnosed by testing for hepatitis B surface antigen, hepatitis core IgG, and HBV DNA, and HCV infection was diagnosed by testing for anti-HCV antibodies and HCV RNA; testing was performed at the central lab of Seoul St. Mary's Hospital. Alcohol-related cirrhosis was clinically diagnosed based on a compatible history of sustained alcohol consumption over 75 g/d in the absence of any other cause for liver disease.

A family history of HCC was determined based on medical records written during personal interviews on admission.

Tumor staging
Tumor staging for HCC was based on a classification system modified from the Union for International Cancer Control staging classification[18-20].

Statistical analysis
The results are presented as frequency (*n*) and percentage for categorical data and median (minimum to maximum) for continuous data.

To compare the general characteristics according to HCC family history, categorical variables were analyzed using either the χ^2 test or Fisher's exact test, as appropriate. Continuous variables were compared using the Mann-Whitney *U*-test. To identify differences in HCC family history according to HBV and HCV infections, statistical analyses were performed by the χ^2 test, Fisher's exact test, or the Mann-Whitney *U*-test, as appropriate.

All statistical analyses were performed using SAS software, Version 9.1 (SAS Institute Inc., Cary, NC). A 2-tailed *P*-value of < 0.05 was considered statistically significant.

RESULTS

Patient characteristics
The demographic features of the HCC patients are summarized in Table 1. A total of 2242 cases with HCC were recruited by a retrospective chart review. The number of male patients (*n* = 1765) was approximately 3.6 times that of female patients (78.7% *vs* 21.3%, respectively). The median age at the time of diagnosis was 57 years (18-89 years), and the median age at the peak incidence of HCC was in the sixth decade (*n* = 722, 32.2%) followed by the seventh (*n* = 640, 28.6%) for all cases. When classified by gender, male patients were most commonly diagnosed with HCC in their fifties, whereas female patients were most commonly diagnosed in their sixties.

The most common single cause of HCC was HBV (*n* = 1683, 75.1%); the second was HCV, and the fourth was alcohol. The group classified as "unknown" was the third largest group and included the patients with non-B, non-C, and non-alcoholic liver cirrhosis and those for whom the cause of HCC could not be identified even after thorough evaluation (Table 1). In patients with chronic hepatitis B, the peak incidence of HCC was observed in the sixth decade of life; however, it was observed 10 years later in patients for whom HCC was caused by chronic hepatitis C or alcohol (data not shown). In 32 (1.4%) of 2242 patients, more than one etiology was identified. HBV co-infection with HCV was the most common combination and affected 17 patients; HBV with alcohol was the second most common and affected 13 patients; the remaining two cases were caused by HCV with alcohol. When the patients were classified based on whether a patient had HBV infection, the HBV-positive group consisted of 1713 patients (76.4%), whereas the HBV-negative group included only 529 (23.6%) patients. The patients were then reclassified into HCV-positive and

Table 1 General characteristics of the study population *n* (%)

		Total *n* = 2242	FHx (-) *n* = 2077 (92.6%)	FHx (+) *n* = 165 (7.4%)	*P* value
Gender	Male	1765 (78.7)	1639 (78.9)	126 (76.4)	0.441
	Female	477 (21.3)	438 (21.1)	39 (23.6)	
Age	Median (min-max)	57.0 (18.0-89.0)	57.0 (18.0-89.0)	52.0 (29.0-79.0)	< 0.0001
	< 20	1 (0.1)	1 (0.1)	0 (0.0)	< 0.001
	20-29	13 (0.5)	12 (0.6)	1 (0.6)	
	30-39	112 (5.0)	99 (4.8)	13 (7.9)	
	40-49	458 (20.4)	410 (19.7)	48 (29.1)	
	50-59	722 (32.2)	661 (31.8)	61 (37.0)	
	60-69	640 (28.6)	606 (29.2)	34 (20.6)	
	70-79	256 (11.4)	248 (11.9)	8 (4.8)	
	⩾ 80	40 (1.8)	40 (1.9)	0 (0.0)	
Etiology	HBV	1683 (75.1)	1548 (74.5)	135 (81.8)	0.236
	HCV	296 (13.2)	280 (13.5)	16 (9.6)	
	Alcohol	70 (3.1)	68 (3.3)	2 (1.2)	
	Combined	32 (1.4)	31 (1.5)	1 (0.6)	
	Others	50 (2.2)	45 (2.2)	5 (3.0)	
	Unknown	111 (5.0)	105 (5.0)	6 (3.6)	
HBV	HBV (+)	1713 (76.4)	1577 (75.9)	136 (82.4)	0.058
	HBV (-)	529 (23.6)	500 (24.1)	29 (17.6)	
HCV	HCV (+)	315 (14.1)	298 (14.3)	17 (10.3)	0.15
	HCV (-)	1927 (85.9)	1779 (86.7)	148 (89.6)	
Stage	I	185 (8.3)	167 (8.0)	18 (10.9)	0.221
	II	655 (29.2)	602 (29.0)	54 (32.7)	
	III	648 (28.9)	610 (29.4)	38 (23.1)	
	IVa and IVb	753 (33.6)	698 (33.6)	55 (33.3)	
Lab,	ALT (U/L)	47.0 (3.0-3505.0)	47.0 (3.0-3505.0)	47.5 (3.0-840.0)	0.987
median (min-max)	TB (mg/mL)	1.1 (0.1-47.6)	1.1 (0.1-47.6)	1 (0.1-26.0)	0.868
	Alb (g/dL)	3.5 (1.2-9.6)	3.5 (1.2-9.6)	3.7 (1.6-5.0)	0.106
	Platelet (x 10^3/μL)	121.5 (8.0-1084.0)	121.0 (18.0-1084.0)	133.0 (8.0-635.0)	0.181
Tumor markers,	AFP (ng/ mL)	69.1 (0.0-529 470.0)	67.00 (0.0-529 470.0)	110.8 (1.1-53 606.0)	0.972
median (min-max)	PIVKA-II (Mau/mL)	88.0 (1.0-16 636.8)	92.0 (1.0-16 636.8)	80.0 (6.0-2000.0)	0.990

AFP: Alpha-fetoprotein; Alb: Serum albumin; ALT: Alanine transaminase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; FHx: Family history; PIVKA-II: Protein induced by vitamin K deficiency-II; TB: Serum total bilirubin.

-negative groups; the HCV-positive group included only 14.1% (*n* = 315) of all patients.

By the modified International Union Against Cancer (UICC) staging classification, only 185 patients (8.3%) were diagnosed at stage I, followed by 655 (29.2%) patients at stage II, 648 (28.9%) patients at stage III, and 753 (33.6%) patients at stage IV. Thus, over half of the patients (62.5%) were diagnosed at advanced stages of HCC, including stages III and IV.

Laboratory data at diagnosis did not indicate severe hepatic dysfunction. The medians of serum alanine transaminase, serum total bilirubin, serum albumin, and platelet count were 47 U/L (3.0-3505.0), 1.1 mg/mL (0.1-58), 3.5 g/dL (1.2-4.98), and 121 500/μL (8000-1 084 000), respectively. With regard to tumor markers, the median serum AFP level was 69.1 ng/mL (0.0-529 470.0), and the median level of serum protein induced by vitamin K deficiency (PIVKA-II) was 88.0 Mau/mL (1.0-16 636.8). No statistical difference between the positive- and negative-family-history groups was found.

Family history of HCC

As shown in Table 1, 165 (7.4%) of 2242 patients had a positive family history of HCC in one or more family members, whereas 2077 (92.6%) patients had no family

history of any HCC. Because the main concern was the influence of a positive family history in the development of HCC, positive histories in the offspring of the patients were not considered. Among those 165 patients with positive family histories, 159 had a positive family history in one or more first-degree relatives, including parents and siblings. Of the remaining six patients (all of these patients as well as their mothers had HBV infection), five had a positive family history of HCC in a brother or sister of the patient's mother, and one had a positive family history in the grandmother and in an uncle on the mother's side. We considered these patients to have positive family histories because vertical transmission of HBV and its oncogenic effects on the affected individual and on his or her family members cannot be omitted in a population with a high prevalence of HBV infection. The number of patients with a positive family history in a single family member was 143. A father, a mother, or a sibling was a single family member with a history of HCC in 25, 29 and 89 patients, respectively. The number of patients with a positive family history in two or more family members were as follows: both mother and father in two, a father and a sibling in two, a mother and a sibling in 10, a mother and two siblings in one, two siblings in one, a grandmother and an uncle in one, a sibling and

Table 2 Distribution of patients with positive family history by age and tumor staging *n* (%)

	n	Age (yr, median)	Range	*P* value
Family members				
Father	29	53	29.0-78.0	0.101
Mother	42	48.5	33.0-79.0	
Siblings	102	39	30.0-48.0	
HBV infection		HBV (+)	HBV (-)	
		(*n* = 136)	(*n* = 29)	
Age	Median	52.0	57.0	0.065
	(min-max)	(30.0-78.0)	(29.0-79.0)	
	< 20	0 (0.0)	0 (0.0)	0.001
	20-29	0 (0.0)	1 (3.4)	
	30-39	12 (8.8)	1 (3.4)	
	40-49	39 (28.7)	9 (31.0)	
	50-59	56 (41.2)	5 (17.5)	
	60-69	26 (19.1)	8 (27.6)	
	70-79	3 (2.2)	5 (17.2)	
	≥ 80	0 (0.0)	0 (0.0)	
Stage	1	17 (12.5)	1 (3.4)	0.056
	2	46 (33.8)	8 (27.6)	
	3	26 (19.1)	12 (41.4)	
	4	47 (33.6)	8 (27.6)	
HCV infection		HCV (+)	HCV (-)	
		(*n* = 18)	(*n* = 147)	
Age	Median	54.0	52.0	0.629
	(min-max)	(29.0-75.0)	(30.0-79.0)	
	< 20	0 (0.0)	0 (0.0)	0.026
	20-29	1 (5.9)	0 (0.0)	
	30-39	1 (5.9)	12 (8.1)	
	40-49	5 (29.4)	43 (29.1)	
	50-59	3 (17.6)	58 (39.2)	
	60-69	6 (35.3)	28 (18.9)	
	70-79	1 (5.9)	7 (4.7)	
	≥ 80	0 (0.0)	0 (0.0)	
Stage	1	18 (12.2)	0 (0.0)	0.335
	2	49 (33.1)	5 (29.4)	
	3	32 (21.6)	6 (35.3)	
	4	49 (33.1)	6 (35.3)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; FHx: Family history.

an uncle in two, and a mother, grandmother, and an aunt in one. Considering 159 the patients positive family history only in the first degree relative, when grouped into HBV-positive and -negative, the median age at diagnosis of HCC was 51.5 and 57.0, respectively ($P = 0.049$).

Age at diagnosis: Regardless of the family history of HCC, both groups had a peak incidence of HCC diagnosis in their fifties. However, the median age at diagnosis was 52 years (range: 29-79 years) for those with positive family histories, which was significantly younger ($P < 0.0001$) than that for those with negative family histories (57; range: 18-89 years). In the positive-family-history group, 61 (37.0%) patients were diagnosed in their fifties, and 48 (29.1%) in their forties. In the negative-family-history group, 661 (31.8%) patients were diagnosed in their fifties, and 606 (29.2%) in their sixties ($P < 0.001$) (Table 1).

Among the patients in the positive-family-history group, the median age at diagnosis in the HBV-positive patients (*n* = 136) *vs* the HBV-negative patients (*n* = 29) was 52 *vs* 57, respectively; this difference was not sig-

nificant ($P = 0.065$). HBV-positive patients were most frequently diagnosed with HCC in their fifties (*n* = 56, 41.2%), followed by their forties (*n* = 39, 28.7%). The median age at diagnosis in the HCV-positive patients (*n* = 18) *vs* the HCV-negative patients (*n* = 147) was 54 *vs* 52 years, respectively; this difference also failed to reach statistical significance ($P = 0.629$) (Table 2).

Risk factors: HBV and HCV were two important causes of HCC development with or without a family history of HCC. Among patients with a positive family history, 135 cases were caused by HBV infection only, and 16 cases were caused by HCV only; one patient had both HBV and HCV infections (Table 1). In the negative-family-history group, 1548 (74.5%) cases were caused by HBV infection, followed by 280 (13.5%) cases of HCV infection, and these percentages were not significantly different from those found in the positive-family-history group ($P = 0.236$) (Table 1).

Among those with a positive family history of HCC, the median age at diagnosis of 136 patients with HBV infection was 52 years (range: 30-78 years), whereas that of 29 patients without HBV infection was 57 years (range: 29-79 years) ($P = 0.132$). With regard to HCV infection among those with a positive family history, the median age at diagnosis was 54 years (range: 29-75 years) in the HCV-infected group and 52 years (range: 30-79 years) in the HCV-noninfected group ($P = 0.562$) (Table 2).

Staging at diagnosis: HCC staging was based on a modified UICC staging system, with UICC stages IVa and IVb defined here as stage IV. Stages Ⅰ, Ⅱ, Ⅲ and IV included 18 (10.9%), 54 (32.7%), 38 (23.0%) and 55 (33.4%) patients, respectively, in the positive-family-history group, and 167 (8.0%), 602 (29.0%), 610 (29.4%) and 698 (33.6%) patients, respectively, in the negative-family-history group ($P = 0.221$). When the stages were reclassified into earlier (stages Ⅰ and Ⅱ) *vs* advanced stages (stages Ⅲ and IV), the early and advanced stages included 72 (43.6%) and 93 (56.4%) patients in the positive-family-history group and 768 (37.0%) and 1308 (63.0%) patients in the negative family history group ($P = 0.090$), respectively.

HBV infection and family history of HCC

As shown in Table 3, 1711 patients had HBV infections, and 134 (7.8%) of these had a positive family history of HCC. The median age of these 134 patients was 52 years (30-78 years), and among those without a family history (*n* = 1577, 92.1%), the median age was 57 years (range: 18.0-89.0 years), showing a significant statistical difference between the two groups ($P < 0.0001$). Among 529 patients without HBV infection, 29 (5.5%) had a positive family history of HCC, and their median age was 57 years (range: 29-79 years), whereas the remaining 500 patients without a family history had a median age of 61 years (range: 22-88 years) ($P = 0.164$). The patients were then divided into two groups based on age: a younger group (ages under 45 years) and an older group (ages 45 years or more). Among the HBV-positive patients, the numbers

Table 3 Characteristics of study subjects based on hepatitis B virus infection and family history *n* (%)

		HBV (+) (*n* = 1713)		*P* value	HBV (-) (*n* = 529)		*P* value
		FHx (+) (*n* = 136)	FHx (-) (*n* = 1577)		FHx (+) (*n* = 29)	FHx (-) (*n* = 500)	
Age, median (range)		52 (30.0-78.0)	57 (18.0-89.0)	< 0.0001	57 (29.0-79.0)	61 (22.0-88.0)	0.164
Age group	< 45	26 (19.1)	197 (12.5)	0.028	5	57	0.244
	⩾ 45	110 (80.9)	1380 (87.5)		24	443	
Stage	1	17 (12.5)	122 (7.7)	0.018	1 (2.2)	45 (9.0)	0.364
	2	46 (33.8)	443 (28.1)		8 (27.6)	159 (31.8)	
	3	26 (19.1)	473 (30.0)		12 (41.4)	137 (27.4)	
	4	47 (34.6)	539 (34.2)		8 (27.6)	159 (31.8)	
non-HBV	HCV				16 (55.2)	280 (56.0)	0.481
	Alcohol				2 (6.9)	68 (13.6)	
	Combined				0 (0.0)	2 (0.4)	
	Others				5 (17.2)	45 (9.0)	
	Unknown				6 (20.7)	105 (21.0)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; FHx: Family history.

of patients in the younger group with positive and negative family histories were 26 and 197, respectively, *vs* 110 and 1380, respectively, in the older group (*P* = 0.028). In the HBV-negative group with 529 patients, no significant difference was observed between the younger and older groups either in positive- (5 *vs* 24) or negative-family-history group (57 *vs* 443).

Considering the tumor staging at diagnosis, the numbers of patients with HBV infection and a positive family history were 17 (12.5%), 46 (33.8%), 26 (19.1%) and 47 (34.6%) in stages Ⅰ, Ⅱ, Ⅲ and Ⅳ, respectively. These frequencies were significantly different (*P* = 0.018) from those among patients with HBV infection and a negative family history, for whom the corresponding numbers were 122 (7.7%), 443 (28.1%), 473 (30.0%) and 539 (34.2%). The patients without HBV infection and with a positive family history at each stage numbered 1 (3.2%), 8 (25.8%), 12 (38.7%) and 8 (32.3%), and those without HBV infection and with a negative family history numbered 45 (9.0%), 159 (31.8%), 137 (27.4%) and 159 (31.8%) in stages Ⅰ, Ⅱ, Ⅲ and Ⅳ, respectively (*P* = 0.425).

DISCUSSION

This is the first extensive investigation of the relationship between a family history of HCC and the risk of HCC development in Korea, with further considerations regarding major risk factors for HCC development. We observed that 7.4% (*n* = 165) of 2242 patients with HCC reported having a positive family history of HCC.

The most significant finding in this study was that the median age at diagnosis was 5 years younger among patients with a positive family history than among those with a negative family history (*P* < 0.0001). Also, the age distribution was significantly different between the groups (Table 1, *P* < 0.001). The age at diagnosis of HCC had been analyzed in a previous study evaluating the association of family history of liver cancer with HCC development in the United States, but in that study, the mean age at diagnosis in patients with a positive family history (mean age: 64.1 years, *n* = 21) and in patients without a

family history (mean age: 59.9 years, *n* = 156) did not differ significantly (*P* = 0.1)[12,21]. It was said that the lack of significant association between HCC and affected parents or offspring in the study can be related to the small numbers[12]. On the other hand, the age difference of 5 years was significant in our study, and was younger in patients with a positive family history, we conclude that the significance was partly influenced by the large number of cases recruited in our study. Considering only the HBV-positive patients, we observed that the age at diagnosis was also significantly younger by 5 years in patients with a positive family history of liver cancer compared with those with a negative family history (*P* < 0.0001). This may be a natural corollary, as several reports have reported an association between the development of HCCs in infants and children and vertical transmission of HBV[22,23]. From these observations, we also concluded that the age of diagnosis with HCC may be influenced by the family history of HCC regardless of whether it is related to HBV infection and that infection with HBV earlier in life is not the only factor affecting the earlier development of HCC in HBV endemic regions[24-26]. This may also imply that a person with a history of HCC in any family member should pay special attention to screening for the development of HCC. Still, the effects of genetic backgrounds in these patients remain to be evaluated in the future.

The age recommendation for HCC surveillance in Asian males with HBV is over 40 years, and the recommendation for Asian females is over 50 years[3,4,16,27]. In our study, when the HBV-positive patients were grouped into younger (ages under 45 years) *vs* older (ages 45 years or more) age groups, the patients with positive family histories were diagnosed with HCC at earlier ages compared with those with negative family histories (*P* = 0.042). These results are not surprising and support the common assumption that prolonged exposure to HBV seems to be a possible explanation for a relatively earlier occurrence of HCC[21,25,26,28-32].

Another important finding in this study was the significant association between tumor staging at diagnosis and positive family history among HBV-infected patients

($P = 0.018$). Because HBV is the most common risk factor for HCC, and the vertical transmission of HBV is a medical concern in Korea (although well controlled), these findings may suggest that earlier surveillance for HCC, perhaps earlier than typically recommended, in HBV-positive patients with a positive family history of HCC may allow these patients to be diagnosed at earlier stages; this would facilitate better control of HCC and better treatment outcomes in Korean HCC patients[33-35].

Despite the current increased efforts in HBV vaccination and in prevention of vertical transmission in Korea, HBV was the most prevalent hepatitis virus in our study population, as is expected in many Asian countries. This may reflect the effect on HCC development of vertical transmission several decades ago, before vaccination and prevention were available in Korea, and future study results may differ from ours.

This single-hospital-based study has limitations. The first is the limitation on HCC patients studied, as a single hospital cannot represent the whole country; fortunately, the general characteristics of our study subjects were not much skewed compared with other reports on HCC epidemiology in Korean patients[30]. Second, the medical records describing family histories of primary liver cancer were solely dependent on each patient's memory. Also, the data collection was not performed prospectively, but by reviewing the medical records retrospectively, which may have led to some misclassification. Because this was, in part, a cohort study, we restricted the cases to those with clearly detailed medical records to minimize any misleading information. Third, it is possible that the findings may have been influenced by the surveillance program for HBV infection in Korea, which may have assisted in diagnosis at early ages and at early stages of HCC among patients with HBV infection and positive family histories. It is possible that chronic HBV carriers were diagnosed with HCC earlier than are patients without HBV infection because many of them had regular follow-ups at a liver clinic. However, at this point, because a patient would not be able to be diagnosed with HCC if he or she did not visit a doctor, this bias may be unavoidable unless the whole population was examined. Finally, the genetic characteristics of those who develop HCC must be evaluated in the future because not all patients with HBV infection are prone to developing HCC.

In conclusion, a positive family history of liver cancer may influence the age at diagnosis of HCC, and this difference was also in patients with HBV infection. Furthermore, we cautiously recommend more intensive checkups earlier in life and at shorter intervals for patients who have positive family histories of liver cancer, as this may foster detection of HCC at more treatable stages.

ACKNOWLEDGMENTS

COMMENTS

Background
Hepatocellular carcinoma (HCC), which is the fifth most common cancer and the third most common cause of cancer-related death worldwide, has some well known risk factors for its development. The three well-known causes for this devastating disease are hepatitis B virus (HBV), hepatitis C virus (HCV), and alcoholic liver disease. Recent reports showed familial clustering of this disease based on the perinanatal transmission of the HBV.

Research frontiers
Although the clustering of HBV infection among family members, which is related to vertical transmission, was reported to be the major cause associated with family histories of liver cancer and HCC, the family history of liver cancer affecting HCC development and its familial aggregation along with the possible confounding effects of important risk factors remains to be determined.

Innovations and breakthroughs
This extensive investigation revealed a significant finding that the median age at diagnosis of HCC was 5 years younger among patients with a positive family history than among those without a family history of HCC. Another important finding in this study was the significant association between tumor staging at diagnosis and positive family history in HBV-infected patients.

Applications
The study suggests that a positive family history of liver cancer may influence the age at diagnosis of HCC, and cautiously proposed that earlier surveillance for HCC, perhaps earlier than typically recommended, especially in HBV-positive patients with a positive family history of liver cancer may allow these patients to be diagnosed at earlier stages, as this may foster detection of HCC at more treatable stages.

Terminology
Familial clustering of cancers: Whether based on genetic background, environmental factors, or vertical transmission of a particular infection, familial clustering of cancers has been reported in many types of cancer, including pancreas, colon, stomach, lung and breast cancers.

Peer review
In this retrospective study, the authors have evaluated the impact of a positive family history on the development of hepatocellular carcinoma in a group of Korean patients. 7.4% of 2242 patients had a positive family history of HCC. The median age at diagnosis was significantly lower in those that had a positive family history. This result was maintained even after analyzing separately the population with non-HBV etiology. The authors conclude that a positive family history may be associated with earlier appearance of HCC, warranting a stricter surveillance.

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BRIEF ARTICLE

Risk modification of colorectal cancer susceptibility by interleukin-8 -251T>A polymorphism in Malaysians

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Abstract

AIM: To investigate the allele and genotype frequencies and associated risk of interleukin (*IL*)-8 -251T>A polymorphism on colorectal cancer (CRC) susceptibility risk.

METHODS: Peripheral blood samples of 255 normal controls and 255 clinically and histopathologically confirmed CRC patients were genotyped for *IL-8* -251T>A polymorphism employing allele-specific polymerase chain reaction. The relative association of variant allele and genotypes with CRC susceptibility risk was determined by calculating the odds ratios (ORs). Corresponding χ^2 tests on the CRC patients and controls were carried out and 95% confidence intervals (CIs) were determined using Fisher's exact test. The allele frequencies and its risk association were calculated using FAMHAP, haplotype association analysis software.

RESULTS: On comparing the frequencies of genotypes of patients and controls, the homozygous variant AA was significantly higher in CRC patients ($P = 0.002$) compared to controls. Investigation on the association of the polymorphic genotypes with CRC susceptibility risk, showed that the homozygous variant *IL-8* -251AA had a significantly increased risk with OR 3.600 (95% CI: 1.550-8.481, $P = 0.001$). In the case of allele frequencies, variant allele A of *IL-8* -251 showed a significantly increased risk of CRC predisposition with OR 1.32 (95% CI: 1.03-1.69, $P = 0.003$).

CONCLUSION: Variant allele and genotype of *IL-8* (-251 T>A) was significantly associated with CRC susceptibility risk and could be considered as a high-risk variant for CRC predisposition.

INTRODUCTION

Colorectal cancer (CRC), the incidence of which has been increasing worldwide for the past few years, represents a significant cause of morbidity and mortality. CRC develops as a result of progressive accumulation of genetic and epigenetic alterations that lead to a series of histo-

pathological changes, initiated by transition from normal mucosa to adenoma to carcinoma. Excluding inherited types of CRC, the susceptibility of a certain individual to development of sporadic CRC remains largely undetermined. Sporadic CRC is a multifactorial disease, therefore, environmental factors, host genotype and immunological factors all could significantly contribute to initiation and even progression of this malignancy.

Recently, chronic inflammation has been linked to increased risk of various types of cancer[1,2]. Epidemiological observations, animal models and clinical studies have established an association between continuous inflammatory conditions and CRC[1,3,4]. Patients with inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, are at increased risk of developing CRC[5]. The associations between inflammatory response genes and IBD make them attractive candidate susceptibility genes for CRC because approximately 1:6 individuals with IBD are estimated to develop colorectal malignancy[6]. Despite this evidence strongly implicating chronic inflammation as a culprit in colorectal carcinogenesis, surprisingly little research has directly addressed the genetic predisposing factors that mediate inflammatory response and favor CRC development.

Genetic polymorphisms have emerged in recent years as important determinants of disease susceptibility and severity. Polymorphic variants of several genes are thought to play a key role in determining how individuals respond at the cellular level to various environment conditions including inflammation. If inflammation constitutes one of the molecular networks underlying susceptibility to CRC, genes that mediate inflammatory responses might be a group of candidate genes for CRC predisposition. Few genes are known to be important for inflammation of the colorectum, and their allelic variants have been shown to have biological effects[7,8]. Interleukin (*IL*)-8 is a chemokine and one of the major mediators of inflammatory responses, and is believed to play a role in the pathogenesis of cancer. Several polymorphisms have been detected in the *IL-8* gene, and a common polymorphism at the -251 position (251T>A) of the promoter region has been associated with transcriptional activity of the gene. A case-control study was designed to investigate the *IL-8* -251T>A polymorphic allele and genotype frequencies in healthy controls and sporadic CRC patients in the Malaysian population, and to determine the influence of the polymorphic genotype of *IL-8* -251T>A on sporadic CRC susceptibility risk.

MATERIALS AND METHODS

Recruitment of subjects

The study was approved by the Research Review Board and Ethics Committee of Universiti Sains Malaysia and Ministry of Health (MOH) Malaysia. In this case-control study, cases comprised 255 CRC patients (139 male and 116 female), recruited from Hospital Universiti Sains Malaysia, and from a few hospitals under the MOH, Malay-

Table 1 Distribution of sex and age in cases and controls

	Patients	Controls
Sex, *n* (%)		
Female	116 (45.5)	140 (54.9)
Male	139 (54.5)	115 (45.1)
Age (mean ± SD)	57.26 ± 7.074	48.91 ± 12.020

sia like Hospital Sultanah Bahiyah, Alor Setar, Kedah and Hospital Raja Perempuan Zainab Ⅱ, Kota Bharu, Kelantan, Malaysia. An equal number of sex- and age-matched (± 5-10 years) normal healthy individuals (115 male and 140 female) were also recruited as controls. The ages of the patients ranged from 27 to 77 years with a mean age of 57.26 years. For the controls, the age ranged from 33 to 78 years with a mean age of 48.91 years. The sex and age distribution of the study subjects are shown in Table 1.

DNA extraction

Peripheral blood samples of 255 normal controls and 255 clinically diagnosed and histopathologically confirmed CRC patients were collected in EDTA tubes, after obtaining written informed consent. The collected samples were stored at -20 ℃ till use. Genomic DNA was extracted using commercial DNA extraction kit (QIAGEN, Hilden, Germany) and the gene of interest was amplified using appropriate primers. Single nucleotide polymorphism -251 T>A in the *IL-8* gene was determined using allele-specific polymerase chain reaction (PCR).

Genotyping

For genotyping, the allele-specific primers used were 5' -CCACAATTTGGTGAATTATCAAT-3' and (251A) or 5'-TGCCCCTTCACTCTGTTAAC-3' (251T). The consensus primer used was 5'-TGCCCCTTCACTCTGT-TAAC-3' (giving a PCR product of 366 bp). The *IL-8* -251T>A polymorphic sequence was amplified using PCR with composition master mix using 100 ng DNA template, primer (0.2 μmol/L), 2.0 mmol/L MgCl2, 10 × buffer, 10 mmol dNTP (0.2 μL) and 5 U *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, United States) with a total volume of 25 μL PCR mixture. The annealing temperature was 56.7 ℃, and 35 PCR cycles were carried out. The PCR products were isolated on 2% agarose gels and visualized with SYBR Green. The *IL-8* -251T>A polymorphic genotypes were categorized into homozygous wild, heterozygous and homozygous variant.

Statistical analysis

The difference in various genotype frequencies of *IL-8* among the cases and controls was calculated. The relative associations of various genotypes with CRC susceptibility risk was determined by calculating the odds ratios (ORs). Corresponding χ^2 tests on the CRC patients and controls were carried out and 95% confidence intervals (CIs) were determined using Fisher's exact test. Statistical analysis was carried out using SPSS version 18. The allele frequen-

Figure 1 Various interleukin-8 -251T>A genotype patterns in sporadic colorectal cancer patients observed after genotyping using allele-specific polymerase chain reaction. The genotype was considered as homozygous wild type [interleukin (*IL*)-8 -251TT] when only one band appeared at the T allele. When two bands appeared, each at the A allele and T allele, it was considered as heterozygous variant (*IL-8* -251TA). The homozygous variant genotype was characterized by the appearance of only one band at the A allele. M: DNA ladder; 1: Homozygous wild type (*IL-8* -251TT); 2: Heterozygous (*IL-8* -251TA); 3 and 4: Homozygous variant (*IL-8* -251AA).

cies and risk associations were calculated using FAMHAP, haplotype association analysis software to derive ORs.

RESULTS

Visualization of PCR product of IL-8 -251T>A

Various *IL-8* -251T>A genotype patterns in CRC patients in the Malaysian population are showed in Figure 1. A sample was considered as homozygous wild type (*IL-8* -251TT) when only one band appeared at the T allele. When two bands appeared at both the T allele and A allele, it was considered as heterozygous variant (*IL-8* -251TA). For the homozygous variant genotype (*IL-8* -251AA), only one band appeared at the A allele.

Frequencies of IL-8 -251T>A genotypes in CRC cases and controls

The frequencies of *IL-8* -251T>A genotypes in cases and controls are shown in Table 2. Among the 255 controls, the homozygous wild TT genotype was observed in 54 (21.18%), the heterozygous TA genotype was observed in 189 (74.12%) and the homozygous variant genotype AA was detected in 12 (4.7%) individuals. In the case of 255 CRC patients, 40 (15.69%) showed homozygous wild-type TT genotype (15.69% *vs* 21.18%, *P* = 0.11), 183 (71.76%) showed heterozygous variant TA genotype (71.76% *vs* 74.12%, *P* = 0.55), and 32 (12.55%) showed homozygous variant AA genotype. On comparing the frequencies of the polymorphic genotypes among the cases and controls, the homozygous variant genotype frequency was significantly higher among CRC cases (12.55% *vs* 4.7%, *P* = 0.002).

Association risk of IL-8 -251T>A genotypes with CRC susceptibility

Table 2 shows the associated risk of *IL-8* -251T>A genotypes with CRC susceptibility in this population. When the association of the polymorphic genotypes with CRC susceptibility risk was investigated, the *IL-8* homozygous variant genotype (AA) showed significantly increased risk with OR 3.600 (95% CI: 1.550-8.481, *P* = 0.001).

	Patients	Controls	OR (95% CI)	*P* value
IL-8 genotype				
Wild type -251TT	40 (15.69)	54 (21.18)	[1] (Ref.)	-
Hetero -251TA	183 (71.76)	189 (74.12)	1.307 (0.808-2.117)	0.250
Variant -251AA	32 (12.55)	12 (4.7)	3.600 (1.550-8.481)	0.001
Allele				
Wild type	263 (51.6)	297 (58.2)	0.76 (0.59-0.97)	-
Variant	247 (48.4)	213 (41.8)	1.32 (1.03-1.69)	0.003

Table 2 Association risk and frequencies of T and A alleles of interleukin-8 -251T>A genotypes with colorectal cancer susceptibility

IL-8: Interleukin-8; OR: Odd ratio; CI: Confidence intervals.

Allele frequencies and risk association of inflammation response genes with CRC susceptibility

The allele frequencies and risk association of T and A alleles in cases and controls in the Malaysian population are showed in Table 2. The frequency of wild-type allele T was 51.6% and the frequency of variant allele A was 48.4% among the CRC cases. In the case of controls, the frequency of the wild-type allele T was 58.2% and 41.8% showed variant allele A. When the risk association of variant allele was examined, variant allele A of *IL-8* showed a significantly higher risk with OR 1.32 (95% CI: 1.03-1.69, *P* = 0.003).

The patient group included patients from different parts of Malaysia and so the clinicopathological features of many of these patients could not be collected. Therefore, these details could not be specified. For the same reason, the association of genotype frequencies with patient prognostic subgroups could not be evaluated.

DISCUSSION

Inflammation, which is part of the immune response, may also induce or exaggerate some diseases through production of proinflammatory cytokines. Inflammatory cytokines are major inducers of chemokines that play a central role in leukocyte recruitment to sites of inflammation. Chemokines have pleiotropic biological effects that can play several roles in cancer progression, including angiogenesis, inflammation, cell recruitment and migration. *IL-8* or chemokine CXC ligand 8 is the prototype member of the CXC chemokine family. Evidence has shown that the individual level of cytokine production is affected by single nucleotide polymorphisms in cytokine genes, and the observed differences in cytokine production among individuals can be at least partially explained by gene polymorphisms. Genetic polymorphisms might directly influence inter-individually in the magnitude of inflammatory response, and this might contribute to an individual's ultimate clinical outcome. Genetic polymorphisms of cytokine genes have been identified to play a role in susceptibility to various diseases including cancer[9]. A common polymorphism in the -251 position (251T>A) of the promoter region of *IL-8* has been identified.

We investigated the frequencies and potential risk mo-

dification of *IL-8* -251T>A polymorphic genotype and allele on CRC susceptibility in the Malaysian population. Compared to controls, the prevalence of homozygous variant AA genotype was significantly higher in CRC patients (4.70% *vs* 12.55%, *P* = 0.002), whereas for the homozygous wild-type genotypes (TT) and heterozygous variant genotypes (TA), there was no significant difference in frequencies between the two groups. In a study by Yang *et al*[10], on the association of *IL-8* -251T>A polymorphism with prostate cancer, there was no significant difference in the distribution of *IL-8* polymorphic genotypes between prostate cancer cases and controls.

It has been suggested that *IL-8* and its receptors are crucial to the development and progression of many malignancies[11]. Genetic polymorphisms of the *IL-8* gene have been implicated in the susceptibility to a range of cancers including oral cancer[12], breast carcinoma[13] and gastric cancer[14]. Our interest was to investigate whether the genetic variants are related to the CRC risk in the Malaysian population. Our results showed that the polymorphism in the *IL-8* gene was significantly associated with the risk of CRC. The -251AA genotype was associated with a significantly increased risk of CRC as compared with the -251TT genotype (OR: 3.600, 95% CI: 1.550-8.481, *P* = 0.001). Similarly, for allele frequencies, such an association was observed. When compared with wild-type allele T of SNP *IL-8* -251, the variant allele A *IL-8* -251 showed significantly increased risk for CRC predisposition with OR 1.32 (95% CI: 1.03-1.69, *P* = 0.003). The strong association that we observed in CRC patients prompts us to suggest that *IL-8* gene -251AA polymorphism could contribute significantly to CRC susceptibility.

A few other molecular genetic epidemiological studies in diverse ethnic populations have found consistent as well as inconsistent results with ours. The *IL-8* -251T>A polymorphism has been associated with the risk of gastric cancer and gastric ulcer in Japanese patients with *Helicobacter pylori* infection[15]. Taguchi *et al*[14] also have reported that *IL-8* -251T>A polymorphism is associated with higher expression of *IL-8* protein, more severe neutrophil infiltration, and increased risk of atrophic gastritis and gastric cancer in the Japanese population. In the study of Gunter *et al*[16], homozygous variant genotype of the *IL-8* -251T>A had a 2.7-fold increased risk of colorectal adenoma compared to the homozygous wild type in the American population. In the French population, Küry *et al*[17] have reported that the heterozygous and homozygous variants of *IL-8* c -352 T>A are associated with an elevated risk of CRC compared to the homozygous major genotype. Li *et al*[18] have found that the risk of gastric cancer in the Chinese population is significantly elevated in patients with the *IL-8* -251AA genotype with OR 2.02 (95% CI: 1.27-3.21). In contrast with our study, Landi *et al*[19] have reported that the *IL-8* -251T>A genotypes have a protective role against CRC predisposition in the Spanish population, and Theoropoulus has reported that this SNP has no effect on CRC

susceptibility risk in the Greek population[8]. A few other studies have suggested that the *IL-8* -251AA genotype is associated with an increased risk of prostate cancer in the Caucasian population[20] and Kaposi's sarcoma in the Dutch population[21], but a decreased risk of CRC[4].

The 251T>A polymorphism at the promoter region of *IL-8* gene is associated with the transcriptional activity of the gene[22], and to influence production and expression of *IL-8*[14,15]. Disrupted gene expression or altered protein formation of the *IL-8* gene may contribute positively or negatively to the establishment or progression of CRC. According to Wei *et al*[22], higher promoter activity of *IL-8* -251AA polymorphism might increase production and expression of *IL-8*, inducing a Th1-predominant immune response and leading to more susceptibility to CRC.

Free radicals generated as a result of oxidative stress produced by inflamed tissues may cause alteration in many metabolic reactions such as regulation of DNA, RNA and lipids, and thus can lead to cancer development[23,24]. Meira *et al*[25] have demonstrated that in inflamed colon cancer tissues, reactive oxygen species (ROS) and reactive nitrogen species are produced from activated inflammatory cells, and that these two species enhance DNA damage and this can cause mutation in areas of inflammation. Moreover, in CRC-associated colitis, chronic inflammation has been reported to cause oxidative damage to DNA, influence mutations in the *p53* gene in the inflamed tissues, and drive the cells to malignant transformation[26]. Oxidative stress produced by inflammatory cells in inflamed tissues in the intestinal tract has been reported to influence the development of CRC in patients with IBD[27]. These studies have clearly highlighted the importance of *IL-8* in modulating inflammation of the colorectum. According to Okada *et al*[28], ROS produced by inflammatory cells cause not only direct DNA damage but also indirect effects such as dysregulation of cell proliferation and apoptosis, stimulation of angiogenesis, and modification of gene/protein expression and protein activities that will cause cancer. Therefore, the relevance of *IL-8* -251T>A polymorphism in CRC susceptibility could be explained by the enhanced transcriptional activity of the gene, resulting in functional alteration of the gene product.

To the best of our knowledge, this is the first study on the association of the *IL-8* gene -251 T>A polymorphism and CRC risk in the Malaysian population. Our results show that the genetic variation of *IL-8* gene influences susceptibility to CRC in the Malaysian population, and suggest inflammation-mediated pathways in the process of colorectal carcinogenesis.

COMMENTS

Background

Colorectal cancer (CRC), the incidence of which has been increasing worldwide for the past few years, represents a significant cause of morbidity and mortality. Genetics has a key role in predisposition to CRC and in its initiation and progression. Identifying predisposing genetic variations is important for our understanding of the carcinogenic process. Recently, chronic inflammation has been linked to increased risk to various types of cancer including CRC. Despite evidence strongly implicating chronic inflammation as a culprit in colorectal carcinogenesis, surprisingly little research has directly addressed the genetic predisposing factors that mediate inflammatory response and favor CRC development. Thus, it was of interest to explore the contribution of single nucleotide polymorphisms (SNPs) in inflammation genes as predisposing factors for CRC susceptibility.

Research frontiers

If inflammation constitutes one of the molecular networks underlying susceptibility to CRC, genes that mediate inflammatory response might be a group of candidate genes for CRC predisposition. Interleukin (IL)-8 is a chemokine and one of the major mediators of inflammatory responses, and is believed to play a role in the pathogenesis of cancer. IL-8 is a proangiogenic cytokine that is overexpressed in many human cancers and its expression promotes tumor growth, angiogenesis and metastasis. A polymorphism at -251 position (251T>A) of the promoter region of IL-8 has been associated with transcriptional activity of the gene. This study was designed to determine the frequencies and influence of the *IL-8* -251T>A polymorphic genotype and alleles on sporadic CRC susceptibility risk in the Malaysian population.

Innovations and breakthroughs

There are not many data available on the contribution of SNPs in inflammation response genes in mediating CRC predisposition risk, especially from the Asian population, and none from the Malaysian population. This is believed to be the first report of an association of genetic variation of *IL-8* with CRC susceptibility risk in the Malaysian population. We observed that the genetic diversity of the *IL-8* gene influences patient susceptibility to CRC and implies the importance of inflammation-mediated pathways in the process of colorectal carcinogenesis. From our results, it is reasonable to pronounce that *IL-8* gene may be an important candidate in the modulation of colorectal inflammation and the *IL-8* 251AA (homozygous variant genotype) could be considered as an important high-risk variant for CRC predisposition in the Malaysian population.

Applications

Early diagnosis is important for successful management of CRC patients and is facilitated by both invasive and noninvasive means of surveillance. Identification of genetic predisposition factors of CRC will help with better understanding of the colorectal carcinogenic process and in the design of diagnostic, therapeutic and preventive strategies. Understanding the genes and pathways that control the earliest steps of the disease and individual susceptibility can contribute to clinical management in the near future. In the future, study can be extended to a population level and individuals with high-risk predisposition genotypes can be identified. Once identified, they can be enrolled in cancer surveillance programs that will help in CRC prevention strategies.

Peer review

In this case-control study to determine the influence of the polymorphic genotype on sporadic CRC susceptibility risk in the Malaysian population, the *IL-8* -251T>A polymorphic allele and genotype frequencies were evaluated in 255 patients with sporadic CRC and 255 normal healthy controls. The frequency of the homozygous variant AA was significantly higher in CRC patients compared to controls. Furthermore, the homozygous variant *IL-8* -251AA was significantly associated with increased risk of CRC predisposition. This study is of interest because previous studies on the relationships between *IL-8* -251T>A polymorphism and CRC have yielded contradictory results.

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BRIEF ARTICLE

Eradication of *Helicobacter pylori* increases childhood growth and serum acylated ghrelin levels

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Abstract

AIM: To determine whether *Helicobacter pylori* (*H. pylori*)-infected children have reduced body weight (BW) and height (BH) growth, and if *H. pylori* eradication may restore growth while improving serum acylated ghrelin.

METHODS: This longitudinal cohort study with one-year follow-up enrolled 1222 children aged 4 to 12 years old into an observation cohort (18 with and 318 without *H. pylori*) and intervention cohort (75 with and 811 without). The 7-d triple therapy was used for eradication in the intervention cohort. The net increases of BW and BH as well serum acylated ghrelin after one-year follow-up were compared between successful eradicated *H. pylori*-infected children and controls.

RESULTS: In the observation cohort, the *H. pylori*-infected children had lower z score of BW (-1.11 ± 0.47 *vs* 0.35 ± 0.69, *P* = 0.01) and body mass index (BMI) (0.06 ± 0.45 *vs* 0.44 ± 0.73, *P* = 0.02) at enrollment and lower net BW gain after one-year follow-up (3.3 ± 2.1 kg *vs* 4.5 ± 2.4 kg, *P* = 0.04) than the non-infected controls. In the intervention cohort, the *H. pylori*-infected children had lower z score of BMI (0.25 ± 1.09 *vs* 0.68 ± 0.87, *P* = 0.009) and serum acylated ghrelin levels (41.8 ± 35.6 pg/mL *vs* 83.6 ± 24.2 pg/mL, *P* < 0.001) than the non-infected controls. In addition to restoring decreased serum ghrelin levels (87.7 ± 38.0 pg/mL *vs* 44.2 ± 39.0 pg/mL, *P* < 0.001), the *H. pylori*-infected children with successful eradication had higher net gains (*P* < 0.05) and increase of z scores (*P* < 0.05) of both BW and BH as compared with non-infected controls after one-year follow-up.

CONCLUSION: *H. pylori*-infected children are associated with low serum acylated ghrelin and growth retardation. Successful eradication of *H. pylori* restores ghrelin levels and increases growth in children.

Yang YJ, Sheu BS, Yang HB, Lu CC, Chuang CC. Eradication of *Helicobacter pylori* increases childhood growth and serum acylat-

INTRODUCTION

Primary infection with *Helicobacter pylori* (*H. pylori*) usually occurs during childhood[1]. This organism has been proven to cause chronic gastritis, peptic ulcer diseases, and had a high correlation with gastric cancer in humans[2,3]. In children, the *H. pylori* prevalence rate was relatively lower than adults[4,5]. Besides the link with gastric diseases, the association between *H. pylori* infection and growth retardation in children has raised clinical attention to this issue and caused some debate recently. Some cross-sectional analyses have indicated that *H. pylori*-infected children had subnormal growth retardation as compared with non-infected children[6-8], but some others did not support such findings[9,10]. Long-term observational studies have reported that children with persistent *H. pylori* infection have reduced body weight (BW) and height (BH) growth than the non-infected peers[11-13]. Therefore, to further support the causal relationship between *H. pylori* infection and growth retardation in children, interventional trials involving *H. pylori* eradication may provide new insights using a rigorous study design.

Ghrelin, a growth-hormone-releasing peptide biosynthesized mainly in the fundic mucosa, regulates appetite and body composition and is affected by inflammatory and atrophic events associated with *H. pylori* infection[14,15]. Previous studies showed conflicting results regarding the correlation between plasma ghrelin levels and *H. pylori* infection after eradication of bacteria[16-18]. This controversy may be caused by the measurement of total plasma ghrelin, which contains both acylated and desacylated forms. Acylated ghrelin is a more potent agonist on the growth-hormone-stimulating receptor than the desacylated form and undergoes a compensatory elevation in patients with chronic atrophic gastritis[19-21]. This study seeks to examine active ghrelin levels and its relationship with growth in patients before and after *H. pylori* eradication.

Although eradication of *H. pylori* can restore body mass index (BMI) and serum albumin in adult patients with infection[22,23], such improvement has not yet been documented in *H. pylori*-infected children. Moreover, it is unclear whether the improving growth parameters after *H. pylori* eradication are subsequently linked to increase serum acylated ghrelin levels. Therefore, this study sought to examine whether *H. pylori* eradication improves BW and BH growth in children in parallel with increases in serum acylated ghrelin levels.

MATERIALS AND METHODS

Subject enrollments in the two cohorts

This study enrolled 1292 students, aged 4 to 12 years old from three elementary schools and their associated pre-

school kindergartens in Tainan City, Taiwan. The participants were consecutively enrolled into two study cohorts. Each participant provided informed consent documentation that was signed by her/his parents.

The first cohort (observation cohort) enrolled 400 children in 2005 to screen for the *H. pylori* infection, and they were then scheduled to return for follow-up growth status by a half-year interval of up to one year. The second group was an interventional cohort which enrolled 892 children in 2006 to screen for the *H. pylori* infection. Moreover, the *H. pylori*-infected subjects were invited to receive one-week of triple therapy for *H. pylori* eradication. As well, the children in the 2nd cohort were scheduled to return for follow-up growth status by a half-year interval of up to one year.

In each cohort, both the enrolled children and their parents were reviewed with a questionnaire to record data on underling medical diseases, *H. pylori* infection status, and a range of demographic variables, including socio-economic status, such as number of family members[8], and annual household income (low income indicated less than $15 000 US/year). The same nursing assistant provided the introduction of questionnaire to the enrolled subjects. Children with pre-established and severe medical/organic conditions predisposing to the failure of thrive, such as genetic/metabolic disorders and cyanotic congenital heart diseases, were not included. The study also excluded children with a known past history to receive anti-*H. pylori* therapy and children underwent eradication therapy or acid suppressors, during the follow-up period in the observation group. In both groups, the control cases were randomly selected (1:4 in the observation and 1:3 in the interventional cohorts) and were matched by age and gender to children with ^{13}C-labeled urea breath test (^{13}C-UBT)-confirmed *H. pylori* infection. Moreover, for the *H. pylori*-infected (confirmed by a positive ^{13}C-UBT) children at entry, the *H. pylori* status was assessed with a ^{13}C-UBT after 6 mo (intervention cohort) and one year follow-up (both cohort).

BMI and z scores of weight, height and BMI

For each participant, the overnight fasting BW and BH were serially measured at enrollment and at the follow-up period on the 6th mo and the 12th mo, respectively. The BMI was defined as BW in kilograms/squared of body length in meter (kg/m^2). The z scores (SD scores) of BW, BH and BMI were calculated using the reference population of 2003 Taiwanese boys and girls based on health-related physical fitness and based on 2006 World Health Organization standards[24]. The net changes of BW, BH and BMI were calculated by the value of each parameter at follow-up minus the corresponding value at enrollment. We also defined the increase of z score means that z scores of BW, BH and BMI were upgrade at the one-year follow-up than at the enrollment (the net change > 0).

Serological screening of H. pylori infection and confirmation by urea breath test

In each enrolled child, the serum was tested for anti-*H. pylori*

Figure 1 The study flow diagram and the case numbers were listed for the different study follow-up periods. *H. pylori*: *Helicobacter pylori*; [13C]-UBT: [13C]-labeled urea breath test.

IgG antibodies (HEL-p TEST™ Ⅱ, AMRAD Bio-tech, Australia) by enzyme-linked immunosorbent assay (ELISA) methods. The serologic kit has been validated with a favorable sensitivity and specificity (> 90%) in detecting *H. pylori* infection in our previous studies[25]. The seropositive children further confirmed by [13C]-UBT to diagnose ongoing *H. pylori* infection[8]. The cut-off value of positive [13C]-UBT was defined as excess $^{13}CO_2/^{12}CO_2$ ratio more than 3.5‰[8,26].

Eradication therapy for H. pylori-infected children
For the *H. pylori*-infected children in the intervention cohort, lansoprazole (1 mg/kg per day, max. 30 mg bid), amoxicillin (50 mg/kg per day, max. 1 g bid), and clar-ithromycin (15 mg/kg per day, max. 500 mg bid) were prescribed for one week[26]. We have educated the partici-pants and their parents for the compliance and report of complications. Successful eradication therapy was defined by a negative result of [13C]-UBT on both the 6th and the 12th mo follow-up, respectively[27].

Serial serum acylated ghrelin levels before and after H. pylori eradications
The serum acylated ghrelin levels of the interventional cohorts at enrollment were compared between children with and without *H. pylori* infection. In addition, the serial serum acylated ghrelin levels of the children with *H. pylori* eradication collected at enrollment, the 6th mo, and the 12th mo follow-up were compared. Each blood sample of child was collected in the morning before breakfast and was incubated in the ice-bath container immediately. The sera were separated by centrifugation within 2-3 h and were stored in a -80 ℃ refrigerator until use. These samples' serum acylated ghrelin levels were analyzed in duplicate by a commercial kit (LINCO Research, St. Charles, Missouri, United States), using ELISA methods.

Statistical analysis
The χ^2 test with the odds ratio (OR) and 95% conference interval (CI) and logistic regression test were applied as an estimate of the possibly related factors between *H. pylori*-infected and non-infected children. The Student's *t* test and one-way analysis of variance with least significant dif-ference test correction were used as appropriate to com-pare the differences of ghrelin, BW, BH, BMI and their net changes during one-year follow-up periods among different study groups. The paired *t* test was used to ana-lyze the difference of the serial serum acylated ghrelin levels before and after eradication therapy within the same study group. A *P* value less than 0.05 was consid-ered statistically significant.

RESULTS
Participants and H. pylori infection
There were 84% (336/400) children in the observation and 99% (886/892) children in the intervention cohorts who completed the questionnaires and provided their sera for the anti-*H. pylori* IgG antibodies tested, respec-tively. In Figure 1, the case numbers of each cohort were serially summarized during the one-year follow-up. One hundred and twenty-one (27 in the observation cohort, 94 in the intervention cohort) were defined with sero-positivity of *H. pylori* infection. Among them, 113 chil-dren received [13C]-UBT, of which only 93 (82%) children were positive (18 in the observation cohort and 75 in the intervention cohort). Accordingly, the overall *H. pylori* prevalence was 7.6% in these two cohorts.

For the 18 *H. pylori*-positive children in the observation cohort, the infection was persisted with a positive [13C]-UBT until the end of follow-up on the 1st year. Among the 75 *H. pylori*-infected children in the intervention co-hort, 57 children were enrolled to receive the 7-d eradica-

Table 1 Comparison of demographic background, baseline body parameters, and the changes of growth after one-year follow-up between the *Helicobacter pylori*-infected and non-infected matched controls of the observation cohort (mean ± SD)

Groups	H. pylori-positive subjects	H. pylori-negative controls	P value
n	18	18	
Age (yr)	9.1 ± 1.6	9.4 ± 1.7	0.57
Sex (female:male)	8:10	27:45:00	0.59
Body weight (kg)			
At enrollment	29.9 ± 7.0	35.0 ± 10.1	0.02
z score at enrollment	-1.11 ± 0.47	0.35 ± 0.69	0.01
The 1st year	33.2 ± 7.5	39.5 ± 11.3	0.007
z score at the 1st year	-0.17 ± 0.45	0.38 ± 0.67	0.002
Net change	3.3 ± 2.1	4.5 ± 2.4	0.04
Body height (cm)			
At enrollment	132.3 ± 11.4	136.8 ± 12.3	0.15
z score at enrollment	-0.17 ± 0.49	0.15 ± 0.73	0.07
The 1st year	138.0 ± 11.4	142.5 ± 12.4	0.15
z score at the 1st year	-0.14 ± 0.48	0.13 ± 0.71	0.1
Net change	5.7 ± 0.9	5.8 ± 1.9	0.81
BMI (kg/m^2)			
At enrollment	16.8 ± 1.6	18.4 ± 2.8	0.03
z score at enrollment	0.06 ± 0.45	0.44 ± 0.73	0.02
The 1st year	17.2 ± 1.7	19.0 ± 3.0	0.01
z score at the 1st year	0 ± 0.38	0.45 ± 0.75	0.01
Net change	0.35 ± 0.8	0.69 ± 1.0	0.14

BMI: Body mass index; *H. Pylori*: *Helicobacter pylori*; Net change: The growth parameters at the 1st year minus that at enrollment.

Table 2 Comparison of the demographic background, baseline body parameters, and the changes of growth among the different groups of the intervention cohort completed the one-year follow-up (mean ± SD)

Groups	H. pylori eradication failure	H. pylori eradication success	H. pylori-negative controls
n	17	34	153
Age (yr)	8.4 ± 1.9	8.7 ± 2.0	9.0 ± 1.7
Sex (female:male)	10:07	19:15	81 : 72
Family peptic ulcer history (%)	44.4	29.4	21
Intra-familial members ≥ 5 (%)	33.3	29.4	32.1
Low income (%)	28.6	53.3	44.1
Baseline serum acylated ghrelin (pg/mL)[a,c]	37.2 ± 31.4	44.2 ± 37.9	83.6 ± 24.2
Body weight (kg)			
At enrollment	32.2 ± 10.6	32.9 ± 11.4	36.1 ± 11.4
z score at enrollment	0.44 ± 1.00	0.35 ± 1.04	0.60 ± 0.87
The 1st year	37.2 ± 11.7	38.7 ± 13.8	41.0 ± 12.6
z score at the 1st year	0.56 ± 0.88	0.49 ± 1.03	0.61 ± 0.88
Net change[c]	5.03 ± 2.77	5.84 ± 3.37	4.84 ± 2.35
Increase of z score (%)[c]	23.5	38.2	17.6
Body height (cm)			
At enrollment	134.6 ± 12.7	134.3 ± 11.7	135.7 ± 12.5
z score at enrollment	0.62 ± 0.99	0.44 ± 0.88	0.33 ± 0.77
The 1st year	141.8 ± 11.8	142.3 ± 12.8	141.5 ± 13.0
z score at the 1st year[a,c]	0.77 ± 0.94	0.63 ± 0.75	0.32 ± 0.79
Net change[c,e]	7.20 ± 2.85	8.00 ± 2.78	5.85 ± 1.81
Increase of z score (%)[c]	35.3	35.3	15.7
BMI (kg/m^2)			
At enrollment[a]	17.1 ± 2.6	17.7 ± 4.1	19.0 ± 3.7
z score at enrollment[c]	0.24 ± 0.75	0.21 ± 1.14	0.68 ± 0.87
The 1st year	18.1 ± 3.1	18.8 ± 4.7	20.0 ± 3.8
z score at the 1st year[c]	0.35 ± 0.82	0.27 ± 1.12	0.67 ± 0.87
Net change	0.98 ± 1.57	1.10 ± 1.56	0.99 ± 1.03
Increase of z score (%)	35.3	32.4	17

Low income: Indicated < $15000 US/year. Increase of z score means that z scores of body weight, height and body mass index (BMI) were upgrade at the one-year follow-up than at the enrollment (the net change > 0). The difference of the body weight, height, BMI and ghrelin level among the three groups were analyzed by oneway analysis of variance model with least significant difference correction. The difference of the up-shift of the z scores of body weight, height and BMI were analyzed by χ^2 test. [a]*P* < 0.05 between *H. pylori*-positive subjects with eradication failure and controls, [c]*P* < 0.05 between *H. pylori*-positive subjects with eradication success and controls.

tion therapy. A total of 6 subjects withdrew or were lost to follow-up. All of the finally eligible subjects had good drugs compliance (taking drugs at least 6 d) and none had major adverse complications. The intention-to-treat and per-protocol eradication rate of *H. pylori*-infected children were 60% (34/57) and 67% (34/51), respectively.

A lower BW, HW and BMI in the H. pylori infected subject
In the observation cohort, there were 72 age- and gender-matched subjects selected to serve as *H. pylori*-negative controls. In Table 1, the *H. pylori*-infected children had lower BW (29.9 ± 7.0 kg *vs* 35 ± 10.1 kg, *P* = 0.02), z score of BW (-1.11 ± 0.47 *vs* 0.35 ± 0.69, *P* = 0.01), BMI (16.8 ± 1.6 kg/m^2 *vs* 18.4 ± 2.8 kg/m^2, *P* = 0.03) and z score of BMI (0.06 ± 0.45 *vs* 0.44 ± 0.73, *P* = 0.02) than those non-infected controls. Moreover, after one-year follow-up, the *H. pylori*-infected children had a significantly lower BW (33.2 ± 7.5 kg *vs* 39.5 ± 11.3 kg, *P* = 0.007), z score of BW (-0.17 ± 0.45 *vs* 0.38 ± 0.67, *P* = 0.002), BMI (17.2 ± 1.7 kg/m^2 *vs* 19 ± 3.0 kg/m^2, *P* = 0.01), and z score of BMI (0 ± 0.38 *vs* 0.45 ± 0.75, *P* = 0.01) than the non-infected ones. Also in Table 1, there was a significantly lower net BW gain in the *H. pylori*-infected children than that in the non-infected controls (3.3 ± 2.1 kg *vs* 4.5 ± 2.4 kg, *P* = 0.04) after one-year follow-up. However, the net changes of BH, and BMI were not different between the children with and without *H. pylori* infections (*P* > 0.05).

In the intervention cohort (57 *H. pylori*-infected and 153 controls), children with *H. pylori* infection had significantly lower BMI (17.7 ± 3.8 kg/m^2 *vs* 19.0 ± 3.7 kg/m^2, *P* = 0.02) and z score of BMI (0.25 ± 1.09 *vs* 0.68 ± 0.87, *P* = 0.009) than controls at the enrollment. In Table 2, there was no difference with regards to patients' demographic background among the eradication failure, eradication success, and control groups at enrollment. In comparison to the observation cohort, the z score of BMI at enrollment was significantly lower in successful eradication group (0.21 ± 1.14 *vs* 0.68 ± 0.87, *P* = 0.007) than in the non-infected controls. The baseline BW and BH were still lower in the *H. pylori*-infected children (either with eradication success or failure) than in controls, although it is not statistically significant.

Successful H. pylori eradication improves body growth of children within one year
There were 34 children with successful *H. pylori* eradica-

A

B

Figure 2 Comparison of the serum acylated ghrelin levels (mean) at enrollment, at 6th mo and at 12th mo follow-ups between the two groups of *Helicobacter pylori*-infected children with success (A) and with failure (B) of eradication therapy. The significance was analyzed by paired *t* test.

tion and 17 children with failure of who completed the one-year follow-up study. Moreover, we completed the one-year follow-up to the 153 age- and sex-matched non-infected controls. One-year after eradication therapy, the *H. pylori*-infected children with successful eradication had significantly higher net increases of BW (5.84 ± 3.37 kg *vs* 4.84 ± 2.85 kg, *P* = 0.04) and BH (8.00 ± 2.78 cm *vs* 5.85 ± 1.81 cm, *P* < 0.001) than the *H. pylori*-negative controls. Moreover, the rates of increase of z scores of BW (38.2% *vs* 17.6%, *P* = 0.02) and BH (35.3% *vs* 15.7%, *P* = 0.02) were significantly higher in children with successful eradication than controls. We further analyzed the enrolled age as a confounder for the increase of z scores of BW and BH by multiple logistic regression analysis. The results confirmed the successful eradication of *H. pylori* was an independent factor to predict the increase of BW (*P* = 0.01) and BH (*P* = 0.01) in the intervention cohort. Although triple therapy failed to achieve successful eradication, these *H. pylori*-infected children still had a higher net increase of BH (7.20 ± 2.85 cm *vs* 5.85 ± 1.81 cm, *P* = 0.01) than the non-infected controls. However, these *H. pylori*-infected children and non-infected controls had no difference in the net increase of BW (5.03 ± 2.77 kg *vs*

4.84 ± 2.35 kg, *P* = 0.78) and increase of z scores of BW (23.5% *vs* 17.6%, *P* = 0.79) during one-year follow-up.

Lower serum acylated ghrelin correlated to *H. pylori* infection and lower BW

In the intervention cohort, the *H. pylori*-infected children had a significantly lower serum acylated ghrelin level (41.8 ± 35.6 pg/mL *vs* 83.6 ± 24.2 pg/mL, *P* < 0.001) than the controls. In Table 2, at enrollment, both the *H. pylori*-infected children with and without eradication success during follow-up had lower serum acylated ghrelin levels than the non-infected controls (both *P* < 0.001). As the *H. pylori*-infected children exhibited lower BW and lower serum acylated ghrelin, the study further tested whether the children with lower BW, within the age ranges, could have a lower serum acylated ghrelin. We compared the serum acylated ghrelin levels of the children between those with BW and z score of BW above and below the selected cut-off point in the different age ranges (Table 3). Only in the *H. pylori*-infected children with age ranges as 8-12 years, the serum acylated ghrelin level was lower in the children with BW below the cut-off point than that with BW above (23.8 ± 22.1 pg/mL *vs* 51.8 ± 40.9 pg/mL, *P* = 0.02).

Improvement of the serum acylated ghrelin level after eradication therapy

In addition to the baseline serum acylated ghrelin levels, the study subjects of the intervention cohort underwent testing for serial serum acylated ghrelin levels after triple therapy. In Figure 2A, for the children with successful *H. pylori* eradication, the serum acylated ghrelin levels were significantly increased after eradication therapy as early as the 6th mo (88.2 ± 17.3 pg/mL *vs* 44.2 ± 38.1 pg/mL, *P* < 0.001), until the 12th mo (87.7 ± 38.0 pg/mL *vs* 44.2 ± 38.1 pg/mL, *P* < 0.001). In Figure 2B, for the children with failure of *H. pylori* eradication, the serum acylated ghrelin levels could be also significantly increased by eradication therapy at the 6th mo (93.2 ± 31.6 pg/mL *vs* 37.2 ± 30.9 pg/mL, *P* < 0.001) and at the 12th mo (80.6 ± 28.8 pg/mL *vs* 37.2 ± 30.9 pg/mL, *P* = 0.003).

DISCUSSION

Extra-gastric diseases related to *H. pylori* infection are emerging in importance, such as iron deficiency anemia and growth retardation in children[28]. Other studies have argued that lower socioeconomic status is conjunction with the presence of *H. pylori* accounts for poor growth in children[29]. For overcoming the influencing bias of poor socioeconomic status, indicated by low income, to child growth, multiple logistic regression confirmed that *H. pylori* infection was closely related to both z scores of BW and BMI independent to socioeconomic status. Accordingly, the current study should have not encountered significant bias of social backgrounds on growth limitation in children.

Based on the data of the observation cohort, the *H. pylori*-

Table 3 The differences of the baseline serum acylated ghrelin levels between the children with body weight above and below the cut-off point selected based on the different age ranges of children (mean ± SD)

	H. pylori infection		Non-*H. pylori* infection	
Age ranges (yr)	4-7	8-12	4-7	8-12
BW cut-off point, kg (*n*)	26 (21)	36 (32)	26 (47)	36 (87)
Baseline serum acylated ghrelin (pg/mL)				
Above or equal to the BW cut-off point	51.3 ± 38.6	51.8 ± 40.9	78.2 ± 12.0	85.9 ± 26.9
Below the BW cut-off point	47.8 ± 36.5	23.8 ± 22.1	82.5 ± 17.2	83.8 ± 31.2
[1]*P* value	0.93	0.02	0.31	0.78
z score of BW cut-off point (*n*)	0.5 (18)	0.5 (35)	0.5 (28)	0.5 (106)
Baseline serum acylated ghrelin (pg/mL)				
Above or equal to the z score of BW cut-off point	53.4 ± 40.6	46.8 ± 38.9	81.3 ± 14.1	81.7 ± 19.7
Below the z score of BW cut-off point	45.9 ± 33.6	27.3 ± 26.5	81.9 ± 16.6	89.6 ± 36.4
[1]*P* value	0.68	0.09	0.91	0.15

[1]The *P* value indicated the difference of serum acylated ghrelin levels between the children with body weight (BW) and z score of BW above or equal to the cut-off point and those with below the cut-off point within the same age ranges, analyzed by the Student's *t* test. The BW (z score of BW) cut-off point was determined by the mean (median) of non-*Helicobacter pylori* (*H. pylori*) infected children within the same age ranges.

infected children had significantly lower BW and BMI than gender- and age-matched controls at enrollment. In addition, the BH of *H. pylori*-infected children was 4.5 cm less than that of the non-infected children. After one-year follow-up, the *H. pylori*-infected children had profoundly lower BW, BMI, and net BW gain than the non-infected children. These findings comparing infected and matched disease-free subjects suggest that *H. pylori* infection exerted a negative effect on childhood growth. One possibility is that the presence of *H. pylori* leads to chronic gastric inflammation and thereby decreases food intake[30]. Alternatively, *H. pylori* infection may modify hormones related with the appetite, such as ghrelin[19-21,31,32].

Besides having a lower BW, the *H. pylori*-infected children had a significantly lower baseline serum acylated ghrelin level than that of the gender- and age-matched controls. These data not only suggest a relationship between *H. pylori* infection and lower BW, but that infection is linked to reduced ghrelin levels. Accordingly, it is important to determine whether children with a lower BW had lower serum acylated ghrelin. Supported by the findings in Table 3, only in the *H. pylori*-infected children with age ranging from 8-12 years, the serum acylated ghrelin levels were lower in children with BW below the cut-off point than that with BW above. This finding indicates a lower serum acylated ghrelin, which is induced by the *H. pylori* infection, could be related to induce a lower BW in children aged 8-12 years old.

Eradication of *H. pylori* in symptomatic adult patients has been reported to increase BMI[22,23], and to restore decreased ghrelin levels induced by *H. pylori* infection[16,17]. However, there is still limited evidence to prove such effects in children. Recently, a trial demonstrated *H. pylori* eradication may result in a significant increase in BMI, but with a decrease in the circulating ghrelin levels in a small group of children[33]. Consistent with these findings, our study supported *H. pylori* eradication to address positive impact on the improvement of childhood growth concomitant with an increase of serum acylated ghrelin

level during the one-year long-term follow-up. Therefore, this study is the first to support that *H. pylori* eradication can restore decreased serum acylated ghrelin in children with lower BW. In the future, a longer follow-up study may help to determine whether ongoing elevations in BMI will ultimately lower serum ghrelin via a feedback loop. Our study was nonetheless sufficient to show that within one-year of *H. pylori* eradication, the children could achieve normal growth stature with concomitant restoration of the decreased serum acylated ghrelin induced by the *H. pylori* infection.

Even though some *H. pylori*-infected children had a failure of triple therapy, there was still existed an increase of BW, BH, and serum acylated ghrelin levels at the 6th and the 12th mo. Triple therapy can decrease bacterial loads or gastric inflammation[15,33]. We have analyzed the 51 pairs of ^{13}C-UBT and ghrelin levels (at enrollment, the 6th and 12th mo follow-up) in 17 children with a failure of triple therapy. The result shows the bacterial loads, indicated by the values of ^{13}C-UBT are not correlated well to the ghrelin levels ($r^2 = 0.03$, $P = 0.25$). Therefore, it is possibly due to transient improvement of gastric inflammation to restore serum acylated ghrelin levels. Lack of endoscopic evidence in children with failure of therapy is the limitation in this study. A longer follow-up period is thus needed to clarify this transient improving effect in children with failure of therapy.

In summary, *H. pylori* infection can be associated with decreased serum acylated ghrelin levels, BW and BH in children. Successful *H. pylori* eradication can restore ghrelin levels and the growth of BW and BH in the infected children with growth retardation.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection in children causes not only gastric inflammation and peptic ulcer diseases but also extragastric disorder. Longitudinal observational have found that children with persistent *H. pylori* infection have reduced body weight (BW) and height (BH) growth than the non-infected

ones. In addition, previous studies showed conflicting results regarding the correlation between plasma ghrelin levels and *H. pylori* infection after eradication of bacteria. Therefore, long-term follow up the childhood growth as well ghrelin levels in *H. pylori*-infected children after eradication therapy can illustrate the causal relationship between *H. pylori* infection and growth retardation in children.

Research frontiers
Growth retardation in *H. pylori*-infected children without any organic diseases remains controversial for eradication therapy. The authors aimed to establish a new indication for treating *H. pylori* infection in children with growth retardation and to explore the serum acylated ghrelin levels correlated to eradication therapy.

Innovations and breakthroughs
This study demonstrated that *H. pylori* infection can be associated with decreased serum acylated ghrelin levels, BW and BH in children. In the interventional study, successful *H. pylori* eradication can restore serum acylated ghrelin levels and the growth of BW and BH in the infected children with growth retardation at the 1-year follow-up.

Applications
This study confirmed the causal relationship of *H. pylori* infection and childhood growth retardation. Therefore, we supposed that eradication therapy should be considered as a treatment strategy in *H. pylori*-infected children with growth retardation, which was not related to other organic diseases.

Terminology
Growth retardation is indicated by poor BW and BH growth as compared to the age- and gender-matched normal population. Eradication therapy means that a treatment strategy to eradicate *H. pylori* from stomach. The first-line regiment consists of one proton pump inhibitor and two antibiotics.

Peer review
This is an interesting study aimed at determining whether *H. pylori*-infected children have reduced growth rates and lower levels of ghrelin compared to uninfected and if *H. pylori* eradication may reverse those changes. The study is well written and well designed.

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BRIEF ARTICLE

Colorectal cancer screening: Comparison of transferrin and immuno fecal occult blood test

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Abstract

AIM: To evaluate the sensitivity and specificity of transfesrrin dipstick test (Tf) in colorectal cancer (CRC) screening and precancerous lesions screening.

METHODS: Eight hundreds and sixty-one individuals at high-risk for CRC were recruited. Six hundreds and eleven subsequently received the three fecal occult blood tests and colonoscopy with biopsy performed as needed. Fecal samples were obtained on the day before colonoscopy. Tf, immuno fecal occult blood test (IFOBT) and guaiac fecal occult blood test (g-FOBT) were performed simultaneously on the same stool. To minimize false-negative cases, all subjects with negative samples were asked to provide an additional stool specimen for a second test even a third test. If the results were all negative after testing three repeated samples, the subject was considered a true negative. The performance characteristics of Tf for detecting CRC and precancerous lesions were examined and compared to those of IFOBT and the combination of Tf, IFOBT and g-FOBT.

RESULTS: A total of six hundreds and eleven subjects met the study criteria including 25 with CRC and 60 with precancerous lesions. Sensitivity for detecting CRC was 92% for Tf and 96% for IFOBT, specificities of Tf and IFOBT were both 72.0% (95% CI: 68.2%-75.5%; χ^2 = 0.4, P > 0.05); positive likelihood ratios of those were 3.3 (95% CI: 2.8-3.9) and 3.4 (95% CI: 2.9-4.0), respectively. In precancerous lesions, sensitivities for Tf and IFOBT were 50% and 58%, respectively (χ^2 = 0.8, P > 0.05); specificities of Tf and IFOBT were 71.5% (95% CI: 67.6%-75.1%) and 72.2% (95% CI: 68.4%-75.8%); positive likelihood ratios of those were 1.8 (95% CI: 1.3-2.3) and 2.1 (95% CI: 1.6-2.7), respectively; compared to IFOBT, g-FOBT+ Tf+ IFOBT had a significantly higher positive rate for precancerous lesions (83% *vs* 58%, respectively; χ^2 = 9.1, P < 0.05). In patients with CRC and precancerous lesions, the sensitivities of Tf and IFOBT were 62% and 69% (χ^2 = 0.9, P > 0.05); specificities of those were 74.5% (95% CI: 70.6%-78.1%) and 75.5% (95% CI: 71.6%-79.0%); positive likelihood ratios of those were 2.5 (95% CI: 2.0-3.1) and 2.8 (95% CI: 2.3-3.5). Compared to IFOBT alone, combining g-FOBT, IFOBT and Tf led to significantly increased sensitivity for detecting CRC and cancerous lesions (69% *vs* 88%, respectively; χ^2 = 9.0, P < 0.05).

CONCLUSION: Tf dipstick test might be used as an additional tool for CRC and precancerous lesions screening in a high-risk cohort.

Key words: Transferrin; Immuno fecal occult blood test; Colorectal cancer; Precancerous lesions; Transferrin dipstick test

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INTRODUCTION

Colorectal cancer (CRC) is one of the major diseases threatening human health. In the United States, CRC is the third most frequently diagnosed cancer among men and women, and the third leading cause of cancer death[1]. In China, the prevalence of CRC has risen in recent years, possibly attributable to changes in the population's lifestyle and dietary habits[2,3]. In most cases, CRC is believed to arise within precancerous lesions that develop slowly over many years[4,5].

Currently, many tools are be used for CRC screening. CRC screening tests recommended by the American Cancer Society (ACS) can be grouped into 2 categories: (1) tests that primarily detect CRC, which include tests that look for blood, such as guaiac fecal occult blood test and fecal immunochemical test, or exfoliated DNA [single-strand DNA (sDNA)] in stools; and (2) tests that can detect cancer and advanced lesions, which include endoscopic and radiological exams, i.e., colonoscopy, double-contrast barium enema (DCBE), and computed tomography colonography (CTC) (or virtual colonoscopy)[6]. However, these tests all have certain limitations.

Several published randomized trials have showed that the most widely accepted test method, fecal occult blood test (FOBT), can reduce CRC incidence[7] and mortality rate[8]. However, guaiac fecal occult blood test (g-FOBT) has been criticized for its high false positive because it detects non-human haem in food[9,10]. Compared with that of g-FOBT, the sensitivity of immuno fecal occult blood test (IFOBT) is significantly higher[11-13]. IFOBT specifically detects human hemoglobin (Hb) in stool by antibody-antigen reaction, which has no restrictions on diet or drug intake. However, Hb is unstable in feces because it can be degraded by bacteria. Furthermore, Hb can not be used to detect lesions that are not accompanied by bleeding[14-17]. Fecal DNA test was developed based on the molecular genetics of CRC. It is suggested that the occurrence of most CRCs has close relationship with chromosomal instability, with mutations progressively accumulating in the *adenomatous polyposis coli* gene, the p53 tumor-suppressor gene, and the K-ras oncogene[18]. Despite relatively high specificity[19], Fecal DNA test has many problems[20], including the lack of adequate fecal DNA makers, complex extraction steps, and so on. Furthermore, population-based studies showing the capability of the method to decrease mortality of CRC have been lacking[21]. Other non-invasive methods include testing for faecal calprotectin, which has high sensitivity but low specificity[22].

DCBE is a preferred method for screening in children, old people and those who can not undergo colonoscopy. However, its false positive and false negative ratios are both higher than those of colonoscopy[15,23]. Colonoscopy can detect CRC in the entire colonic lumen and is the most sensitive and specific test. A report showed that the incidence and mortality of CRC rate were reduced to 67% and 65%, respectively, after colonoscopy screening in an average-risk cohort[24]. However, colonoscopy is invasive and has risks to certain extent[25]. High costs and painful procedure has prevented colonoscopy from being used as a method for large-scale screening of CRC. Practically it is only used for final diagnostic test of positive patients. In 2008, two additional tests have been added to CRC screening guidelines of the ACS[26]: sDNA and CTC. CTC is a minimally invasive method for examination of the whole colon. It is safe and the entire colon can be examined thoroughly. A recently study shows that for ≥ 10 mm colorectal lesions, the sensitivity of CTC is similar to that of colonoscopy. However, for < 10 mm and flat neoplasms, the sensitivity of CTC is lower than colonoscopy[27]. Additionally, CTC can not perform biopsy and is an expensive procedure. For these reasons, our study sought to develop a method to improve the sensitivities and specificities of CRC and precancerous lesions screening.

Transferrin (Tf), which is present in plasma by the release of neutrophil-specific granules, is undetectable in normal human gastrointestinal tract. Detection of Tf in feces or contents in the stomach indicates bleeding in gastrointestinal tract. Unlike hemoglobin, Tf is resistant to degradation by digestive enzymes and bacteria. Thus, compared to hemoglobin, Tf is more stable in feces[28]. It has been reported that fecal Tf is elevated in patients with colorectal tumor, compared to healthy individuals[29]. Recently, a number of proteomic studies showed that Tf could be used as a marker expressing in a number of cancers[30,31]. Saitoh *et al*[32] and Hirata *et al*[33] compared fecal Tf with IFOBT in clinical studies and found that Tf was as useful as IFOBT in diagnosing colorectal diseases. However, these two studies did not analyze patients with precancerous lesions. Sheng *et al*[34] compared fecal Tf with IFOBT for their sensitivities in detecting CRC and precancerous lesions in CRC patients. However, the subjects of this study were CRC patients, and specificity was not analyzed.

So far, Tf has not been recommended as a method for CRC screening by the ACS. Based on the above studies, we assumed that the sensitivity and specificity of Tf in detecting CRC and precancerous lesions were equal or superior to IFOBT. Using a combination of the three measurements (g-FOBT, Tf and IFOBT) appears to increase the sensitivity of diagnosis in high-risk population. In order to investigate whether Tf can be applied in the screening of CRC and precancerous lesions, we conducted this study to compare the effectiveness of Tf and IFOBT in the detection of colorectal cancer and precancerous lesions.

MATERIALS AND METHODS

Study materials

The stool specimen collection, colonoscopy and pathologic examination were performed in the Eighth Hospital of Wuhan City which is a hospital specializing in anorectal diseases. G-FOBT, IFOBT and Tf kits were purchased from Baso Diagnostics Inc and WHPM Inc.

Study group

From January 2010 to September 2010, 861 subjects at high-risk (a personal history of curative-intent resection of CRC or intestinal polyps; family history of colorectal cancer; having the following two or more: chronic diarrhea, chronic constipation, abdominal pain, dark stool, blood or mucus on stool) were recruited. The inclusion criteria were as following: age over 14 years, male or female. Subjects with age < 14 years were excluded. All participants provided written informed consent and were instructed on diet and drug restrictions three days before and during the period of stool collection.

Fecal samples collection and IFOBT and Tf analysis

All fecal samples were collected the day before colonoscopy and processed in accordance with manufacturer's instructions. We applied the fecal sample on the strip and the result was read out within 5 min (the result was invalid after 5 min). A red bar in control area (C) only was considered as negative. A red bar in both the testing area (T) and the control area (C), was considered as positive. If there was no red bar in the control area(C), the test was considered invalid. Tf, IFOBT and g-FOBT were performed simultaneously on the same stool. To minimize false-negative cases, all subjects with negative samples were asked to provide an additional stool specimen for a second test; if the second test still gave negative result, a third test would be conducted. As long as one of the three tests showed positive results, the subject was considered to have a positive sample. If the results were all negative after testing three repeated samples, the subject was considered a true negative. Approximately 10% of the samples were repeated and the concordance was 100%.

Statistical analysis

The positive rate of Tf alone, IFOBT alone, Tf combined with IFOBT (Tf + IFOBT), Tf and IFOBT combined with g-FOBT (Tf + IFOBT + g-FOBT), as well as their respective specificity, likelihood ratio, odd ratio and 95% confidence interval were calculated to compared the sensitivity of Tf, IFOBT, Tf+ IFOBT and Tf + IFOBT + g-FOBT in detecting CRC and precancerous lesions. χ^2 and McNemar's test were conducted to determine the significance of difference. $P < 0.05$ in a two-tailed test was considered statistically significant. Analyses were performed using SPSS version 17.0.

RESULTS

Subject enrollment flow is described in Figure 1. Of the

Figure 1 Flow diagram of the study. [1]Includes polyps that were less than 3 mm in diameter, broadbased, sessile and fat.

861 participants in this study, 250 subjects who have taken neither FOBTs nor colonoscopy, or have taken only one of the tests were excluded in this survey. Six hundred and eleven subsequently received both FOBTs and colonoscopy with biopsy performed as needed. Among them, 286 were found to have abnormalities by colonoscopy, while 447 were classified as low risk population including no abnormalities (325 cases) and benign lesions (122 cases). Benign lesions included chronic enteritis, chronic schistosomiasis bowel disease, intestinal diverticula, colorectal erosive inflammation (a total of 112 cases) and inflammatory intestinal mucosa by biopsy (10 cases). One hundred and seventy-four subjects were found to have polyps or neoplasm. Pathological examination showed CRC (25 cases), precancerous lesions (60 cases), inflammatory intestinal mucosa (10 cases); Polyps (79 cases) that were less than 3 mm in diameter, broad-based, sessile and flat were not subjected to biopsy. Precancerous lesions included tubular adenoma, villous adenoma, tubular villous adenoma and hyperplastic polyp with moderate-severe dysplasia (with histological confirmation).

The overall demographic information of 611 subjects (Table 1). There were 310 men and 301 women among the participants, with a median age of 50 years (range 14-85 years). Among them, 10 men and 15 women had CRC, with a median age of 62 years; 35 men and 25 women had precancerous lesions, with a median age of 56 years.

The positive rate of g-FOBT, Tf, Tf+ IFOBT, and g-FOBT+ Tf+ IFOBT in fecal samples from five groups of participants is shown in Table 2. In CRC, the positive rates of Tf and IFOBT were 92% and 96%, respectively ($\chi^2 = 0.4$, $P > 0.05$). In precancerous lesions, the positive

Table 1 Patient demographics

Charac-teristic	No. of participants	Colorectal cancer	Precancerous lesions	Polyp	Abnor-mality	Low risk
Total	611	25	60	79	286	447
Sex						
Male	310	10	35	49	152	216
Female	301	15	25	30	134	231
Age, yr						
Median	50	62	56	53	53	48
Range	14-85	39-85	24-84	25-84	17-85	14-81

rates for Tf and IFOBT were 50% and 58%, respectively ($\chi^2 = 0.8$, $P > 0.05$); compared to IFOBT, g-FOBT+ Tf+ IFOBT had a significantly higher positive rate for precancerous lesions (83% *vs* 58%, respectively; $\chi^2 = 9.1$, $P < 0.05$). In CRC and precancerous lesions, the positive rates for Tf and IFOBT were 62% and 69% ($\chi^2 = 0.9$, $P > 0.05$), whereas g-FOBT+ Tf+ IFOBT also provided significantly higher positive rate compared to IFOBT alone (88% *vs* 69%, respectively; $\chi^2 = 9.0$, $P < 0.05$). For Tf alone, a difference in positive rate was observed for detecting CRC and precancerous lesions (92% *vs* 50%, respectively; $\chi^2 = 13.3$, $P < 0.05$).

The performance characteristics of various tests examined by our study (Table 3). For detecting CRC, The specificities of Tf and IFOBT were both 72.0% (95% CI: 68.2%-75.5%); positive likelihood ratios of those were 3.3 (95% CI: 2.8 - 3.9) and 3.4 (95% CI: 2.9-4.0), respectively. For detecting precancerous lesions, specificities of Tf and IFOBT were 71.5% (95% CI: 67.6%-75.1%) and 72.2% (95% CI: 68.4%- 75.8%); positive likelihood ratios of those were 1.8 (95% CI: 1.3-2.3) and 2.1 (95% CI: 1.6-2.7), respectively. For detecting both CRC and precancerous lesions, specificities of Tf and IFOBT were 74.5% (95% CI: 70.6%-78.1%) and 75.5% (95% CI: 71.6%-79.0%); positive likelihood ratios of those were 2.5 (95% CI: 2.0-3.1) and 2.8 (95% CI: 2.3-3.5), respectively. In these tests, the specificity of Tf and IFOBT for detecting CRC was the same. Likelihood ratio can accurately reflect how likely it is that patients with CRC will test positive. The likelihood ratio showed that Tf and IFOBT detected CRC (3.3 and 3.4, respectively) more effectively than they detected precancerous lesions (1.8 and 2.1, respectively).

DISCUSSION

The data from our study demonstrated that the sensitivities and specificities of Tf and IFOBT were similar in the detection of colorectal cancer and precancerous lesions in high-risk cohort. These results suggest that when using Tf alone, the sensitivity and specificity have no visible difference compared to using IFOBT alone; when combining these three methods, the sensitivity can be enhanced.

There had been several comparative studies of Tf and IFOBT previously. Saitoh *et al*[32] found that the sensitivities of Tf and IFOBT for detecting CRC were similar

(53.8% and 61.5%, respectively). The study used enzyme-linked immunosorbent assay (ELISA) kit for fecal Tf and Latex agglutination for IFOBT. Hirata *et al*[33] found that the sensitivities of Tf and IFOBT were both 50%, whereas combining both methods gave a slightly higher sensitivity of 61.1%. The study measured the Tf and Hb quantitatively by sandwich ELISA. Both studies analyzed the sensitivity for detecting colorectal diseases (colon cancer, colorectal polyps, ulcerative colitis, Crohn's disease, *etc.*) but not precancerous lesions. In addition, in both previous studies, each patient was tested only once with one stool specimen. In contrast, our study strove to minimize false negative results by testing up to three stool specimens from a single patient, hence achieving a more accurate estimation of sensitivity. Sheng *et al*[34] found that the positive ratio of Tf and IFOBT for detecting colorectal cancer were 80% and 75%, respectively. For detecting precancerous lesions, the positive ratios were 72% (Tf) and 44% (IFOBT). The difference is statistically significant. Combining the two methods gave a positive ratio of 78% in detecting precancerous lesions. Three possible reasons might explain the differences between Sheng *et al*[34] and our study. First, the tested subjects were different. The previous study tested CRC patients. Our study tested those who are at high-risk. Second, the sample size was different. Our study had 611 samples, compared to 110 in the previous study. Third, the design of the studies was different. The previous study took only one stool specimen from an individual patient and retested the sample if the result was negative. We took at least one specimen from every participant and up to three specimens from those showing negative results. None of the three previous studies analyzed the specificities of colorectal cancer and precancerous lesions detection. The difference in specificity may be caused by variation in other factors, such as degradation of hemoglobin, samples, experiment and the quality of reagents, *etc.*

The study shows that Tf and IFOBT both have false positive and false negative results in colorectal cancer and precancerous lesions screening. IFOBT specifically detects the Hb in stool by antibody-antigen reaction. Anti-Hb antibody do not react with animal blood, fruits and vegetables in the testing material, and do not confer per-oxidase activity, which obviously reduce the false positive rate. However, the test has several problems, including (1) some participants' hemoglobin may not be recognized by the anti-Hb antibody used in the test; (2) hemoglobin can be degraded by bacteria, resulting in the loss of antigen; (3) the symptom of bleeding in early colorectal lesions is intermittent; and (4) the massive bleeding causes an excessive amount of antigen to be present in the reaction system and hence the "pre-band phenomenon". These are all possible causes of false negative results in the detection using IFOTB. Tf, a type of β1 globulin with a molecular weight of 77 KD, transports extracellular iron into cells through membrane receptor-mediated endocytosis[35]. Tf can resist degradation caused by digestive enzymes and bacteria, and is more stable than hemoglobin in stool. But Tf can only be detected at a concentration

Table 2 Positive rate of three fecal occult blood tests in fecal samples from colorectal cancer patients, precancerous lesions subjects, polyp subjects, abnormality subjects and low risk subjects *n* (%)

Disease (N)	g-FOBT		Tf		IFOBT		Tf+ IFOBT		g-FOBT+ Tf+ IFOBT	
	+[1]	-[1]	+[1]	-[1]	+[1]	-[1]	+[1]	-[1]	+[1]	-[1]
CRC (25)	25 (100)	0 (0)	23 (92)	2 (8)	24 (96)	1 (4)	25 (100)	0 (0)	25 (100)	0 (0)
Precancerous lesions (60)	36 (60)	24 (40)	30 (50)	30 (50)	35 (58)	25 (42)	40 (67)	20 (33)	50 (83)	10 (17)
Polyp (79)	35 (44)	44 (56)	29 (37)	50 (63)	20 (25)	59 (75)	34 (43)	45 (57)	49 (62)	30 (38)
Abnormality (286)	153 (53)	133 (47)	126 (44)	160 (56)	128 (45)	158 (55)	162 (57)	124 (43)	203 (71)	83 (29)
Low risk (447)	148 (33)	299 (67)	105 (23)	342 (77)	109 (24)	338 (76)	154 (34)	293 (66)	221 (49)	226 (51)

[1]*n* (*n*/N × 100%). Tf: Transferrin; IFOBT: Immuno fecal occult blood test; g-FOBT: Guaiac-fecal occult blood test; CRC: Colorectal cancer.

Table 3 Sensitivity, specificity, positive likelihood ratio and odd ratio of three fecal occult blood tests for detection of colorectal cancer, precancerous lesions and colorectal cancer + precancerous lesions

Test	No. of neoplasms detected	Sensitivity%		Specificity%		Likely ratio(+) (95% CI)	Odd ratio (95% CI)
		n/total	% (95% CI)	*n*/total	% (95% CI)		
CRC							
g-FOBT	25	25/25	100 (86.7-100)	367/586	62.6 (58.6-66.5)	2.7 (2.4-3.0)	-
Tf	25	23/25	92 (75.0-97.8)	422/586	72 (68.2-75.5)	3.3 (2.8-3.9)	29.6 (6.8-126.9)
IFOBT	25	24/25	96 (80.5-99.3)	422/586	72 (68.2-75.5)	3.4 (2.9-4.0)	61.8 (8.3-460.2)
Tf+ IFOBT	25	25/25	100 (86.7-100)	358/586	61.1 (57.1-65.0)	2.6 (2.3-2.8)	-
g-FOBT+ Tf+ IFOBT	25	25/25	100 (86.7-100)	266/586	45.4 (41.4-49.4)	1.8 (1.7-2.0)	-
Precancerous lesions							
g-FOBT	60	36/60	60 (47.4-71.4)	343/551	62.3 (58.1-66.2)	1.6 (1.3-2.0)	2.5 (1.4-4.3)
Tf	60	30/60	50 (37.7-62.3)	394/551	71.5 (67.6-75.1)	1.8 (1.3-2.3)	2.5 (1.5-4.3)
IFOBT	60	35/60	58.3 (45.7-70.0)	398/551	72.2 (68.4-75.8)	2.1 (1.6-2.7)	3.6 (2.1-6.3)
Tf+ IFOBT	60	40/60	66.7 (54.1-65.3)	338/551	61.3 (57.2-65.3)	1.7 (1.4-2.1)	3.2 (1.8-5.6)
g-FOBT+ Tf+ IFOBT	60	50/60	83.3 (72.0-90.7)	256/551	46.5 (42.3-50.6)	1.6 (1.4-1.8)	4.3 (2.2-8.7)
CRC+ precancerous lesions							
g-FOBT	85	61/85	71.8 (61.4-80.2)	343/526	65.2 (61.0-69.2)	2.1 (1.7-2.5)	4.8 (2.9-7.9)
Tf	85	53/85	62.4 (51.7-72.0)	392/526	74.5 (70.6-78.1)	2.5 (2.0 -3.1)	4.8 (3.0-7.8)
IFOBT	85	59/85	69.4 (59.0-78.2)	397/526	75.5 (71.6-79.0)	2.8 (2.3-3.5)	7.0 (4.2-11.5)
Tf+ IFOBT	85	65/85	76.5 (66.4-84.2)	338/526	64.3 (60.1-68.2)	2.1 (1.8-2.5)	5.8 (3.4-9.9)
g-FOBT+ Tf+ IFOBT	85	75/85	88.2 (79.7-93.5)	256/526	48.7 (44.4-53.0)	1.7 (1.5-1.9)	7.1 (3.6-14.1)

Tf: Transferrin; IFOBT: Immuno fecal occult blood test; g-FOBT: Guaiac-fecal occult blood test; CRC: Colorectal cancer; CI: Confidence interval.

greater than 10 ng/mL. The ratio of hemoglobin and Tf is 5.4:1 in specimens containing blood. Thus, if the subject has low level of Tf, or the bleeding is very trivial, the testing threshold can not be reached and false negative results will be the outcome. Our study tested the stool specimen repeatedly, therefore reduced the error rate. All subjects underwent standard colonoscopic examination with biopsy performed as needed. In this way, an accurate test was performed to examine the sensitivities and the specificities of the three methods.

The results of this study have a significant implication for CRC screening. A number of studies showed that early detection based on fecal occult blood test helped decrease CRC mortality by 15%-25%[4,7,36]. Mandel *et al*[7,37] found that screening once every year or once every two years with g-FOBT or IFOTB can decrease the mortality of CRC and CRC related diseases, compared to no screening. In our test, for 65 subjects, IFOBT showed negative result while Tf were positive. Hence, Tf is appropriate for the screening of CRC and precancerous lesions. Positive likelihood ratio, which involves both sensitivity and specificity of screening, can fully evaluate the

diagnosing value of screening. It is very stable and not subject to morbidity. Results of our study demonstrated that the positive likelihood ratio of Tf detecting CRC was similar with that of IFOBT in various populations, which indicates that Tf has a similar value with IFOBT and is fit for the CRC screening in an average- risk population. Further, the findings of the analysis suggest that a combination of Tf, IFOBT and g-FOBT enables compensation of the inadequacy of single tests, which will reduce false negative rate and improve the positive ratio. So, in order to enhance the sensitivities of detecting CRC and precancerous lesions, all three methods should be used simultaneously.

Our study does have some limitations, and the first is its study subject. The sensitivity and specificity of Tf had been calculated in this study, those of that in an average-risk group are yet to be further determined. Prospective studies in an average-risk group are needed to validate these results. Nevertheless, hardly everyone at average-risk group can undergo colonoscopy, leading that the specificities of fecal occult blood tests can not be evaluated. We prepare to apply computed tomographic virtual

colonoscopy to screen patients who are at average-risk for CRC. The second is the range of age in this study is very wide. The third major limitation of our study is that the three stool occult blood tests were all qualitative and certain amount of deviation was existed compared to quantitative test.

In conclusion, Tf dipstick test can be applied to screen for CRC and precancerous lesions and the efficacy is approximately the same as that of IFOBT in high risk cohort. By combining g-FOBT, Tf and IFOBT, the sensitivity can be improved significantly while the specificity is sacrificed. Large-scale and prospective clinical studies will be needed to determine whether Tf dipstick test can be used as a screening method for CRC and precancerous lesions in different screening population.

COMMENTS

Background

Fecal occult blood test (FOBT) is a simple and convenient tool for colorectal cancer (CRC) screening. Immuno fecal occult blood test (IFOBT) has limited sensitivities and specificities for detecting CRC and precancerous lesions.

Research frontiers

FOBT, a non-invasive method, can reduce CRC incidence and mortality rate. However, hemoglobin (Hb) is unstable in feces because it can be degraded by bacteria. Furthermore, Hb can not be used to detect lesions that are not accompanied by bleeding. Transferrin (Tf), which is present in plasma by the release of neutrophil-specific granules, is undetectable in normal human gastrointestinal tract. Tf can resist degradation caused by digestive enzymes and bacteria, and is more stable than hemoglobin in stool.

Innovations and breakthroughs

Tf dipstick test was found to be as sensitive and specific as IFOBT in the detection of CRC and precancerous lesions in high-risk cohort. Combining guaiac fecal occult blood test, IFOBT and Tf enhanced the sensitivity.

Applications

Tf dipstick test can be applied to screen for CRC and precancerous lesions and the efficacy is approximately the same as that of IFOBT in high risk cohort.

Terminology

Transferin (Tf), a type of β1 globulin with a molecular weight of 77 KD, transports extracellular iron into cells through membrane receptor-mediated endocytosis. Detection of Tf in feces or contents in the stomach indicates bleeding in gastrointestinal tract.

Peer review

The study seeks to evaluate biomarkers for colorectal cancer screening. To develop non-invasive method such as fecal test for cancer screening is clinically relevant. The study has tested a reasonable size of cohorts and found combined test of several markers let to significantly increased sensitivity of detecting cancerous lesions compared to the commonly used method. The results suggest that the transferrin dipstick test might be used as an additional tool for colorectal cancer and precancerous lesions screening in a high-risk cohort.

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BRIEF ARTICLE

Health-related quality of life evaluated by tumor node metastasis staging system in patients with hepatocellular carcinoma

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Abstract

AIM: To investigate and evaluate the change in health-related quality of life (HRQoL) by tumor node metastasis (TNM) staging system in patients with hepatocellular carcinoma (HCC).

METHODS: A total of 140 patients diagnosed with HCC between June 2008 and April 2009 in our department were enrolled to this study. One hundred and thirty-five (96.5%) patients had liver cirrhosis secondary to hepatitis B virus (HBV) infection, 73 (54.07%) of them being HBV DNA positive; the other etiologies of liver cirrhosis were alcoholic liver disease (1.4%), hepatitis C (1.4%) or cryptogenic (0.7%). All subjects were fully aware of their diagnosis and provided informed consent. HRQoL was assessed before treatment using the functional assessment of cancer therapy-hepatobiliary (FACT-Hep) questionnaire. Descriptive statistics were used to evaluate demographics and disease-specific characteristics of the patients. One-way analysis of variance and independent samples t tests were used to compare the overall FACT-Hep scores and clinically distinct TNM stages. Scores for all FACT-Hep items were analyzed by frequency analyses. The mean scores obtained from the FACT-Hep in different Child-Pugh classes were also evaluated.

RESULTS: The mean FACT-Hep scores were reduced significantly from TNM Stage Ⅰ to Stage Ⅱ, Stage ⅢA, Stage ⅢB group (687 ± 39.69 *vs* 547 ± 42.57 *vs* 387 ± 51.24 *vs* 177 ± 71.44, *P* = 0.001). Regarding the physical and emotional well-being subscales, scores decreased gradually from Stage Ⅰ to Stage ⅢB (*P* = 0.002 *vs* Stage Ⅰ; *P* = 0.032 *vs* Stage Ⅱ; *P* = 0.033 *vs* Stage ⅢA). Mean FACT-Hep scores varied by Child-Pugh class, especially in the subscales of physical well-being, functional well-being and the hepatobiliary cancer (*P* = 0.001 *vs* Stage I; *P* = 0.036 *vs* Stage Ⅱ; *P* = 0.032 *vs* Stage ⅢA). For the social and family well-being subscale, only Stage ⅢB scores were significantly lower as compared with Stage Ⅰ scores (*P* = 0.035). For the subscales of functional well-being and hepatobiliary cancer, there were significant differences for Stages ⅡⅠ, ⅢA and ⅢB (*P* = 0.002 *vs* Stage Ⅰ).

CONCLUSION: HRQoL of patients with HCC worsens gradually with progression of TNM stages. The most impaired subscales of HRQoL, as measured by FACT-Hep, were physical and emotional well-being.

Key words: Hepatocellular carcinoma; Tumor node metastasis staging; Functional assessment of cancer therapy-hepatobiliary; Health-related quality of life; Cross-sectional study

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INTRODUCTION

In recent years, there has been increased interest in quality of life as it pertains to patients' health status. Many different questionnaires, such as the functional assessment of cancer therapy-hepatobiliary (FACT-Hep), the Health-Related Quality of Life (HRQoL), and the Short Form (36) Health Survey are becoming key instruments in the evaluation of patients' health status. HRQoL results may be more relevant than length of life, as patients are frequently more concerned about life quality than longevity.

The incidence of hepatocellular carcinoma (HCC) in China is increasing. HCC is now the second leading cause of death of cancers[1]; about 15%-40% of the hepatitis B virus carriers may develop cirrhosis and HCC[2]. Approximately 80% of the patients diagnosed with HCC are unable to undergo surgical resection or transplantation[3]. Non-surgical treatment, such as transcatheter chemo-embolization or chemotherapy may improve the patients' prognosis to varying degrees[4-8]. Symptoms can be extremely variable in advanced HCC; the compensated patients may be symptomatic for months or decades. The impact is significant on patients' functioning and well-being. Patients may experience anxiety because of emotional concerns associated with the disease and treatment. Complications and extra-hepatic manifestations of advanced disease may reduce the quality of life, as therapeutic interventions may restrain outdoor activities. These challenges may negatively affect the quality of life, including physical, emotional, and functional well-beings[9].

Assessment of HRQoL with cancer has become an important outcome indicator during the last two decades[10]. The FACT-Hep is the most widely used evaluation tool focusing on hepatobiliary cancer such as HCC, pancreatic cancer and cancers of the gallbladder[11]. In clinical studies, the FACT-Hep performs well in assessing the quality of life of patients with HCC, and is considered to be of utmost importance for improving survival rates and quality of life[11].

The FACT-Hep[12] is a new and important index for evaluation of prognosis of the patients and the results of clinical trials, complementing the traditional end-points assessment, such as tumor response rate and survival time[13-15].

As HCC worsens, quality of life generally decreases, but two unanswered questions remain. First, FACT-Hep has not been used to assess prognosis with a large series of HCC patients at different tumor node metastasis (TNM) stages. Second, quality of life is a broad concept, and no data are available to examine the impact of disease on patients' self-perception and the factors which are associated with poor HRQoL on the FACT-Hep.

The aim of this study was to determine the HRQoL of patients at different TNM tumor stages of HCC by FACT-Hep, and to determine the factors associated with impaired HRQoL. The TNM Classification of Malignant Tumors was based on physical exam findings, imaging studies (ultrasound, computed tomography or magnetic resonance imaging) and other tests[16]. For solid tumors, such as HCC, TNM is the most commonly used staging system.

MATERIALS AND METHODS

Patients

Most patients admitted to our department had primary liver cancers. On admission, patients' quality of life was evaluated using the Chinese version of the FACT-Hep. During an 11-mo period from June 2008 to April 2009, we studied 140 patients with HCC (133 males, 7 females; aged 28-75 years). Patients completed the study questionnaire following the diagnosis of HCC, and prior to therapeutic intervention. All subjects were fully aware of their diagnosis and provided informed consent to participate. The ethical committees of the participating centers approved the study. The TNM staging system[17] (edition 6 published in 2002; and instituted in 2003) was used in this study. Patients were selected based on the demographic characteristics or clinical status.

Inclusion criteria

(1) Patients aged $\geq$ 18 years; (2) diagnosis of HCC was established by imaging examinations (ultrasound or computed tomography) and confirmed by α1-variable fetoprotein levels exceeding 10 times the normal values, or liver biopsy; (3) patients who had no prior history of malignancy with encephalopathy and no cognitive impairment (as judged by the attending clinician); (4) patients who should speak, read, understand and write Chinese; (5) Karnofsky $\geq$ 60. The expected survival was at least six months; (6) patients who voluntarily agreed and were able to make the decision to participate in the study; and (7) patients who provided written informed consent.

Exclusion criteria

(1) Illiteracy; (2) current psychosis or homicidal ideation; (3) serious visual or auditory disease; (4) evidence of cognitive impairment or psychiatric disturbance that would prevent informed consent; (5) physical condition too poor to complete the required the 20-25 min of questionnaires; and (6) patient's family requests that the patient's condition should be kept a secret from the patients.

FACT-Hep

The FACT-Hep is a 45-item, self-report instrument designed to measure HRQoL in patients with HCC. The FACT-Hep consists of 27-item FACT-General (FACT-G), which assesses generic HRQoL concerns using five subscales, and the newly validated 18-item hepatobiliary subscale, which assesses specific symptoms of hepatobiliary

Table 1 Patient characteristics *n* (%)

Factors		*P* value
Age (yr), median (range)	52 (28–75)	0.3509
Sex		< 0.001
Male	133 (95.0)	
Female	7 (5.0)	
Level of education		< 0.0001
Primary school	20 (14.0)	
Secondary school	56 (40.0)	
Commercial or vocational school	64 (46.0)	
Tumor, node, metastasis stage		
I	49 (35.0)	0.0023
II	35 (25.0)	
IIIA	29 (20.7)	
IIIB	27(19.3)	
IIIC	0	
IV	0	
Etiology		
Hepatitis B	135 (96.5)	
Hepatitis C	2 (1.4)	
Alcoholic	2 (1.4)	
Cryptogenic	1 (0.7)	
Child-Pugh class		
A	84 (60.0)	
B	29 (20.7)	
C	27 (19.3)	

Table 2 Functional assessment of cancer therapy-hepatobiliary mean scores (*n* = 140)

FACT-Hep subscales	mean ± SD	Sum of scores	*F*	*P* value
PWB	16.47 ± 4.27	276.86	16.99	0.000
SFWB	14.52 ± 3.35	37.08	1.71	0.184
EWB	12.74 ± 3.39	131.33	7.11	0.001
FWB	17.57 ± 3.93	165.32	5.84	0.004
HepCS	41.46 ± 9.52	754.79	5.72	0.004

PWB: Physical well-being; SFWB: Social and family well-being; EWB: Emotional well-being; FWB: Functional well-being; HepCS: Hepatobiliary cancer subscale; FACT-Hep: Functional assessment of cancer therapy-hepatobiliary.

that the patient complete the FACT-Hep again.

Statistical analysis

SPSS 13.0 software was used to process and analyze the data. Descriptive statistics were used to evaluate demographic and disease-specific characteristics. One-way analysis of variance and independent samples *t* tests ($P < 0.05$) were used to compare FACT-Hep scores between clinically distinct groups.

RESULTS

Sample size

FACT-Hep questionnaires were issued to 145 patients. Two patients dropped out of the study due to disease exacerbation and three questionnaires were omitted from analysis because of missing data (> 5 questions). In total, 140 questionnaires were completed (with a completion rate of 98.19%). The average time for finishing a FACT-Hep was 13.50 ± 2 min.

Patient characteristics

Of the 140 patients, 133 were male (95.0%) and the mean age at diagnosis was 52.34 ± 9.73 years (range: 28-75). The TNM tumor stages were as follows: Stage I : 49 cases; Stage II: 35 cases; Stage IIIA: 29 cases; and Stage IIIB: 27 cases. All patients had cirrhosis. One hundred and thirty-five (96.5%) subjects had liver cirrhosis secondary to hepatitis B virus (HBV) infection, with 73 (54.07%) of them being HBV DNA positive; the other etiologies of liver cirrhosis included alcoholic liver disease (2), hepatitis C (2) or cryptogenic (1). Demographic and clinical characteristics are shown in Table 1.

FACT-Hep scores and TNM stage

Median FACT-Hep scores decreased as TNM stage advanced, and FACT-Hep scores were strongly associated with TNM stage (Figure 1).

Differences in FACT-Hep items

Mean FACT-Hep subscale scores were: PWB 16.47 ± 4.272; SFWB 14.52 ± 3.351; EWB 12.74 ± 3.394; FWB 17.90 ± 4.06; and HepCS 41.46 ± 9.52. The overall mean differences are presented in Table 2.

cancer and side effects of treatment. The five FACT-Hep subscales are: (1) physical well-being (PWB, 7 questions); (2) social and family well-being (SFWB, 7 questions); (3) emotional well-being (EWB, 6 questions); and (4) functional well-being (FWB, 7 questions); and (5) hepatobiliary cancer subscale (HepCS, 20 questions).

The FACT-Hep shows a high internal consistency at initial assessment (Cronbach's alpha range: 0.72-0.94) and retesting (Cronbach's alpha range: 0.81-0.94)[11]. Measurement stability is also high for all aggregated scales (test-retest correlation range: 0.84-0.91; interclass correlation coefficient range: 0.82-0.90)[11]. The FACT-Hep can be used independently as a brief measure of disease-related symptoms and functioning in assessing HRQoL of patients with HCC[11]. In this study, the FACT-Hep was translated into Chinese and adjusted appropriately based on the local cultural background. Early research using the Chinese version of the FACT-Hep showed a better reliability and validity (a reliability > 0.5 and a validity > 0.73)[11]. The present study aimed to assess the relationship between the HRQoL in HCC patients as measured by the Chinese version of the FACT-Hep and TNM tumor stage.

All FACT items were rated on 5-point scales ranging from 1 to 5. Converse items should be unified before analysis. The PWB, FWB, SFWB and EWB were summed to get the FACT-G total score. The FACT-G and HepCS scores were summed to obtain the FACT-Hep total score. Higher scores on all subscales of the FACT-Hep reflect higher functioning and fewer symptoms.

Patients completed the FACT-Hep after receiving uniform written instructions from a medical doctor (Li MD) or nurse (Dong HJ). Following FACT-Hep, another doctor (Lang QB) reviewed the forms for missing items. If there were more than three missing items, we requested

Table 3 Functional assessment of cancer therapy-hepatobiliary subscale results by tumor node metastasis stage of hepatocellular carcinoma and by Child-Pugh class (mean ± SD)

	n	PWB	SFWB	EWB	FWB	HepCS
TNM stage						
I	49	21 ± 2.15[d,f]	15.81 ± 3.68	15.11 ± 2.79[d,f]	19.96 ± 3.20[d,f]	48.52 ± 8.36[d,f]
II	35	19.07 ± 3.41[b,f]	14.69 ± 2.82	13.38 ± 3.01[b]	19.24 ± 2.21[b]	45.45 ± 10.01[b]
III A	29	16.29 ± 2.24[b,d]	14.66 ± 3.3	13.06 ± 3.08[b]	18.37 ± 3.98[b]	41.29 ± 7.05[b]
III B	27	12.41 ± 2.88[b,d,f]	13.61 ± 3.32[a]	10.84 ± 3.15[b,d,f]	14.69 ± 3.39[b,d]	35.35 ± 7.44[b,d]
Child-Pugh class						
A	73	19.56 ± 2.79	15.29 ± 3.34	14.12 ± 2.89	19.58 ± 3.26[j]	46.12 ± 8.79[j]
B	40	16.65 ± 2.79[h]	14.25 ± 3.08	11.8 ± 3.05	16.48 ± 2.98[h]	38.88 ± 6.61[h]
C	27	10.52 ± 2.41[h]	12.85 ± 3.21[h]	10.41 ± 3.49[h,j]	13.78 ± 3.48[h,j]	32.7 ± 7.38[h,j]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* Stage I ; [d]$P < 0.01$ *vs* Stage II ; [f]$P < 0.01$ *vs* Stage III A; [h]$P < 0.01$ *vs* Child A; [j]$P < 0.01$ *vs* Child B. PWB: Physical well-being; SFWB: Social and family well-being; EWB: Emotional well-being; FWB: Functional well-being; HepCS: Hepatobiliary cancer subscale.

Figure 1 Mean functional assessment of cancer therapy-hepatobiliary scores by tumor node metastasis stage for hepatocellular carcinoma patients (*n* = 140, mean ± SD). [b]$P < 0.01$ *vs* Stage I ; [d]$P < 0.01$ *vs* Stage II ; [f]$P < 0.01$ *vs* Stage III A. FACT-Hep: Functional assessment of cancer therapy-hepatobiliary.

Child-Pugh classification and FACT-Hep

Scores for each FACT-Hep item worsened with increasing severity of hepatic cirrhosis, based on the Child-Pugh classification (Table 3). Further analyses of factors impacting specific HRQoL were performed using percent analysis.

Physical well-being: Lack of physical strength was reported by 86% of patients; nausea by 51%; illness affecting the role at home by 83%; pain by 66%; recent uncomfortable feelings by 92%; and frequent bed rest by 55%. Patients had variable decreases in daily exercise capacity.

Social and family well-being: Patients kept close contact with friends (92%); received emotional support at home (59%); had support from friends (69%); had family who understood the patient's condition (49%); communicated about their condition with family (69%); and had close ties with a lover (43%). Only five patients answered questions regarding their sex lives (3.5%).

Emotional well-being: Sorrow and sad emotions were reported by 83% of patients; fear of death by 79%; and concern about disease progression by 74%. Only 53% of patients completed the item regarding current treatment and losing confidence in overcoming the current disease.

Functional well-being: Working ability was reported by 41%; sense of satisfaction with work by 51%; enjoyment of life by 55%; acceptance of the disease by 59%; insomnia by 95%; enjoyment from hobbies by 63%; and quality of life satisfaction by 52%.

Hepatobiliary cancer subscale: Patients reported stomach distension (65%); weight loss (57%); abnormal intestinal function (87%); digestive dysfunction (87%); diarrhea (64%); good appetite (36%); concern regarding appearance (61%); shoulder and back pain (71%); constipation (54%); fatigue (84%); ability to independently accomplish daily affairs (47%); jaundice 60 cases (43%); fever (36%); itching (27%); food taste changes (48%); cold sensitivity (61%); dry mouth (76%); stomach pain (63%); and swollen ankles (20%). No patients had bile drainage tubes.

DISCUSSION

Our study showed that patients with HCC had a perceived health status which varies by TNM stage for most FACT-Hep items. This conclusion was based on questionnaires widely used in Chinese clinical studies which indicate that the quality of life is an important prognostic factor and predicts survival time in patients with HCC[18].

HCC, an end-stage complication of liver disease, is expected to affect quality of life, but limited results have been reported previously[19]. Both disease-related and treatment-related symptomatic relief has been the primary goal in advanced HCC management because of the low survival rate of the patients. Alleviating clinical symptoms and improving quality of life have become targets for HCC treatment[20]. Quality of life and related factors in patients with liver cancer have been reported in some studies as indicators of treatment efficacy[21-23]. The FACT-Hep has been widely used in clinical studies to assess HRQoL[11]. Although traditional clinical diagnostic indicators (survival time and tumor response rates) and patients' subjective feelings are the primary components of quality of life for patients with HCC, the FACT-Hep can provide more comprehensive clinical evaluations[24,25].

There are several staging systems for HCC, such as the Japan Integrated Staging score, the new barcelona

clinic liver cancer (BCLC) staging classification, and the Tokyo score. These proposed staging systems consider both tumor size and liver function for HCC evaluation[26].

We classified patients' disease status using the BCLC and found that advancing BCLC stage was associated with a decreasing trend for FACT-Hep scores, because BCLC staging is related to liver function. Patients with cirrhosis had lower FACT-Hep scores (lower scores represent lower quality of life, Table 3). Previous studies have assessed the relationship between liver function and HRQoL deterioration and fatigue in patients with quantified inflammatory activity and degree of fibrosis[27]. Additionally, quality of life in patients with cirrhosis secondary to primary biliary cirrhosis or chronic hepatitis C has been reported previously[28-30].

The FACT-HepG mean scores showed that HRQoL in HCC patients significantly declined from TNM Stage I to Stage II to Stage IIIA to Stage IIIB. Thus, FACT-Hep scores could reflect varying levels of HCC disease severity.

Our results demonstrate that HCC has a significant and potentially adverse impact on physical health and psychological well-being, causing disruptions to patients' normal lives. Because the SFWB status of patients with HCC was impaired, family and friends' emotional support becomes particularly important. Although these symptoms may appear minor in the clinical setting, these factors may significantly predict the poor quality of life. Unfortunately, relevant information about sex life on the FACT-Hep questionnaire was limited and controversial; only five patients completed these questions. This limited response may be due to Chinese cultural norms regarding discussion of sexual activity. Throughout the five FACT-Hep items, we found that EWB was variably impaired. Thus, it appears that patients who have an established diagnosis of HCC experience a wide range of negative emotional symptoms such as sorrow and fear of death. Patients reported various levels of FWB based on the disease stage. On the HepCS, patients most commonly reported abnormal intestinal function, digestive dysfunction and fatigue.

Some limitations must be considered in evaluating these results. To avoid the variability of different therapeutic effects, we chose a simple analysis of untreated patients on admission. However, we believe that this approach may more accurately reflect the relationship between FACT-Hep results and TNM stage. Additionally, it may be more significant, in both clinical work and clinical perspective studies, to use the FACT-Hep as a tool to evaluate the patients' quality of life prior to treatment. Careful consideration of digestive dysfunction and emotional support is needed, as symptoms greatly impact HRQoL. Only 3.5% of patients responded to questions regarding sexual activity on the FACT-Hep scale. Cultural influences may preclude the use of these questions and it may be more instructive to focus on HCC patients' psychological states in tumor remission and long-term quality of life, which are other important elements of FACT-Hep. Regarding sleep, the sleep disorders in patients with advanced HCC may be due to the increased burden of physical and psychological factors.

Frequency percents of depression, anxiety and other psychiatric symptoms were significantly higher than other factors. This may be related to the disease itself, which is often associated with a range of negative emotional responses at the time of diagnosis. Furthermore, the poor prognosis, short survival time, need for repeated treatment, and high treatment cost may directly affect patients' mental health status, as demonstrated by the FACT-Hep results for stress, anxiety, fear of death and disease progression.

In conclusion, this study demonstrated that advancing TNM tumor stages were associated with the declining patient quality of life. These results may be useful for physicians to adjust HCC management according to patients' physical condition, extent of disease, and quality of life. Results of this study could be used to provide improved health services to meet the needs of HCC patients at different TNM tumor stages prior to initiating the treatment. In addition, more attention should be paid to the sad emotions, digestive dysfunction, fatigue and insomnia. Despite these findings, more definite evidence of the benefits of FACT-Hep questionnaire is required to justify its use. We plan to perform a long-term follow-up of these HCC patients to closely monitor the quality of life changes and to explore and predict their trends.

COMMENTS

Background
The incidence of hepatocellular carcinoma (HCC) in China is increasing, and HCC is now the second leading cause of death of cancers. Although many therapies have improved survival rates, symptoms become extremely variable in advanced HCC disease; compensated patients may be symptomatic for months or decades. In recent years, there has been increased interest in quality of life as it pertains to patients' health status. Many different questionnaires, such as the functional assessment of cancer therapy-hepatobiliary (FACT-Hep), the health-related quality of life (HRQoL), and the short form (36) health survey are becoming key components in the evaluation of patients' health status. HRQoL results may be more relevant than length of life, as patients are frequently more concerned about life quality than longevity.

Research frontiers
The Chinese version of the FACT-Hep is a new and important tool for the evaluation of prognosis and clinical trials, complementing the traditional end-point methods such as tumor response rate and survival time. This study demonstrated that advancing tumor node metastasis (TNM) stages were associated with the declining patient quality of life. These results may be useful for physicians to adjust HCC management according to patients' physical condition, extent of disease, and quality of life.

Innovations and breakthroughs
As HCC disease worsens, quality of life generally decreases, but two unanswered questions remain. First, FACT-Hep has not been used to assess prognosis with a large series of HCC patients at different TNM tumor stages. Second, quality of life is a broad concept, and no data are available to examine the impact of disease on patients' self-perception and the factors associated with poor HRQoL on the FACT-Hep.

Applications
This study demonstrated that advancing TNM tumor stages were associated with the declining patient quality of life. These results may be useful for physicians to adjust HCC management according to patients' physical condition, extent of disease, and quality of life. Results of this study could be used to provide improved health services to meet the needs of HCC patients at different TNM tumor stages prior to initiating the treatment

Terminology

The FACT-Hep is a 45-item, self-report instrument designed to measure HRQoL in patients with HCC. The FACT-Hep consists of 27-item FACT-G, which assesses generic HRQoL concerns using five subscales, and the newly validated 18-item hepatobiliary subscale, which assesses specific symptoms of hepatobiliary cancer and side effects of treatment. The five FACT-Hep subscales are: (1) physical well-being (7 questions); (2) social and family well-being (7 questions); (3) emotional well-being (6 questions); and (4) functional well-being (7 questions); and (5) hepatobiliary cancer subscale (20 questions).

Peer review

The paper quantifies the quality of life of 140 consecutive and unselected patients with HCC. They have used the FACT-Hep questionnaire and classified their patients according to TNM staging system.

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BRIEF ARTICLE

Effect of intensive *vs* conventional insulin therapy on perioperative nutritional substrates metabolism in patients undergoing gastrectomy

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Abstract

AIM: To investigate the effect of intensive *vs* conventional insulin therapy on perioperative nutritional substrates metabolism in patients undergoing radical distal gastrectomy.

METHODS: Within 24 h of intensive care unit management, patients with gastric cancer were enrolled after written informed consent and randomized to the intensive insulin therapy (IIT) group to keep glucose levels from 4.4 to 6.1 mmol/L or the conventional insulin therapy (CIT) group to keep levels less than 10 mmol/L. Resting energy expenditure (REE), respiratory quotient (RQ), resting energy expenditure per kilogram (REE/kg), and the lipid oxidation rate were monitored by the indirect calorimeter of calcium citrate malate nutrition metabolism investigation system. The changes in body composition were analyzed by multi-frequency bioimpedance analysis. Blood fasting glucose and insulin concentration were measured for assessment of Homeostasis model assessment of insulin resistance.

RESULTS: Sixty patients were enrolled. Compared with preoperative baseline, postoperative REE increased by over 22.15% and 11.07%; REE/kg rose up to 27.22 ± 1.33 kcal/kg and 24.72 ± 1.43 kcal/kg; RQ decreased to 0.759 ± 0.034 and 0.791 ± 0.037; the lipid oxidation ratio was up to 78.25% ± 17.74% and 67.13% ± 12.76% supported by parenteral nutrition solutions from 37.56% ± 11.64% at the baseline; the level of Ln-HOMA-IR went up dramatically ($P < 0.05$, respectively) on postoperative days 1 and 3 in the IIT group. Meanwhile the concentration of total protein, albumin and triglyceride declined significantly on postoperative days 1 and 3 compared with pre-operative levels ($P < 0.05$, respectively). Compared with the CIT group, IIT reduced the REE/kg level (27.22 ± 1.33 kcal/kg *vs* 29.97 ± 1.47 kcal/kg, $P = 0.008$; 24.72 ± 1.43 kcal/kg *vs* 25.66 ± 1.63 kcal/kg, $P = 0.013$); and decreased the Ln-HOMA-IR score ($P = 0.019, 0.028$) on postoperative days 1 and 3; IIT decreased the level of CRP on postoperative days 1 and 3 ($P = 0.017, 0.006$); the total protein and albumin concentrations in the IIT group were greater than those in the CIT group ($P = 0.023, 0.009$). Postoperative values of internal cell fluid (ICF), fat mass, protein mass (PM), muscle mass, free fat mass and body weight decreased obviously on postoperative 7th day compared with the preoperative baseline in the CIT group ($P < 0.05$, respectively). IIT reduced markedly consumption of fat mass, PM and ICF compared with CIT ($P = 0.009$ to 0.026).

CONCLUSION: There were some benefits of IIT in decreasing the perioperative insulin resistance state, reducing energy expenditure and consumption of proteins and lipids tissue in patients undergoing gastrectomy.

BRIEF ARTICLE

GABA stimulates human hepatocellular carcinoma growth through overexpressed GABAA receptor theta subunit

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Abstract

AIM: To investigate the function of gamma-aminobutyric acid (GABA) and gamma-aminobutyric acid A receptor θ subunit (GABRQ) in hepatocellular carcinoma (HCC).

METHODS: Semiquantitative polymerase chain reaction was used for detecting the expression of GABRQ receptor among HCC cell line HepG2, normal liver cell line L-02, non-malignant Chang's liver cells, 8 samples of HCC tissues and paired non-cancerous tissues. HepG2 cells were treated with GABA at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L), and their proliferating abilities were analyzed with the methyl thiazolyl tetrazolium assay, cell cycle analysis and tumor implanted in nude mice. Small interfering RNA was used for knocking down the endogenous GABRQ in HepG2. Proliferating abilities of these cells treated with or without GABA were analyzed.

RESULTS: We identified the overexpression of GABRQ in HCC cell lines and half of the tested HCC tissues. Knockdown of endogenous GABRQ expression in HepG2 attenuated HCC cell growth, suggesting its role in HCC cell viability. We studied the effect of GABA in the proliferation of GABRQ-positive cell lines *in vitro* and *in vivo*, and found that GABA increased HCC growth in a dose-dependent manner. Notably, the addition of GABA into the cell culture medium promoted the proliferation of GABRQ-expressing HepG2 cells, but not GABRQ-knockdown HepG2 cells, which means that GABA stimulates HepG2 cell growth through GABRQ.

CONCLUSION: GABRQ play important roles in HCC development and progression and could be a promising molecular target for the development of new diagnostic and therapeutic strategies of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common

primary liver cancer and one of the most common malignancies in the world, accounting for approximately one million deaths per year[1,2]. Although liver resection and local ablation are regarded as potentially curative treatment[3], its prognosis is poor. Most of the patients are diagnosed with advanced disease at presentation for which palliative therapy forms the mainstay of treatment[4].

To improve this situation, the development of novel molecular therapies against effective targets is an urgent issue. Toward this direction, we previously used a method combining an in silico screen and experimental verification to identify genes that are differentially expressed in cancers compared with their corresponding normal tissues[5]. Among genes that are overexpressed in HCC cells, we focused on the gamma-aminobutyric acid (*GABA*) gene. Gamma-aminobutyric acid A receptor θ subunit (GABRQ) is a subunit of gamma-aminobutyric acid A (GABAA) receptors that may associate with other GABAA receptor subunits to form a functional chloride channel which mediates inhibitory synaptic transmission in the mature central nervous system (CNS). GABA primarily functions as an inhibitory neurotransmitter in the mature CNS by activating the GABA receptor, but it can also modulate the proliferation, migration and differentiation of neuronal cells during CNS development[6-9] and the proliferation of peripheral non-neuronal cells[10,11]. GABA and GABAA receptors are also present in peripheral tissues, including cancerous cells, but their precise functions are poorly defined.

This study demonstrates that GABRQ is overexpressed in HCC and that GABA promotes the proliferation of cancer cells through GABRQ.

MATERIALS AND METHODS

Cell lines
HCC cell line HepG2 and normal liver cell lines Chang's liver and L-02 were maintained by our lab and cultured in Dulbecco-modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Cells were maintained at 37 ℃ atmosphere of humidified air with 5% CO_2.

Collection of tissues
All samples of HCC tissues and paired non-cancerous tissues (5 cm away from tumor) were obtained during surgical resection from the Xiangya Hospital of Central South University. Written consent was obtained from the patients, who agreed to the collection of tissue samples. The resected tissue samples were immediately cut into small pieces and snapfrozen in liquid nitrogen until use. All tumor tissue and paired non-cancerous tissue samples were pathologically confirmed.

Semiquantitative polymerase chain reaction
RNA isolated from cells was reverse-transcribed and amplified using the One-Step reverse transcription polymerase chain reaction (RT-PCR) System (Fermentas, Vilnius, Lithu-

ania). The sets of primers for GABRQ receptor subunit are Sense 5'-TCGAGTTCTCCTCTGCTGTG-3', Antisense 5'-TATGCAGATCCAGGGACAA-3' (465 bp); Sense 5'-AATCCCATCACCATCTTCCA-3' and antisense 5'-CCTGCTTCACCACCTTCTTG-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 580 bp). After heating at 95 ℃ for 1 min, samples were exposed to 30 cycles (GAPDH, 25 cycles) of 95 ℃ for 30 s, 60 ℃ for 30 s and 68 ℃ for 1 min 30 s with a final extension at 68 ℃ for 10 min. Reaction products were separated on 1.5% agarose gels containing ethidium bromide and the level of amplification was analyzed using a Phosphor Imager.

RNA interference
To knockdown GABRQ expression, we used pGCsi-U6/Neo/GFP vector encoding a small hairpin RNA directed against the target gene in HepG2.The target sequences for GABRQ were: 5'-taGCAAGGAGGTGTATTTCTA-3' (Si-1), 5'-caGCTATGGTGTTCGCTTTAA-3'(Si-2), 5'-caGGCTGATGACAGTA TTATT-3'(Si-3), 5'-aaGGAT-GCTTTCGTGCATGAT-3'(Si-3). As a negative control, we used shRNA vector without hairpin oligonucleotides (Si-Mock).

Cell transfection
Human HCC cell line HepG2 was plated onto 6-well plates, and transfected with these small interfering RNA (siRNA) expression vectors using FuGENE6 (Roche) according to the instructions of the manufacturer, followed by 800 μg/mL of neomycin selection. The cells were harvested 10 d later to analyze the knockdown effect on GABRQ by RT-PCR using the primers shown above and by flow cytometry using rabbit anti-human polyclonal antibody against GABRQ (Chemicon).

Impact of GABRQ-siRNA on the growth of hepatocellular carcinoma cells
HepG2/Si-1, HepG2/Si-Mock cells were seeded with serum-free medium at a density of 10^3 cells/well in 96-well plates (*n* = 6), grown overnight, washed in phosphate-buffered saline (PBS), and incubated with 10% FBS with or without 40 μmol/L GABA DMEM at 37 ℃, 5% CO_2, for varying periods and exposed to fresh media every other day. During the last 4 h of each day's culture, the cells were treated with methyl thiazolyl tetrazolium (MTT, 50 μg per well, Sigma, United States). The generated formazan was dissolved in dimethyl sulfoxide (DMSO) and the ODs at 490 nm were measured for detecting the cell viability.

The effect of GABRQ silencing on the colony formation of HepG2 cells was analyzed by colony formation assay. HepG2/Si-1, HepG2/Si-Mock cells at 100 cells per well in 6-cm plates were incubated with serum-fee medium for 24 h, and then cultured in 10% FBS with or without 40 μmol/L GABA DMEM at 37 ℃, 5% CO_2, for 3 wk. The cell colonies were washed twice with PBS, fixed by 4% paraformaldehyde for 15 min and stained with Giemsa for 30 min. Individual clones with more than 50

Table 1 Cell cycle of HepG-2/Si-Mock and HepG-2/Si-1 (mean ± SD, n = 3)

	Si-1	Si-Mock
G0/G1 (%)	53.95 ± 3.22	49.95 ± 3.56
G2/M (%)	22.38 ± 2.79	21.61 ± 3.83
S (%)	24.29 ± 3.32	28.74 ± 3.85[a]

[a]$P < 0.05$ *vs* Si-1.

cells were counted. Clone forming efficiency for individual type of cells was calculated, according to the number of colonies/number of inoculated cells × 100%.

To evaluate the impact of GABRQ silencing on the HepG2 cells and the effect of GABA stimulation on the HepG2 cells, cell cycle was examined by flow cytometry analysis. HepG2/Si-1, HepG2/Si-Mock cells were incubated with serum-fee medium for 24 h, and then cultured in DMEM with 10% FBS with or without 40 μmol/L GABA, then harvested at 70%-80% confluence and resuspended in fixation fluid at a density of 10^6/mL; 1500 μL propidium iodide (PI) solution was added, and the cell cycle was detected by FACS Caliber (Becton-Dickinson).

Effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells

To study the effect of GABA on the proliferation of GABRQ-expressing HCC cells, cell proliferation was tested *in vitro*. In the MTT assay, HepG2 cells were seeded with serum-free medium at a density of 10^3 cells/well in 96-well plates (n = 6), grown overnight, washed in PBS, and incubated with GABA (Sigma-Aldrich) at serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L) in appropriate medium supplemented with 1% FBS. The samples were tested every 24 h for 6 d. MTT was added (50 μg/well) for 4 h. Formazan products were solubilized with DMSO, and the optical density was measured at 490 nm.

In the flow cytometry assay, HepG2 cells were incubated with serum-fee medium for 24 h, and then cultured in DMEM with 10% FBS and serial concentrations (0, 1, 10, 20, 40 and 60 μmol/L) GABA for 48 h. Cells were harvested and resuspended in fixation fluid at a density of 10^6/mL, 1500 μL PI solution was added, and the cell cycle was detected by FACS Caliber (Becton Dickinson).

Tumor formation in nude mice

The influence of GABRQ silencing and GABA stimulation on the tumor development of HCC *in vivo* was examined. Briefly, HepG2, HepG2/Si-Mock and HepG2/Si-1 cells were treated with or without GABA (40 μmol/L) for 24 h first, and then the cells (3 × 10^6) were suspended in 0.2 mL of extracellular matrix gel and injected subcutaneously in the left back flank of the animals. The 8-wk-old BALB/c nude (nu/nu) mice (Slac Laboratory Animal Center, Shanghai, China) were divided into six groups: (1) the mice were injected with HepG2 and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2, n = 4); (2) the mice were injected with HepG2 and treated with GABA injections (40 μmol/L in 150 μL of 0.9%

NaCl) into the implanted tumor (HepG2 + GABA, n = 4); (3) the mice were injected with HepG2/ Si-Mock and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2/Si-Mock, n = 4); (4) the mice were injected with HepG2/Si-Mock and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the implanted tumor (HepG2/Si-Mock + GABA, n = 4); (5) the mice were injected with HepG2/Si-1 and treated with 0.9% NaCl injection (150 μL) into the implanted tumor (HepG2/Si-1, n = 4); and (6) the mice were injected with HepG2/Si-1 and treated with GABA injections (40 μmol/L in 150 μL of 0.9% NaCl) into the implanted tumor (HepG2/Si-1 + GABA, n = 4). The same operator carried out the injections every other day starting from "day 0" when the tumors were implanted. Tumor variables were measured every 3 d by an electronic caliper, and tumor volume was calculated using a standard formula[12,13]: tumor volume = width^2 × length × 0.5. At the end of the experiment, all mice were sacrificed and individual tumor weights were measured.

Statistical analysis

All data were expressed as mean ± SD. Differences among groups were determined by analysis of variance analysis and comparison between two groups was analyzed by the Student's t test using the GraphPad Prism soft-ware version 4.0 (GraphPad Software, Inc, San Diego, CA). A value of $P < 0.05$ was used to indicate statistical significance.

RESULTS

Expression of GABRQ receptors

We documented GABRQ mRNA expression in HepG2, Chang's liver and L-02 cell lines as well as in 8 pairs of HCC and adjacent non-tumor tissues. The results of semi-quantitative RT-PCR show GABRQ receptor subunit was detected in HepG2 and in Chang's liver cells, but not in normal cell line L-02 (Figure 1A). GABRQ receptor subunit was also detected in HCC tissues (6/8), but not in adjacent non-tumor tissues (Figure 1B).

Impact of GABRQ-siRNA on the growth of hepatocellular carcinoma cells

To investigate the biological significance of GABRQ overexpression in HCC cells, we constructed four siRNA expression vectors (Si-1, Si-2, Si-3 and Si-4) specific to GABRQ transcripts and transfected them into HepG2 cells that endogenously expressed high levels of GABRQ, as shown in Figure 1. A knockdown effect was observed by RT-PCR when we transfected Si-1, but not Si-2, Si-3, Si-4 or a negative control Si-Mock (Figure 2A). MTT assay (Figure 2B) revealed a drastic reduction in the number of cells transfected with Si-1 compared with Si-Mock for which no knockdown effect was observed. Cell proliferation was detected by flow cytometry; results showed HepG2 cells with GABRQ siRNA blocked the cell cycle in G1 phase, which may inhibit the growth of HepG2 cells (Table 1). This result was consistent with the MTT analysis.

Figure 1 Expression of gamma-aminobutyric acid A receptor θ subunit in different cell lines, in liver cancerous tissues (Ca) and adjacent tissues of liver cancers (N) by reverse transcription polymerase chain reaction. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Figure 2 The impact of gamma-aminobutyric acid A receptor θ subunit-siRNA on the growth of hepatocellular carcinoma cells. A: Reverse transcription polymerase chain reaction (RT-PCR) verified the RNAi effect on gamma-aminobutyric acid A receptor θ subunit (GABRQ); B: Methyl thiazolyl tetrazolium assay HepG2 cells transfected with Si-1 and negative vectors to GABRQ. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Cell cycle of HepG-2 treated with gamma-aminobutyric acid at serial concentrations (mean ± SD, $n = 3$, μmol/L)

	0	1	10	20	40	60
G0/G1 (%)	40.9 ± 2.92	39.6 ± 2.73	33.8 ± 2.79	31.7 ± 2.56	30.2 ± 2.17	47.2 ± 2.93
G2/M (%)	18.1 ± 0.84	19.8 ± 0.97	20.7 ± 0.85	21.3 ± 0.79	20.3 ± 1.18	18.5 ± 1.23
S (%)[a]	31.0 ± 1.89	30.6 ± 1.94	35.5 ± 2.28	37.0 ± 2.17	39.5 ± 2.34	34.3 ± 2.02

[a]$P < 0.05$ *vs* HepG-2 treated with gamma-aminobutyric acid.

A RT-PCR verified the knockdown effect on GABRQ expression by Si-1, but not by Si-2, Si-3, Si-4 and a negative control Si-Mock in HepG2 cells. GPDH was used to quantify RNAs; Figure 2B illustrates MTT assay of HepG2 cells transfected with Si-1 vectors to GABRQ and a negative control vector (Si-Mock). Y-axis: Average value of absorbance at 490 nm, measured with a microplate reader ($n = 6$, $P < 0.05$).

Effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells

Results displayed in Figure 3A show the addition of GABA in the culture media enhanced the proliferation of HepG2 cells in a dose-dependent manner. The promoting effect on HCC cell proliferation was more evident with the GABA concentration ranging from 1 μmol/L to 40 μmol/L. When the GABA concentration was increased to 60 μmol/L, the promoting effect became insignificant.

The promoting effect on HCC cell proliferation was also detected by flow cytometry analysis. After treating with GABA at serial concentrations, the G0/G1-phase fraction of HepG2 cells significantly decreased; on the contrary, S-phase cells significantly increased, especially at the concentration of 20 μmol/L and 40 μmol/L (Figure 3B, Table 2); this result was consistent with the results above.

In the nude mice implanted with tumors (injected with HepG2 cells), the development of solid HCC tumors was monitored for 40 d. As a result, a significant difference in tumor weight was found in GABA-treated (at the concentration of 40 μmol/L) mice compared with mice injected with 0.9% NaCl only (Figure 3C and D).

Effect of GABA on the growth of hepatocellular carcinoma cells after down-regulated expression of GABRQ

To examine the function of GABRQ as a GABA receptor on the growth of GABRQ-expressing HCC cells, we treat-

Figure 3 The effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells. A: The effects of serial concentration gamma-aminobutyric acid (GABA) on proliferation of HepG-2 cells; B: Cell cycle measured by flow cytometry; C: Forty days after tumor cell injection, mice were sacrificed and tumor weight was recorded ($n = 4$, $P < 0.01$); D: Comparison of tumor weight (the left was tumor of mice injected with 0.9% NaCl only, the right was the tumor of mice treated with 40 μmoL/L GABA).

Table 3 Cell cycle of HepG-2/Si-Mock and HepG-2/Si-1 treated with or without gamma-aminobutyric acid at concentration of 40 μmol/L (mean ± SD, $n = 3$)				
	Si-Mock	**Si-Mock + GABA**	**Si-1**	**Si-1 + GABA**
G0/G1 (%)	49.95 ± 3.16	46.21 ± 2.98	53.95 ± 2.62	48.68 ± 2.49
G2/M (%)	21.61 ± 3.43	20.82 ± 2.43	22.38 ± 2.19	21.45 ± 2.26
S (%)	28.74 ± 3.35	33.64 ± 3.76[a]	24.29 ± 2.72[b]	27.43 ± 1.95

[a]$P < 0.05$, [b]$P < 0.01$ *vs* Si-Mock.

ed HepG2/Si-1 and HepG2/Si-Mock cells with or without GABA (40 μmol/L). The results are shown in Figure 4A: GABA enhanced the growth of HepG2/Si-Mock compared with the HepG2/Si-Mock without GABA. On the other hand, the proliferating ability of HepG2/Si-1, which did not express GABRQ, was not enhanced by GABA. In the nude mice injected with HepG2/Si-Mock, the tumor weight of the mice treated with GABA was much larger than that of the mice treated without GABA, while the mice injected with HepG2/Si-1 did not present such differences (Figure 4C and D).

To further explore GABA stimulation of HepG2 cell growth through GABRQ, we examined the effects of Si-1 and Si-Mock on cell cycle. After treatment with 40 μmol/L GABA, the G0/G1-phase fraction of HepG2/Si-Mock cells significantly decreased; in contrast, S-phase

cells significantly increased, but this event did not occur in HepG2/Si-1 cells (Table 3, Figure 4B).

The other illustration of growth effect of reduced GABRQ expression in HepG2 cells was achieved in a colony formation assay (Figure 4E and F). As a result, the average colony number of Si-1 cells was decreased compared with Si-Mock cells. After treatment with 40 μmol/L GABA, the numbers of cell colonies of HepG2/Si-Mock cells significantly increased, but this did not occur in HepG2/Si-1 cells. These data indicate that GABA stimulates HepG2 cell growth through GABRQ.

DISCUSSION

In this study, we validated the overexpression of GABRQ in more than half of the tested HCC tissues compared with the adjacent non-tumor liver tissues; GABRQ was expressed in malignant liver cell lines HepG2 and moderately expressed in normal cell line Chang's liver, but not in normal cell line L-02, implicating that GABRQ may be a good molecular target for the diagnosis of HCC. Functional analysis using siRNA of GABRQ strongly supported its involvement in the development and progression of HCC. In our study, the proliferation rate of HepG2 cells after GABRQ knockdown was significantly reduced, whereas proliferation of Si-Mock cells was not inhibited. This result indicated that GABRQ may increase the pro-

Figure 4 The effect of gamma-aminobutyric acid on the growth of hepatocellular carcinoma cells after down-regulated expression of gamma-aminobutyric acid A receptor θ subunit. A: The effects of gamma-aminobutyric acid (GABA) on proliferation of HepG2/Si-Mock and HepG2/Si-1 cells; B: Analysis of cell cycles of HepG-2 cells by flow cytometry; C: Forty days after tumor cell injection, mice were sacrificed and tumor weight was recorded (n = 4, P < 0.01 for Si-Mock *vs* Si-Mock + GABA; n = 4, P < 0.01 for Si-1 *vs* Si-1 + GABA); D: Comparison of tumor weight (the left two ones were tumor of mice injected HepG2/Si-Mock and HepG2/Si-1 with 0.9% NaCl, the right two ones were the tumor of mice HepG2/Si-Mock and HepG2/Si-1 treated with 40 μmoL/L GABA); E and F: Colony formation assay (n = 3, P < 0.05 for HepG-2/Si-Mock *vs* HepG-2/Si-Mock + GABA).

liferation ability of hepatocytes. Primarily, GABA and GABA receptors function as an inhibitory neurotransmitter in the mature CNS, but their precise functions in non-neuronal cells or tumor cells are unknown. Joseph *et al*[14]

reported that GABA could inhibit colon cancer migration associated with the norepinephrine-induced pathway. On the other hand, another report showed that GABA and GABAB receptor pathways could be involved in

prostate cancer metastasis or invasion through the regulation of metalloproteinase production[15]. Therefore, it is controversial whether GABA-associated pathways could act positively or negatively in the regulation of cancer cell behavior. However, our findings in this study can clearly indicate evidence supporting the theory that GABA and GABAA receptor with GABRQ promote HCC cell proliferation.

By comparing the proliferative activity of the GABRQ-knockdown HepG2 cells treated with GABA, we found that GABA stimulated HepG2 cell growth through GABRQ. The proliferating ability of the cells treated with GABA was not enhanced compared with the cells without GABA treatment. Previous studies suggest that GABA stimulates collagen synthesis and proliferation of human fibroblasts[16]. Biju *et al*[17] reported that, in N-nitrosodiethylamine-induced neoplasia in the rat liver, GABAB receptors were increased and that the GABAB receptor agonist baclofen increased epidermal growth factor-mediated DNA synthesis in hepatocytes. Thus, GABA-associated pathways also could act positively in the regulation of cancer cell behavior. Our findings in this study also support the theory that GABA and GABRQ promote HepG2 cell proliferation *in vivo* and *in vitro*. Interestingly, GABAA receptor antagonist bicuculline methiodide could also promote the proliferation of HepG2 cells (data not shown), indicating that it might activate some other signal pathways[18].

Although GABA usually induces hyperpolarization in adult neurons, GABA has been shown to exert depolarizing responses in the immature CNS structures and CNS tumors[19,20]. In particular, GABA increased the proliferation of immature cerebellar granule cells through the activation of GABAA receptors and voltage-dependent calcium channels[21,22]. Takehara *et al*[23] reported that GABA stimulated pancreatic cancer growth through GABRP by increasing intracellular Ca^{2+} levels and activating the mitogen-activated protein kinase/extracellular signal-regulated kinase cascade. Also, Minuk *et al*[24] reported that human HCC tissues were depolarized compared with adjacent non-tumor tissues. From the results above, we deduce that GABA may promote the HepG2 cell proliferation through GABRQ by voltage-dependent calcium channels. Interestingly, GABA inhibited the growth of the GABRQ-knockdown HepG2 cells. This indicates that GABA activates some other receptors to inhibit the proliferation without GABRQ, which is identical to some previous reports[25-27].

In conclusion, compared with adjacent non-tumor tissues, HCC tissues have increased GABRQ receptor expression. Knockdown of GABRQ expression in receptor-expressing malignant hepatocytes results in attenuated *in vitro* and *in vivo* tumor growth. Moreover, GABA promotes hepatocyte proliferation through GABRQ. These findings highlight the importance of elucidating the role of GABAergic activity in the pathogenesis of HCC. They also raise the potential for new therapeutic and diagnostic approaches to human HCC.

COMMENTS

Background
Gamma-aminobutyric acid A receptor θ subunit (GABRQ) is a subunit of the gamma-aminobutyric acid A (GABAA) receptors that may associate with other GABAA receptor subunits to form a functional chloride channel which mediates inhibitory synaptic transmission in the mature central nervous system (CNS). gamma-aminobutyric acid (GABA) functions as an inhibitory neurotransmitter for activating GABA receptors.

Research frontiers
Recently, abnormal levels of gene and protein expression of some GABA receptor subunits have been detected in many malignant tumors. This research indicates that GABAergic system may play an important role in the pathogenesis and development of malignant tumors.

Innovations and breakthroughs
This study demonstrated the overexpression of GABRQ in hepatocellular carcinoma (HCC), which has not been previously described, and illustrated that GABA stimulates HCC cell proliferation through GABRQ.

Applications
Further characterization of GABRQ will provide new insights into the role of GABRQ in the molecular pathogenesis and therapy of HCC.

Terminology
GABA stands for gamma-aminobutyric acid, which is an inhibitory neurotransmitter. GABRQ stands for gamma-aminobutyric acid A receptor θ subunit.

Peer review
The authors have analyzed the expression and the role of GABRQ in hepatocellular carcinoma. The manuscript is well-written and the study is conducted appropriately in order to understand the molecular mechanisms that control hepatocarcinogenesis, and also raise the potential for new therapeutic and diagnostic approaches to human HCC.

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BRIEF ARTICLE

Phosphatase and tensin homolog expression related to cetuximab effects in colorectal cancer patients: A meta-analysis

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Abstract

AIM: To investigate the correlation between expression of phosphatase and tensin homolog (PTEN) and cetuximab effects in colorectal cancer.

METHODS: We searched PubMed, EMBASE and ASCO to identify eligible studies. Finally, 8 randomized control studies were included in the meta-analysis. STATA 10.0 Software was used to investigate heterogeneity among individual studies and to summarize all the studies. Risk ratios (RRs) and hazard ratios (HRs) with 95% confidence intervals (CIs) were used to assess the strength of the association.

RESULTS: Compared with 20 of 266 patients with loss of PTEN, 206 of 496 patients with intact PTEN protein expression had a better objective response rate to cetuximab-based therapy (RR, 4.75; 95% CI, 2.59-8.72; $P < 0.001$). PTEN positivity was associated with better progression-free survival (PFS) (HR, 0.675; 95% CI, 0.473-0.964; $P = 0.031$) but not with better overall survival (OS) (HR, 0.608; 95% CI, 0.411-0.899; $P = 0.013$). In patients with KRAS wild-type status, PTEN positivity did not predict a longer PFS or OS (PFS: HR, 0.707; 95% CI, 0.440-1.138; $P = 0.154$; OS: HR, 0.943; 95% CI, 0.646-1.377; $P = 0.761$).

CONCLUSION: Expression of PTEN is related to the effect of cetuximab in colorectal cancer patients and should be considered in treatment with cetuximab.

Shen Y, Yang J, Xu Z, Gu DY, Chen JF. Phosphatase and tensin homolog expression related to cetuximab effects in colorectal cancer patients: A meta-analysis. *World J Gastroenterol* 2012; 18(21): 2712-2718 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i21/2712.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i21.2712

INTRODUCTION

Colorectal cancer (CRC) is the fourth common malignancy and the second leading cause of cancer death in Western countries[1]. More than half of CRC patients will develop metastatic lesions (mCRC), which are often found in the liver[2]. Although novel pharmaceutical and surgical interventions have been introduced to treat mCRC, the 5-year survival rate for mCRC remains below 10%[3,4]. Recently cetuximab, a monoclnonal antibody that targets the epidermal growth factor receptor (EGFR) has

been proven to be efficacious in mCRC patients[3]. Cetuximab binds to EGFR *via* its ligand-binding domain to inhibit the activation of EGRF signaling. In clinical trials, cetuximab has been reported to achieve a response rate of 10% as a single agent and of 23%-25% in combination with chemotherapy[5,6]. The addition of cetuximab to chemotherapies enhances their antitumor activity[7]. The proposed mechanisms include: reducing tumor cell proliferation, angiogenesis, and DNA repair capacity; increasing apoptosis; and inducing cell cycle arrest at treatment-sensitive points[5]. These effects may enhance and restore tumor sensitivity to cytotoxic agents[8].

In CRC patients, EGFR is overexpressed in 75% of the tumors and its overexpression is associated with worse outcome[3,9]. EGFR was accordingly an obvious candidate for targeted therapy in this malignancy[5]. The tumor suppressor phosphatase and tensin homolog (PTEN) is an important negative regulator of cell-survival signaling[1]. To date, there is evidence to suggest that loss of expression of PTEN has negative association with the prognosis of CRC, especially mCRC. Loss of PTEN expression results in increased phosphatidylinositol phosphate-3 concentration, which induces subsequent protein kinase B hyperphosphorylation, thus protecting cancer cells from apoptotic stimuli[10-12]. In Addition, underexpression of PTEN confers resistance to cetuximab-induced apoptosis[10].

It is important to reveal the relation between the expression of PTEN and the prognosis of mCRC patients treated with cetuximab, as this will be helpful for adopting appropriate targeted therapy for patients[13]. At present, there are many studies which have reported the clinical outcomes of cetuximab in mCRC patients with loss of expression of PTEN. Hence, we carried out a meta-analysis to analyze the relation between the expression of PTEN and prognosis of CRC patients treated with cetuximab.

MATERIALS AND METHODS

Eligibility criteria
The purpose of this research was to systematically review the published articles of cetuximab-based chemotherapy in CRC (both primary and metastatic). Studies which reported the patients' PTEN status and compared the prognosis, were included in the analysis. The primary outcomes of interest were overall survival (OS) and progression-free survival (PFS). Care was taken to include only primary data or data that superseded earlier work.

Identification of studies
The search for studies was performed using the electronic database PubMed with the keywords "colorectal cancer", "cetuximab" and "PTEN". We also referred to the electronic database ASCO and EMBASE. All studies matching the eligibility criteria were retrieved and their bibliographies were checked for other relevant publications. Review articles and bibliographies of other relevant stud-

ies were identified through hand-searching to identify the additional studies. Data from review articles, case reports, abstracts, and letters were not included. Pharmaceutical industries and authors were not contacted. Characteristics of the studies were extracted from published articles and summarized in a consistent manner to aid comparison[14].

Statistical analysis
The meta-analysis was conducted by using Stata software (version 10.0; StataCorp Lakeway, College Station, TX, United States). Before performing the analyses, data of each published study were carefully checked and verified for coherence with the original publications. The strength of the association between status of PTEN and response of cetuximab-based therapy was measured by the risk ratio (RR) with 95% confidence intervals (CIs). Individual trial level time-to-event data was summarized by the hazard ratio (HR) with 95% CIs. Pooled estimations of RR and HR were obtained by calculating a weighted average of RR and HR from each study.

Statistical heterogeneity between studies was evaluated with the χ^2 test with significance set at a P value of 0.05. The percentage of total variation across the studies, with higher values indicating a greater degree of heterogeneity, was measured by the I^2 statistic. If the P value was $\leqslant 0.05$, the assumption of homogeneity was deemed invalid, and the DerSimonian-Laird method[15] (random-effects model) was used after exploring the causes of the heterogeneity; otherwise, the Mantel-Haenszel method[16] (fixed-effects model) was used. In the absence of heterogeneity, the fixed-effects and random-effects models provided similar results. I^2 lay between 0% and 100%, and a value of 0% indicated no observed heterogeneity, while larger values indicated increasing heterogeneity[17].

Findings of the meta-analysis are depicted in classical Forest plots, with point estimates and 95% CIs for each trial and overall size of the squares proportional to the effect size[18]. It was statistically significant when the two-tailed P value < 0.05. Publication bias was adjusted using the trim-and-fill method, and assessed by visual inspection of funnel plots (Figure 1)[19].

RESULTS

Description of studies
After exclusion of duplicate and irrelevant studies (Figure 2), our search yielded 8 eligible published studies that were retrieved for more detailed evaluation and meta-analysis[3,5,9,10,20-23]. The main characteristics of these selected studies are summarized in Table 1, and the description of PTEN status listed in Table 2. Most of the patients received a cetuximab-based therapy as second-line or later therapy after chemotherapy failure. All 8 studies including a total of 698 patients, of whom 513 were allocated to cetuximab plus irinotecan and others to cetuximab only or with various regimens as shown in detail in Table 1. The outcome measures of the above studies were evaluated based on the Response Evaluation Criteria in Solid Tu-

Table 1 The main characteristics of the 8 selected studies

First Author	Year	Type of study	n	Chemotherapy regimen						
				Ctx only	Ctx plus iri	Ctx plus folfiri	Ctx plus folfox	Pan	Ctx plus oxa	Ctx plus oxa and cap
Sartore-Bianchi *et al*[3]	2009	Cohort study	110	14	74	0	0	22	0	0
Negri *et al*[5]	2009	Retrospective study	50	0	36	0	0	0	14	0
Laurent-Puig *et al*[9]	2009	Retrospective study	173	3	141	28	0	0	0	0
Loupakis *et al*[10]	2009	Retrospective cohort study	102	2	100	0	0	0	0	0
Perrone *et al*[21]	2009	Cohort study	32	0	32	0	0	0	0	0
Frattini *et al*[20]	2007	Cohort study	27	0	23	0	0	0	0	4
Razis *et al*[22]	2008	Retrospective study	72	1	13	27	18	-	-	-
Sartore-Bianchi *et al*[23]	2009	Cohort study	132	15	94	0	0	23	0	0

CTX: Cetuximab; Pan: Panitumumab; oxa: Oxaliplaten; cap: Capecitabine.

Table 2 Description of phosphatase and tensin homolog status

No.	Title of the study	Method
1	Analysis of PTEN, BRAF and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer	IHC
2	PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients	FISH
3	PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer	IHC
4	PTEN status in advanced colorectal cancer treated with cetuximab	FISH
5	PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies	IHC
6	PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients	IHC
7	Potential value of PTEN in predicting cetuximab response in colorectal cancer: An exploratory study	FISH
8	Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer	IHC

PTEN: Phosphatase and tensin homolog; BRAF: V-raf murine sarcoma viral oncogene homolog; EGFR: Epidermal growth factor receptor; IHC: Immunohistochemistry; FISH: Fluorescence *in situ* hybridization.

Figure 1 Begg's funnel plot of publication bias.

Figure 2 Selection of the studies.

mor criteria, PFS and OS. Patients with stable disease or progression of disease were defined as non-responders. Results are presented for the comparisons with the available data.

Analysis of status of the PTEN homolog and objective response

Five articles documented the response rate of cetuximab-based therapy (Figure 3A). There were 266 patients with loss of PTEN and 496 patients with normal expression of PTEN. In total, compared with 20 of 266 patients with

loss of PTEN, 206 of 496 patients with intact protein expression had an objective response rate to cetuximab-based therapy (RR, 4.75; 95% CI, 2.59-8.72; $P < 0.001$). There was no heterogeneity between trials ($P = 0.637$, $I^2 = 0.0\%$). We also analyzed the response to cetuximab-based therapy in metastatic and primary colorectal tumors. Cetuximab-based therapy achieved significantly higher RR among patients with PTEN expression for metastatic tumors (RR, 6.46; 95% CI, 2.94-14.19; $P < 0.001$). In contrast, among 128 assessable primary tumors, 32 of 87 PTEN-positive and 5 of 41 PTEN-negative patients were

A

Study	PTEN-positive n/N	PTEN-negtive n/N	RR (fixed)	Weight %	RR (fixed) 95% CI
01 Primary colorectal cancer					
Fotios Loupakis, 2009	11/49	4/36		72.29	2.020 (0.700, 5.834)
FV Negri, 2009	21/38	1/5		27.71	2.763 (0.468, 16.324)
Subtotal (95% CI)	87	41		100	2.226 (0.893, 5.548)

Test for heterogeneity: $\chi^2 = 0.09$, d.f. = 1 ($P = 0.765$), $I^2 = 0.00\%$
Test for overall effect: $Z = 1.72$ ($P = 0.086$)

02 Metastatic colorectal cancer					
Fotios Loupakis, 2009	12/33	1/22		15.56	8.000 (1.119, 57.215)
FV Negri, 2009	14/20	0/4		10.47	6.905 (0.490, 97.254)
Andrea Sartore-Bianchi, 2009	17/49	1/32		15.69	11.102 (1.553, 79.369)
Evangelia Razis, 2008	18/43	3/23		50.69	3.209 (1.055,9.762)
M Frattini, 2007	10/16	0/11		7.6	14.824 (0.958, 229.366)
Subtotal (95% CI)	161	92		100	6.462 (2.943, 14.192)

Test for heterogeneity: $\chi^2 = 2.21$, d.f. = 4 ($P = 0.697$), $I^2 = 0.00\%$
Test for overall effect: $Z = 4.65$ ($P = 0.000$)

Fotios Loupakis, 2009	23/82	5/58		44.06	3.254 (1.314, 8.057)
FV Negri, 2009	35/58	1/9		13.02	5.431 (0.846, 34.874)
Andrea Sartore-Bianchi, 2009	17/49	1/32		9.1	11.102 (1.553, 79.369)
Evangelia Razis, 2008	18/43	3/23		29.41	3.209 (1.055, 9.762)
M Frattini, 2007	10/16	0/11		4.41	14.824 (0.958, 229.366)
Subtotal (95% CI)	248	133		100	4.749 (2.586, 8.719)

Test for heterogeneity: $\chi^2 = 2.54$, d.f. = 4 ($P = 0.637$), $I^2 = 0.00\%$
Test for overall effect: $Z = 5.02$ ($P = 0.000$)

0.001 0.01 0.1 1 10 100 1000

Legend: *n* = Number of responses
N = Number of patients

Favours PTEN-negtive   Favour PTEN-positive

B

Study	HR (fixed) 95% CI	Weight %	HR (fixed)
01 PFS			
Fotios Loupakis, 2009		29.04	0.490 (0.200, 0.750)
Andrea Sartore-Bianchi, 2009		70.96	0.770 (0.501, 1.167)
Subtotal (95% CI)		100.00	0.675 (0.473, 0.964)

Test for heterogeneity: $\chi^2 = 1.27$, d.f. = 1 ($P = 0.259$), $I^2 = 21.6\%$
Test for overall effect: $Z = 2.16$ ($P = 0.031$)

02 OS			
Fotios Loupakis, 2009		34.79	0.760 (0.380, 1.430)
Andrea Sartore-Bianchi, 2009		65.21	0.540 (0.332, 0.874)
Subtotal (95% CI)		100.00	0.608 (0.411, 0.899)

Test for heterogeneity: $\chi^2 = 0.67$, d.f. = 1 ($P = 0.414$), $I^2 = 0.0\%$
Test for overall effect: $Z = 2.49$ ($P = 0.013$)

0.02 0.05 1 2 5

Legend: PFS = Progression-free survival
OS = Overall survival

An HR less than 1 indicates a survival advantage

C

Study	HR 95% CI	Weight %	HR 95% CI
01 PFS fixed-model			
Fotios Loupakis, 2009		23.05	0.450 (0.120, 0.870)
Andrea Sartore-Bianchi, 2009		76.95	0.810 (0.470, 1.390)
Subtotal (95% CI)		100.00	0.707 (0.440, 1.138)

Test for heterogeneity: $\chi^2 = 1.04$, d.f. = 1 ($P = 0.308$), $I^2 = 3.9\%$
Test for overall effect: $Z = 1.43$ ($P = 0.154$)

02 OS random-model			
Fotios Loupakis, 2009		28.41	0.500 (0.150, 1.260)
Andrea Sartore-Bianchi, 2009		34.87	0.430 (0.220, 0.810)
Pierre Laurent-Puig, 2009		36.71	1.800 (1.100, 3.100)
Subtotal (95% CI)		100.00	0.759 (0.268, 2.153)

Test for heterogeneity: $\chi^2 = 12.93$, d.f. = 1 ($P = 0.002$), $I^2 = 84.5\%$
Test for overall effect: $Z = 0.52$ ($P = 0.604$)

0.01 0.02 0.05 1 2 5 10

Legend: PFS = Progression-free survival
OS = Overall survival

An HR less than 1 indicates a survival advantage

Figure 3 Analysis of status of the phosphatase and tensin homolog homolog. A: Analysis of status of the phosphatase and tensin homolog (PTEN) homolog and objective response; B: Analysis of status of the PTEN homolog and survival; C: Combined analysis of the PTEN homolog and Kirsten rat sarcoma 2 viral onco-gene homolog (KRAS) status and survival. RR: Risk ratio; CI: Confidence interval; HR: Hazard ratio; OS: Overall survival; PFS: Progression-free survival.

responders, and there was no significant difference observed (RR, 2.226; 95% CI, 0.893-5.548; P = 0.086). There was no evidence for heterogeneity between the studies (P = 0.697, I^2 = 0.0%; P = 0.765, I^2 = 0.0%; respectively).

Analysis of status of the phosphatase and tensin homolog and survival

Only two trials involving 170 patients were included in this comparison, because none of the other eligibility criteria had sufficient follow-up data listed (Figure 3B). The HR summarizes survival for PTEN-positive compared with PTEN-negative patients after cetuximab-based therapy, with an HR of less than 1 indicating a survival advantage for expression of PTEN in colorectal tumors. As for PFS, PTEN positivity was associated with better survival (HR, 0.675; 95% CI, 0.473-0.964; P = 0.031). The analysis for OS confirmed that loss of PTEN was significantly associated with poor clinical outcome (HR, 0.608; 95% CI, 0.411-0.899; P = 0.013). There was no significant intertrial heterogeneity for the end points of PFS (P = 0.259, I^2 = 21.6%) or OS (P = 0.414, I^2 = 0.0%).

Combined analysis of the PTEN homolog and KRAS status and survival

The studies selected for this analysis are listed in Figure 3C. The HR summarizes survival for PTEN-positive/wt-KRAS *vs* PTEN-negative/wtKRAS, with an HR of less than 1 indicating a survival advantage for PTEN-positive/wtKRAS. Overall, among patients with KRAS wild-type status, PTEN positivity did not predict a longer PFS or OS (PFS: HR, 0.707; 95% CI, 0.440-1.138; P = 0.154; OS: HR, 0.943; 95% CI, 0.646-1.377; P = 0.761). Heterogeneity was not found among trials for the analysis of PFS (P = 0.308, I^2 = 3.9%). However, there was marked intergroup heterogeneity for the combined analysis of OS making it difficult to obtain a clear conclusion (P = 0.002, I^2 = 84.5%). To adjust for this bias, the trim-and-fill method was implemented. The adjusted estimates for OS were obtained by using the random-effects model (HR, 0.759; 95% CI, 0.268-2.153; P = 0.604). In the results from the data, there was no difference between the fixed-effects and the random-effects model, indicating the reliability of this meta-analysis, so we can reach a real conclusion.

DISCUSSION

Nowadays, there is a trend towards individualized treatment in tumor therapy. The optimized application of cetuximab has paved a way for individualized treatment of CRC[24]. In recent years, cetuximab has been widely used in the patients with mCRC, and most of the patients have better prognosis than those treated with combined chemotherapy alone. However, personalized cancer medication is based on the genetics of individual colorectal tumors[24]. Hence, the effects of molecular alterations, especially the activating mutations in the KRAS protein, and the corresponding therapeutic effect of cetuximab have been widely discussed[25-27]. KRAS mutation testing is used

in the setting of EGFR-targeted therapy for metastatic disease worldwide[28]. Nevertheless, an intact KRAS is necessary but not sufficient to obtain benefit from EGFR inhibition[29-32]. Alterations in other downstream effectors of EGFR, such as BRAF and PIK3CA/PTEN have been found to give rise to cetuximab resistance[1,33]. Therefore, there is a deep need to reveal possible interactions between targeted agents, so that we can better select patients likely to respond to cetuximab-based treatment[28,29,34].

In this study, we focused on the association between the alteration of PTEN protein expression and the therapeutic effects of cetuximab in CRC patients. In addition, patients treated with panitumumab were also listed in the study search, because the two EGFR inhibitors, cetuximab (the chimeric IgG1 monoclonal antibody) and panitumumab (the humanized IgG2 monoclonal antibody), are currently approved in medication for CRC[34,35]. Both of the molecules bind to the EGFR, leading to inhibition of its downstream signaling and providing some clinical benefit.

PTEN is a tumor suppressor protein, which works as a negative regulator of PI3K/PTEN/Akt, which is a cell-survival signaling pathway[36]. Loss of PTEN expression was associated with the aggressive capacity of CRC, and that understanding the biologic mechanisms responsible for regulation of PTEN expression may allow better translational treatment of CRC patients. Furthermore, CRC patients with loss of PTEN expression show resistance to cetuximab[1].

In our selected studies, patients with normal PTEN expression had higher RR in all CRC with cetuximab-based therapy (especially in mCRC). Also we revealed that patients with PTEN normal expression with cetuximab treatment have better prognosis than those without cetuximab treatment and statistical analysis (OS and PFS) also presents significant differences (P < 0.05). In these studies we concluded that PTEN be proposed as an independent predictive factor[1] of cetuximab efficacy. We suggested that PTEN could help to predict prognosis and efficacy of cetuximab. Diagnostic evaluation of PTEN expression might provide additional guidelines for the treatment strategies for CRC patients and valuable prognostic information.

On the other hand, we did a combined analysis of PTEN and KRAS status on OS and PFS. Unfortunately, among patients with wild-type KRAS, PTEN positivity did not predict longer PFS and OS. Only one report showed the interaction between KRAS mutations with or without expression of PTEN in CRC. Thus, we could not perform a meta-analysis. The conclusion obtained in the report was that the PFS and OS of PTEN-positive patients with KRAS mutations were not significantly longer than in all other patients who presented with KRAS mutations and were PTEN-negative[10]. Survival analyses by Loupakis *et al*[10] demonstrated that BRAF mutations (HR, 3.75; P = 0.015) but not PIK3CA mutations (HR, 1.20; P = 0.672), were significantly associated with decreased OS, whereas neither of these alterations was significantly

associated with PFS. Further clinical data are necessary to identify a certain genes-alteration signature to predict the therapeutic effects of cetuximab-based therapy.

There are some limitations in this meta-analysis. First, the numbers of published studies were not adequate for a comprehensive analysis. Second, only 3 trials reported data of PFS and OS, and a lack of the original data in some studies limited our evaluation of survival, which may cause serious confounding bias. Third, although significant heterogeneity in some end-point variables were at least partly overcome by random-effects analysis, there was still heterogeneity between the relevant studies for inclusion, which may have affected the final results.

In conclusion, our meta-analysis showed an important role of PTEN status in determining the application of cetuximab-based targeted therapy. More clinical trials are warranted in this field to obtain more accurate results. Further improvement in the tailoring of EGFR targeted therapies needs more studies on molecular dissection of the EGFR-initiated oncogenic signaling cascade.

COMMENTS

Background
Cetuximab as a monoclnonal antibody (mAb) that has been used in colorectal cancer (CRC) patients. However, the responses vary in different individuals. Phosphatase and tensin homolog (PTEN) is an important negative regulator and its downregulation has been found in many CRC patients. The relationship between PTEN expression and the effects of cetuximab in CRC patients is still uncertain. The aim of this meta-analysis was to obtain a correlation.

Research frontiers
Cetuximab is a mAb that targets the epidermal growth factor receptor (EGFR). It binds to EGFR *via* its ligand-binding domain to inhibit the activation of EGRF signaling. Cetuximab has been reported to achieve a response rate of 10% as a single agent and of 23%-25% in combination chemotherapy. PTEN is an important negative regulator of cell-survival signaling and underexpression of PTEN confers resistance to cetuximab-induced apoptosis.

Innovations and breakthroughs
Many studies have reported the clinical outcomes of cetuximab in CRC patients with loss expression of PTEN. An exact conclusion has not been achieved mainly because of the limitation of sample size. This is the first study to report the relation between the expression of PTEN and prognosis of CRC patients treated with cetuximab.

Applications
This study may be helpful for adopting appropriate target therapy of cetuximab in patients with CRC.

Terminology
PTEN is the tumor suppressor phosphatase and tensin homolog that plays as an important negative regulator of cell-survival signaling.

Peer review
This is an interesting manuscript presenting a systematic analysis of the impact of PTEN expression on CRC response to cetuximab. It has clear limitations due to the quality of published papers. However, its findings are interesting.

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BRIEF ARTICLE

Aberrant methylation and downregulation of *sall3* in human hepatocellular carcinoma

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Abstract

AIM: To investigated whether *sall3* transcription was regulated by promoter CpG island hypermethylation in hepatocellular carcinoma (HCC).

METHODS: The cell lines Huh7, HepG2, SK-HEP1, SM-MC7721, Bel7402, QGY7703 and a cohort of 38 HCC tissue specimens and corresponding nontumorous tissues were subjected to analysis for *sall3* promoter CpG island methylation and mRNA transcription. *sall3* promoter CpG island methylation levels were determined using the MassARRAY platform and mRNA transcription levels of the gene were detected by quantitative real-time polymerase chain reaction.

RESULTS: The levels of *sall3* mRNA were decreased by more than twofold in 33 of 38 tumor tissues compared to adjacent noncancerous tissues. Among these 33 tumor tissues with lower levels of *sall3* mRNA, 24 showed higher levels of methylation. Based on these results, we hypothesized that the decrease in *sall3* mRNA transcription level was likely due to promoter CpG island hypermethylation. Changes in *sall3* mRNA transcription and promoter CpG island methylation were determined in the above six cell lines after treatment with 0, 0.1, 0.5 and 2.5 μmol 5-aza-2-deoxycytidine, a demethylating agent. Promoter CpG island methylation levels decreased in a dose-dependent manner in all six cell lines, while the mRNA transcription level increased dose-dependently in Huh7, HepG2, SK-HEP1 and SMMC7721 cells and irregularly in Bel7402 and QGY7703 cells.

CONCLUSION: These results indicated that promoter CpG island hypermethylation contributes to the downregulation of *sall3* mRNA transcription in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, representing a major public health issue, especially in Asia[1]. The pathogenesis of HCC involves chronic hepatitis virus infection and activation of oncogenes and/or inactivation of tumor suppressor genes by mutations and epigenetic modification[2]. Epigenetic inactivation of tumor suppressor genes by DNA hypermethylation plays an important role in carcinogenesis[3]. Many groups have reported that promoter hypermethylation of CpG islands is associated with development, stage, recurrence, progression and survival in HCC[4,5]. In many cases, aberrant methylation of promoter regions within genes is correlated with a loss of gene expression[6]. Furthermore, in contrast to mutations, epigenetic changes may be reversible, raising the possibility of developing therapeutics based on restoring a normal epigenetic state in cancer-associated genes[7,8].

sal was originally identified as a region-specific homeotic gene in *Drosophila*[9]. *sall3* is one of four mammalian members of the sal-like (sall) gene family (*sall1*, *sall2*, *sall3* and *sall4*), which are involved in embryonic development[10]. *sall3* is one of several genes deleted in 18q deletion syndrome, characterized by hearing loss, mental retardation, midfacial hypoplasia, delayed growth, and limb abnormalities[11]. Loss of the *sall3* gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality[12]. Recently, it was reported that sall3 can interact with DNMT3A and shows the ability to inhibit CpG island methylation in HCC[13]. However, when scanning the nucleotide sequences of *sall3*, we found a CpG island in the promoter. It has been reported that *sall3* gene methylation levels are significantly increased in bladder cancer compared to nontumorous controls, and may be a new biomarker for the sensitive and specific detection of bladder cancer[14]. Furthermore, it has been reported that the *sall3* gene CpG island has a higher frequency of hypermethylation in HCC tumors compared with adjacent noncancerous tissues as determined by a qualitative methylation method[15]. However, the decreased *sall3* mRNA transcription levels in human HCC tissues and whether this is caused by promoter CpG island hypermethylation have not been fully examined.

Here, we show that sall3 mRNA transcription was downregulated in most (33/38) tumor tissues examined compared with adjacent noncancerous tissues. Most (24/33) downregulation of mRNA transcription was strongly associated with hypermethylation of the promoter CpG island. This association was further confirmed by subsequent cell line experiments; treatment of the cell lines with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine reversed promoter CpG island hypermethylation and restored *sall3* mRNA transcription. These results indicated that promoter CpG island hypermethylation is the main reason for the downregulation of *sall3* mRNA transcription in HCC.

MATERIALS AND METHODS

Tissue specimens and cell lines

Thirty-eight paired clinical samples of HCC, including tumor tissues and adjacent noncancerous tissues, were collected from surgical specimens at the Department of Hepatobiliary Surgery, Nanfang Hospital (16 cases), and the Cancer Institute of Sun Yat-sen University (22 cases), both in Guangzhou, China. All specimens were obtained immediately after surgical resection and were stored at -70 ℃ until DNA/RNA extraction.

Written informed consent was obtained from all patients prior to inclusion in the study. The study protocol was approved by the Nanfang Hospital Ethics Committee at Southern Medical University and the Sun Yat-sen Cancer Center Ethics Committee at Sun Yat-sen University.

Cell culture and 5-aza-CdR treatment

Six HCC cell lines (Huh7, HepG2, SMMC-7721, Bel-7402, SK-HEP1, QGY7703) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin and incubated in a 5% CO_2 atmosphere at 37 ℃. The demethylating agent, 5-aza-2-deoxycytidine (5-aza-CdR; Sigma, St. Louis, MO), was freshly prepared in ddH_2O. HepG2 cells (3×10^5 cells/well) and other hepatoma cells (1×10^5 cells/well) in exponential growth phase were seeded in 6-well plates. After 24 h of culture, cells were treated with 5-aza-CdR at 0, 0.1, 0.5 and 2.5 mol for 3 d. The culture medium was replaced every 24 h with fresh media containing 5-aza-CdR. Total RNA and genomic DNA were extracted for real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and DNA methylation level analysis.

Detection of sall3 CpG Island DNA hypermethylation

Genomic DNA was extracted from cells and HCC samples using a QIAamp DNA Minikit (Qiagen, Valencia, CA). Genomic DNA (1 μg) was modified with sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA). DNA methylation levels of clinical samples and cell lines were determined using the MassARRAY platform (Sequenom, San Diego, CA) as described previously[16]. Briefly, two fragments covering 38 CpG sites from *sall3* were amplified from bisulfite-modified DNA. A 10-mer tag sequence was added to the forward primer, and a T7-promoter tag was added to the reverse primer to balance the PCR primer length. The primers used were 5'-AGGAAGAGAGGGATTGTTTGGATTTGATTT-TAATTT-3' (sense) and 5'-CAGTAATACGACTCAC-TATAGGGAGAAGGCTCACAAATAACCTCCTA-AAACTTCCC-3' (antisense); 5'-AGGAAGAGAGTTT-TAAGGTTGGTTTTTATTTTGTTT-3' (sense) and 5'-CAGTAATACGACTCACTATAGGGAGAAGGCTT-CTCAAAAATAATCTCAAACCCCTA-3' (antisense). Methylation data for individual units (1-3 CpG sites per unit) were analyzed using the EpiTyper software (Sequenom).

Figure 1 Average methylation levels were calculated from 38 tumors (T) and adjacent non-cancerous tissues (N) on 24 CpG units from *sall3* CpG island respectively. The data was analyzed by Wilcox rank sum test ([a]$P < 0.05$ *vs* non-cancerous tissues). Error bar, 95% confidence interval.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from cell lines and tissue samples using the Trizol reagent (Invitrogen), according to the manufacturer's protocol. First-strand cDNA was generated using a SYBR PrimeScript RT-PCR Kit (TaKaRa, Kyoto, Japan). *sall3* mRNA expression was detected by qRT-PCR using a SYBR Premix Ex *Taq* Kit (TaKaRa) on an ABI 7500 Real-Time PCR System (Applied Biosystems). β-actin was used as an internal control. The primers used were as follows: *sall3* forward primer: 5'-GCT-GCCTTCTCAGTTATTTGACC-3', reverse primer: 5'-TGACCGTTCACTTCCATTTTGA-3'; β-actin forward primer: 5'-TTGTTACAGGAAGTCGCTTGCC-3', reverse primer: 5'-ATGCTATCACCTCCCCTGTGTGT-3'. Relative levels of *sall3* mRNA were calculated and expressed as $2^{-\Delta\Delta Ct}$[17].

Statistical analysis

qRT-PCR results in different groups were analyzed by Student's *t* test. The methylation levels of Oct-6 in HCC tumors and adjacent noncancerous tissues were compared using the Wilcox rank sum test. All tests were two-sided. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

sall3 promoter CpG island aberrant methylation and sall3 mRNA transcription in human HCC tissues

To determine whether *sall3* promoter CpG island hypermethylation changes leads to decreased *sall3* mRNA transcription in human HCC tissues, the methylation levels of the *sall3* promoter CpG island in 38 HCC tumors and adjacent noncancerous tissues were examined using the MassARRAY platform (Sequenom). Of the 38 tumors, 27 (71%) showed higher methylation levels at the *sall3* CpG island compared with adjacent noncancerous tissue, while methylation levels were similar at the *sall3* CpG

island in tumor tissue and adjacent noncancerous tissue in 11 cases (29%). None of the tumors showed lower methylation levels at the *sall3* CpG island compared with adjacent noncancerous tissues. Average methylation levels for each CpG unit in the 38 tumor tissues and adjacent noncancerous tissues are listed in Figure 1. Among the total of 24 CpG units (1-3 CpG sites per unit), the average methylation levels of 20 CpG units in 38 tumors were significantly higher than those in adjacent noncancerous tissues.

To determine whether aberrant CpG island DNA methylation in HCC tissues may be correlated with the decreased *sall3* mRNA transcription, *sall3* mRNA levels in 38 paired samples were determined by qRT-PCR (Figure 2). The relative ratio between methylation and mRNA expression levels of *sall3* are negatively correlated in 26 of 38 tumor tissues *vs* corresponding adjacent noncancerous tissues (Figure 3). Of the 38 tumor tissues, 33 (86.8%) showed a decreased *sall3* mRNA level compared to adjacent noncancerous tissues (Figure 2). Among these 33 tumor tissues with lower mRNA levels, 24 showed higher methylation levels and a negative association was found between CpG island methylation and mRNA expression (Figure 3); nine tumor tissues showed methylation levels that were not significantly different from adjacent noncancerous tissues. Of the 38 tumor tissues examined, three (7.9%) showed higher *sall3* mRNA expression than adjacent noncancerous tissues (Figure 2). Methylation level was similar to the adjacent noncancerous tissue in one of these three tumor tissues, while the methylation levels in the remaining two tumor tissues were higher than those in the adjacent noncancerous tissue. Of the 38 tumor tissues, two (5.3%) showed similar *sall3* mRNA expression to adjacent noncancerous tissues (Figure 2). Of these two tumor tissues, one showed no significant difference in methylation level compared with adjacent noncancerous tissues, while the methylation level in the other was higher than that the in the adjacent noncancer-

Figure 2 *sall3* mRNA is analyzed in 38 tumor tissues (T) and adjacent non-cancerous tissues (N). Error bars, SD from triplicates.

Figure 3 Correlation between methylation levels and mRNA expression of *sall3* in 24 tumor tissues and corresponding adjacent noncancerous tissues.

ous tissue. Together, these results indicated a negative correlation between *sall3* promoter CpG island hypermethylation and *sall3* mRNA transcription in 24 tumor tissues and their adjacent noncancerous tissues (Figure 3). *sall3* mRNA transcription in other tissues showed no association with *sall3* promoter CpG island hypermethyl-

ation, suggesting regulatory mechanisms other than those involved in HCC.

sall3 promoter CpG island hypermethylation correlates its mRNA transcription in human HCC cell lines
To determine whether decreased *sall3* mRNA transcrip-

2723

Figure 4 Quantitative methylation analysis on each CpG unit of sall3 CpG island in HCC cells after 5-Aza-CdR treatment or control (A-F).

Figure 5 Relative sall3 expression in six hepatocellular carcinoma cells after 5-AZA-CdR treatment. Error bars, SD from triplicates.

tion levels in human HCC tissues are due to promoter CpG island hypermethylation, six human HCC cell lines (Huh7, HepG2, SK-HEP1, SMMC7721, Bel7402 and QGY7703) were exposed to 0, 0.1, 0.5 or 2.5 μmol 5-aza-CdR, an inhibitor of DNMTs, for 72 h. As expected, after treatment with 0.1, 0.5 or 2.5 μmol 5-aza-CdR, promoter CpG island methylation levels showed dose-dependent downregulation in all six cell lines (Figure 4). *sall3* mRNA levels also showed dose-dependent upregulation

in Huh7, HepG2, SK-HEP1 and SMMC7721 cells, while it was irregularly upregulated in Bel7402 and QGY7703 cells (Figure 5). These data indicated that *sall3* promoter CpG island hypermethylation is likely responsible for the decrease in *sall3* mRNA transcription level.

DISCUSSION

HCC is one of the most common gastrointestinal ma-

lignancies, ranking fifth in the occurrence of common cancers, and third in common causes of cancer-related death[18]. Early-stage HCC is potentially curable with surgical resection or hepatic transplantation[19,20]. However, most patients present with advanced disease and are not amenable to surgical resection or transplantation. Therefore, they have a poor prognosis[21], and improved methods for early diagnosis are urgently required. DNA methylation is a gene expression regulatory mechanism and plays a fundamental role in carcinogenic processes[22,23]. Aberrant CpG island methylation of tumor-related genes is an early and frequent event in the process of carcinogenesis, and DNA methylation status of tumor-related genes is a potential diagnostic marker[24-26]. As it is possible to restore the function of methylated tumor suppressor genes, combinations of epigenetic modifiers and other therapeutic agents may also become a promising alternative to conventional treatments[27-29].

Although an increasing number of genes undergoing aberrant CpG island methylation have been reported in HCC[4,5,30,31], the methylated genes have not yet been fully characterized. Yu *et al*[14] reported that *sall3* was a novel target of aberrant methylation in bladder cancer. Xia *et al*[15] reported that the hypermethylation frequency in tumor tissues was significantly higher than those in adjacent noncancerous tissues, but whether the decreased transcription of *sall3* was caused by hypermethylation of the promoter CpG island in HCC remained unknown.

In the present study, we found that the levels of *sall3* mRNA were decreased in 33 of 38 tumor tissues compared with those in adjacent noncancerous tissues. Twenty-four of these 33 tumor tissues with reduced *sall3* mRNA levels showed elevated methylation levels, suggesting that the decreased *sall3* mRNA transcription levels were likely caused by promoter CpG island hypermethylation. We also confirmed that six HCC cell lines (Huh7, HepG2, SK-HEP1, SMMC7721, Bel7402 and QGY7703) were hypermethylated at the *sall3* promoter. Using the demethylating agent 5-aza-CdR, the transcription of *sall3* could be restored in these cells when the *sall3* promoter region was partially demethylated. Taken together with the results in HCC tissue samples, it is clear that promoter hypermethylation in *sall3* was strongly associated with the reduced transcription of *sall3* mRNA. However, in more than 25% of the HCC cases (9/33), decreased mRNA transcription was observed with similar CpG island methylation levels. In addition, there were three tumor tissues with promoter hypermethylation without decreased mRNA transcription compared with corresponding nontumorous tissues; the levels of mRNA transcription were increased in two of these cases and similar in the remaining one case. These results suggested that other regulatory mechanisms unrelated to promoter hypermethylation may also be involved. Further studies are needed to clarify this issue. However, the overall strong association between promoter hypermethylation and decreased mRNA transcription suggests a causative role of aberrant *sall3* promoter methylation and decreased mRNA transcription in most cases of HCC.

In conclusion, we showed that *sall3* mRNA transcrip-tion was downregulated in most (33/38) tumor tissues compared with adjacent noncancerous tissues in HCC. Most cases (24/33) with downregulated mRNA transcription were strongly associated with promoter CpG island hypermethylation. This association was further confirmed by subsequent experiments in cell lines; treatment of the cell lines Huh7, HepG2, SK-HEP1, SMMC7721, Bel7402 and QGY7703 with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine reversed promoter CpG island hypermethylation and restored *sall3* mRNA transcription. These results indicated that promoter CpG island hypermethylation is the main reason for downregulation of *sall3* mRNA transcription in HCC. These finding regarding *sall3* mRNA transcription and DNA methylation associated with human HCC provide new insights into the pathogenesis of HCC and may serve as a powerful molecular marker for detecting HCC in biopsy tissues of HCC patients.

COMMENTS

Background
Inactivation of tumor suppressor genes by promoter CpG island hypermethylation plays a key role in cancer pathogenesis.

Research frontiers
Aberrant CpG island methylation of tumor-related genes is a early and frequent event in carcinogenetic process and DNA methylation status of tumor-related genes is a potential diagnostic marker. Using demethylating agents to restore the function of methylated tumor suppressor genes also becomes a promising alternative to conventional treatments.

Innovations and breakthroughs
sall3 mRNA transcription was downregulated in most tumor tissues compared with adjacent noncancerous tissues. Downregulation of mRNA transcription was strongly associated with hypermethylation of the promoter CpG island. This association was further confirmed by subsequent cell line experiments; treatment of the cell lines with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine reversed promoter CpG island hypermethylation and restored sall3 mRNA transcription.

Applications
The finding of sall3 mRNA transcription and DNA methylation associated with human hepatocellular carcinoma (HCC) provide new insights into pathogenesis of HCC and may serve as a powerful molecular marker for detecting HCC in biopsies tissues of HCC patients.

Terminology
To concisely and accurately describe, define or explain the specific, unique terms that are not familiar to majority of the readers, but are essential for the readers to understand the article. DNA methylation typically occurs at CpG sites (cytosine-phosphate-guanine sites, that is, where a cytosine is directly followed by a guanine in the DNA sequence). This methylation results in the conversion of the cytosine to 5-methylcytosine. Hypermethylation typically occurs at CpG islands in the promoter region and is associated with gene inactivation. Demethylation is the chemical process resulting in the removal a of methyl group (CH_3) from a molecule.

Peer review
This is a nice human study conducted by a group of competent researchers. The study is nicely done. Also, the paper is well written.

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CASE REPORT

A pediatric non-protein losing Menetrier's disease successfully treated with octreotide long acting release

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Abstract

Pediatric Menetrier's disease (MD) is an uncommon, acute, self-limited hypertrophic gastropathy characterized by enlarged gastric folds associated with epithelial hyperplasia and usually accompanied by protein losing gastropathy. Gastric cytomegalovirus infection is found in one third of MD children and its treatment is often associated with remission. Diagnosis often requires full-thickness biopsy due to inability to detect typical histological findings with conventional endoscopic biopsy. We report an uncommon case of non self-limited pediatric MD needing endoscopic mucosal resection for diagnosis which was then successfully treated with octreotide long-acting release (LAR). To the best of our knowledge, this is the first pediatric MD case successfully treated with octreotide LAR. Our experience suggests octreotide LAR as treatment for refractory MD before gastrectomy.

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Key words: Menetrier's disease; Octreotide; Endoscopic mucosal resection; Children

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INTRODUCTION

Menetrier's disease (MD) is a rare hypertrophic gastropathy characterized by enlarged gastric rugal folds and epithelial hyperplasia, accompanied by protein losing gastropathy[1]. Primarily seen in adults, MD can have a long clinical course and is associated with considerable morbidity and even mortality, which are related to surgical resection and potential risk for malignant transformation[2-4]. Pediatric MD often presents as oedema and hypoalbuminemia due to protein loss through the abnormal gastric mucosa and usually has a benign self-limited course with symptoms resolving within 5 wk[4,5]. Diagnosis often requires full-thickness biopsy in conjunction with endoscopic view of enlarged gastric folds[6] while histology includes foveolar hyperplasia with cystic

dilation of pits, accompanied by glandular atrophy of the gastric body[1,2]. Gastric cytomegalovirus (CMV) infection is found in one third of MD children and its treatment is often associated with remission[7,8]. We describe the first case of non self-limited and non protein losing MD, diagnosed with endoscopic mucosal resection (EMR) and successfully treated with octreotide long-acting release (LAR).

CASE REPORT

A 4-year-old boy was referred to our Unit for severe iron deficiency anemia (haemoglobin level: 4.7 g/dL and iron studies consistent with iron-deficiency anemia) already treated with blood transfusion in a territorial hospital. The child had a normal physical development with regular growth curve. Liver and spleen diseases were excluded on a clinical and biochemical basis. A grandfather underwent total gastrectomy due to MD. At upper gastrointestinal endoscopy there were marked thickened gastric folds with overlying erosions and exudate involving the corpus and fundus, whereas the antrum and pylorus were normal (Figure 1). Mucosal biopsies showed mild foveolar hyperplasia, dilation of some glands and were negative for *Helicobacter pylori* and CMV. The latter was also negative in the gastric juice, serum and urine. There was no clinical and laboratory evidence of protein-losing enteropathy and serum gastrin level was normal.

After 2 mo of unsuccessful treatment with omeprazole at 2 mg/kg per day, an EMR was done in the area of greatest gastric folds thickening to obtain a wider sample for a histological diagnosis. Thus, a diagnosis of MD was made. based on the presence of marked epithelial hyperplasia with tortuous and cystically dilated foveolar glands, discontinuous atrophy of stomach glands and significant reduction of parietal cells; a strong small vessel congestion with a diffuse edema was also observed within the lamina propria (Figure 2).

Following a lack of response to conventional therapies and due to preliminary reports on successful use of octreotide in adult MD[9-13], the patient was given ten doses of octreotide (50 μg subcutaneously two times a day), which was well tolerated. He then began octreotide LAR (5 mg intramuscularly every 28 d). Two months later haemoglobin and iron levels were normal. Six months later therapy was stopped and a rapid reduction of the haemoglobin level ensued. Thus, we continued octreotide with long-term normalization of the haemoglobin levels. Fifteen months later gastric folds were less prominent and no erosions at endoscopic follow-up were seen; however the histology was unchanged.

DISCUSSION

MD is characterized by hypertrophic folds in the body of the stomach, foveolar hyperplasia and hypoproteinemia[1]. While spontaneous remission is rare in adults and gastrectomy is not uncommonly required in refractory disease[2,3], pediatric MD is commonly an acute, self-limited,

Figure 1 Endoscopic view of markedly thickened gastric folds, with overlying erosions and exudates involving fundus (A) and corpus (B).

Figure 2 Histopathology from endoscopic mucosal resection (HE staining; A: 40×, B: 20×) shows elongated, tortuous and cystically dilated foveolar glands, discontinuous atrophy of gastric glands and significant reduction of parietal cells.

protein-losing gastropathy[5]. However, a hyperplastic hypersecretory variant of MD with normal or increased acid secretion and no protein loss is reported: it often

requires full-thickness biopsy due to an inability to detect typical histological findings with conventional endoscopic biopsy[6].

Therapy in children is supportive and includes adequate hydration, antisecretory agents (histamine 2 receptor antagonists and proton pump inhibitors) and albumin replacement[3-5]. Treatment of CMV, when detected, is usually associated with remission[7,8].

Interestingly, transforming growth factor α, one of the ligands for the epidermal growth factor receptor (EGF-R), has been implicated in the mechanisms underlying MD[14]: it causes dose-dependent *in vitro* proliferation of gastric epithelial cells and reduction of acid secretion, which are hallmarks of the disease[15,16].

Treatment with an experimental monoclonal antibody against EGF-R has been successful in 3 severe MD cases[17,18]. There is also evidence that somatostatin decreases the number of EGF binding sites at the cell surface[19]; thus, octreotide may modulate EGF-R signalling at several levels[20,21]. These mechanisms and preliminary reports on successful use of octreotide in adults with MD[9-13] prompted us to use this agent in our patient.

In conclusion, we describe an uncommon case of pediatric MD with atypical presentation (only anemia) and chronic unremitting course. MD should be considered in all children with thickened gastric folds at endoscopy, even in the absence of typical clinical and laboratory manifestations. EMR is a good alternative to full thickness biopsy for diagnosing disorders associated with gastric wall thickening such as MD.

To the best of our knowledge, this is the first pediatric MD case successfully treated with octreotide LAR. Our experience suggests octreotide LAR as treatment for refractory MD before gastrectomy.

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CASE REPORT

Infliximab stopped severe gastrointestinal bleeding in Crohn's disease

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Abstract

To report the result of rapid ulcer healing by infliximab in Crohn's patients with severe enterocolic bleeding. During 2005 and 2010, inflammatory bowel disease database of King Chulalongkorn Memorial and Samitivej hospitals were reviewed. There were seven Crohn's disease (CD) patients (4 women and 3 men; mean age 52 ± 10.4 years; range: 11-86 years). Two of the seven patients developed severe gastrointestinal bleeding (GIB) as a flare up of CD whereas the other five patients presented with GIB as their first symptom for CD. Their mean hemoglobin level dropped from 12 ± 1.3 g/dL to 8.7 ± 1.3 g/dL in a 3-d period. Median packed red blood cells units needed for resuscitation was 4 units. Because of uncontrolled bleeding, surgical resection was considered. However, due to the poor surgical candidacy of these patients (*n* = 3) and /or possible development of short bowel syndrome (*n* = 6), surgery was not pursued. Likewise angiographic embolization was not considered in any due to the risk of large infarction. All severe GIBs successfully stopped by one or two doses of intravenous infliximab. Our data suggests that infliximab is an alternative therapy for CD with severe GIB when surgery has limitation or patient is a high risk.

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INTRODUCTION

Although severe gastrointestinal bleeding (GIB) is an uncommon complication of inflammatory bowel disease (IBD), severe GIB occurs in 0.1% of ulcerative colitis[1] and 1.2%-1.3% of Crohn's disease (CD)[1,2]. This in turn sometimes progresses to a potential life-threatening condition. Approximately one third of CD patients developed GIB as a flare up and another one fourth of CD patients presented with GIB as an initial symptom[3]. Bleeding sources were mostly found in the colon (50%-85%) and the small bowel (15%-50%). Unfortunately, one third of CD related GIBs were severe and surgery was required because of refractory bleeding especially after failed conventional medical and endoscopic treatment[1,3]. Therefore treatment for severe hemorrhage in IBD remains a challenge. Recently, there has been only a handful of case reports of severe CD related GIB controlled with tumor

necrosis factor (TNF)-α antibody (infliximab). We report the largest number (n = 7) of CD patients presenting with severe GIB who were successfully treated with infliximab without the need for surgery.

CASE REPORT

There were seven CD patients (4 women and 3 men; mean age 52 ± 10.4 years; range: 11-86 years). Two of the seven patients developed severe GIB as a flare up of CD whereas the other five patients presented with GIB as their first symptom for CD (Tables 1 and 2).

In a group with flared CD (n = 2), one patient was diagnosed as colonic CD for 2 mo. She was steroid dependent who required oral prednisolone 35 mg/d and azathioprine 1.5 mg/kg per day. She was admitted because of severe bleeding per rectum and developed orthostatic hypotension. She required 4 units of pack red cell for resuscitation during those 3 d of hospitalization. Another patient was diagnosed as ileocolonic CD for 7 mo. She had been taking budesonide 9 mg/d and mesalamine 2 g/d to control her CD before admission. She developed acute abdominal pain, fever and severe hematochezia. Her hemoglobin (Hb) dropped from 12 to 10 g/dL within 2 d. A unit of pack red cell was required to maintain hemoglobin level.

In patients who presented with hematochezia as their first CD symptom (n = 5), three of the five patients had had abdominal pain and watery diarrhea for 10-14 d prior to the present of hematochezia. The other two presented initially with hematochezia without prior warning gastrointestinal (GI) symptoms. All of those denied the use of non-steroidal anti-inflammatory drugs (NSAIDs) prior to the presentation. Skin signs and symptoms that suggestive of Behçet's disease were not recognized in any.

The average baseline Hb was 12 ± 1.3 g/dL in all patients. Coagulogram and platelets count were normal. The average C-reactive protein level was high (mean 14 ± 18 mg/L; normal 0-6). Endoscopy and ileo-colonoscopy were performed as the initial investigations. One patient with suspected proximal ileal bleeding underwent a double balloon enteroscopy. Endoscopic findings showed multiple discrete deep ulcers with either active oozing or visible vessel in all seven patients. Of these, two patients with visible vessel found on the ulcer underwent endoscopic hemostasis with hemoclipping. However, recurrent hematochezia developed in both and repeat endoscopy failed to indentify other source of bleeding despite the inactive status of previously clipped vessels. Bleeding sources located in the small bowel and mainly in the ileum without colonic source in five patients, while the other had pure colonic lesion. One patient had ulcers in both ileum and colon. Biopsies from Ileum and colon were done in all patients and they revealed acute and chronic inflammation. No granuloma was identified. All specimens were negative for inclusion body and *Mycobacterium tuberculosis* (by polymerase chain reaction).

Despite, an intravenous dexamethasone 5 mg was given at every 6 h for 3-5 d, all patients still had persistent hematochezia. Their mean Hb level dropped from 12 ± 1.3 g/dL to 8.7 ± 1.3 g/dL in a 3-d period. Median packed red blood cells units needed for resuscitation was 4 units. Because of uncontrolled bleeding, surgical resection was considered. Due to the poor surgical candidacy of these patients (n = 3) and/or possible development of short bowel syndrome (n = 6), surgery was not pursued. Likewise angiographic embolization was not considered in any due to the risk of large infarction from multiple areas of embolization. Then infliximab (5 mg/kg) was infused instead. Infliximab rapidly stopped bleeding definitely within 24 h in 6 patients. Another patient developed recurrent bleeding after 3 d of the first dose of infliximab. Subsequently, bleeding ceased promptly after the second dose of infliximab that administered at day tenth. Median doses of infliximab were two. All underwent a follow-up ileo-colonoscopy that revealed a significant improvement of ileal and colonic ulceration (Figures 1 and 2). At the 30-d follow up, no patients reported recurrent bleeding.

DISCUSSION

In our case series, we identified CD patients with severe GIB presented as either the first manifestation or a flare up of disease. None of our patients had histories or findings suggestive of NSAIDs induced ulcers or Behçet's disease. The common location of ulcers in our series involved ileum, ileocolic region, colon and a combination of all areas. The most common endoscopic findings were extensive multiple deep ulcers with or without active oozing. Infliximab was used as a last resort for controlling bleeding after failure of standard treatments. In fact, surgical treatment was considered in all cases, but it was not opted as mentioned earlier. Almost all patients responded promptly within 24 h after a single dose of infliximab. Only one patient with ileocolonic CD needed the second dose to achieve definite hemostasis after the recurrent bleeding.

Management of severe GIB in CD is problematic since there are multiple lesions with the possibility of bleeding from multiple sites. Endoscopy should be attempted in all patients, but only a quarter of patients that the bleeding sites could be precisely identified[3]. Medical therapies such as steroids, azathioprine and mesalamine also have been reported to control bleeding, but the prompt response is uncertain. Surgical resection is also a crucial therapy[4-6]. Recurrent bleeding was significantly lower in surgically treated patients (5.7%) compared with medically treated patients (38.5%). However, there is a significant rate of post operative and perioperative mortality at 6.9%[7]. In addition, the risk of developing short bowel syndrome after resection should be considered because these patients may have an extensive small bowel involvement[8-10]. Radiological intervention, one of the alternative treatments for small bowel bleeding[11], can accidentally contribute to small bowel infarction after multiple area of embolization. From those five series reported on GIB related to CD (n = 101), an angiographic embolization

Figure 1 Deep ileal ulcer (A) and completely healed ileal ulcer 6 wk after infliximab (B).

Figure 2 Ileal ulcer with hemoclips (A) and healing ileal ulcer 6 wk after infliximab (B). Note the clips still presented.

Table 1 Clinical characteristics and outcomes of infliximab treatment in 2 Crohn's disease patients with severe gastrointestinal bleeding as a flare-up disease

No.	Age (yr)	Sex	Duration of CD	Location	Current treatment	Presenting symptom	Dropped rate of Hb (g/dL)	PRBC (unit)	Characteristic of lesion	Infliximab therapy	Bleeding controlled in	Follow-up (mo)
1	11	F	2 mo	Colon	Prednisolone 35 mg/d azathioprine	GIB (1 d)	from 11 to 8 in 3 d	4	Multiple deep colonic ulcers without oozing	Infliximab 5 mg/kg (single dose)	1 d	12
2	19	F	7 mo	Ileocolon	Budesonide 9 mg/d 5-ASA	GIB (1 d)	from 12 to 10 in 3 d	1	Multiple ileal and colonic ulcers with oozing	Infliximab 5 mg/kg (d0, d10)	10 d	10

CD: Crohn's disease; GIB: Gastrointestinal bleeding; Hb: Hemoglobin; 5-ASA: 5-aminosalicylates; PRBC: Packed red blood cell; F: Female.

Table 2 Clinical characteristics and outcomes of infliximab treatment in 5 Crohn's disease patients with severe gastrointestinal bleeding as a first presentation

No.	Age (yr)	Sex	Presenting symptom	Dropped rate of Hb (g/dL)	PRBC (unit)	Location	Characteristic of lesion	Infliximab therapy	Bleeding controlled in	Follow-up (mo)
1	59	M	Diarrhea and abdominal pain (10 d) GIB (1 d)	from 10 to 8 in 3 d	6	Ileum	Multiple ileal ulcers with oozing and one visible vessel	Infliximab 5 mg/kg (d0, week 2)	1 d	24
2	86	M	GIB (1 d)	from 12 to 8.5 in 4 d	7	Ileum	Multiple ileal ulcers with oozing	Infliximab 5 mg/kg (d0, week 2)	1 d	36
3	71	F	Diarrhea and abdominal pain (10 d) GIB	from 13 to 10 in 3 d	3	ileum	Multiple ileal ulcers with oozing	Infliximab 5 mg/kg (d0, week 2)	1 d	12
4	50 yr	F	Diarrhea and abdominal pain (14 d) GIB (1 d)	from 14 to 10 in 3 d	2	Ileum and jejunum	Multiple ileal and jejunum ulcers with oozing	Infliximab 5 mg/kg (d0, week 2)	1 d	36
5	71 yr	M	1st episode GIB from ileal ulcer (1 mo) Recurrent GIB (1 d)	from 11 to 6.5 in 5 d	6	Ileum	Multiple ileal ulcers with oozing and one visible vessel	Infliximab 5 mg/kg (single dose)	1 d	24

CD: Crohn's disease; GIB: Gastrointestinal bleeding; Hb: Hemoglobin; 5-ASA: 5-aminosalicylates; PRBC: Packed red blood cell; M: Male; F: Female.

Table 3 Successful control severe lower gastrointestinal bleeding in Crohn's disease with infliximab

Study	Sex	Age (yr)	Location	Duration of disease	Current treatment	Presenting symptom	PRBC (unit)	Characteristic of lesion	Infliximab therapy	Bleeding controlled in	Follow-up (mo)
Belaihe *et al*[26], 2002	F	28	Ileocolon CD	3 yr	Budesonide azathioprine	Lower GIB	5	Multiple deep ulcers at colon without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	14 d	5
	F	59	colon CD	9 yr	Prednisolone, metronidazole, ciprofloxacin	Lower GIB	4	Multiple deep ulcers at colon without bleeding stigmata	Infliximab 5 mg/kg (single dose)	4 d	4
Papi *et al*[7], 2003	M	50	Ileocolon CD S/P resection and ileocolonic anastomosis due to bleeding	9 mo	Prednisolone azathioprine	Lower GIB with hypovolumic shock	NA	Deep ulcers at ileocolon anastomosis without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	12
	M	68	Ileum CD S/P ileal resection due to stricture	24 yr	Mesalamine	Melena	4	Large ulcer at ileocolon anastomosis without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	3
Tsujikawa *et al*[25], 2004	M	31	Ileocolon CD S/P ileocolectomy due to ulcer bleeding	12 yr	Salazosulfap yrimidine	Lower GIB	NA	Multiple ulcers at ileocolon anastomsosis and ileum without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	4
Ando *et al*[22], 2009	F	16	Colonic CD	1 yr	Mesalamine prednisolone	Lower GIB with hypovolumic shock	6	Multiple deep ulcers at colon with diffuse mucosal inflammation without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	3 d	12
Meyer *et al*[24], 2009	F	19	Ileocolonic CD	6 yr	Mesalamine prednisolone	Lower GIB with hypovolumic shock	4	Multiple ulcers at terminal ileum without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6)	NA	6
Julián Gómez *et al*[23], 2010	M	44	Ileocolon CD S/P total colectomy with ileostomy due to toxic megacolon	NA	NA	Postop small bowel resection due to obstruction bleeding	10	Multiple deep ulcers at small bowel without bleeding stigmata	Infliximab 5 mg/kg (d0, week 2, week 6) and maintenance dose	5 d	3
Alcalde Vargas *et al*[21], 2011	M	27	Ileocolon CD	2 yr	Mesalamine	Massive lower GIB	8	Multiple ulcers entire colon and abundant dark red blood at terminal ileum	Infliximab 5 mg/kg (single dose)	4 d	NA
	F	36	Colon and perianal CD	NA	Amoxicillin-clavulanate metronidazole	Massive lower GIB	12	Multiple deep ulcers entire colon and blood clots	Infliximab 5 mg/kg (single dose)	6 d	NA
	M	24	Colon CD	1 mo	Prednisolone	Massive lower GIB	5	Multiple deep ulcers entire colon and spontaneous bleeding mucosa	Infliximab 5 mg/kg (single dose)	4 d	NA

CD: Crohn's disease; GIB: Gastrointestinal bleeding; PRBC: Packed red blood cell; M: Male; F: Female; NA: Not available.

was attempted only in one patient[1-3,12,13]. Unfortunately, that such patient subsequently necessitated surgery due to small bowel infarction after an ileocecal artery embolization[3]. Angiography with intra-arterial vasopressin infusion in case where embolization is not possible has previously been proven to be successful in two CDs related GIB[14,15]. However, many side effects and complications could develop from this technique including hypertension, coronary vasoconstriction, cardiac arrhythmia, and bowel infarction. To decrease the risk for bowel infarction, it is advisable to use superselective angiographic embolization[16]. Although the risk of bowel infarction may be decreased, this serious complication cannot be ignored. In experienced centers, bowel infarction still developed in 5%-24% of lower GIB patients who treated with superselective mesenteric arterial embolization[17,18].

The pathogenesis of hemorrhagic type CD remains unclear. One possible hypothesis is transmural inflammations leading to mucosal ulcers erode to blood vessels. On endoscopic examinations, all of our patients had diffuse deep ulcers and majority of them (86%) had active oozing. Since severe hemorrhage usually develops from ulcers eroding into blood vessels, any treatment that can rapidly heal the mucosa is an ideal therapeutic tool to control and prevent recurrent hemorrhage. Anti-TNF-α (infliximab) has been shown to induce rapid mucosal healing[19,20]. Therefore, infliximab has a possible role in treating severe hemorrhagic CD. Moreover, the identification for precise bleeding site is not required since infliximab can systematically heal multiple small bowel ulcers.

To date, eleven CDs related GIB treated with infliximab from the seven series has been reported (Table

3)[7,21-26]. Severe hematochezia was presented in eight flare-up CD patients and the other three presented with hematochezia as their initial CD manifestation. Four patients previously had undergone for surgical treatment including ileocolectomy and total colectomy[7,23,25,26]. Colon and ileocolon were the most common locations of GIB. Ileocolonoscopy showed multiple discrete deep ulcers in all. Majority of patients had more than one potential site of bleeding[7,21-26]. The high risk bleeding stigmata was found in only one patient that presented with diffuse spontaneous mucosal bleeding[21]. One patient underwent a total colectomy and small bowel resection, but the bleeding recurred[23]. No patient underwent angiographic therapy. Infliximab was administered as a last resource for uncontrolled bleeding. Most of patients responded to the first dose of infliximab. Only one patient required the second dose of infliximab on day fourteenth to control recurrent bleeding[26]. Six patients received three doses of infliximab and another five received only a single dose of infliximab. Maintenance with infliximab was considered only in one patient[23]. Surgery was not pursued in any[4,15-20].

To our knowledge, we report the largest cases series of severe GIB in CD in which infliximab had been used. Infliximab was able to control hemostasis as a result of rapid ulcer healing. Definite hemostasis was achieved after the first or second dose of infliximab. Nevertheless, more further prospective studies are required to confirm the utilization of infliximab for severe GIB in CD.

In conclusion, infliximab may be a good alternative treatment to control severe bleeding related to small bowel and colonic ulcers in active CD especially in patients with high risk for surgery and/or high risk to develop a short bowel syndrome.

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LETTERS TO THE EDITOR

Dual therapy for third-line *Helicobacter pylori* eradication and urea breath test prediction

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Abstract

We evaluated the efficacy and tolerability of a dual therapy with rabeprazole and amoxicillin (AMX) as an empiric third-line rescue therapy. In patients with failure of first-line treatment with a proton pump inhibitor (PPI)-AMX-clarithromycin regimen and second-line treatment with the PPI-AMX-metronidazole regimen, a third-line eradication regimen with rabeprazole (10 mg q.i.d.) and AMX (500 mg q.i.d.) was prescribed for 2 wk. Eradication was confirmed by the results of the ^{13}C-urea breath test (UBT) at 12 wk after the therapy. A total of 46 patients were included; however, two were lost to follow-up. The eradication rates as determined by per-protocol and intention-to-treat analyses were 65.9% and 63.0%,

respectively. The pretreatment UBT results in the subjects showing eradication failure; those patients showing successful eradication comprised 32.9 ± 28.8 permil and 14.8 ± 12.8 permil, respectively. The pretreatment UBT results in the subjects with eradication failure were significantly higher than those in the patients with successful eradication (P = 0.019). A low pretreatment UBT result ($\leqslant$ 28.5 permil) predicted the success of the eradication therapy with a positive predictive value of 81.3% and a sensitivity of 89.7%. Adverse effects were reported in 18.2% of the patients, mainly diarrhea and stomatitis. Dual therapy with rabeprazole and AMX appears to serve as a potential empirical third-line strategy for patients with low values on pretreatment UBT.

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TO THE EDITOR

Eradication of *Helicobacter pylori* (*H. pylori*) has been reported as an effective strategy in the treatment of peptic ulcers and gastric mucosa-associated lymphoid tissue lymphomas and also prevents the recurrence of gastric cancer after endoscopic resection[1-7]. The first-line regimen for the treatment of *H. pylori* infection in Japan is triple therapy with a proton pump inhibitor (PPI), amoxicillin (AMX) and clarithromycin (CLR) administered for 7 d. Failure of this first-line therapy against *H. pylori* infection has been reported in approximately 20% of infected patients[8,9]. With the increase in the frequency of CLR-resistant *H. pylori*, there is rising concern about the potential decline in the eradication rate of this infection[10]. Although therapy with PPI-AMX-metronidazole (MNZ) administered for 1 wk has been found to be effective as a second-line regimen in patients failing the

first-line regimen, approximately 10% of patients fail to respond to even second-line treatment, necessitating the establishment of an alternative third-line strategy for the effective eradication of *H. pylori*[3,11].

Although *H. pylori* bacteria easily develop resistance to CLR and MNZ, *H. pylori* has been considered to seldom become resistant to AMX. AMX is the preferred antibiotic because it is bactericidal and resistance is rare; therefore, it can be used again after treatment failure[8]. A number of studies have suggested that good success rates for *H. pylori* eradication could be obtained with AMX and PPI dual therapy if the effective PPI dose and frequency of administration were increased[12]. The majority of patients who experience two eradication failures have the rapid metabolizer genotype of CYP2C19. Because omeprazole and lansoprazole are extensively metabolized by CYP2C19 in this genotype, their plasma concentrations will not attain levels sufficient to inhibit acid secretion, and therefore, antibiotics such as AMX will be less stable in the stomach, resulting in a lower eradication rate[13]. The PPI rabeprazole is a substitute of benzimidazole. CYP2C19 is less involved in the metabolism of rabeprazole than in that of omeprazole and lansoprazole[14]. Moreover, rabeprazole has a greater and more rapid acid-inhibitory effect than does omeprazole. Several reports on the pharmacokinetics and pharmacodynamic characteristics of PPIs have indicated that a sufficient plasma concentration of PPIs can be achieved in patients with the rapid metabolizer genotype of CYP2C19 by frequent PPI dosing[12,15]. Furuta *et al*[16] recently reported an excellent eradication rate of 87.8% following dual therapy with rabeprazole 4 times/day and AMX as a third-line rescue. However, their study was completed at only one or two centers. Our study was designed as a prospective, multicenter trial with the participation of 16 Japanese hospitals affiliated with the National Hospital Organization to investigate the efficacy of dual therapy with 4 times daily dosing of rabeprazole and AMX as empiric third-line rescue therapy.

A total of 46 patients (26 males, 20 females; age 60.7 ± 12.9 years, mean ± SD) referred to us between January 2009 and January 2012 were enrolled. Endoscopic examinations were conducted before treatment in all patients, and *H. pylori* positivity was confirmed by histology, stool antigen test, *H. pylori*-specific IgG antibodies or the ^{13}C-urea breath test. All patients had a history of two treatment failures (first-line treatment used: triple therapy with PPI-AMX-CLR for 7 d; second-line treatment used; triple therapy with PPI-AMX-MNZ for 7 d). The exclusion criteria in this study were (1) age < 18 years; (2) presence of clinically significant underlying disease (hepatic or renal disease, diabetes mellitus); (3) history of gastric surgery; and (4) allergy to any of the drugs used in the study. *H. pylori* eradication failure was defined as a positive ^{13}C-urea breath test (UBT) at the end of 12 wk after completion of treatment. The ^{13}C-urea used was 100 mg ^{13}C-labelled urea, produced by Otsuka pharmaceutical Co., LTD, Japan. The procedure was modified from the European standard protocol for the detection of *H. pylori*[17]. We

Table 1 Demographic characteristics of the patients and the results of eradication therapy

Characteristics	Total ($n = 46$)	Eradication success ($n = 29$)	Eradication failure ($n = 15$)	P value
Age (mean ± SD, yr)	60.7 ± 12.9	59.8 ± 13.4	60.8 ± 12.1	0.813
Sex (male/female)	26/20	15/14	10/5	0.530
Diagnosis (GU/DU/CG)	23/15/8	15/10/4	8/3/4	0.450
Pretreatment UBT	20.4 ± 21.2	14.8 ± 12.8	32.9 ± 28.8	0.019
Eradication rate (ITT) %	63.0			
Eradication rate (PP) %	65.9			

GU: Gastric ulcer; DU: Duodenal ulcer; CG: Chronic gastritis; UBT: Urea breath test; ITT: Intention-to-treat; PP: Per protocol.

chose 2.5 permil for cut-off level of the rise in the delta value of $^{13}CO_2$ at 15 min after the ingestion of ^{13}C-urea.

The treatment regimen was rabeprazole 10 mg q.i.d. and AMX 500 mg q.i.d. administered for 2 wk. Participants were requested to return at the conclusion of the therapy for an interview regarding any adverse events. Successful *H. pylori* eradication was defined as a negative UBT at the end of 12 wk after completion of treatment. Statistical analyses were performed using the chi-square, Fisher's exact and Student's *t* tests, as appropriate. *P* values of less than 0.05 were accepted as representing statistical significance. The study was conducted with the approval of the Ethics Committee of the National Hospital Organization Tokyo Medical Center, and informed consent was obtained from all patients prior to the examinations. The clinical trial registration number of the University Hospital Medical Information Network was R000003204.

Of the 46 patients enrolled, 2 dropped out of the study, leaving 44 patients in the per protocol (PP) set. *H. pylori* eradication was confirmed in 29 patients, representing an eradication rate of 63.0% [95% confidence intervals (CI): 47.6%-76.8%] by intention-to-treat (ITT) analysis and 65.9% (95% CI: 50.1%-79.5%) by PP analysis (Table 1). Patient compliance with the prescribed treatment was excellent. Adverse events were recorded in 8 patients (18.2%; 95% CI: 8.2%-32.7%). Six patients had mild diarrhea or soft stools but went on to complete the study. Two patients developed stomatitis.

Because the numerical results of the UBT are a function of the total urease activity within the stomach, they represent a quantitative index of the density of gastric *H. pylori* colonization[18]. As a low pretreatment UBT value could be one of the predictive factors for eradication success, the pretreatment UBT value was analyzed. The pretreatment UBT results in the subjects with eradication failure and in those with successful eradication were 32.9 ± 28.8 and 14.8 ± 12.8 (permil, mean ± SD), respectively. The results of the statistical analysis showed that the pretreatment UBT results in the subjects with eradication failure were significantly higher than those in the patients with successful eradication ($P = 0.019$, effect size 0.81). We plotted original receiver operator characteristic (ROC)

curves for the pretreatment UBT results to establish the appropriate cutoff value. According to the ROC curves, the optimal cutoff value in our population was 28.5. When patients were assigned to two groups (UBT results $\leq$ 28.5 permil and > 28.5 permil), the eradication rates were 81.3% (26/32) and 25.0% (3/12), respectively ($P = 0.001$). A low pre-treatment UBT value ($\leq$ 28.5 permil) predicted the success of the eradication therapy with a sensitivity of 89.7%, specificity of 60.0%, positive predictive value of 81.3%, negative predictive value of 75.0% and accuracy of 79.5%.

Currently, a standard third-line therapy still remains to be established. *H. pylori* isolates after two eradication failures are often resistant to both MNZ and CLR. The alternative candidates for third-line therapy are fluoro-quinolones-AMX-PPI, rifabutin-AMX-PPI, and high-dose PPI/AMX therapy[2,19-21]. Gisbert *et al*[22] conducted a prospective multicenter study to evaluate the outcomes of treatment with a third-line levofloxacin-based regimen. The patients were treated for 10 d with a regimen consisting of omeprazole, levofloxacin and AMX. The eradication rates as determined by PP and ITT analyses were 66% and 60%, respectively. However, resistance to fluoroquinolones has been shown to be easily acquired, and in countries with a high rate of use of these drugs, the resistance rates are relatively high. González Carro *et al*[23] evaluated the efficacy of a third-line rifabutin-based triple therapy. The patients were treated with PPI, rifabutin and AMX for 10 d. The eradication rates as determined by PP and ITT analyses were 62.2% and 60.8%, respectively. However, it has been suggested that the use of rifabutin be reserved for the treatment of multidrug-resistant *Mycobacterium tuberculosis* strains[24].

Our results for the dual therapy with 4 times daily dosing of rabeprazole and AMX for 14 d, which yielded eradication rates in the PP and ITT analyses of 65.9% and 63.0%, were as successful as other empiric third-line therapy regimens. In particular, a low pretreatment UBT result ($\leq$ 28.5 permil) predicted the success of the eradication therapy with a positive predictive value of 81.3%, sensitivity of 89.7% and specificity of 60.0%, so the dual therapy appeared to serve as a promising option for empiric third-line rescue therapy in patients with a low pretreatment UBT value.

We recently reported the resistant rates of *H. pylori* to AMX. The resistance rates to AMX (MIC $\geq$ 0.06 μg/mL) in the groups with no history of eradication treatment, a history of one treatment failure, and a history of two treatment failures were 13.6%, 26.5% and 49.5%, respectively. The MIC_{90} of AMX increased by 2-fold after each eradication failure[25]. Resistance to AMX in *H. pylori* was gradually induced after unsuccessful eradication. Because the AMX resistance rate after two treatment failures was relatively high, the eradication rate of the present study was lower than that of previous report by Furuta *et al*[16]. Therefore, antimicrobial susceptibility testing of *H. pylori* is desirable before the selection of a suitable third-line therapy, although the culture-based antibiotic susceptibil-

ity testing for *H. pylori* is expensive, time-consuming, and not always available on a routine basis[26]. There are several limitations to our study. First, our eradication study was single armed using the dual therapy, and different doses or superiority over quinolone-based therapy was not evaluated. Second, we did not examine the *in vitro* susceptibility in patients treated with the dual therapy. Thus, *in vitro* resistance to AMX was not elucidated. These issues should be re-evaluated in future studies.

Finally, although we did not achieve excellent eradication success, the dual therapy appeared to serve as a promising option for empiric third-line rescue therapy in patients with low pretreatment UBT values. The antimicrobial susceptibility testing of *H. pylori* is desirable before the selection of a suitable third-line therapy in patients with high pretreatment UBT values.

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World Journal of Gastroenterology

Events Calendar 2012

January 13-15, 2012
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Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
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April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

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Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
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September 8-9, 2012
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ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3　**Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4　**Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462　PMCID:2516377　DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5　**Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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]

Volume with supplement

7　**Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8　**Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9　Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10　**Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11　**Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12　**Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13　**Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14　**Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15　Morse SS. Factors in the emergence of infectious dis-eases. 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

Statistical data

Write as mean $\pm$ SD or mean $\pm$ SE.

Statistical expression

Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as υ (in Greek), sample number as n (in italics), and probability as P (in italics).

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Italics

Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
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Contents

Contents

Contents

ACKNOWLEDGMENTS	**I**	Acknowledgments to reviewers of *World Journal of Gastroenterology*

APPENDIX	**I**	Meetings
	I-VI	Instructions to authors

ABOUT COVER

Yamamoto H, Adachi Y, Taniguchi H, Kunimoto H, Nosho K, Suzuki H, Shinomura Y. Interrelationship between microsatellite instability and microRNA in gastrointestinal cancer.

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AIM AND SCOPE

FLYLEAF	**I-IX**	Editorial Board

EDITORIAL

Update on risk scoring systems for patients with upper gastrointestinal haemorrhage

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Abstract

Upper gastrointestinal haemorrhage (UGIH) remains a common medical emergency worldwide. It is increasingly recognised that early risk assessment is an important part of management, which helps direct appropriate patient care and the timing of endoscopy. Several risk scores have been developed, most of which include endoscopic findings, although a minority do not. These scores were developed to identify various end-points including mortality, rebleeding or clinical intervention in the form of transfusion, endoscopic therapy or surgery. Recent studies have reported accurate identification of a very low risk group on presentation, using scores which require simple clinical or laboratory parameters only. This group may not require admission, but could be managed with early out-patient endoscopy. This article aims to describe the existing pre- and post-endoscopy risk scores for UGIH and assess the published data comparing them in the prediction of outcome. Recent data assessing their use in clinical practice, in particular the early identification of low-risk patients, are also discussed.

Key words: Upper gastrointestinal haemorrhage; Bleeding; Endoscopy; Risk assessment; Scoring systems; Blatchford; Rockall

INTRODUCTION

Upper gastrointestinal haemorrhage (UGIH) continues to be a major cause of hospital admission and mortality throughout the world. A recent United Kingdom national prospective audit of 6750 patients with UGIH reported a median five day length of stay and 10% mortality[1]. In that audit, peptic ulcer disease and variceal bleeding accounted for 36% and 11% patients respectively.

Management of UGIH consists of appropriate resuscitation and assessment, with timely endoscopy to diagnose and if necessary treat the underlying lesion. Similar to other common medical conditions, risk scores have been developed to try and identify those at lower or higher risk of poor outcome. Two recent international consensus documents have emphasised the importance of risk assessment in patients with UGIH[2,3].

An ideal risk score is one that is easy to calculate, accurate for relevant outcomes and can be measured early after presentation with UGIH. Most risk scores require endoscopy although others do not. If a low risk group can be identified soon after presentation, it may allow non-admission of this group with arrangements made for out-patient endoscopy. Higher risk groups require in-patient endoscopy for full evaluation and therapy. This

review describes the existing risk scores for UGIH (clinical and endoscopy based) and gives an update on data regarding their use in clinical practice.

METHODOLOGY

A Medline and PubMed search was undertaken using the keywords: upper gastrointestinal haemorrhage, bleeding, endoscopy, risk assessment and scoring systems. The period covered was 2000-2011 although earlier major publications were used for this review, including those referenced by articles and guidelines within the search period.

It is well recognised that patients with variceal bleeding constitute a specific and high risk group, with outcome largely dependent on the severity of underlying liver disease as assessed by the Childs-Pugh score or model for end stage liver disease (MELD)[4]. This review was not designed to describe scores specifically designed for patients with variceal bleeding and will not describe assessment of this subgroup in detail.

The review is split into assessment and comparisons of risk scores for UGIH which require endoscopy, and those which do not (pre-endoscopy scores) which can be calculated early after presentation. Where studies have directly compared scores for specific end-points, the area under the receiver operator curves (AUROC) are given if available. Finally there is a section describing the optimum clinical use of scores, focusing on the important issue of early identification of low-risk patients who may be suitable for discharge or even non-admission.

RISK SCORES REQUIRING ENDOSCOPY

The most commonly used risk scoring system in UGIH is the Rockall score, which was described in 1996 following analysis of data from a large English audit[5] (Table 1). The score was developed to assess the risk of death following presentation with UGIH and incorporates patient age, haemodynamics, comorbidities and endoscopic findings. Due to the importance of underlying liver disease or failure in prognosis, most generic scoring systems for UGIH including the Rockall score incorporate this as a score component.

The American Baylor score was developed in 1993 to predict rebleeding after endoscopic therapy for non-variceal UGIH[6]. It includes five clinical and endoscopic variables. The Cedar Sinai predictive index is another American score which was derived after a structured literature review to predict outcome and length of hospital stay after UGIH[7]. It includes endoscopic findings, haemodynamics, comorbidities and time from symptoms.

The Spanish Almela score was developed to identify a low risk non-variceal group suitable for out-patient management and includes components from the history, haemodynamics and endoscopic findings[8]. An Italian 10 point score was recently developed to predict mortality after non-variceal bleeding[9]. Several other endoscopy based guidelines and clinical prediction models

Table 1 Rockall score				
Component score	0	1	2	3
Age (yr)	< 60	60-79	≥ 80	-
Haemodynamics:				
Pulse (bpm)	< 100	≥ 100	-	-
Systolic BP (mmHg)	≥ 100	≥ 100	< 100	-
Comorbidities	None	-	IHD, cardiac failure, other major comorbidity	Renal or liver failure, disseminated malignancy
Diagnosis	MW or no lesion and no stigmata	All other diagnosis	Malignant lesions of UGIT	-
Stigmata of haemorrhage	No stigmata or dark spot on ulcer	-	Blood in UGIT, adherent clot, visible/ spurting vessel	-

A score of ≤ 2 identifies a low-risk patient suitable for early discharge. UGIT: Upper gastrointestinal tract; IHD: Ischaemic heart disease; MW: M-Weiss tear; GI: Gastrointestinal; BP: Blood pressure.

for UGIH have been reported from America[10,11], Hong Kong[12] and Italy[13].

COMPARISONS OF ENDOSCOPY BASED RISK SCORES

The Rockall score has been externally validated in several countries[14-17]. It has been also been shown to be superior to the Baylor and Cedar-Sinai scores in identifying low risk patients among a cohort with non-variceal bleeding[14]. In this study, all three scores were better at predicting mortality than rebleeding. The AUROC figures for mortality for the Rockall, Cedar-Sinai and Baylor scores were 0.85, 0.81 and 0.78 respectively, with the corresponding figures for rebleeding 0.68, 0.67 and 0.59. The Italian 10-point score was recently reported to be superior to the Rockall score for predicting 30-d mortality (AUROC 0.81 vs 0.66), but this requires external validation[9].

At present, the Rockall score is the most widely used and studied post-endoscopy score to predict outcome. No other endoscopy based score has yet been validated to be of proven superiority in clinical use.

PRE-ENDOSCOPIC RISK SCORES

An abbreviated pre-endoscopic or "admission-Rockall" score is often used, omitting the last two (endoscopic) components of the full Rockall score. However there has been debate about its accuracy and general clinical applicability. The Glasgow Blatchford Score (GBS) was developed in 2000 to predict the need for hospital based intervention (transfusion, endoscopic therapy, or surgery) or death following UGIH[18] (Table 2). Romagnuolo et al[19] described a modified GBS (due to unavail-

Table 2 Glasgow blatchford score	
Admission risk marker	**Score**
Blood urea (mmol/L)	
6.5-8	2
8-10	3
10-25	4
> 25	6
Hb (g/L)	
Men	
120-130	1
100-120	3
< 100	6
Women	
100-120	1
< 100	6
Systolic BP (mmHg)	
100-109	1
90-99	2
< 90	3
Pulse ⩾ 100/min	1
History and comorbidities	
Melaena	1
Syncope	2
Hepatic disease[1]	2
Cardiac failure[2]	2

[1]History of or clinical/laboratory evidence of liver disease; [2]History of or clinical/echocardiographic evidence of cardiac failure. A score of zero identifies low-risk patients suitable for non-admission. BP: Blood pressure.

ability of serum urea or history of syncope) from Canadian data which predicted high risk endoscopic stigmata, rebleeding and mortality.

The Cambridge score[20] and artificial neural networks (ANNs)[21,22] are other reported pre-endoscopic scoring systems. The former requires 14 clinical and laboratory variables and has not been externally validated. The latter require analysis of even more variables using computer software and are only applicable to non-variceal UGIH. Partly for these reasons the scores are not widely used.

COMPARISONS OF PRE-ENDOSCOPIC RISK SCORES

Six recent studies from United Kingdom and Taiwan have shown the GBS to be superior to the admission Rockall score in predicting need for clinical intervention or death[18,23-28]. Interestingly, a large United Kingdom multicentre study indicated the GBS was also superior to the full (post-endoscopy) Rockall score for predicting these combined outcomes, with AUROC figures for the GBS, full Rockall and admission Rockall scores 0.90, 0.81 and 0.71 respectively[23]. Another recent United Kingdom study comparing the GBS and admission Rockall scores for the same end-points has reported similar AUROC figures at 0.92 and 0.75, respectively[28].

In a larger (*n* = 1555 patients) follow-up publication from the United Kingdom multi-centre study group, AUROC figures for mortality were similar using the GBS, full Rockall and admission Rockall scores at 0.74, 0.79

and 0.76 and respectively[26]. An even higher mortality AUROC figure of 0.81 was recently reported using the GBS in a large study from Singapore and Malaysia[29].

The United Kingdom multicentre follow-up study reported similar figures for the GBS and full Rockall scores in predicting need for endoscopic therapy or surgery, with both superior to the admission Rockall score. AUROC figures for this end-point were 0.79, 0.76 and 0.63 respectively. In a recent large study from Hong Kong, the GBS was again shown to be a better predictor of need for endoscopic therapy than the admission Rockall score, with an AUROC of 0.72[30]. In this study, the admission Rockall score was unable to predict need for endoscopic therapy.

Superiority of a modified GBS over the admission Rockall score in predicting high risk endoscopic stigmata or rebleeding has been reported from North America[19]. The GBS has also been shown to be superior to both the full and admission Rockall scores in predicting need for transfusion (AUROC figures 0.92, 0.75 and 0.69 respectively), presumably because the GBS includes admission haemoglobin as a component variable[26].

Two recent studies assessing relatively complex ANNs have reported them to be superior to the admission Rockall and equivalent to the full Rockall score in predicting endoscopic therapy and superior to the full Rockall score in predicting mortality in non-variceal UGIH[21,22]. The larger of these studies revealed AUROC figures of 0.95 and 0.67 in predicting mortality using the ANN and the Rockall score respectively[22]. This is an impressive figure, but the complexity of ANNs is a significant limitation.

Whilst these studies suggest that some pre-endoscopic scores are equivalent or better at predicting outcome compared with the full Rockall score, all higher risk patients require in-patient endoscopy to diagnose and possibly treat underlying pathology. However pre-endoscopic scores may allow early identification of a low risk group who may not require in-patient endoscopy. As indicated above, studies from several countries have suggested that the relatively simple GBS is superior to the admission Rockall score in predicting clinically relevant end points. Interestingly the GBS also appears to perform well in comparison to the (post endoscopy) full Rockall score. Other pre-endoscopy scores have either not been externally validated or appear too complex for routine clinical use.

OPTIMUM CLINICAL USE OF SCORES FOR UPPER GASTROINTESTINAL HAEMORRHAGE

The major existing risk scores for UGIH are summarised in Table 3. It is usually recommended that all patients with UGIH, except a very low-risk group, are admitted and have endoscopy within 24 h[2,3]. There is no clear evidence of benefit if endoscopy is undertaken earlier than 24 h, although a small group of patients with massive bleeding and haemodynamic compromise will

Table 3 Summary of major published risk scores for upper gastrointestinal haemorrhage

Score[Ref.]	Endoscopy required?	Number of variables	Suitable for unselected upper GI bleeding patients?
Full Rockall[5]	Yes	6[1]	Yes
Baylor[6]	Yes	5	No
Cedars Sinai[7]	Yes	6	Yes
Admission Rockall[5]	No	4[1]	Yes
Glasgow Blatchford[18]	No	5[2]	Yes
ANN[21]	No	20	No

[1]Comorbidities variable describes 5 specific conditions; [2]History and co-morbidities variable describes 4 specific situations. GI: Gastrointestinal; ANN: Artificial neural network.

require emergency endoscopy. The decision on urgent endoscopy in this emergency group is usually based on clinical judgement rather than a specific score, however the recent study from Singapore and Malaysia suggested survival benefit for patients with a GBS of ≥ 12 who were endoscoped within 12 h[29]. This approach requires further study.

The most helpful use of a score in clinical practice is possibly identification of a low risk group who are suitable for early discharge or even non-admission. Interestingly, most scores seem to perform better in patients at low rather than higher risk[14].

Early identification of low-risk patients using endoscopy based scores

Patients with a Rockall score of ≤ 2 are generally accepted as being at low-risk of poor outcome, but calculation requires endoscopy[5,31]. Of the initial cohort used to develop the score, 26% patients met these criteria[5]. Long-streth reported safe early discharge using endoscopic and clinical guidelines to identify low risk patients[32]. Interestingly 32% of patients defined as low risk in this study had a Rockall score > 2. Two relatively small randomised studies suggested that early discharge of selected patients deemed "low risk" using endoscopic and clinical parameters did not affect outcome and offers cost savings[13,33].

Local evaluation of the Cedars-Sinai predictive index reported that 70% patients achieved low risk status after endoscopy, and hospital stay was significantly reduced[7]. The Almela score identified over one third of non-variceal UGIH patients as suitable for early discharge following endoscopy[8]. There were five deaths in this early discharge group, although none were related to UGIH.

Although endoscopic resources vary internationally, it is interesting that the recent United Kingdom national audit revealed that only 52% hospitals had 24 h emergency endoscopy cover and only 50% patients admitted with UGIH had their endoscopy within 24 h[34]. At weekends, American and United Kingdom data show that endoscopy is significantly delayed[35,36]. Therefore the ability to identify low risk patients prior to endoscopy who may be suitable for out-patient management is very attractive.

Early identification of low-risk patients using pre-endoscopy scores

A GBS of zero has been reported to have > 99% sensitivity in identification of those who do not require intervention, rebleed or die in studies from Hong Kong (China)[30], United States[37], Japan[38], Taiwan (China)[27] and United Kingdom[18,23,25,28]. The proportion of patients with a GBS of zero in the above studies ranged from 5%-22%, probably due to differences in local populations and healthcare organisation. Several studies have assessed extending the definition of low risk patients suitable for out-patient management to those with GBS ≤ 1 or ≤ 2, but safety of this approach requires further study[24,28,38,39].

An admission Rockall score of zero is often cited as identifying low risk patients, and identified 15% patients in the initial report[4]. However, studies have shown that up to 18% in this "low risk" group have clinically relevant end-points including endoscopic therapy, rebleeding and death[23,25,28,36]. Whilst no score will be perfect in clinical use for identifying low risk patients, most clinicians would prefer to err on the safe side and use a score with high sensitivity, to avoid discharging patients who may require intervention or die.

CONCLUSION

Risk scores are of critical importance in UGIH, allowing early discharge of low risk patients and appropriate therapy for higher risk patients. The Rockall score is the most widely used and studied post-endoscopy score. The GBS is more accurate than the admission Rockall score for early (pre-endoscopic) prediction of clinically relevant outcomes, and is highly sensitive in identifying low risk patients suitable for out-patient management. Whilst other UGIH risk scores have been described, they require external validation and further comparative studies with the established GBS and Rockall scores.

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Interrelationship between microsatellite instability and microRNA in gastrointestinal cancer

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Abstract

There is an increasing understanding of the roles that microsatellite instability (MSI) plays in Lynch syndrome (by mutations) and sporadic (by mainly epigenetic changes) gastrointestinal (GI) and other cancers. Deficient DNA mismatch repair (MMR) results in the strong mutator phenotype known as MSI, which is the hallmark of cancers arising within Lynch syndrome. MSI is characterized by length alterations within simple repeated sequences called microsatellites. Lynch syndrome occurs primarily because of germline mutations in one of the MMR genes, mainly *MLH1* or *MSH2*, less frequently *MSH6*, and rarely *PMS2*. MSI is also observed in about 15% of sporadic colorectal, gastric, and endometrial cancers and in lower frequencies in a minority of other cancers where it is often associated with the hypermethylation of the *MLH1* gene. miRNAs are small noncoding RNAs that regulate gene expression at the posttranscriptional level and are critical in many biological processes and cellular pathways. There is accumulating evidence to support the notion that the interrelationship between MSI and miRNA plays a key role in the pathogenesis of GI cancer. As a possible new mechanism underlying MSI, overexpression of *miR-155* has been shown to downregulate expression of MLH1, MSH2, and MSH6. Thus, a subset of MSI-positive (MSI+) cancers without known MMR defects may result from *miR-155* overexpression. Target genes of frameshift mutation for MSI are involved in various cellular functions, such as DNA repair, cell signaling, and apoptosis. A novel class of target genes that included not only epigenetic modifier genes, such as *HDAC2*, but also miRNA processing machinery genes, including *TARBP2* and *XPO5*, were found to be mutated in MSI+ GI cancers. Thus, a subset of MSI+ colorectal cancers (CRCs) has been proposed to exhibit a mutated miRNA machinery phenotype. Genetic, epigenetic, and transcriptomic differences exist between MSI+ and MSI– cancers. Molecular signatures of miRNA expression apparently have the potential to distinguish between MSI+ and MSI– CRCs. In this review, we summarize recent advances in the MSI pathogenesis of GI cancer, with the focus on its relationship with miRNA as well as on the potential to use MSI and related alterations as biomarkers and novel therapeutic targets.

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Key words: Microsatellite instability; MicroRNA; DNA mismatch repair; Frameshift mutation; MicroRNA processing

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INTRODUCTION

A type of genetic instability characterized by length alterations within simple repeated microsatellite sequences, termed microsatellite instability (MSI), occurs in a majority of patients with Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer) by mutations and in a subset of sporadic gastrointestinal (GI) and other cancers by mainly epigenetic changes[1-10]. Genetic and epigenetic inactivation of DNA mismatch repair (*MMR*) genes results in the mutator phenotype, mutations in cancer-related genes, and cancer development (Figure 1). MSI underlies a distinctive carcinogenic pathway because MSI-positive (MSI+) cancers exhibit many differences in clinical, pathological, and molecular characteristics relative to MSI-negative (MSI-) cancers irrespective of their hereditary or sporadic origins. The differences in genotype can be explained because deficient MMR leads to a strong mutator phenotype with a very specific mutation spectrum. MSI accumulates frameshift mutations in repeated sequences located in coding regions of target tumor suppressor genes. The peculiar genotype of MSI+ cancers also includes specific patterns of gene regulation. MSI+ GI cancers often show an aberrant epigenetic pattern such as hypermethylation of various genes, including the key *MMR* gene, *MLH1*. The differences in genotype and phenotype between MSI+ and MSI- GI cancers are likely to be causally linked to their differences in biological and clinical features. Diagnostic characterization of the MSI status thus has implications in basic and clinical oncology. MiRNAs are small RNA molecules that regulate gene expression at the posttranscriptional level and are critical for many cellular functions[11-20]. There is accumulating evidence to support the notion that the interrelationship between MSI and miRNA plays a key role in the pathogenesis of GI cancer.

MSI BY THE OVEREXPRESSION OF MIR-155 OR MIR-21

Various pathogenic events, including germline and somatic mutations, promoter methylation, and reduced histone acetylation[21], lead to inactivation of core MMR proteins. A vast majority of MSI+ cancers can be explained by mutation and/or epigenetic inactivation of the core MMR proteins[22]. The etiologies of the remaining MSI+ cancers remain poorly understood. In an unselected se-

ries of 1066 colorectal cancer (CRC) patients, 135 (13%) were MSI+[22]. Of these, 23 (6%) had germline mutations in one of the *MMR* gene, and 106 (79%) showed methylation of the *MLH1* promoter. Approximately 5% of these MSI+ cancers displayed loss of expression of at least one of the core MMR proteins without a well-defined genetic or epigenetic cause[22].

Vareli *et al*[23] have shown that overexpression of *miR-155* significantly downregulates the core MMR proteins; namely, MLH1, MSH2, and, MSH6 in CRC cell lines, thus inducing an MSI. The downregulation of MLH1 and MSH2 proteins by *miR-155* lead to destabilization of the respective heterodimeric complex proteins and a mutator phenotype[24]. An inverse correlation between *miR-155* overexpression and the expression of MLH1 and MSH2 was further demonstrated in human CRC tissues. Most MSI+ cancers without a known cause of MMR inactivation show *miR-155* overexpression. However, not all CRCs with increased miR-155 expression were MSI+. It is also possible that miR-155 affects other related DNA repair proteins, thus enhancing the phenotypic effect of MMR defects. Although further confirmation is required, the results suggest that *miR-155* overexpression is an additional mechanism underlying the development of MSI in cancer (Figure 2)[23].

The reduced expression of a single allele of the adenomatous polyposis coli and transforming growth factor (*TGF*)-β receptor I gene has been linked to CRC[25,26]. Thus, incomplete repression of MMR proteins by *miR-155* is not unique to tumor suppressor genes in cancer. Recently, miR-NAs have been suggested to act as transactivating elements involved in allele and gene expression regulation[27,28]. These results support the notion that miRNAs play a role in the non-Mendelian regulation of *MMR* genes.

miR-21 is overexpressed in various types of human cancers, including CRC[29]. Valeri *et al*[30] reported that *miR-21* directly targets the 3' untranslated region (UTR) of *MSH2* and *MSH6* mRNA, resulting in downregulation of protein expression. The inverse correlation between *miR-21* overexpression and MSH2 expression was shown in CRC tissues. Cells that overexpress *miR-21* showed significantly reduced 5-fluorouracil (5-FU)-induced G2/M damage arrest and apoptosis that is characteristic of defective MMR. Because *miR-21* expression could increase in cell lines continuously exposed to 5-FU[31], cancer cells may develop a secondary resistance to 5-FU through *miR-21* overexpression. Thus, *miR-21*-dependent downregulation of *MSH2-MSH6* may be responsible for both primary and secondary resistance to 5-FU.

TARGET CANCER-RELATED GENES OF MSI

The instability in cancer-related genes at coding microsatellites causes frameshift mutations and functional inactivation of affected proteins, thereby providing a selective growth advantage to deficient MMR cells[32]. For instance, *TGF-β receptor II* and the pro-apoptotic gene *BAX* are

Figure 1 A model of DNA mismatch repair and molecular pathways for microsatellite instability+ colorectal cancers. A: A model of the proposed mechanism of mismatch repair (MMR) proteins, illustrating patterns of relevant heterodimerization; B: The models for colorectal cancer (CRC) carcinogenesis are presented in parallel for Lynch syndrome and sporadic cases. HNPCC: Hereditary nonpolyposis colorectal cancer; MSI: Microsatellite instability; CIMP: CpG island methylator phenotype; MSI: Microsatellite instability.

Figure 2 Cancer progression of sporadic microsatellite instability+ colorectal cancers. The model for microsatellite instability (MSI)+ colorectal cancer progression is presented based on levels of MSI and chromosomal instability (CIN), and genetic, epigenetic and miRNA alterations. SSA/P: Sessile serrated adenomas/polyps; CIMP: CpG island methylator phenotype; MMR: Mismatch repair.

Figure 3 Representative target genes in microsatellite instability+ gastrointestinal cancers. A number of cancer-related genes mutated in microsatellite instability+ gastrointestinal cancers have been reported. The relevance of each mutation is not necessarily proven.

frequently inactivated by slippage-induced frameshift mutations in mononucleotide tracts present in their gene coding regions[33,34]. These findings have provided proof for the causative link between MSI and mutations in cancer-related genes, and they were also convincing examples of the differences between the mutator and suppressor pathways for cancer. These genes have also been mutated in cancers in the suppressor pathway, but at decreased frequencies and not by slippage-linked frameshifts[35,36].

A number of cancer-related genes mutated in MSI+ cancers have been reported (Figure 3). Mutations that promote cancer cell growth are assumed to be the driving force during MSI+ carcinogenesis and are designated as real common target genes (Figure 2)[37]. Mutations of microsatellite-harboring genes that do not contribute to carcinogenesis are designated as bystander genes. However, it is not always clear which mutations are "driver mutations" and which are "passenger mutations"[9,38]. The Selective Targets database (SelTarbase) (http://www.seltarbase.org) of human mononucleotide-microsatellite mutations and their potential impact to carcinogenesis and immunology has been developed[39]. The database includes a comprehensive database of all human coding, untranslated, noncoding RNA and intronic mononucleotide repeat tracts and is useful for basic and clinical oncology.

Because MSI+ cancers accumulate many mutations, disruption of cell growth and survival regulation can be accomplished in different cancers by mutations in different genes of the same signaling pathways[40]. Therefore, genes with infrequent and/or monoallelic mutations should not be regarded as irrelevant. Thus, the relevance of microsatellite-specific mutations in MSI+ cancers can be proven only when there is supporting evidence for functionality irrespective of mutation incidence[2].

Target cancer-related genes of MSI+ cancers can be functionally categorized as tumor suppressors and genes involved in DNA repair, cell cycle, cell proliferation, apoptosis and others (Figure 3). Interestingly, every human *MMR* gene except *MLH1* contains a mononucleotide repeat of at least A7[41]. Thus, frameshift mutations of *MSH3* and *MSH6* led to the concept of "the mutator that mutates the other mutator" (Figure 2)[42].

The spectrum of mutations of target genes could affect cancer biology, therapeutic response, and prognosis of patients. Most putative MSI target genes have been proposed mainly on the basis of high mutation frequency detected within their coding regions. However, genes containing microsatellites that are located within noncoding regulatory regions, such as introns, promoters, and 5' and 3' UTRs, could be also mutated in MSI+ cancers. Alterations within untranslated mononucleotide repeat tracts can alter transcription level or transcript stability. It has been suggested that some intronic repeat mutations in genes, such as *ATM*, *MYB*[43], and *MRE11*[44], play a

Figure 4 MiRNA biogenesis and genes mutated in microsatellite instability+ gastrointestinal cancers. A consequence of perfect complementarity between miRNA and mRNA is mRNA cleavage and degradation. Imperfect alignment represses gene translation. ORF: Open reading frame; RISC: RNA-induced silencing complexes.

role in MSI carcinogenesis[45]. Decreased matrix metallo-proteinase (MMP)-3 expression due to insertions and/or deletions in the *MMP-3* promoter region led to a decrease in the levels of the active MMP-9 form, which may explain the less invasive potential of MSI+ cancer cells[46] and the propensity for a good prognosis in the case of MSI+ CRCs[47].

Genomic copy number changes are frequently observed in cancers. It is well known that MSI+ cancers show less genomic copy number changes and are mostly diploid[48]. However, genes responsible for chromosomal instability (CIN) could be mutated in MSI+ cancers, and these defects may be selected during the course of cancer progression (Figures 2 and 3)[49]. Furthermore, mutations of *RAD50* and *MRE11* are reportedly associated with defects in nonhomologous end-joining, leading to chromosomal alterations during cancer progression[45].

Altered histone modifications that affect chromatin structures are also involved in carcinogenesis[49]. Epigenetic modifier genes could also be MSI target genes. Ropero *et al*[50] detected frameshift mutations in the histone deacetylase (*HDAC*) 2 gene in MSI+ GI cancers. This *HDAC2* mutation made mutation-positive cancer cells more resistant to the antiproliferative and proapoptotic

effects of certain HDAC inhibitors such as trichostatin A, but not to others such as butyric acid and valproic acid. Since HDAC inhibitors may serve as therapeutic agents for cancer, these findings support the use of *HDAC2* mutation status in future pharmacogenetic treatment[50].

MIRNA PROCESSING MACHINERY GENES AS MSI TARGET GENES

MiRNAs are small noncoding RNAs that regulate gene expression at the posttranscriptional level and are critical in many biological processes and cellular pathways (Figure 4)[11-20]. MiRNA expression profiles of human cancers have been characterized by an overall mature miRNA downregulation[51-53]. The causes of the aberrant miRNA expression patterns in cancer involve DNA copy number amplification or deletion[54], inappropriate transactivation, transcriptional repression by oncogenic and/or other factors[55], failure of miRNA post-transcriptional regulation[56], and genetic mutation[57] or transcriptional silencing associated with hypermethylation of CpG island promoters[58-62].

The control of the miRNA biosynthesis pathway is important in the spatiotemporal pattern of miRNA expression in cells. Thus, impaired miRNA processing pathways

may themselves be targets of genetic and/or epigenetic disruption in cancer[63,64]. Recently, it has been reported that mitogenic signaling can be translated into changes in cell viability and proliferation through the miRNA biogenesis pathway. A *TAR RNA-binding protein 2* (*TARBP2*) gene encodes TRBP, an essential functional partner of DICER1 (Figure 4)[65,66]. TARBP2 is phosphorylated under normal growth conditions, which increases the stability of TARBP2 and DICER[67]. Upon growth factor stimulation, MAPK/ERK pathway increases TARBP2 phosphorylation, leading to a coordinated increase in levels of growth-promoting miRNA and a decrease in the expression of *let-7* tumor suppressor miRNA. In contrast, pharmacological inhibition of MAPK/ERK resulted in an anti-growth miRNA profile[67]. These results further suggest the important role of miRNA processing mediated by TARBP2 in preservation of a normal, untransformed cell state[17].

Melo *et al*[68] have found truncating heterozygous mutations in *TARBP2* in MSI+ cancer cell lines and in primary sporadic and hereditary MSI+ GI cancers (Figure 4). *TARBP2* mutations diminished TRBP protein expression, resulting in impaired miRNA processing and enhanced cellular transformation. The TRBP impairment was associated with a secondary defect in DICER1 activity by destabilization of the DICER1 protein. Thus, *TARBP2* mutations may explain overall miRNA downregulation in a subset of MSI+ cancers. Because the restoration of efficient miRNA production by the reintroduction of TRBP can suppress cancer cell growth, these findings are important for the development of new therapeutic strategies for the treatment of cancer[68].

MUTATIONS OF THE *EXPORTIN 5* GENE

Because of the nuclear retention of certain precursor miRNAs (pre-miRNAs), mature miRNA expression levels are not consistent with pre-miRNA expression levels in various human cancer cell lines[17]. Thus, defects in the nuclear export of pre-miRNAs may be one of the mechanisms underlying the global impairment of mature miRNAs in human cancer. The *exportin 5* (*XPO5*) mediates nuclear export of pre-miRNA (Figure 4). Melo *et al*[69,70] have identified inactivating heterozygous mutations of *XPO5* in MSI+ cancer cell lines and in primary sporadic and hereditary MSI+ GI cancers. The mutant form of XPO5 does not comprise a C-terminal region that is important for the formation of the pre-miRNA/XPO5/Ran-GTP ternary complex. Thus, the XPO5 defect trapped certain pre-miRNAs in the nucleus, reduced miRNA processing, and impaired miRNA-target inhibition. It is important to note that the restoration of XPO5 functions rescued the disturbed export of critical tumor-suppressive pre-miRNAs, which results in tumor suppression[69].

Interestingly, although the heterozygous *XPO5* mutation decreased accumulation of a fraction of detectable miRNAs, many others were not affected. It seems that XPO5 does not bind to pre-miRNAs universally but has

certain substrate preferences, which are possibly mediated by sequence or structure[38]. Strategies directed toward stimulating the activity of miRNA processing factors and restoring the production of mature growth inhibitory miRNAs may have therapeutic value[69].

MUTATED MIRNA MACHINERY PHENOTYPE AS A NEW CANCER PHENOTYPE

Recent works have suggested that the other component of the miRNA biogenesis pathway, DICER1, is a haploinsufficient tumor suppressor[71,72]. Therefore, it appears that at least three components of the miRNA biogenesis pathway are haploinsufficient tumor suppressors, with TARBP2 and XPO5, but not DICER, mutations prevalent in MSI+ cancers[38]. In addition, the miRISC components *AGO2*, *TNRC6A*, and *TNRC6C* can also be mutated in MSI+ cancers (Figure 4)[73], although the functional significances remain to be determined[38,70].

From these observations, a new cancer phenotype known as mutated miRNA machinery phenotype (MMMP) has been proposed for MSI+ CRCs having mutations in the miRNA machinery genes and the deregulated miRNAome. Although larger studies are required to fully characterize and validate this feature as a criterion for classification, a broader miRNAome-modifying approach may be effective for cancer patients with MMMP[15].

TRANSCRIPTOMIC DIFFERENCES BETWEEN MSI+ AND MSI- CRCS

As molecular markers, gene expression profiles are being developed for many cancers. Array technology has identified a number of genes that are expressed differentially between MSI+ and MSI- CRCs[74-76]. By using supervised analysis of cDNA microarray data, Giacomini *et al*[77] identified a robust expression signature distinguishing MSI+ and MSI- CRC cell lines. By using high-density oligonucleotide microarrays, Kruhoffer *et al*[78] constructed a gene signature that distinguished MSI+ and MSI- CRCs. The authors further constructed a signature that distinguished sporadic and hereditary cases of MSI+ CRCs. Identification of a signature for MMR deficiency would be relevant, both biologically and clinically[78].

As for miRNAs, Lanza *et al*[79] analyzed 16 MSI+ and 23 MSI- CRCs for genome-wide expression of miRNA and mRNA. On the basis of combined miRNA and mRNA expression, a molecular signature comprising 27 differentially expressed genes, including 8 miRNAs, could correctly distinguish MSI+ and MSI- CRCs. Among the differentially expressed miRNAs, various members of the oncogenic *miR-17-92* family were significantly upregulated in MSI- cancers. Among these, *miR-17-5p*, *miR-20*, *miR-25*, *miR-92-1*, *miR-92-2*, *miR-93-1*, and *miR-106a* were significantly upregulated in MSI- when compared with

Figure 5 Targeted therapies based on molecular alterations in microsatellite instability+ colorectal cancers. Microsatellite instability+ cancers may be managed more effectively with novel targeted therapies based on molecular alterations. MSI: Microsatellite instability; HDAC: Histone deacetylase; PI3K/mTOR: Phosphoinositide 3-kinase/mammalian target of rapamycin; XPO5: Exportin 5.

MSI+ CRCs. Because members of the *miR-17-92* family act as oncogenes, these results may explain, at least in part, the less aggressive behavior of MSI+ CRCs when compared with their MSI- counterparts.

Earle *et al*[80] analyzed 22 MSI-H, including 6 Lynch syndrome, 8 MSI-L, and 25 MSS CRCs for a selected panel of 24 miRNAs. Relative expression of *miR-26b*, *miR-31*, *miR-92*, *miR-155*, *miR-196a*, and *miR-223* were significantly different among MSI subgroups, and *miR-31* and *miR-223* were overexpressed in CRCs of patients with Lynch syndrome. These findings indicate that miRNA expression in CRC is associated with MSI status, including Lynch syndrome and MSI-L, and that miRNAs may play significant roles in these MSI subgroups in addition to having possible effects on cancer characteristics.

Slattery *et al*[81] analyzed 70 CRCs for 866 miRNAs using microarrays. At the 1.5-fold level, 143 miRNAs were differentially expressed in MSI+ CRCs. *MiR-139-3p*, *miR-223*, and *miR-370* were upregulated and *miR-24-2*, *miR-424*, *miR-552*, and *let-7g* were downregulated at a level of 1.5-fold or greater in MSI+ CRCs when compared with MSI- CRCs.

Thus, differentially expressed miRNAs are likely to be relevant, both biologically and clinically, although their functional significances remain to be determined. High levels of *miR-21* in the stroma of CRCs reportedly predict short disease-free survival in stage Ⅱ CRC patients; however, the levels are not associated with the MSI status[82].

DEFECTIVE MMR AS A NOVEL THERAPEUTIC TARGET

MSI+ cancers may be managed more effectively with novel targeted therapies based on molecular alterations (Figure 5). A combination of treatments that target both primary alterations of DNA *MMR* gene and secondary alterations, such as frameshift mutations of target genes, may be also effective. A synthetic lethal relationship, where the simultaneous inhibition of two different regulatory pathways leads to cell death, is a recent therapeutic strategy[83]. Therefore, identification of synthetic lethal interactions with MMR deficiency could potentially lead to the identification of specific therapeutic targets[84]. The inhibition of poly (adenosine diphosphate-ribose) polymerase (PARP) is a potential synthetic lethal therapeutic strategy for the treatment of cancers with specific DNA repair defects, such as a BRCA1 or BRCA2 mutation[85].

A subset of MSI+ CRCs may also be suitable for this strategy. A novel PARP inhibitor, ABT-888, showed preferential activity on MSI+ CRC cell lines harboring mutations in both *MRE11* and *RAD50* genes when compared with MSI- cell lines that were wild type for both genes[86]. Recently, Vilar *et al*[87] reported that MSI+ CRCs deficient in double strand break (DSB) repair due to *MRE11* mutations show a higher sensitivity to PARP-1 inhibition. A phase Ⅱ study assessing the efficacy of a PARP-1 inhibitor, olaparib, in CRCs stratified by MSI status is ongoing. Further clinical studies regarding combinations of a PARP-1 inhibitor with other DSB-inducing therapies, such as radiation or irinotecan, are warranted in MSI+ CRCs with *MRE11* mutations. Although these results need to be confirmed in other settings, they suggest that specific mutations such as *MRE11* can be used to exploit the concept of synthetic lethality in MSI+ cancers, which has been successful in BRCA1-mutant breast cancers[88].

Methotrexate reportedly induces oxidative DNA damage and is selectively lethal to cancer cells with MSH2 defects[84,89]. Thus, a synthetic lethal relationship between deficient MSH2 and treatment with methotrexate led to a phase Ⅱ trial, incorporating measurement of 8-oxoG

DNA lesions as a biomarker, in metastatic CRC patients with germline mutation or loss of MSH2.

Because MSI+ CRCs often harbor a near diploid stable karyotype, these cancers may be sensitive to mitotic inhibitors, such as taxanes and kinesin-5 inhibitors[90]. To determine the effect of CIN and MSI on the efficacy of the microtubule-stabilizing agent patupilone/EPO960, a phase Ⅱ study called CIN and Anti-Tubulin Response Assessment in CRC is ongoing. It is assumed that MSI+ CRC patients will benefit more than MSI- CRC patients.

Gene expression signatures can also be used for new MSI+ cancer therapies. Fourteen of the 164 compounds were shown to target MSI+ cancer cell lines using combined gene expression data sets and a connectivity map[91]. Rapamycin, LY-294002, 17-(allylamino)-17-demethoxygeldanamycin, and trichostatin A were the most convincing candidate compounds. MSI+ cell lines with *MLH1* hypermethylation were preferentially targeted by rapamycin and LY-294002 when compared with MSI- cells. These results underscore the relevant role of the PI3K/AKT/mTOR pathway and its therapeutic application in MSI+ cancer, although its clinical significance needs confirmation.

MiRNA-based cancer therapy is limited mainly to targeting a single miRNA[92,93]. However, if most cancers are characterized by a defect in miRNA production and global mature miRNA downregulation[51-53], restoration of the global miRNAome may be an attractive approach in cancer therapy. Melo *et al*[94] have found that the small molecule enoxacin, a fluoroquinolone used as an antibacterial compound, enhances the miRNA-processing machinery by binding to TRBP. Enoxacin was shown to inhibit the growth of a variety of cancer cells. The enhanced miRNA-processing activity by enoxacin did not depend on general fluoroquinolone activity but on the unique chemical structure of enoxacin. These results highlight the key role of disturbed miRNA expression in carcinogenesis, and suggest the potential of novel miRNA-based cancer therapy to restore the disrupted miRNAome of cancer cells.

Finally, it remains to be determined whether *XPO5* mutations can be exploited therapeutically. Since it is difficult to directly restore XPO5 activity, restoring miRNA accumulation by alternative methods may be a more realistic strategy. Given that only a few deregulated tumor suppressor miRNAs appear to be critical for the tumor-promoting effect of *XPO5* mutations, it may be possible to supply those miRNAs exogenously as miRNA duplexes that would not need to undergo nuclear export. It may also be possible to find a subset of important target genes of deregulated tumor suppressor miRNAs, which may be responsive to inactivation by conventional pharmacological methodologies or novel biologics.

CONCLUSION

The biological and clinical implications of MSI in GI cancers continue to develop. Recent findings, such as overexpression of *miR-21* and *miR-155* and mutations of *TARBP2* and *XPO5* in MSI+ GI cancers, further suggest the important interrelationship between MSI and miRNA in MSI carcinogenesis. The clinicopathological, genetic, epigenetic, prognostic, and therapeutic characteristics of MSI+ cancers are becoming clear, but remain to be fully determined. Analysis of MSI status in cancer patients is warranted as a screening for Lynch syndrome; it could be a potential predictive marker of response to chemotherapy. Since molecular targeting therapeutics are being used in clinical settings and trials, it seems important to clarify if molecular target genes are differentially regulated between MSI+ and MSI- cancers and if the MSI status has the prognostic or predictive significance in metastatic CRC. Further analysis is required to gain insight into MSI carcinogenesis, for a better understanding of disease pathogenesis, and for the development of new diagnostic and/or therapeutic approaches targeting essential pathogenetic alterations.

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Toxic hepatitis in occupational exposure to solvents

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Abstract

The liver is the main organ responsible for the metabolism of drugs and toxic chemicals, and so is the primary target organ for many organic solvents. Work activities with hepatotoxins exposures are numerous and, moreover, organic solvents are used in various industrial processes. Organic solvents used in different industrial processes may be associated with hepatotoxicity. Several factors contribute to liver toxicity; among these are: species differences, nutritional condition, genetic factors, interaction with medications in use, alcohol abuse and interaction, and age. This review addresses the mechanisms of hepatotoxicity. The main pathogenic mechanisms responsible for functional and organic damage caused by solvents are: inflammation, dysfunction of cytochrome P450, mitochondrial dysfunction and oxidative stress. The health impact of exposure to solvents in the workplace remains an interesting and worrying question for professional health work.

INTRODUCTION

Some studies have suggested that exposure to organic solvents may induce liver toxicity[1,2] because most chemicals are metabolized in the liver and toxic metabolites generated through the metabolism are the main cause of liver damage.

Work activities with hepatotoxin exposure are numerous and include chemists, dry cleaners, farm workers, painters, health care workers, nurses, and printers. Organic solvents are used in various industrial processes such as spray painting, paint manufacturing, degreasing, metal processing, aeronautical and auto manufacturing maintenance and manufacturing, as well as various chemical storage facilities. Exposure to hepatotoxins can occur through intentional or accidental ingestion in food or absorption of toxic contaminants through the skin. Contamination includes the ingestion of water, skin absorption *via* water baths, and volatilization of solvents, and heated bathrooms with a shower of water.

Although a number of industrial chemicals are known

to be hepatotoxins, liver disease from occupational exposure is rarely suspected or diagnosed[3].

Three conditions must be fulfilled for the diagnosis of professional toxic hepatitis: (1) Liver damage should take place after occupational exposure to a substance; patient occupational history and the workplace in question is necessary; (2) Liver enzymes must increase to at least double the upper limit of normal levels; and (3) Tertiary conditions, such as other causes of liver disease, must be excluded[4,5].

The most important factors contributing to toxicity liver are protein binding, species differences, points of binding inside the liver intracellular, nutritional condition, genetic factors, interaction with medications in use, alcohol abuse and interaction, and age. For the age factor, it has been shown that age susceptibility clearly plays a role. For instance, neonatal rats are less susceptible to carbon tetrachloride and bromobenzene toxicity as compared to adult animals[6].

The hepatotoxic effects of some of the solvents were recognized as early as 1887. Very little is known about the frequency of occupational liver injury by solvents. It is still difficult to assess the damage from exposure due to difficult controls in the workplace[7-9]. Clinical presentation of occupational liver disease may be acute/subacute or chronic, but is often insidious.

Occupational toxic hepatitis can be divided into three types: hepatocellular, cholestatic and mixed (Table 1).

Liver damage is likely to be more severe in the hepatocellular type than in the cholestatic or mixed type; a patient with elevated bilirubin levels in hepatocellular liver injury indicates serious liver disease.

Patients with the cholestatic or mixed type are likely to develop chronic disease more frequently than those with the hepatocellular type.

The solvents suspected to be responsible for liver occupational disease are: dimethylformamide (DMF), dimethylacetamide (DMA), trichloroethylene (TCE), tetrachloroethylene, carbon tetrachloride, xylene, toluene, and chloroform, whose organoleptic properties and main uses are schematically presented in Table 2.

The solvents that follow are the most extensively used in the chemical industry.

DIMETHYLFORMAMIDE

DMF is an organic compound with the formula $(CH_3)_2$-NC(O)H that takes the form of a colorless, water-soluble liquid. Pure dimethylformamide is odorless, whereas technical grade or degraded dimethylformamide often has a fishy smell due to the impurity of dimethylamine. Its name is derived from the fact that it is a derivative of formamide, the amide of formic acid.

Dimethylformamide has been termed the universal solvent and is used commercially as a solvent for vinyl resins, adhesives and epoxy formulations (the latter for use in laminated printed circuit boards); for purification and/or separation of acetylene, acid gases and aliphatic hydro-

carbons; and in the production of polyacrylic or cellulose triacetate fibres and pharmaceuticals. It is also used as a catalyst in carboxylation reactions; in organic synthesis; as an industrial paint; as a carrier for gases, and in inks and dyes in printing and fibre-dyeing applications[10-12].

It is widely used for resins and polar polymers and in applications such as protective coatings, films, printing inks and adhesives. It is also used in the pharmaceutical industry in the formulation of pesticides, and in the manufacture of synthetic leathers[13,14].

Occupational exposure to dimethylformamide may occur in the production of organic chemicals, resins, fibers, paints, inks and adhesives. Exposure can also occur during the use of ink coatings, adhesives, in the synthetic leather industry, and in the repair of aircraft.

In 100 workers occupationally exposed to this solvent for at least one year (mean exposure of 5 years; range = 1-15 years), a statistically significant incidence of hepatic impairment was found, as indicated by elevated gamma-glutamyl transpeptidase levels and digestive disturbances[15].

Symptoms of irritation occurring during work with DMF include watery eyes, dry throat, and coughing. The exposed workers also reported a reduced sense of smell and dry coughs. Workers exposed to DMF also reported facial flushing and palpitations after ingesting alcohol. This condition is related to alcohol intolerance, characterized by a disulfiram-type reaction.

DIMETHYLACETAMIDE

DMA is an organic compound with the formula CH_3C-(O)N(CH_3)_2$ that is widely used in the synthetic fiber and resin industries[16].

It is colorless, water miscible, has a high boiling point, and is commonly used as a polar solvent in organic chemistry, as a solvent for vinyl resins, cellulose derivatives, polyacrylonitrile, linear polyesters and styrene. It is also used as in catalyst and solvent elimination, cyclization, alkylation reactions and halogenations.

DMA is a widely used solvent in acrylic and elasthane fiber spinning, and is also used as: a solvent in the production of X-ray contrast media; a solvent in the production process of antibiotics like cephalosporins (such as cefadroxil, cefalexin and cefradine); and a solvent and

Table 1	Clinical-diagnostic types of occupational toxic hepatitis				
Type of disease	ALT	ALP	γ-GT	Bilirubin	Bile acids
Hepatocellular	> 2 ULN	N	> 2	Elevated levels	Elevated levels
Cholestatic	N	> 2 ULN	> 4	Normal or moderate level	Elevated levels
Mixed	> 2 ULN	≥ ULN	> 2	Normal or moderate level	Elevated Levels

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GT: Glutamyl transferase; ULN: Upper limit of normal; N: Normal value. Normal value, ALT 9-63 U/L; ALP 38-126 U/L; γ-GT 7-50 U/L; Bilirubin 0.20-1.5 mg/dL; Bile acids < 10 μmol/L.

Table 2 Main features of the solvents described

Solvents	Organoleptic properties	Main uses	Main mode of absorption
Dimethylformamide	Water-soluble liquid	Production of organic chemicals, resins, fibers, paints, inks and adhesives	Inhalation
	Colorless	Vinyl resins, adhesives and epoxy formulations	Skin
	Odorless	Purification and/or separation of acetylene	
	Polar polymer	Production of polyacrylic or cellulose triacetate fibres and pharmaceuticals	
		Industrial paint	
		Protective coatings, films, printing inks and adhesives	
		Pharmaceutical industry	
		Formulation of pesticides	
Dimethylacetamide	Colorless	Organic chemistry	Inhalation
	Water miscible	Vinyl resins	Skin
	High boiling point	Cellulose derivatives	Gastric
	Polar	Polyacrylonitrile	
	Greasy	Linear polyesters and styrene	
		Production process of antibiotics like cephalosporins	
		Production of X-ray contrast media	
		Manufacture of polyimide resins, polysulfones and cellophane	
Trichloroethylene	Non-flammable liquid	Volatile anesthetic (in the past)	Inhalation
	Clear	Food industry (e.g., the decaffeination of coffee and the	Skin
	Pleasant smell	preparation of flavoring extracts from hops and spices)	Gastric
	Volatile		
	Organic compound		
Tetrachloroethylene	Colorless liquid	Dry cleaning and metal cleaning,	Inhalation
	Volatile	veterinary anthelmintic, textile industry,	Skin
	High stabile	automotive and other metalworking industries,	Gastric
	Non-flammable	dry-cleaning industry	
Carbon tetrachloride	Liquid	Refrigerant	Inhalation
	Easily evaporates	Pesticide	Skin
	Sweet smell		Gastric
	Unpleasant smell (> 10 ppm)		
Xylene	Flammable liquid	Resins, gums, rubber cleaners, degrease paints, lacquers, varnishes,	Inhalation
	Light-colored or colorless, strong odor	adhesives, cements, inks, gasoline	Skin
Toluene	Refractive liquid	Paints, coatings, synthetic fragrances, adhesives, inks, cleaning	Inhalation
	Colorless	agents, pharmaceuticals, dyes, cosmetic nail products, and	Skin
	Flammable soluble in water	synthesis of organic chemicals	
	Pungent odor		
Chloroform	Colorless	Pharmaceutical industry	Inhalation
	Sweet-smelling	Dyes and pesticides	
	Dense liquid	Reagent	
		Anesthetic	

reaction medium in the manufacture of polyimide resins, polysulfones and cellophane.

The hepatic toxicity of DMA is well known in animals, with reports of an increase in liver weight, steatosis, hepatic focal cystic degeneration, transaminasemia, biliary hyperplasia and centrilobular single cell necrosis[17].

It was discovered that a worker in a polyurethane plastic producing plant who was accidentally exposed to DMA mixed with ethylenediamine developed chemical induced hepatitis with several toxic features[18].

There was another report of a male worker in a factory of synthetic stretch fabric who was exposed to mixed solvents, including DMA, in a confined space continuously for 4-6 h/d for three days developing hepatic injury with other clinical manifestations of acute DMA intoxication. Toxic hepatitis following excessive skin exposure to DMA was reported among workers from a new production line of acrylic fiber[19,20].

TRICHLOROETHYLENE

TCE is a volatile organic compound with the chemical formula C_2HCl_3. A chlorinated hydrocarbon, it is used as an industrial solvent, and takes the form of a clear non-flammable liquid with a sweetish smell resembling chloroform.

Until 1975, it was used as a volatile anesthetic (however, it produced depression of the central nervous system) and inhaled obstetrical analgesic in millions of patients, as well as an extractant in food-processing. It is now used for vapor degreasing and as a solvent[21].

TCE is a solvent for a wide variety of organic materials, but is also used in the food industry for the decaffeination of coffee and the preparation of flavoring extracts from hops and spices.

Higher concentrations can cause tachypnea, and many types of cardiac arrhythmias which are exacerbated by epinephrine (adrenaline).

TCE has also been used as an inhaled patient controlled analgesic agent, mainly for the treatment of trigeminal neuralgia.

It was found that 10% of workers exposed to TCE became jaundiced with massive hepatic necrosis[22].

The data in humans, although limited, clearly suggests a toxic effect on human livers. Case reports describe TCE as inducing hepatitis and liver necrosis[23].

TETRACHLOROETHYLENE

Also, known under the name tetrachloroethene, tetrachloroethene is a chlorocarbon with the formula $Cl_2C=CCl_2$. It is a colorless liquid that is volatile, highly stable, and nonflammable, and mainly used as a solvent in dry cleaning and metal cleaning. It is also used for veterinary anthelmintic, processing and finishing in the textile industry, as an extraction solvent, grain fumigant, heat-exchange fluid, and in the manufacture of fluorocarbons.

Tetrachloroethylene is an excellent solvent for organic materials. It is also used to degrease metal parts in the automotive and other metalworking industries and appears in certain consumer products including spot removers, paint strippers, silicone lubricants, and food[24,25]. Its toxicity presents itself as different effects in the central nervous system, kidneys and liver. Symptoms of toxicity from exposure include fatigue, dizziness, headache, vomiting and nausea, signs of hepatic or renal failure, and pulmonary edema[26].

Tetrachloroethylene causes irritation of the eyes and nose mucosal. Severe exposure can lead to behavior alteration, coma and death[24,26].

CARBON TETRACHLORIDE

Carbon tetrachloride, also known by numerous other names, is an organic compound with the formula CCl_4 that takes the form of a clear liquid that very easily evaporates. Most carbon tetrachloride that finds its way into the environment is therefore found as a gas.

Carbon tetrachloride does not burn easily. It normally has a sweet smell, but this can change to a more unpleasant odor when the concentration of carbon tetrachloride reaches 10 parts per million parts of air (ppm).

In the 20th century, carbon tetrachloride was commonly used as a dry cleaning solvent, as a refrigerant, and in lava lamps[27]. One specialty use of carbon tetrachloride was by stamp collectors to reveal watermarks on the backs of postage stamps without damaging the stamp. However, once it became apparent that carbon tetrachloride exposure had severe adverse health effects, safer alternatives such as tetrachloroethylene were found for these applications, and its use in these roles declined from about 1940 onward.

Carbon tetrachloride was used as a pesticide to kill insects in stored grain but, in 1970, it was banned in consumer products in the United States. Before the Montreal Protocol, large amounts of carbon tetrachloride were used to produce the Freon refrigerants R-11 (trichlorofluoromethane) and R-12 (dichlorodifluoromethane). However, these refrigerants were identified as playing an important role in ozone depletion, and therefore their use was banned. Carbon tetrachloride is still used to manufacture less destructive refrigerants however.

Carbon tetrachloride is one of the most powerful solvents toxic to the liver, and is widely used in scientific research to assess liver damage and hepatoprotective agents[28].

Indeed, carbon tetrachloride has been known for many years to be toxic to the liver. It has been shown to produce hepatic damage including necrosis and fatty degeneration in various experimental animal species[29,30]. Several experiments have also shown that single doses can cause areas of necrosis in the liver within minutes[31,32], as well as liver enzyme abnormalities known to indicate liver damage[33,34].

XYLENE

Xylene is a clear, light-colored or colorless, flammable liquid which evaporates rapidly and is also called "xylol," "dimethylbenzene," or "mixed xylenes". Its odor is strong and sweetish like other aromatic solvents.

Xylene may be found in: solvents for gums, resins, rubber cleaners, degreaser paints, lacquers, varnishes, adhesives, cements, epoxy resins, inks, dyes, and gasoline.

The xylene in commercial use is composed of a mixture of the three isomers ortho-xylene, meta-xylene, and para-xylene; the meta-isomer predominates in these mixtures. O-Xylene and m-xylene are clear, colorless, flammable liquids that have characteristically sweet, balsamlike odors. At low temperatures, the para-isomer occurs in the form of clear, colorless plates[35].

TOLUENE

Toluene occurs as a colorless, flammable, refractive liquid that is slightly soluble in water, has a sweet and pungent odor, and has the chemical formula $C_6H_5CH_3$[36].

It is used to produce benzene and as a solvent in paints, coatings, synthetic fragrances, adhesives, inks, and cleaning agents. Toluene is also used in the production of polymers used to make nylon, plastic soda bottles, polyurethanes, and for pharmaceuticals, dyes, cosmetic nail products, and the synthesis of organic chemicals[36]. Exposed to toluene may occur from breathing ambient or indoor air, from the use of common household products (paints, paint thinners, adhesives, synthetic fragrances and nail polish), and cigarette smoke. The deliberate inhalation of paint or glue may result in high levels of exposure to toluene, as well as other chemicals, in solvent abusers[36].

Toluene exposure may also occur in the workplace, especially in occupations such as printing or painting, where toluene is frequently used as a solvent. Automobile emissions are the principal source of toluene in the ambient air. Toluene may be released to the ambient air

during the production, use, and disposal of industrial and consumer products that contain toluene. Levels of toluene measured in rural, urban, and indoor air averaged 1.3, 10.8, and 31.5 micrograms per cubic meter (μg/m^3), respectively[36].

Toluene is metabolized by the liver; however, the liver does not appear to be a primary target for toluene toxicity. Indeed, the central nervous system (CNS) is the primary target for toluene toxicity in both humans and animals for acute and chronic exposures. CNS dysfunction (which is often reversible) and narcosis have been frequently observed in humans acutely exposed to low or moderate levels of toluene by inhalation; symptoms include fatigue, sleepiness, headaches, and nausea. CNS depression and death have occurred at higher levels of exposure.

A study of printing factory workers who were exposed to toluene at a concentration of less than 200 ppm showed minimal changes to liver enzymes[37]. A study by Svensson *et al*[38] looked at 47 rotogravure workers occupationally exposed to toluene and showed elevation of liver enzymes and chemical hepatitis.

Hepatotoxicity has been observed in the literature in individuals exposed to xylene and toluene[39].

CHLOROFORM

Chloroform is a volatile organic compound that takes the form of a colorless liquid with a non-irritating odor and a slightly sweet taste. It will burn only when it reaches very high temperatures. In the past, chloroform was used as an inhaled anesthetic during surgery, but it is not used for that purpose today. Today, chloroform is used to make other chemicals and can also be formed in small amounts when chlorine is added to water. Other names for chloroform are "trichloromethane" and "methyl trichloride".

It is produced as a byproduct of water chlorination and the bleaching of paper. Chloroform may also be elicited as a vehicle exhaust. In 1923, Meyer and Pessoa showed the toxicity of chloroform to the human liver[40].

MECHANISMS OF HEPATOTOXICITY

The pathophysiologic mechanisms of hepatotoxicity are still being explored, but are characterized by organic and functional damage of the liver. The principal alterations are: (1) Disruption of the hepatocyte; with a decrease in ATP levels. Disassembly of actin fibrils at the surface of the hepatocyte with blistering and rupture of the membrane; (2) Disruption of the transport proteins; toxins may affect transport proteins at the canalicular membrane and can interrupt bile flow. It also detects interruption of transport pumps; (3) Cytolytic T-cell activation: the covalent binding of a toxin to the P-450 enzyme acts as an immunogen, activating T cells and cytokines and stimulating a multifaceted immune response; (4) Apoptosis of hepatocytes; activation of the apoptotic pathways by the tumor necrosis factor-alpha receptor of Fas may trig-

ger the cascade of intercellular caspases, which results in programmed cell death; and (5) Bile duct injury; toxic metabolites excreted in bile may cause injury to the bile duct epithelium[41]. In cultured rat hepatocytes, the hydrophobic bile acid glycochenodeoxycholate at pathophysiologically relevant concentrations (20-100 mmol/L) induces apoptosis, as documented by cell shrinkage, nuclear condensation and lobulation, caspase activation, DNA fragmentation, and phosphatidylserine externalization. Thus, bile acids provide a valuable model to dissect the mechanisms of liver cell apoptosis and the role of apoptosis in liver injury from endogenous toxicants. Apoptosis occurs by one of two pathways: (1) a death receptor pathway; and (2) the mitochondrial pathway.

The main pathogenic mechanisms responsible for functional and organic damage caused by solvents are: inflammation, dysfunction of cytochrome P450, mitochondrial dysfunction and oxidative stress.

INFLAMMATION

Inflammation plays an important role in classical chemical toxicities[42,43]. Hepatic non-parenchymal cells, the Kupffer, sinusoidal endothelial, and fat-storing or Ito (stellate) cells, and recruited leukocytes, i.e., monocytes and neutrophils, contribute to the pathogenesis of hepatic toxicity.

Proinflammatory cytokines, chemokines, reactive oxygen and nitrogen species, that promote oxidative stress in the damage induced by toxic substances, are produced by Kupffer cells and neutrophils.

In response to a direct action of the chemical, the Kupffer cells are activated resulting in the production of proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF) α, and chemokine receptor chemokines.

Each of these factors can upregulate expression of β-2 integrin [cluster of differentiation (CD)11b/CD18] and prime neutrophils for reactive oxygen species (ROS) formation. C5a also stimulates Kupffer cells to release ROS.

The aforementioned cytokines can regulate genes for the production of factors that induce and/or promote apoptosis, or that stimulate the proliferation of hepatocytes.

These cytokines can mediate many pathological effects, including inflammatory cell infiltrates, lipogenesis, fibrogenesis and cholestasis[44].

For example, TNF α/IL-6 induction occurs within minutes following CCl$_4$ exposure and is responsible for the activation of nuclear transcription factors including AP-1, NFκB, and STAT 3[45,46], which regulate genes involved in cell growth. Liver toxicity induced by solvents is presented schematically in Figure 1.

In addition, cytokines activate the expression of adhesion molecules on endothelial cells and hepatocytes. If primed neutrophils receive a chemotactic signal from the parenchyma, they will transmigrate and adhere to hepatocytes. This leads to the final activation of neutrophil with degranulation (protease release) and adherence-dependent

Figure 1 Hypothetical role of inflammation in chemical-induced hepatotoxicity. TNF: Tumor necrosis factor; IL: Interleukin; PMN: Prime neutrophils.

Figure 2 Effects of solvents on cytochrome P450.

oxidant stress, which causes cell necrosis. Mediators generated during cell injury, such as lipid peroxidation products (LPO) and chemokines, become chemotactic signals for further neutrophil activation and transmigration[47].

Therefore, both neutrophils and Kupffer cells, directly or through activation of a complement, are activated by solvents and drugs toxicity, tissue trauma, ischemia-reperfusion, sepsis, and other pathophysiological events. In particular, Kupffer cells release cytotoxic mediators, such as proinflammatory mediators, and reactive oxygen species, such as cytokines and chemokines. Functionally, complement factors (e.g., C5a) and cytokines prime and activate neutrophils to promote their recruitment into the hepatic vasculature. Chemotactically-stimulated neutrophils extravasate and adhere to parenchymal cells. Through the release of reactive oxygen and proteases, they induce necrotic cell death adhesion molecules on neutrophils (β-2 integrins, especially CD11b/CD18) and ICAM-1 on endothelial cells and hepatocytes, which are are essential for neutrophil margination, extravasation, and oxidant production.

Cytokines can induce hepatic adhesion molecule and chemokine formation, which in turn is modulated by oxidant stress[48].

CYTOCHROME P450

Cytochrome P450 (P450 or CYP) plays an important role in the biotransformation of many endogenous compounds and xenobiotics. The most common enzyme system for oxidation of xenobiotics is cytochrome P450,

also known as CYP450 monooxygenase, hydroxylase or oxidase[49,50].

The liver is the major source of P450, although it is expressed in various extrahepatic tissues like the kidney[51].

P450 isoform CYP2E1 is the most abundant isoform in the human liver and therefore is responsible for the metabolism of a wide variety of exogenous and endogenous substrates[52,53]. CYP2E1 dependent ethanol metabolism produces oxidative stress through generation of ROS, a possible mechanism by which solvents are hepatotoxic[54,55].

The cyt p450 is a membrane protein for the disposal of xenobiotics. It is mainly associated with the smooth membranes of the ER of liver cells, while the enzymes for changes to endogenous substances (prostaglandins, cholesterol, *etc.*) are mainly associated with mitochondria.

For example, toxic substances that are known to be substrates of cytochrome P450 enzymes include acetaminophen, cyclophosphamide, doxorubicin and diclofenac.

The detoxification process *via* enzymatic systems, principally the glutathione and cytochrome P450, is different genetically and therefore plays a significant role in the way some individuals detoxify solvents differently from others. It has been shown that all conditions resulting in reduced activity of cytochrome P450, reduced the ability to detoxify solvents and, alternatively, increase the percentage of fat in the liver (e.g., malnutrition). In the study of toxic hepatitis there is a need to evaluate the potential inhibition and inductions of some of the substances involved, and assess the potential interaction with the myriad of drugs that are substrates of CYP[56,57] (Figure 2).

The metabolite responsible for the liver damaging effect of carbon tetrachloride is a C Chloride Ⅲ which is formed from carbon tetrachloride[31-58].

The studies by Brady *et al*[59] and Lindros *et al*[60] have shown that the enzymes involved in the bioactivation of carbon tetrachloride are cytochrome P-450, localized in the liver endoplasmic reticulum.

MITOCHONDRIAL DYSFUNCTION

Various endogenous and exogenous substances impair mitochondrial β-oxidation to cause micro-vesicular

steatosis through oxidative stress and damage to mitochondrial proteins, lipids, and DNA. In humans, these oxidative lesions cause mitochondrial DNA (mtDNA) deletions[61].

In normal mitochondria, enzymes involved in the import and β-oxidation of fatty acids or the tricarboxylic acid cycle are encoded by nuclear DNA[62]. Import polypeptides and enzymes involved in the β-oxidation of long-chain fatty acids are in the inner membrane, while those involved in the β-oxidation of medium- and short-chain chain fatty acids or the tricarboxylic acid cycle are in the matrix, together with mtDNA[62].

mtDNA is a circular, double-stranded molecule. Each cell contains many copies of this DNA, as there are several mtDNA copies in a single mitochondrion and many mitochondria per cell[62,63]. MtDNA is extremely sensitive to oxidative damage owing to its proximity to the inner membrane (the main cellular source of ROS), the absence of protective histones, and incomplete repair mechanisms in mitochondria[62-64].

Many solvents (cationic and amphiphilic) are able to concentrate in mitochondria as a result of the mitochondrial membrane potential[65]. Accumulation of these solvents within liver mitochondria inhibits fatty acid β-oxidation (causing steatosis) and electron transfer along the respiratory chain[65]. Overly reduced respiratory chain intermediates react with oxygen to form the superoxide anion. ROS oxidize fat deposits[65]. Similarly, in alcohol abuse, increased ROS formation causes extensive peroxidation of fat deposits and frequent steatohepatitis[66].

OXIDATIVE STRESS

The oxidative damage caused by free radicals is thought to be a basic mechanism underlying many pathological conditions, including hepatotoxicity by solvents.

Oxidative stress develops when there is an imbalance between the pro-oxidant and antioxidant ratio, leading to the generation of ROS. Environmental contaminants such as solvents, herbicides, and insecticides are known to modulate antioxidant defensive systems and cause oxidative damage in organisms by ROS production[67,68].

Oxidative damage accumulates more in mitochondria than in the rest of the cells because electrons continually leak from the respiratory chain to form damaging ROS (Smith, R., 1999).

ROS, such as hydrogen peroxide (H_2O_2), superoxide anion O_2-, and hydroxyl radical (OH•) at supranormal levels, can react with biological macromolecules potentially leading to enzyme inactivation, LPO, DNA damage and cell death, but at low concentrations their effects are less pronounced[69].

These free radicals are capable of damaging many cellular components such as DNA, proteins and lipids[70].

CLINIC OF TOXIC HEPATITIS

Toxic effects on the liver have been studied as early as

1887 and it was determined that there must be a change in the rate of the metabolism of these compound in order to create toxic products, otherwise toxicity will not occur. This chain of events is obligatory from a pharmacokinetic point of view for the majority of solvents[7-9].

There are a few clinical features associated with occupational liver disease including fatigue, appetite loss, arthralgia, hypertransaminasemia, hypergamma glutamyl transferase (GT), and splenomegaly. As there are many causes of liver injury, it is essential to exclude other aetiologies. Other aetiologies include viral hepatitis, biliary diseases, alcohol abuse, and non-alcoholic fatty liver disease.

Clinical presentation of occupational liver disease may be acute/subacute or chronic but is often insidious. Since some of the solvents may cause chronic health effects, it may take decades to study and document such events. Some hepatotoxins are capable of causing malignancy. The most famous example is vinyl chloride which was once thought to be safe and was used for many years until it was found to cause liver tumors.

Signs and symptoms of toxic hepatitis occurring may include: jaundice, itching, and abdominal pain in the upper right portion of the abdomen, fatigue, loss of appetite, nausea and vomiting, rash, weight loss, and dark or tea-color urine.

In acute toxic hepatitis the patient's condition is similar to viral hepatitis and rapidly deteriorates, resulting in marked liver dysfunction, encephalopathy and coagulopathy. The features of toxic hepatitis are: apoptosis of hepatocytes, ischemic liver injury, sepsis, and cholestasis. Hepatocyte apoptosis and necrosis, when massive, result in fulminant hepatic failure[71].

Acute exposure and toxicity has been associated with liver necrosis, and liver steatosis, and chronic exposure has been associated with liver cirrhosis. The mechanism of injury is most likely the result of metabolic changes by the liver.

Liver damage in the form of hepatomegaly, jaundice, and elevation in levels of several hepatic transaminases and bilirubin has also been reported.

Orthotropic liver transplantation (OLT) has improved the survival of these patients (49% undergo OLT), yet 37% die while awaiting OLT.

Steatosis (fat accumulation in the liver) is important in order to look at the effect of solvents, which are known to be toxic to the liver. Steatosis is the result of anomalous transport of lipids and, as a consequence, accumulation of lipids in the liver. For that reason, exposure to hepatotoxic solvents is clinically associated with liver steatosis, among others, and is a good clinical marker of solvent hepatotoxicity (having ruled out other factors).

After steatosis, necrosis is the second most common effect of liver damage as a result of hepatotoxic solvents. It is the result of destruction of the cell architecture, as well as damage of the biochemical pathways.

Chronic effects on the liver in long-term occupational exposure to low levels of organic solvents remain undetermined.

Indeed, epidemiological studies in this field were difficult for several reasons.

These include: (1) the cause of chronic liver injury can be attributed to a number of factors and not only isolated in occupational exposure; and (2) failure to control chronic liver damage among workers because of vague symptoms and signs, and lack of specificity and sensitivity of conventional liver enzyme tests[72].

Many cases of liver cirrhosis with no known aetiology raise the suspicion that some may be of occupational origin.

DIAGNOSIS OF TOXIC HEPATITIS

Laboratory

Liver damage can be of two types: hepatocellular damage (death of liver cells), in which alanine aminotransferase and aspartate aminotransferase are altered; and cholestatic damage (bile stasis) with an increase of parameters such as alkaline phosphatase and γ-GT. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities are routinely used as clinical endpoints indicative of hepatotoxicity. Toxic hepatic damage includes necrosis and fatty degeneration.

The liver is one of the target organs for toxins, thus biological effects monitoring is required medical surveillance of workers exposed to hepatotoxin. It is also important to evaluate platelet count abnormalities and serum bilirubin in patients.

The evaluation of plasma enzyme showed the advantage of being in the past been well tested in clinical practice, but the main disadvantage is that the enzymes are not organ specific and this can cause occasional diagnostic problems in clinical practice[73,74].

Alanine aminotransferase is considered to be liver-specific in the rat[75]. Aspartate aminotransferase activity is high in the rat liver, along with the kidney, pancreas and erythrocytes[76], which means that elevated serum AST is indicative of tissue and cellular damage, but is not specific for hepatotoxicity.

As a general rule, clinically significant liver injury is often defined as ALT > 3 times the upper limit of normal (ULN)[77].

A factor that may portend a worse prognosis than the elevation of transaminases litter alone is associated with the increase with aminotransferase[78,79].

Serum levels of γ-GT have been recognized as a marker of hepatobiliary disease. Conditions that could cause increases in γ-GT levels are different; hepatotoxic agents and other non-hepatic factors such as renal, pulmonary, and myogenic (including cardiac) disorders. Indeed γ-GT is not merely a sensitive marker for liver and bile disorders, but it may also serve as a risk marker for a multiplicity of other chronic diseases; the metabolic syndrome (namely, obesity, hypertension, lipid metabolism, and in particular type 2 diabetes) for example[80,81].

The measurement of total bile acids in serum may be a more sensitive indicator of hepatic function and has the advantage of being organ specific. Measurement of bile

acids has not been standard clinical practice, however, and interpretation of high results might present difficulties.

Bile acids are synthesized from cholesterol in the liver, and secreted to the duodenum *via* the bile duct[82].

Secreted bile acids are reabsorbed almost completely into the intestine and returned to the liver (Hofman, 2007). Thanks to efficient enterohepatic circulation of bile acids, less than 10% of intestinal bile acids are eliminated in the feces. The secretion of bile acids into the systemic circulation is small. The blood concentration of bile acids is less than 10 μmol/L under normal conditions, but is increased in the case of a disorder of the liver or biliary tract[82]. Hepatotoxicity implicated from occupational and drug exposure has also been associated with elevations in total bile acid concentrations. Retention of bile constituents within the hepatocyte during cholestasis is associated with hepatocyte apoptosis.

In cholestatic disease, endogenously generated bile acids produce hepatocellular apoptosis by stimulating Fas translocation from the cytoplasm to the plasma membrane, where self-aggregation occurs, to trigger apoptosis. In cholestasis, secretion is impaired, resulting in elevated concentrations[83] of toxic bile acids (TBA) within hepatocytes. At pathophysiologic concentrations, TBA trigger translocation of intracellular Fas bearing vesicles to the plasma membrane where they self-aggregate in the absence of ligand. Activated Fas receptor complexes on the plasma membrane then cause caspase 8 activation and an apoptotic cascade.

Evaluation of the concentration of bile acids is not widely used as a routine screening test. Recently, a simpler and more rapid method is the direct enzymatic assay of urine sulfated bile acids (USBA)[84].

Mild forms of toxic hepatitis may not cause any symptoms and may be detected only by blood tests. Histological samples obtained from workers exposed to solvents that had only mild biochemical abnormalities have shown prominent fatty change, or steatosis, with degrees of inflammation and fibrosis, which suggest that parenchymal changes may be an early feature of solvent induced liver injury.

Diagnostic imaging

The diagnosis of toxic hepatitis improves with the use of imaging techniques (ultrasound, contrast enhanced ultrasonography, computed tomography, magnetic resonance imaging). In most cases the findings are not characteristic, but history and laboratory investigations allow us to make a diagnostic hypothesis. Indeed, they are essential in determining the clinical-pathological example for the evaluation of steatosis[85].

Routine evaluation includes ultrasound (US) and contrast enhanced ultrasound (CEUS), as reliable methods of first instance. Assessment may include, where necessary, computed tomography (CT) scans, magnetic resonance imaging (MRI) and liver biopsy.

Presently, the most widely used method for assessment of fatty liver is abdominal ultrasound, as it has sev-

eral advantages. It is not invasive, is readily available, and provides reliable information.

CT and MRI techniques are sensitive for the evaluation of steatosis, but are more expensive and less readily available[86].

However, it is necessary to clarify that recent studies have shown that abdominal ultrasound is not accurate in cases of chronic liver disease with fibrosis. In the latter case, it is important to confirm the steatosis and hepatic fibrosis assessment CT[87].

Toxic hepatitis is characterized by different degrees of steatosis and fibrosis, which can lead to cirrhosis.

Various degrees of steatosis can be defined as: (1) "light steatosis", presence of slight "bright liver" and no deep attenuation; (2) "moderate steatosis", presence of mild "bright liver" and with deep attenuation; and (3) "severe steatosis", presence of diffusely severe "bright liver" and deep attenuation without visibility of the diaphragm.

Chronic toxic hepatitis can progress to cirrhosis and liver failure. Early diagnosis of cirrhosis is important to prevent the development of severe liver failure. The diagnosis of cirrhosis of the liver requires a histological demonstration for the evaluation of abnormal nodules of regeneration and fibrosis. Therefore, liver biopsy is still considered the gold standard instrument. However, liver biopsy is limited by a number of disadvantages, such as availability of expert practitioners, cost, invasiveness of the procedure, and risk of complications (bleeding, pneumothorax, pain perforation and bile peritonitis)[88,89].

CONCLUSION

Many occupational activities can cause abnormalities in liver function tests without any symptoms suggestive of liver disease.

In patients with abnormalities in liver function tests without an obvious cause, a careful history including not only drugs, but also herbal remedies, should first be obtained.

History taking should also include occupational activity, exposition time, alcohol consumption and underlying chronic liver disease.

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ORIGINAL ARTICLE

Simple and reproducible hepatectomy in the mouse using the clip technique

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Abstract

AIM: To investigate the reliability of massive hepatectomy models by using clip techniques.

METHODS: We analyzed anatomical findings in 100 mice following massive hepatectomy induced by liver reduction > 70%. The impact of various factors in the different models was also analyzed, including learning curves, operative time, survival curves, and histopathological findings.

RESULTS: According to anatomical results, models with 75%, 80%, and 90% hepatectomy produced massive hepatectomy. Learning curves and operative times were most optimal with the clip technique. Each hepatectomy performed using the clip technique produced a reasonable survival curve, and there were no differences in histopathological findings between the suture and clip techniques.

CONCLUSION: Massive hepatectomy by the clip technique is simple and can provide reliable and relevant data.

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Key words: Hepatectomy; Animal model; Clip; Microsurgery; Surgical technique

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INTRODUCTION

New insights into mechanisms in the hepatology field have been established from experimental animal models. The rodent hepatectomy model is mainly employed to examine liver regeneration, liver failure, and tumor metastasis, and the 70% hepatectomy by *en bloc* ligation of the lobes is well established in the rat[1,2]. However, mouse

models allow for the use of gene-altered or knockout strains and for laboratory assays, due to the development of specific agents and antibodies[3]. At present, there are a number of murine hepatectomy models[4-10], of which the 70% hepatectomy (termed 2/3 partial hepatectomy) is most common. The introduction of microsurgical techniques has also enabled high rates of successful surgery, especially in individualized dissection and ligation of vessels[6,11,12]. However, murine hepatectomy remains challenging because of the delicate nature of the liver, the lack of intravenous access, and the risk of hemorrhage[9], resulting in high rates of mortality and morbidity[5,13]. In a previous paper, the rate of complications was reported as approximately 30%[6,14,15]. Currently, innovative methods for the hepatectomy model have been documented, such as the ligation method[7,9,14,15] and clip technique[5].

Despite the widespread use of the 70% hepatectomy[4-7], alternative hepatectomy models with resected volumes > 70% (so-called, "massive hepatectomy") are required to provide more clinically relevant experiments on liver regeneration and hepatic failure. Nikfarjam *et al*[5] reported benefits of the hemostatic clip in hepatectomy models with 37% and 70% of resected volumes in the mouse. Herein, we describe detailed surgical procedures of our institutional hepatectomy models with > 70% resection using suture hemostatic clip in the mouse and compare various factors between suture and clip techniques. Then, we discuss the usefulness of this simple and reproducible technique in hepatectomy models of > 70% resection.

MATERIALS AND METHODS

Animals
Inbred C57BL/6 mice (male, 10-20 wk of age, approximately 25 g body weight) were used (C57BL/6NHsd; Harlan Laboratories, Indianapolis, IN, United States). All mice were maintained under specific pathogen-free conditions. All experimental protocols were approved by IACUC at Mayo Clinic (Protocol No. 33307 and 24907).

Hepatectomy by suture technique
General anesthesia was provided with an anesthesia system (VetEquip Inc., Pleasanton, CA, United States). Inhalational anesthesia was induced and maintained by isoflurane (Isoflurane USP, 250 mL; Webster Veterinary, Sterling, MA, United States). Isoflurane with oxygen flow at 5 L/min was used in the induction phase, and was reduced to 0.5-2.0 L/min in the maintenance phase. Body weight was measured after anesthesia. The abdominal wall was shaved and prepped with povidone-iodine. A transverse incision was performed and hemostasis was obtained using an electrocautery scalpel of bipolar type (Bipolar forceps, Martin ME 102 Electrosurgical unit and Bipolar Accessory Set for ME 102; Harvard Apparatus, Holliston, MA, United States) and that of monopolar type (Bovie, low temperature cautery kit; Aaron Medi-

cal, Clearwater, FL, United States). Mice that received only laparotomy served as a control group in the study. The gastrointestinal tract was moistened with warm saline, covered with gauze, and positioned to the outside of the left abdominal cavity. The liver was handled delicately with cotton-tipped applicators (Hardwood Products Company, Guilford, ME, United States). A surgical microscope at 5-10 × magnification (Surgical Scope M680, Type 10445496; Leica Microsystems Inc., Bannockburn, IL, United States) was used. Even though high magnification (12.5-20 × magnification) is not required, a sufficient light source was indispensable. We used fine-tipped and delicate scalpels which are suitable for ultra-microsurgery, i.e. structures including nerve or vessels < 0.5 mm in diameter[16]. The murine liver was divided into three lobes (right, left, and caudate lobes) and arranged into six segments[4-6,14,17]: right anterior segment (RAS), right middle segment (RMS), right posterior segment (RPS), left anterior segment (LAS), left posterior segment (LPS), and omental segment (OS) (Figure 1A and B). The falciform and triangular ligaments were sharply cut. The infra-hepatic vena cava runs into the RMS and RPS. The infra-hepatic inferior vena cava was skeletonized. The LPS was freed from the diaphragm and OS. The LAS and LPS were mobilized off the hepatic hilum and gastroduodenal tract.

We did not use Pringle's maneuver during the operation. Hepatectomy by suture technique requires a proximal hepatic margin for suture bait. An atraumatic clamp (Micro Vascular Clip, RS-6470 or RS-6472; Roboz Surgical Instrument Co., Gaithersburg, MD, United States) was placed on the hepatic segment, and the optimal proximal hepatic margin was 2-3 mm from the division of each segment. The clamped segment was sharply cut with a 1 mm distal hepatic margin (Figure 1C). Monofilament polypropylene sutures (7-0 Prolene, BV-1, 8304H-X, or 8-0 Prolene, BV130-5, 8732H; Ethicon, Inc., Somerville, NJ, United States) were bilaterally placed without involvement of a clamp. These bilateral sutures were ligated, and each suture was grasped with a curved bulldog clamp (Cooley Bulldog clamps, curved, serrated, MB586R or MB587R; V.Mueller, Aesculap Inc., Center Valley, PA, United States). The liver was sutured by a loose continuous suture that initially involved the clamp, and the last suture reached the right side. The clamp was removed and the suture was tightened and ligated with the stay suture (Figure 1D). The presence of bleeding points was carefully checked using a cotton swab. An additional suture was made with a monofilament nylon suture (10-0 Ethilon, BV130-3, 2820G; Ethicon, Inc.) for well-focused hemostasis if required. Microfibrillar collagen (Avitene; C.R. Bard, Inc., Murray Hill, NJ, United States) was used in hemostasis.

The intraperitoneal cavity and organs were washed with warm saline. The peritoneum and fascia were closed with a continuous suture using absorbable thread (5-0 Coated Vicryl Plus; Ethicon, Inc.). The skin layer was closed separately using the same method.

Figure 1 Hepatic segments and hepatectomy by suture or clip techniques. A: The murine liver is divided into six segments (RAS, RMS, RPS, LAS, LPS, and OS); B: The gallbladder is detected between the RAS and LAS; C: Atraumatic clamp was placed on hepatic segment, with optimal proximal hepatic margin of 2-3 mm from division of each segment. Clamped segment was sharply cut with 1 mm distal hepatic margin (red line). Sutures not involving the clamp were placed bilaterally. Bilateral sutures were ligated, and each suture was grasped with a curved bulldog clamp. By using suture of left-side ligation, cut surface of liver was sutured from left side to right side with loose continuous suture that involved a clamp, and the last suture reached the right side; D: Clamp was removed (dotted red arrow) and hepatic margin was immediately treated by tightening the continuous suture. The thread of tightened continuous suture was ligated with stay suture of right side without over-tightening. Continuous suture was made from right side to left side. The last suture was ligated with stay suture of left side without over-tightening; E: The relevant segment was cut with a 3-5 mm distal hepatic margin (red line); F: Ischemic change of distal hepatic margin at 24 h after 80% hepatectomy was shown. Although liver remnants after massive hepatectomy showed color change due to vacuolization, distal hepatic margins clearly showed necrotic changes. Color change between liver remnant and distal hepatic margin was more enhanced at autopsy; G: Percentages of total volume of each segment were 21.2% ± 3.3% for RAS, 11.5% ± 2.1% for RMS, 14.8% ± 4.0% for RPS, 11.9% ± 1.7% for LAS, 31.6% ± 3.6% for LPS, and 9.0% ± 1.6% for OS; H: Traditional 2/3 hepatectomy of RMS + RPS + OS (35.4% ± 4.0%) is shown. Additional resection of OS (blue line) will make a 75% hepatectomy (RMS + RPS, 26.4% ± 3.8 %); I: Hepatic remnant in 80% hepatectomy was RMS + OS (20.6% ± 2.6%). Additional resection of OS (blue line) will make an RMS-remnant 90% hepatectomy (RMS, 14.8% ± 4.0%); J: OS-remnant 90% hepatectomy is shown (OS, 9.0% ± 1.6%). Dilation of portal vein due to portal hypertension is confirmed in reverse proportion to volume of hepatic remnant (yellow arrows). LAS: Left anterior segment; LPS: Left posterior segment; OS: Omental segment; RAS: Right anterior segment; RMS: Right middle segment; RPS: Right posterior segment.

Hepatectomy by hemostatic clip technique

The preparation and mobilization of each liver segment was similarly performed as described above. M-sized hemostatic clips were used (Horizon Ligation System; Teleflex Medical, Durham, NC, United States). The hepatic segment was retracted using tissue forceps when the clip was applied. A hemostatic clip was applied near the point of division of each segment (Figure 1E), and the proximal hepatic margin was approximately 1 mm from a segmental division. The segment was then cut with a distal hepatic margin of 3-5 mm. An additional suture for complete hemostasis was not typically required. Even during surgery, color change of the clipped segment due to ischemia was confirmed, and the color change between remnant liver and distal hepatic margin was observed at autopsy (Figure 1F).

Post-operative care

A warmer (RightTemp, RTHS-SM; Kent Scientific Co., Torrington, CT, United States) was used to maintain body temperature immediately after surgery, and a heating pad (E12107; Sunbeam, Gainesville, FL, United States) was used to warm the cage until mice completely recovered from anesthesia and surgery. Each mouse was kept separately. Postoperative observation was performed every 2 h for the first 12 h after surgery, and thereafter every 4 h. An analgesic agent (0.1 μg/g body weight, i.m.; buprenorphine 100 μg/mL; Cerilliant, Round Rock, TX, United States) was routinely given intramuscularly every 12 h for 5 d after surgery. Antibiotics (30 μg/g body weight, i.p.; Cephalexin Hydrate; MP Biomedicals, Cleveland, OH, United States) was administered every 12 h for 24 h after surgery. For each mouse, 1 mL of lactate Ringer's solution (Lactated Ringer's Injection USP; B. Braun Medical Inc., Irvine, CA, United States) was routinely administered every 6 h for 24 h.

Assessments and data analysis

We weighed each segment in a total of 100 mice, and segmental percentages were calculated as segmental weight (g)/whole liver weight (g) for each mouse. The extent of the hepatectomy is based on the segmental volumes. Of note, the gallbladder can be easily detected in the mouse between the RAS and the LAS (Figure 1B), while the rat does not have a gallbladder.

The learning curve for surgery is important for reliability of the hepatectomy model. To compare hepatectomy models using the suture technique *vs* the clip technique, we examined learning curves and operative time.

Rare complications of outflow block or biliary obstruction due to surgical clips were previously reported[18,19]. To confirm that the surgical issues were due to the clip itself, it was proposed that the clip may secondarily obstruct outflow or biliary ducts near the proximal hepatic margin or hepatic remnant. At first, we checked histopathological findings of the hepatic remnant near the proximal hepatic margin and the distal hepatic margin in the hepatectomy model by suture and clip techniques.

Focal and/or patchy necrosis is an important histopathological finding after hepatectomy[4,20]. To calculate the rate of the appearance of focal and/or patchy necrosis in hepatectomy models by the suture and clip techniques, we examined ten cases for each technique on the same day. Histopathological analyses and calculations were performed at 6 h after hepatectomy. These examinations were repeated three times.

In this study, data are presented as mean ± SD. Student's *t* test was used for the comparisons of unpaired variables between the two groups. A survival curve was made by the Kaplan-Meier method, and the log-rank test was used for the comparisons of survival rates between two groups. Statistical calculations were performed using StatView Version 5.0 (SAS, Cary, NC, United States). A *P* value < 0.05 was considered statistically significant.

RESULTS

Measurement of the weight of each segment

The percentages of total volume for each of the segments were 21.2% ± 3.3% for RAS, 11.5% ± 2.1% for RMS, 14.8% ± 4.0% for RPS, 11.9% ± 1.7% for LAS, 31.6% ± 3.6% for LPS, and 9.0% ± 1.6% for OS (Figure 1G).

Hepatectomy models with > 70% resection in the mouse

Based on our results in the measurement of segmental weights, we calculated the liver volume of resection and the hepatic remnant. The hepatic remnant in the 75% hepatectomy was RMS + RPS (26.4% ± 3.8%); while for traditional 2/3 hepatectomy the remnant was RMS + RPS + OS (35.4% ± 4.0%) (Figure 1H). Hepatic remnant in the 80% hepatectomy was RMS + OS (20.6% ± 2.6%) (Figure 1I). We had two surgical options for 90% hepatectomy by sparing either OS (9.0% ± 1.6%) or RMS (11.5% ± 2.1%) (Figure 1J). Portal venous dilatation inversely proportional to the volume of the hepatic remnant was observed.

Learning curves

For each technique, ten cases were performed on the same day as one unit for training. The required units that showed no statistical differences in survival curves after hepatectomy with those at the next unit were analyzed. The survival curves of hepatectomy after the training period were compared between the two techniques.

Survival curves of each batch of 10 cases at early term in training in the 80% hepatectomy by the suture technique, and statistical results, are shown in Figure 2A. The *P* values between the first 10 cases and the second 10 cases, between the second 10 cases and the third 10 cases, between the third 10 cases and the fourth 10 cases, and between the fourth 10 cases and the fifth 10 cases were 0.0445, 0.0471, 0.4790, and 0.8941, respectively. The first and second units showed statistical differences in comparison with the next unit. Survival curves of each batch of 10 cases at early term in training in the 80% hepatectomy

Figure 2 **Survival outcomes in 80% hepatectomy after initial training.** A: In 80% hepatectomy by suture technique, survival curves of each 10 cases at early term in training are shown; B: In 80% hepatectomy by the clip technique, survival curves of each 10 cases at early term in training are shown; C: In each technique, operative time was investigated in 20 cases of 80% hepatectomy. These data were corrected by experienced surgeons. Operative times of 80% hepatectomy by suture and clip techniques were significantly different ($P <$ 0.0001). NS: Not significant.

Figure 3 **Actual survival curves after each hepatectomy with > 70% resection.** A: Actual survival curves of each hepatectomy by suture technique; B: Actual survival curves of each hepatectomy by clip technique. NS: Not significant.

comparison with the second unit.

In the 80% hepatectomy, the fifth batch of 10 cases (i.e., 10 cases after the experience of 40 cases) in the suture technique and the fourth batch of 10 cases (i.e., 10 cases after the experience of 30 cases) in the clip technique showed similar survival curves (P = 0.8516), even though 30 cases in the suture technique and 20 cases in the clip techniques seemed to be enough statistically. Our institution has similar results in the 90% hepatectomy model.

Operative time

Operative time data were also collected from experienced surgeons in 20 cases of 80% hepatectomy for each technique. Even with experienced surgeons, operative times for hepatectomy using the suture technique and the clip technique were significantly different ($P <$ 0.0001) (Figure 2C). Operative times were clearly shortened in the clip techniques.

Survival curves after hepatectomy with > 70% resection

Actual survival curves of each hepatectomy are shown in Figure 3A and B. Twenty cases were followed for each hepatectomy by suture (Figure 3A) or clip technique (Figure 3B).

In the 90% hepatectomy, either the OS or RMS was

by the clip technique, and statistical results, are shown in Figure 2B. The P values between the first 10 cases and the second 10 cases, between the second 10 cases and the third 10 cases, between the third 10 cases and the fourth 10 cases, and between the fourth 10 cases and the fifth 10 cases were 0.0363, 0.6854, 0.9127, and 0.9007, respectively. Only the first unit showed statistical differences in

Figure 4 Histopathological findings of hepatic remnant near proximal hepatic margin and distal hepatic margins. Findings at 6 h after 80% hepatectomy are shown by hematoxylin and eosin staining at 100 × magnification. A: No findings suggested obstructions in hepatic remnant near proximal hepatic margin of hepatectomy by suture technique; B: In distal hepatic margin of hepatectomy by suture technique, severe ischemia and massive necrosis are confirmed; C: No findings suggested obstructions by hemostatic clip itself in clip-touched hepatic remnant near proximal hepatic margin of hepatectomy by clip technique; D: In distal hepatic margin of hepatectomy by clip technique, severe ischemia and massive necrosis are confirmed.

left as the remnant. Although not shown, we observed no significant differences in survival curves between the OS-remnant and the RMS-remnant following the 90% hepatectomy using suture and clip techniques ($P = 0.3784$ and 0.3588, respectively).

In the 90% hepatectomy, although not shown, there were no significant differences in survival curves after hepatectomy by suture technique between the OS-remnant ($n = 10$) and the RMS-remnant ($n = 10$) ($P = 0.3784$). Similarly, there were no significant differences in survival curves after hepatectomy by clip technique between the OS-remnant ($n = 10$) and the RMS-remnant ($n = 10$) ($P = 0.3588$).

Histopathological findings of hepatic remnant and distal hepatic margin

Histopathological findings of the hepatic remnant near the proximal hepatic margin at 6 h after 80% hepatectomy by suture technique are shown in Figure 4A. No findings suggested obstructions by the suture, except for the changes consistent with hepatectomy. Histopathological findings of the distal hepatic margin showed severe ischemic and massive necroses at 6 h after 80% hepatectomy by the suture technique (Figure 4B).

Histopathological findings of the clip-touched hepatic remnant near the proximal hepatic margin at 6 h after 80% hepatectomy by the clip technique are shown in Figure 4C. Again, no findings suggested obstructions

by the hemostatic clip. Histopathological findings of the distal hepatic margin at 6 h after 80% hepatectomy by clip technique are shown in Figure 4D, showing severe ischemic and massive necroses as expected.

Focal and/or patchy necrosis after hepatectomy

Focal and patchy necrosis is usually observed as early as one hour after hepatectomy, as reported by others[20,21]. In our studies, we observed similar occurrence of focal necrosis following all types of massive hepatectomy. At 6 h after 70%, 80%, and 90% hepatectomies, there was no difference in the rate of appearance of focal and/or patchy necrosis between the suture technique and the clip technique ($P = 0.6202$; data not shown).

DISCUSSION

A number of different survival curves have been previously reported following hepatectomy with > 70% resection in the mouse. Differences in study design and institutional methodology, as well as various complications, may explain these discrepancies[4]. As such, we recommend that survival curves should be checked beforehand according to institutional methodology and study design in each laboratory. In our institution we have two types of 90% hepatectomy models available, while other models of approximately 90% hepatectomy exist[1,7]. Anatomical analysis of each institution's animal strain is crucial for de-

velopment of reliable models. All mice that receive 90% hepatectomy eventually die after surgery, although 90% hepatectomy has some advantages, especially in studies of liver failure and hepatic surgery. Inderbitzin *et al*[8] reported that the OS exhibits different behaviors according to the degree of liver-volume, and will not work in small-volume hepatectomy. Currently, our massive hepatectomy models employed the OS in only the 80% hepatectomy model, because we have a surgical option in the 90% hepatectomy model. In our experience, the OS of some mice exhibit few branches from veins, except for the portal vein, because swelling due to portal hypertension was often mild even after 90% hepatectomy, and color change was often not enhanced after ligation of the portal venous trunk. Overall, we consider that RMS-remnant 90% hepatectomy will provide more relevant data from the viewpoint of strict effects (*i.e.*, liver damage) due to portal hypertension.

The outcomes of murine hepatectomy can vary greatly if surgical procedures are not performed properly. It is difficult to strictly estimate the stability of surgery, because the learning term may be still not enough, even if statistical analysis showed no differences. Based on our experience, we speculate that approximately 40 cases are required in hepatectomy by the suture technique for initial achievement of reliable survival curves, and that approximately 50 or more cases are required to start the study. By contrast, approximately 30 or 40 cases seem to be enough in hepatectomy by the clip technique for reliable samples. Our results of operative time for hepatectomy clearly indicated that the procedures in the suture technique were more complicated than those in the clip method. Overall, we suggest that the clip technique is advantageous even in hepatectomy models with > 70% resection because of its simplicity.

The hepatocyte is the first cell to start the proliferation reaction after hepatectomy[4]. Although histopathological injury can be confirmed from several hours after hepatectomy, some important signals for liver regeneration start in the later phase. As such, a suitable hepatectomy model must be selected according to experimental purpose. Jin *et al*[22] reported no focal and/or patchy necrosis even after hepatectomy models with 70% and approximately 90% resections, although this necrosis is an important finding after hepatectomy[4,20]. These histopathological findings are confirmed several hours after hepatectomy[4,20]. However, in our study a few mice that received 80% or 75% hepatectomy did not demonstrate necrosis, while all mice that received 90% hepatectomy showed necrosis in the early postoperative period. These discrepancies may be consistent with survival curves because a few mice that received 80% or 75% hepatectomy survived long term. These survivors can be used to provide liver samples with long-term observation after hepatectomy with > 70% resection and are useful for many studies that require long recovery times after hepatectomy with > 70% resection. Based on our results of survival curves and histopathological findings in comparison with suture technique, clip method seems to provide reliable

and relevant data, even in murine hepatectomy models with > 70% resection.

Focal and patchy necroses are also observed as secondary findings after biliary stasis and/or congestion by outflow obstruction. Some surgical problems, including inflow and outflow obstructions, massive bleeding, and bile stasis, will invalidate results. All mice move well after complete recovery, and it is possible that the clip may slide following aggressive movements after recovery from anesthesia and surgery, although our results showed similar phenomena between the clip and suture techniques. During surgery, the hepatic margin may be changed according to clip size. Large-sized clips require more proximal hepatic margin for flow safety and more distal hepatic margin for complete hemostasis. Although we often treat the RAS and LAS in an *en bloc* manner using an ML-sized clip, only one of the smallest feasible clips to one segment should be used to prevent unexpected surgical issues. Surgeons should also note any unexpected findings after hepatectomy procedures. A dilated infrahepatic inferior vena cava is a sign of disturbed flow of the inferior vena cava, because clipping of the RAS, LAS and LPS may cause a twist of the supra-hepatic inferior vena cava, while clipping of the RMS and RPS may cause stenosis of the hepatic vena cava. Segmental congestion and partial color change in the liver also suggest outflow and/or inflow obstruction. During autopsy, unexpected color changes of the liver, massive coagula, and bile leakage are informative for unstable postoperative course. To produce reliable samples, mice that indicate unexpected signs during surgery or autopsy should not be used to take samples.

In conclusion, our clip technique for murine hepatectomy models with > 70% resection (*i.e.*, 75%, 80%, and 90% hepatectomy models) is simple and requires only basic surgical skills. Moreover, this technique provides reproducible results in comparison with the suture technique.

ACKNOWLEDGMENTS

COMMENTS

Background
New insights into mechanisms in the hepatology field have been established from experimental animal models. Reliable models for massive hepatectomy in the mouse are required for experimental liver research.

Research frontiers
Despite the widespread use of the 70% hepatectomy, alternative hepatectomy models with resected volumes > 70% (so-called, "massive hepatectomy") are required to provide more clinically relevant experiments on liver regeneration and hepatic failure.

Innovations and breakthroughs
In 2004, Nikfarjam *et al* reported benefits of the hemostatic clip in hepatectomy models with 37% and 70% of resected volumes in the mouse. Herein, the

authors describe detailed surgical procedures of our institutional hepatectomy models with > 70% resection using suture hemostatic clips in the mouse and compare various factors between suture and clip techniques. The clip technique for murine hepatectomy models with > 70% resection is simple and requires only basic surgical skills.

Applications

The clip technique provides reproducible results in comparison with suture technique, even for massive hepatectomy in the mouse. This technique has an advantage in the simplicity of surgical procedures.

Peer review

The authors presented mouse experimental models for hepatectomy exceeding 70%. They evaluated two techniques of hepatectomy, which is suture technique and clip technique. They concluded that clip technique was superior to suture technique. The manuscript was well organized and well written.

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ORIGINAL ARTICLE

Regional lymphadenectomy for gallbladder cancer: Rational extent, technical details, and patient outcomes

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Abstract

AIM: To define the rational extent of regional lymphadenectomy for gallbladder cancer and to clarify its effect on long-term survival.

METHODS: A total of 152 patients with gallbladder cancer who underwent a minimum of "extended" portal lymph node dissection (defined as *en bloc* removal of the first- and second-echelon nodes) from 1982 to 2010 were retrospectively analyzed. Based on previous studies, regional lymph nodes of the gallbladder were divided into first-echelon nodes (cystic duct or pericholedochal nodes), second-echelon nodes (node groups posterosuperior to the head of the pancreas or around the hepatic vessels), and more distant nodes.

RESULTS: Among the 152 patients (total of 3352 lymph nodes retrieved, median of 19 per patient), 79 patients (52%) had 356 positive nodes. Among node-positive patients, the prevalence of nodal metastasis was highest in the pericholedochal (54%) and cystic duct (38%) nodes, followed by the second-echelon node groups (29% to 19%), while more distant node groups were only rarely (5% or less) involved. Disease-specific survival after R0 resection differed according to the nodal status (*P* < 0.001): most node-negative patients achieved long-term survival (median, not reached; 5-year survival, 80%), whereas among node-positive patients, 22 survived for more than 5 years (median, 37 mo; 5-year survival, 43%).

CONCLUSION: The rational extent of lymphadenectomy for gallbladder cancer should include the first- and second-echelon nodes. A considerable proportion of node-positive patients benefit from such aggressive lymphadenectomy.

INTRODUCTION

Oncological resection for gastrointestinal cancers con-

ventionally involves *en bloc* resection of the primary tumor and the regional lymphatic basin. However, in cases of gallbladder cancer, most surgeons have developed a negative attitude toward regional lymphadenectomy because of reported poor outcomes after radical resection for node-positive disease[1-5]. In contrast, some surgeons including us advocate aggressive lymphadenectomy[6-10]. Thus, the role of regional lymphadenectomy in treating gallbladder cancer remains controversial.

The National Comprehensive Cancer Network (NCCN) guidelines[11] recommend portal lymph node dissection for pathologic T1b (pT1b) or more advanced gallbladder cancer, where the extent of lymphadenectomy is described as "porta hepatis, gastrohepatic ligament, retroduodenal." The guidelines also state, "Patients with nodal disease outside this area are unable to undergo resection"[11]. In contrast, some Japanese and Western groups advocate more extensive lymphadenectomy including some peripancreatic (head only) node groups[6,12-14]. Thus, the extent of regional lymphadenectomy for gallbladder cancer remains nonstandardized worldwide.

Previously we explored the routes and directions of gallbladder lymphatic drainage in a dye injection study[6]; lymph flow originating from the gallbladder descends around the bile duct, and *via* the first-echelon nodes (cystic duct or pericholedochal nodes) into the second-echelon nodes located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries, before finally flowing into the paraaortic nodes[6]. Thereafter, we adopted *en bloc* removal of the first- and second-echelon nodes (designated as "extended" portal lymph node dissection) as a standard procedure for pT2 or more advanced gallbladder cancer[6,10,15].

This retrospective study sought to define a rational extent of regional lymphadenectomy for gallbladder cancer and to clarify outcomes after such a procedure in 152 patients subjected to radical resection. The study goal was to establish the role of regional lymphadenectomy in treating gallbladder cancer.

MATERIALS AND METHODS

Patient population

From May 1982 through December 2010, a total of 173 consecutive Japanese patients with gallbladder cancer underwent a radical resection at the Niigata University Medical and Dental Hospital. A radical resection was defined as removing both the primary tumor and the regional lymph nodes of the gallbladder. Cancer arising in the cystic duct was included as gallbladder cancer[16]. We excluded 13 patients due to an invasive primary malignancy in other organs and 8 patients who did not undergo removal of the second-echelon nodes, leaving 152 patients in this study. They included 96 women and 56 men ranging in age from 37 years to 86 years (median, 68 years).

Radical resection procedures

A variety of radical resection procedures were performed

Table 1 Radical resection procedures for 152 patients with gallbladder cancer

Procedure	No. of patients
Extended cholecystectomy (*n* = 93)	
C + WR + BD + N[1]	54
C + WR + N	21
C[2] + BD + N	12
C[2] + N	6
More extensive resection (*n* = 59)	
C + ERH + BD + N	27
C + Central hepatectomy[3] + BD + N	3
C + Extended left hepatectomy[4] + BD + N	1
C + Right trisectionectomy + BD + N	1
C + WR + PD + N	15
C + ERH + PD + N	7
C + ERH + PPPD + N	3
C[2] + PD + N	2

[1]Designated as "extended" radical cholecystectomy (modified Glenn operation) at our department since 1982; [2]Cholecystectomy with full-thickness dissection: cholecystectomy combined with removal of the cystic plate; [3]Resection of Couinaud segments IV, V, and VIII; [4]Left hemihepatectomy extended to an inferior portion of the right anterior section. C: Cholecystectomy; WR: Wedge resection of the gallbladder fossa; BD: Bile duct resection; N: Regional lymphadenectomy; ERH: Extended right hepatectomy (right hemihepatectomy extended to an inferior portion of Couinaud segment 4); PD: Whipple pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

for gallbladder cancer in the patients analyzed; the choice of procedure for each patient depended on the extent of tumor spread (Table 1). An "extended" radical cholecystectomy, which was instituted at our department in 1982[6,10,15], was the most common operation used among our study cohort; it involved a cholecystectomy, wedge resection of the gallbladder fossa with a rim of non-neoplastic liver tissue (about 2 cm in thickness or more), resection of a suprapancreatic segment of the extrahepatic bile duct (bile duct resection), and "extended" portal lymph node dissection in an *en bloc* fashion. This "extended" radical cholecystectomy is a modification of the "radical cholecystectomy" (Glenn operation) as proposed by Glenn *et al*[17,18] in 1954; we now also call it the "modified Glenn operation"[15].

Some patients with early-stage disease, comorbid disease(s), or advanced age had a less aggressive resection, by omitting the bile duct resection and/or wedge hepatectomy (Table 1). Although pT1 tumors do not warrant radical resection[19], 14 patients with pT1 tumors underwent a radical resection because pT2 or more advanced tumor was not ruled out preoperatively. In contrast, patients with late-stage disease often required more extensive resections such as major hepatectomy (removal of two sections or more extended hepatectomy), pancreaticoduodenectomy (Whipple procedure or pylorus-preserving procedure), or combined major hepatectomy and pancreaticoduodenectomy (Table 1)[12,20].

Despite an aggressive attitude toward lymphadenectomy, we maintain a rather modest attitude toward hepatectomy for gallbladder cancer, and consequently, the most common hepatectomy procedure in our series was

Table 2 Topographical distribution of 3352 lymph nodes evaluated in 152 patients with gallbladder cancer

Node group	No. of patients with node group evaluated	No. of lymph nodes evaluated		No. of patients with positive nodes	No. of positive nodes	
		Range per patient (median)	Total		Range per patient (median)	Total
First-echelon node groups						
Pericholedochal[1]	152	0-9 (2)	410	43	1-9 (1)	75
Cystic duct	152	0-2 (1)	109	30	1-2 (1)	33
Second-echelon node groups						
Retroportal	152	0-9 (3)	458	23	1-5 (2)	47
Posterior superior pancreaticoduodenal[2]	150	0-9 (2)	341	20	1-7 (1)	37
Hepatic artery	148	0-12 (3)	536	20	1-10 (1)	50
Right celiac[3]	128	0-8 (2)	320	15	1-4 (1)	28
Hilar (porta hepatis)	121	0-5 (0)	37	0	NA	0
More distant node groups						
Superior mesenteric	50	0-14 (2)	171	4	1-5 (2)	10
Posterior inferior pancreaticoduodenal	38	0-7 (1)	56	3	1-5 (2)	8
Anterior superior pancreaticoduodenal	29	0-5 (0)	19	1	1-1 (1)	1
Anterior inferior pancreaticoduodenal	27	0-4 (0)	15	2	1-3 (2)	4
Perigastric	52	0-23 (2)	205	4	1-1 (1)	4
Paraaortic node groups	93[4]	1-28 (6)	675	15	1-16 (2)	59

[1]Also referred to as retroduodenal nodes in the NCCN guidelines[11]; [2]Also referred to as posterosuperior pancreaticoduodenal nodes in our previous study[6] or superior retropancreaticoduodenal nodes by Uesaka *et al*[22]; [3]Also referred to as posterior common hepatic nodes by Uesaka *et al*[22]; [4]Includes 55 who underwent a paraaortic lymph node dissection and 38 who underwent a sampling of paraaortic nodes for staging of the disease.

Figure 1 Topographical distribution of the regional lymph nodes of the gallbladder, shown after a full Kocher maneuver. Solid ellipses represent individual lymph nodes: first-echelon nodes (red), second-echelon nodes (blue), and more distant nodes (black). The yellow painted area represents the head of the pancreas, and the dashed line indicates the border of the uncinate process of the pancreas. Arabic numerals indicate each of the first- and second-echelon node groups: (1) pericholedochal, nodes along the common bile duct; (2) cystic duct, node(s) along the cystic duct; (3) retroportal, nodes posterior to the portal vein and cephalad to the uncinate process; (4) posterior superior pancreaticoduodenal, nodes on the posterosuperior aspect of the head of the pancreas; (5) hepatic artery, nodes along the common or proper hepatic artery; (6) right celiac, nodes located right of the celiac axis and posterior to the common hepatic artery; and (7) hilar, nodes within the porta hepatis. The pale green area indicates the extent of a typical "extended" portal lymph node dissection (*en bloc* removal of the first- and second-echelon node groups) in the study department. GB: Gallbladder; IVC: Inferior vena cava; Ao: Aorta; IMA: Inferior mesenteric artery.

(nonanatomic) wedge resection of the gallbladder fossa. However, cases of deep hepatic invasion through the gallbladder fossa and/or invasion of the right portal pedicle (i.e., extension into the triangle of Calot) required a right hemihepatectomy extended to an inferior part of Couin-aud segment 4 (designated as extended right hepatectomy in this study) for tumor clearance. Five patients underwent other hepatectomy procedures (Table 1).

There were also 25 patients in this study who underwent a combined resection of contiguous tissues comprising the transverse colon (*n* = 12), portal vein (*n* = 9), duodenum (*n* = 5), stomach (*n* = 2), and inferior vena cava (*n* = 2). Of the 152 patients, 127 underwent an initial radical resection and 25 underwent a radical second resection after a prior simple cholecystectomy for presumed benign disease.

Regional lymph nodes of the gallbladder

According to earlier studies[6,8,15,21,22], we classified the regional lymph nodes of the gallbladder into three categories as detailed in Table 2: first-echelon nodes, second-echelon nodes, and more distant nodes. The topographical distribution of the first- and second-echelon node groups is illustrated in Figure 1. Briefly, the first-echelon nodes are located along the cystic duct or the common bile duct, and the second-echelon nodes are located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries. Although the hilar (porta hepatis) nodes were not regarded as significant regional nodes[6,21,22], we categorized them as second-echelon nodes for convenience (Table 2) because these nodes are located within the hepatoduodenal ligament, and thus are usually harvested during regional lymphadenectomy[11]. Perigastric nodes are not regarded as regional, but they were analyzed in this study since they were included in the Whipple procedure collection. Paraaortic lymph nodes were considered the terminal station for the regional lymphatic system of the gallbladder[6,21,22].

Figure 2 Computed tomography revealing enlarged peripancreatic nodes with heterogeneous contrast enhancement (N). Note that the posterior superior pancreaticoduodenal node (N, left) and the retroportal node (N, right) are adhered to the head of the pancreas (P). The latter node shifts the portal vein (arrowhead) in a right-upper direction. Histological examination confirmed metastases in 10 regional nodes, some of which had invaded the pancreatic parenchyma. This patient remains well with no evidence of disease at 15 years after a pancreaticoduodenectomy combined with wedge resection of the gall-bladder fossa. The arrow indicates common bile duct; V: Inferior vena cava; A: Aorta.

Figure 3 Photographs taken during an "extended" portal lymph node dissection. A: The posterior superior pancreaticoduodenal artery (PSPDA; arrowheads) is shown following dissection of the posterior superior pancreaticoduodenal nodes. The common hepatic artery is secured with the red loop; B: The superior border of the uncinate process (arrowheads) is exposed, ensuring that dissection of the retroportal nodes is complete. The common bile duct was already transected at the level of PSPDA. The blue loops secure the portal vein, whereas the red loops secure the hepatic arteries. A forceps is picking up the node-bearing adipose tissues dissected *en bloc*. L: Liver; HDL: Hepatoduodenal ligament; GDA: Gastroduodenal artery; IVC: Inferior vena cava; Panc: Head of the pancreas; Du: Duodenum; St: Stomach.

Regional lymphadenectomy procedures for gallbladder cancer

In the current study, regional lymphadenectomy for gall-bladder cancer was divided into two categories according to the extent of nodal dissection: "extended" portal lymph node dissection and "peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy"[15]. The former was defined as *en bloc* removal of the first- and second-echelon node groups (without pancreaticoduodenectomy), while the latter was defined as *en bloc* removal of more distant node groups with pancreaticoduodenectomy in addition to the first- and second-echelon node groups. The extent of an "extended" portal lymphadenectomy is illustrated in Figure 1.

Of all the study patients, 125 underwent an extended portal lymph node dissection (without pancreaticoduodenectomy), while the remaining 27 patients underwent a peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy (Table 1). In addition, 55 of the total number of patients who showed suspected (or confirmed) regional nodal disease also underwent a para-aortic (mainly interaortocaval) lymph node dissection, provided that they were fit enough.

It should be noted that despite our consistent policy of aggressive lymphadenectomy, the degree of regional lymphadenectomy varied somewhat in individual patients at the discretion of the individual surgeons, primarily due to the current study spanning a long period of time. For example, elderly patients or those with comorbid diseases tended to undergo a less aggressive lymphadenectomy.

Details of "extended" portal lymph node dissection

The following describes the technical details of our "extended" portal lymph node dissection (combined with both wedge hepatectomy of the gallbladder fossa and bile duct resection) for gallbladder cancer. After laparotomy, scrutinizing distant metastases by meticulous inspection and palpation is mandatory. Any suspicious nodules in the liver or on the peritoneal surface should be excised and submitted to frozen section examination, while any plan of radical resection should be abandoned if distant metastases are confirmed histologically. If no distant metastases are found, a full Kocher maneuver should be conducted (Figure 1) to enable the peripancreatic nodal status to be appropriately assessed. We favor extended portal lymphadenectomy in the absence of clinically evident peripancreatic (head only) nodal disease, while pancreaticoduodenectomy is often required for peripancreatic (head only) nodal disease adherent to or invading the pancreatic parenchyma (Figure 2)[15].

"Extended" portal lymph node dissection usually starts by dissecting the posterior superior pancreaticoduodenal nodes, where a layer of node-bearing adipose tissue is carefully peeled from the posterosuperior surface of the head of the pancreas. Several superior duodenal vessels are then divided to expose the superior aspect of the head of the pancreas. After the lesser omentum is incised, the common hepatic artery is secured with tape and skeletonized with dissecting nodes around the artery; the hepatic-gastroduodenal artery junction is also exposed. The posterior superior pancreaticoduodenal artery (PS-PDA), usually the first branch of the gastroduodenal artery, which traverses the common bile duct, can now be identified (Figure 3A). This ensures that dissection of the posterior superior pancreaticoduodenal nodes is complete. We often sacrifice PSPDA, because its tiny branches often cause unexpected bleeding due to injury during the nodal dissection. Transection of the common bile duct, just above or at the level of PSPDA, ensues.

The peritoneum covering the hepatoduodenal ligament is incised longitudinally along the proper hepatic artery, which is then skeletonized. The right hepatic artery is also skeletonized by dividing its ductal branch. The portal vein is then secured with tape and skeletonized by dissecting the retroportal nodes, which are then dissected from the superior border of the uncinate process. Next, the right celiac nodes are dissected from the right of the celiac artery. At this stage, the whole node-bearing tissues dissected *en bloc* are freed from the hepatic vessels and the head of the pancreas (Figure 3B); exposure of the superior border of the uncinate process ensures that dissection of the retroportal nodes has been completed (Figure 3B). A wedge hepatectomy of the gallbladder fossa ensues. Following parenchymal division, the cystic plate is exposed as a dense fibrous plate connecting with the portal pedicle of the right hemiliver, and then is divided at its base. The adipose tissue within the triangle of Calot, underneath the cystic plate, contains a cystic node(s), and is dissected downward by dividing the cystic artery at its origin. The extended portal lymph node dissection is now complete. Finally, the common hepatic duct is transected just below the confluence of the right and left hepatic ducts, and the surgical specimen including the gallbladder, gallbladder fossa, bile duct, and node-bearing tissues is harvested *en bloc*.

Retrieval of lymph nodes from fresh surgical specimens

Immediately after resection, the surgeon(s) retrieved lymph nodes from the node-bearing adipose tissues of the fresh surgical specimen, which were then divided by the surgeon(s) into individual node groups according to their locations.

Pathological examination

Pathological findings were documented using the American Joint Committee on Cancer (AJCC) cancer staging manual (7th edition)[16]. The primary tumor was pT1 in 14 patients, pT2 in 60, pT3 in 54, and pT4 in 24. Resection margin status was judged as no residual tumor (R0) or microscopic/macroscopic residual tumor (R1-2). The lymph nodes retrieved were examined histologically for metastases using a representative 3-μm thick section cut from each node.

The number of positive lymph nodes as well as the total lymph node count (TLNC) was recorded for each patient. Here, TLNC represented the sum of regional and paraaortic (if any) nodes evaluated in the patient; the number of positive lymph nodes represented the sum of positive regional and paraaortic (if any) nodes in the patient.

Patient follow-up after resection

Of 152 patients, 6 died during their hospital stay for the definitive resection, giving an in-hospital mortality rate of 4%. Patients discharged to home were followed regularly in outpatient clinics every 1-6 mo for at least five years, with a median follow-up time of 142 mo (range, 0.5 mo to 351 mo). Adjuvant chemotherapy was administered to 53 patients at the discretion of the individual surgeons.

Statistical analysis

Medical records and survival data were obtained for all 152 patients. Continuous variables were compared with the Mann-Whitney *U* test. Only deaths from tumor recurrence were treated as failure cases in the analysis of disease-specific survival (DSS), whereas those from other causes were recorded as censored cases. The survival time in each patient was defined as the interval between the date of definitive resection and the date of last follow-up or death. Survival curves were constructed using the Kaplan-Meier method, and differences in survival were evaluated with the log rank test. The IBM SPSS Statistics 19 software (IBM Japan, Inc., Tokyo, Japan) was used for all statistical evaluations. All tests were two-tailed and $P < 0.05$ was used to indicate statistical significance.

RESULTS

Topographical distribution of lymph nodes harvested by regional lymphadenectomy

A total of 3352 lymph nodes taken from the 152 study patients were evaluated. TLNC ranged from 3 to 78 (median, 19) per patient and varied according to the type of regional lymphadenectomy: 27 patients who underwent a peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy had a greater TLNC (median, 36; range, 7 to 78) compared with 125 patients who underwent an extended portal lymph node dissection (median, 17; range, 3 to 53) ($P < 0.001$).

The topographical distribution of the analyzed lymph nodes included 519 first-echelon nodes and 1692 second-echelon nodes (Table 2). There were significantly more second-echelon nodes per patient (median, 10; range, 2 to 29) than first-echelon nodes (median, 3; range, 0 to 10) ($P < 0.001$).

Topographical distribution of positive lymph nodes

Of the 152 study patients, 79 (52%) had a total of 356 positive lymph nodes. The number of positive nodes per patient ranged from 1 to 41 (median, 2). None of the 14 patients with pT1 tumor had nodal disease, whereas 79 (57%) of 138 patients with pT2 or more advanced tumor had nodal disease.

The topographical distribution of all positive lymph nodes is shown in Table 2. Among the 79 node-positive patients, the prevalence of nodal disease was highest in the pericholedochal (43 of 79; 54%) or cystic duct (30 of 79; 38%) node group, followed by the retroportal (29%), posterior superior pancreaticoduodenal (25%), hepatic artery (25%), and right celiac (19%) node groups. The hilar nodes were not involved in any of our patients. The prevalence of nodal disease was 5% or less in more distant node groups. Of note, paraaortic nodal involvement was found in 15 (19%) patients.

Of 27 patients with a single positive node, 20 (74%)

Table 3 Site of nodal involvement in 27 patients with a single positive node

Node group involved	No. of patients
Cystic duct	11
Pericholedochal	9
Posterior superior pancreaticoduodenal	3
Retroportal	2
Hepatic artery	1
Paraaortic	1

Table 4 Five-year survivors with nodal disease according to type of regional lymphadenectomy

No. of positive nodes	No. of 5-year survivors		
	Extended portal LND	Peripancreatic (head only) LND with PD	Total
1	12	1	13
2-3	4	1	5
≥ 4	1	3	4

LND: Lymph node dissection; PD: Pancreaticoduodenectomy (Whipple procedure or pylorus-preserving procedure).

had nodal disease in either the pericholedochal or cystic duct node group, suggesting that initial nodal involvement occurred primarily in these node groups (Table 3).

Route of lymphatic spread

Analysis of the topographical distribution of positive lymph nodes (Tables 2 and 3) can be used to derive the route of lymphatic spread from gallbladder cancer. In our study patients, gallbladder cancer primarily spread to the first-echelon nodes, then to the second-echelon nodes (other than the hilar nodes), and finally to the paraaortic nodes, while other distant node groups were only rarely involved.

Long-term outcome after regional lymphadenectomy

For all 152 patients, DSS was significantly worse in 22 patients undergoing an R1-2 resection than in 130 patients undergoing an R0 resection ($P < 0.001$; Figure 4); all of the patients undergoing an R1-2 resection died of recurrence within 5 years of resection. This indicates that an R0 resection is a prerequisite for long-term survival after radical resection.

We then focused on a subgroup of 130 patients who had undergone an R0 resection for survival analysis; they comprised 68 node-negative and 62 node-positive patients. DSS after R0 resection differed according to the nodal status ($P < 0.001$; Figure 5), with most node-negative patients achieving long-term survival (median survival time, not reached; 5-year survival rate, 80%). Of the 62 node-positive patients, 22 survived for more than 5 years after an R0 resection, 37 expired within 5 years, and the remaining 3 had survived within 5 years at the time of last follow-up (median survival time, 37 mo; 5-year survival rate, 43%) (Figure 5). These findings suggested that regional lymphadenectomy could achieve

No. of patients at risk

R0 resection	130	100	87	81	76	69	59	46	41	39	34
R1-2 resection	22	10	5	4	2	0	0	0	0	0	0

Figure 4 Kaplan-Meier disease-specific survival estimates stratified for residual tumor (R) status in all 152 patients. The median survival was not reached with a 5-year survival rate of 63% in patients undergoing an R0 resection, whereas it was 13 mo with a 5-year survival rate of 0% in patients undergoing an R1-2 resection.

No. of patients at risk

A	68	60	56	54	52	47	41	32	27	26	23
B	62	40	31	27	24	22	18	14	14	13	11

Figure 5 Kaplan-Meier disease-specific survival estimates stratified for nodal status in 130 patients who underwent an R0 resection. A, without nodal disease; B, with nodal disease.

an acceptable rate of long-term survival even in patients with nodal metastasis, provided that an R0 resection is feasible.

Five-year survival with node-positive disease

The 22 patients with node-positive disease who survived for more than 5 years (Figure 5) comprised 13 with a single positive node, 5 with 2-3 positive nodes, and 4 with ≥ 4 positive nodes (Table 4). Of the 5-year survivors with ≥ 4 positive nodes, three underwent a peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy for marked peripancreatic

nodal disease (Figure 2), suggesting that the addition of pancreaticoduodenectomy is effective for selected patients with peripancreatic nodal disease.

DISCUSSION

The high propensity for lymphatic spread in gallbladder cancer[6,8,10,15,23] renders adequate lymphadenectomy indispensable for improving patient outcomes after resection. However, what constitutes adequate lymph node dissection for these patients remains unresolved and prompted the current study. Since the 1980s, we have routinely harvested at least the first- and second-echelon nodes (Figure 1) during curative-intent resection for pT2 or more advanced gallbladder cancer. As a result, and to the best of our knowledge, we report herein the largest single-institutional series of 5-year survivors (22 patients) with nodal disease ever analyzed. The study findings also indicated that a considerable proportion of node-positive patients could benefit from regional lymphadenectomy, providing that an R0 resection is feasible, and would seem to justify continuing our policy of aggressive lymphadenectomy for gallbladder cancer.

In this study cohort, initial nodal involvement occurred primarily in the cystic duct or pericholedochal nodes (Table 3). Although these node groups are widely accepted as first-echelon nodes of the gallbladder, further possible routes of lymphatic spread have been poorly defined[6,21-23]. In 1991, an autopsy study by Ito and colleagues[21] implicated the cholecystoretropancreatic pathway, which descends along the common bile duct into the retroportal nodes, as the main lymphatic pathway of the gallbladder. Soon after, we identified the same pathway in a dye injection study and showed the second-echelon nodes located posterosuperior to the head of the pancreas or around the hepatic vessels; the dye solution finally drained into the interaortocaval nodes near the left renal vein[6]. In 1996, Uesaka *et al*[22] reported similar findings using vital staining. These studies therefore uniformly confirmed that lymph from the gallbladder first flows in a hepatofugal direction around the common bile duct and into the first-echelon nodes, before reaching the second-echelon nodes (other than the hilar nodes), and finally the paraaortic nodes[6,21,22]. In addition, the prevalence of nodal disease in our series was high in both the first-echelon and second-echelon node groups (other than the hilar nodes), while the other node groups were only rarely involved (Table 2). Thus, the rational extent of regional lymphadenectomy for gallbladder cancer should include at least the first- and second-echelon node groups as defined in the current study (Figure 1).

Despite a number of 5-year survivors with nodal disease reported in the Japanese literature[8,10,12,13], such survivors are exceptionally rare in the Western literature[1,2,5,24]. Since the first proposal by Glenn *et al*[17] in 1954, portal lymph node dissection has been regarded as the standard lymphadenectomy procedure for localized gallbladder cancer throughout the world[15]. However, the scope of portal lymph node dissection differs considerably among institutions. In 2005, Dixon *et al*[24] from the University of Toronto described "a complete portal lymph node dissection, with thorough skeletonization of the portal structures, down to and including the suprapyloric lymph node overlying the hepatic-gastroduodenal artery junction," while Ito and colleagues from the Memorial Sloan-Kettering Cancer Center (MSKCC) in 2011[5] reported a portal lymphadenectomy including "the lymph nodes in the hepatoduodenal ligament and those along with the common hepatic artery." The NCCN guidelines[11] described the extent of portal lymphadenectomy as "porta hepatis, gastrohepatic ligament, retroduodenal." From the above descriptions, we thus assume that the portal lymphadenectomy performed in North America may leave behind some of the second-echelon nodes, particularly the retroportal, posterior superior pancreaticoduodenal, and right celiac node groups, which were frequently involved in the current series (Table 2). This may explain, in part, why portal lymph node dissection only rarely achieves 5-year survival in cases of node-positive gallbladder cancer[1,2,5,24].

Although the AJCC staging manual (6th edition)[25] recommended "analysis of a minimum of three lymph nodes" for accurate staging of gallbladder cancer, a recent population-based study by Coburn *et al*[26] disclosed that among patients in the United States with resectable T1-T3 disease, only 5.3% had retrieval of three or more lymph nodes. In addition, a recent report from MSKCC of 122 patients who underwent a portal lymph node dissection cited a median TLNC of only 3[5]. In contrast, TLNC was much greater (median, 19) in the current series. Insufficient lymph node retrieval with portal lymphadenectomy may also explain, in part, the poor survival of node-positive patients in the Western literature[5,26].

Thorough dissection of the second-echelon nodes is challenging even in the hands of expert hepatobiliary surgeons. In particular, complete removal of the posterior superior pancreaticoduodenal, retroportal, and right celiac node groups mandates a meticulous technique. As described in the Materials and Methods section, there are some measures that can be taken to facilitate adequate dissection of the second-echelon nodes: first, a full Kocher maneuver (Figure 1) is essential for assessing the peripancreatic nodal status; second, identification of PSPDA (Figure 3A) ensures that dissection of the posterior superior pancreaticoduodenal nodes has been completed; and third, exposure of the superior border of the uncinate process (Figure 3B) ensures complete dissection of the retroportal nodes.

In our institution, we prefer to perform bile duct resection in fit patients with pT2 or more advanced gallbladder cancer; indeed, 66 (71%) of the 93 patients who underwent an extended cholecystectomy had a bile duct resection (Table 1). Although the presence of ductal involvement is an absolute indication for such an approach, controversy exists regarding bile duct resection for tumors without clinically evident ductal involvement[27]. Our rationale for bile duct resection for tumors with no evident ductal in-

volvement is to facilitate regional lymphadenectomy, to remove the pericholedochal lymphatic vessels and nodes simultaneously, and to remove the possible presence of microscopic ductal (periductal) involvement as suggested by Shimizu *et al*[28]. Another justification is to avoid the occurrence of ischemic biliary stricture after aggressive periductal nodal dissection[29]. The suprapancreatic segment of the extrahepatic bile duct gets its arterial blood supply mainly from ductal branches of both PSPDA ("retroduodenal artery" by Northover *et al*[30]) and the right hepatic artery[30]. As described in the Materials and Methods section, during our extended portal lymph node dissection, the PSPDA (Figure 3A) was often sacrificed with division of the ductal branch of the right hepatic artery. In addition, skeletonization of the bile duct may inadvertently injure the periductal arterial plexus with "the 3 o'clock and 9 o'clock arteries"[30]. Thus, we believe that simultaneous bile duct resection is a safer option if such aggressive periductal nodal dissection is required[29].

Indications for pancreaticoduodenectomy for gallbladder cancer include direct invasion of the pancreaticoduodenal region and evident peripancreatic (head only) nodal disease (Figure 2)[10,12,15]. While the former indication is widely accepted, it seems that most Western surgeons hesitate to undertake this procedure for the purpose of lymph node dissection because they believe that peripancreatic nodal disease is beyond the scope of resection[1,11,16,27]. Shirai *et al*[12] in 1997 and Sasaki *et al*[13] in 2006 independently reported the effectiveness of pancreaticoduodenectomy for selected cases of peripancreatic nodal disease. This was also suggested by the current study. In 2002, Doty *et al*[14] also suggested that the addition of pancreaticoduodenectomy could result in an R0 resection by removing extensive peripancreatic nodal disease in a select group of patients. Western surgeons should therefore be more open than ever to performing pancreaticoduodenectomy for gallbladder cancer.

The main limitations of the current study revolved around the retrospective nature of the analysis and considerable variability in the degree of nodal dissection among individual patients. However, the unique nature of this study has more clearly defined the role of regional lymphadenectomy for gallbladder cancer than earlier studies, although the survival of node-positive patients remains unsatisfactory (Figure 5). Since 2009, we have therefore routinely administered adjuvant chemotherapy (using gemcitabine and/or S-1 for 6-12 mo) to patients with nodal disease (especially those with multiple positive nodes) to improve survival. In addition, we now use "extended" portal lymphadenectomy for both gallbladder and bile duct cancers, with the latter also showing some success (unpublished data). Thus, it seems that "extended" portal lymph node dissection is applicable to a wide range of biliary tract malignancies.

In conclusion, gallbladder cancer first spreads to the first-echelon nodes (cystic duct or pericholedochal nodes), then to the second-echelon nodes located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries, and finally to the paraaortic nodes. The rational extent of regional lymphadenectomy for pT2 or more advanced tumors should include the first- and second-echelon node groups. Such aggressive lymphadenectomy can achieve an acceptable rate of long-term survival even in patients with nodal metastasis, provided that a potentially curative (R0) resection is feasible. The addition of pancreaticoduodenectomy may also be beneficial in selected patients with peripancreatic (head only) nodal disease. This study confirmed that regional lymphadenectomy plays a key role in radical surgery for gallbladder cancer.

COMMENTS

Background
The extent of regional lymphadenectomy for gallbladder cancer has not been standardized worldwide. Since 1982, the authors have consistently adopted an aggressive lymphadenectomy strategy. What constitutes adequate lymph node dissection for gallbladder cancer remains unresolved and prompted the current study.

Research frontiers
The study aims to define the rational extent of regional lymphadenectomy for gallbladder cancer and to clarify its effect on long-term survival.

Innovations and breakthroughs
The authors clearly define the rational extent of regional lymphadenectomy for pT2 or more advanced gallbladder cancer as at least the first- and second-echelon node groups. They also demonstrate that such aggressive lymphadenectomy can achieve an acceptable rate of long-term survival even in node-positive patients.

Applications
The authors imply that "extended" portal lymph node dissection is applicable to a wide range of biliary tract malignancies (both gallbladder and bile duct cancers).

Terminology
First-echelon nodes mean lymph nodes located along the cystic duct or the common bile duct; second-echelon nodes mean lymph nodes located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries; "extended" portal lymph node dissection mean *en bloc* removal of the first- and second-echelon node groups.

Peer review
This paper provides detailed description of their surgical technique for gallbladder cancer, "extended" portal lymph node dissection, of which this group has been conducting over the years. Documentation of detailed surgical technique is important not only as a material and method of a study, but even more as a tool to compare studies, and as guidance to the next generation of surgeons. They boast the largest single-institutional number of 5-year survivors with nodal disease (22 cases), suggesting the effectiveness of such aggressive lymphadenectomy.

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ORIGINAL ARTICLE

Globulin-platelet model predicts minimal fibrosis and cirrhosis in chronic hepatitis B virus infected patients

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Abstract

AIM: To establish a simple model consisting of the routine laboratory variables to predict both minimal fibrosis and cirrhosis in chronic hepatitis B virus (HBV)-infected patients.

METHODS: We retrospectively investigated 114 chronic HBV-infected patients who underwent liver biopsy in two different hospitals. Thirteen parameters were analyzed by step-wise regression analysis and correlation analysis. A new fibrosis index [globulin/platelet (GP) model] was developed, including globulin (GLOB) and platelet count (PLT). GP model = GLOB (g/mL) × 100/PLT (× 10^9/L). We evaluated the receiver operating characteristics analysis used to predict minimal fibrosis and compared six other available models.

RESULTS: Thirteen clinical biochemical and hematological variables [sex, age, PLT, alanine aminotransferase, aspartate aminotransferase (AST), albumin, GLOB, total bilirubin (T.bil), direct bilirubin (D.bil), glutamyl-transferase, alkaline phosphatase, HBV DNA and pro-thrombin time (PT)] were analyzed according to three stages of liver fibrosis (F0-F1, F2-F3 and F4). Bivariate Spearman's rank correlation analysis showed that six variables, including age, PLT, T.bil, D.bil, GLOB and PT, were correlated with the three fibrosis stages (FS). Correlation coefficients were 0.23, -0.412, 0.208, 0.220, 0.314 and 0.212; and *P* value was 0.014, < 0.001, 0.026, 0.018, 0.001 and 0.024, respectively. Univariate analysis revealed that only PLT and GLOB were significantly different in the three FS (PLT: *F* = 11.772, *P* < 0.001; GLOB: *F* = 6.612, *P* = 0.002). Step-wise multiple regression analysis showed that PLT and GLOB were also independently correlated with FS (R^2 = 0.237). By Spearman's rank correlation analysis, GP model was significantly correlated with the three FS (*r* = 0.466, *P* < 0.001). The median values in F0-F1, F2-F3 and F4 were 1.461, 1.720 and 2.634. Compared with the six available models (fibrosis index, AST-platelet ratio, FIB-4, fibrosis-cirrhosis index and age-AST model and age-PLT ratio), GP model showed a highest correlation coefficient. The sensitivity and positive predictive value at a cutoff value < 1.68 for predicting minimal fibrosis F0-F1 were 72.4% and 71.2%, respectively. The specificity and negative predictive value at a cutoff value < 2.53 for the prediction of cirrhosis were 84.5% and 96.7%. The area under the curve (AUC) of GP model for predicting minimal fibrosis and cirrhosis was 0.762 [95% confidence interval (CI): 0.676-0.848] and 0.781 (95% CI: 0.638-0.924). Although the differences were not statistically significant between GP model and the other models (*P* all > 0.05), the AUC of GP model was the largest among the seven models.

CONCLUSION: By establishing a simple model using available laboratory variables, chronic HBV-infected patients with minimal fibrosis and cirrhosis can be diagnosed accurately, and the clinical application of this model may reduce the need for liver biopsy in HBV-infected patients.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a global public health problem and it is estimated that approximately 350 000 000 people are infected with this virus in the world. More often, it may progress into liver cirrhosis and hepatocellular carcinoma (HCC). Although anti-virus therapy has reduced greatly the risk of cirrhosis and HCC, some positive hepatitis B surface antigen (HBsAg) carriers with less HBV-DNA may eventually develop cirrhosis and HCC[1,2]. Liver biopsy is not common. Patients with more than a 2-fold increase in aminotransferase levels were regarded as having severe liver necroinflammation. However, liver fibrosis was difficult to evaluate by noninvasive methods.

Liver fibrosis is known as the major problem causing morbidity and mortality in chronic HBV-infected patients. Once diagnosed, fibrosis should be treated as early as possible by appropriate methods. Although liver biopsy is being used as the gold standard in diagnosing the degree of fibrosis, not many patients are willing to undergo a liver biopsy because of its invasiveness and there might be inter- and intra-observer variability in the evaluation of specimens obtained[3], and a risk of complications (even if it is low) causing discomfort[4,5].

Some noninvasive methods are expected and used in patients with hepatitis C or B virus infection. The best results were obtained with liver stiffness measurement by means of transient elastography (TE) (FibroScan), or with FibroTest-ActiTest (Biopredictive, Labcorp)[4] and Fibrospect Ⅱ (Prometheus)[5]. However, all these noninvasive methods are expensive and/or require equipments not widely used. Therefore, it is necessary to screen for simpler and cheaper methods for the examination of hepatic fibrosis. Poynard *et al*[6-10] investigated the correlations between the serum aminotransferases level, age, hyaluronic acid level, collagen level, platelet count and different fibrosis stages (FS), but did not draw clear con-clusions. Several scoring systems like age-platelet count (PLT) ratio (AP index), aspartate aminotransferase (AST)-platelet ratio (APRI), age-AST model, fibrosis index (FI), FIB-4 and fibrosis-cirrhosis index (FCI), using different thresholds, have been proposed to detect presence or absence of fibrosis or cirrhosis in patients infected with hepatitis C virus (HCV) or HBV[11-18]. HBV-infected patients are prone to fibrosis; however, there have been few studies on the relationship between noninvasive fibrosis biomarker and liver biopsy. For this purpose, in this study we developed a new noninvasive serum model by assessing several clinicopathological features. We also compared and evaluated the diagnostic accuracy of this noninvasive model consisting of the variables of FI, APRI, FIB-4, FCI, age-AST model and AP index.

MATERIALS AND METHODS

Patients

This is a retrospective cross-sectional study and was carried out from March 2008 to March 2011. Screening of patients was conducted at the Department of Liver Disease (Ruikang Hospital of Guangxi Traditional Chinese Medical University, Nanning, China) and Department of Infectious Disease (The First Affiliated Hospital of Guangxi Medical University, Nanning, China). A total of 114 patients were enrolled to the study (male/female 91/23; mean age 38.32 ± 11.36 years, range 15-67 years. Diagnosis of chronic HBV-infected patients was established based on the presence of positive results of surface antigen (> 0.5 ng/mL) and/or e antigen (> 0.05 NCU/mL) lasting more than six months. Clinical, biochemical and hematologic data were recorded from each patient at the time of liver biopsy. Patients with the following conditions were excluded: presence of other causes of liver disease such as hepatitis A/C/E virus infection, HCC, prior nucleoside medication, prior interferon therapy, fatty liver disease, alcohol intake > 30 g/wk (female) and 60 g/wk (male), insufficient liver tissue for staging of fibrosis, clinical or ultrasonographic evidence of cirrhosis.

Histological staging

Ultrasonographic-guided liver biopsy was performed according to a standardized protocol. The liver biopsy procedure, its advantages and possible adverse effects were explained to the patients. Informed consent was obtained from the patients about the possible transmission of HBV infection. Specimens of 15-20 mm liver tissues were fixed, paraffin-embedded, stained with hematoxylin-eosin and Masson's trichrome. A minimum of six portal tracts was required for diagnosis. Liver biopsy was evaluated with or without knowing the history of the patients. Five fibrosis degrees of histological staging were defined according to "The program of prevention and cure for viral hepatitis", generally used in hospitals of China[19], as F0 (no fibrosis), F1 (mild fibrosis without septa), F2 (moderate fibrosis with few septa), F3 (severe fibrosis

Table 1 Characteristics of the study population

Features	mean ± SD	Minimum	Maximum
Gender (male/female)	91/23		
HBV DNA (copies/mL)			
(< 1000/ ≥ 1000)	44/70		
(< 10^3/10^3-10^5/ ≥ 10^5)	44/32/38		
E antigen (+/-)	52/62		
Age (yr)	38.32 ± 11.36	15	67
PLT(× 10^9/L)	174.44 ± 62.68	50.6	334
T.Bil (g/dL)	0.88 ± 0.49	0.27	3.14
D.Bil (g/dL)	0.29 ± 0.23	0.08	1.39
ALT (IU/L)	69.32 ± 116.40	10.00	983
AST (IU/L)	48.60 ± 64.56	13	594
ALB (g/dL)	4.15 ± 0.43	2.85	5.26
GLOB (g/dL)	2.98 ± 0.52	1.75	4.47
ALP (IU/L)	80.10 ± 27.38	24	154
GGT (IU/L)	59.46 ± 66.92	5	430
PT (s)	12.01 ± 1.58	9.3	18.1

HBV: Hepatitis B virus; GLOB: Globulin; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; T.bil: Total bilirubin; D.bil: Direct bilirubin; ALB: Albumin; GGT: Glutamyltransferase; ALP: Alkaline phosphatase; PT: Prothrombin time.

with numerous septa without cirrhosis) and F4 (cirrhosis). In our study, we simplified the FS as F0-F1 (minimal fibrosis), F2-F3 (advanced fibrosis) and F4 (cirrhosis).

Blood tests

All tests were performed in the patients after a fasting period of 12 h. The routine liver function tests included alanine aminotransferase (ALT), AST, total bilirubin (T.bil), direct bilirubin (D.bil), albumin, globulin (GLOB), glutamyltransferase (GGT), alkaline phosphatase (ALP), PLT, HBV-DNA and prothrombin time (PT). Serum tests were performed using an automatic biochemistry analyzer. The viral load was measured by real-time polymerase chain reaction, with a detection limit of 1000 copies/mL. All biochemical tests and their scores were evaluated without knowledge of liver biopsy results. Thirteen clinical, biochemical and hematological variables were used for the analysis: sex, age, PLT, ALT, AST, albumin, GLOB, T.bil, D.bil, GGT, ALP, HBV DNA and PT.

Statistical analysis

The data was analyzed using statistical package SPSS version 13.5 for Windows. A P value of 0.05 was considered statistically significant. All data was presented as mean values or number of patients. Spearman's rank correlation was used to assess the significant correlation between variables and liver FS. The Student t test or variance analysis was used to compare arithmetic means and parameters, while χ^2 test was used to compare categorical data.

A predictive model, named Globulin/platelet (GP) model, was constructed by modeling the values of the independent variables and their coefficient of regression. The diagnostic value of the model was assessed by calculating the areas under the receiver operating characteristic (ROC) curves. An area under the curve (AUC) of 1.0 rep-

resentes an ideal test, whereas 0.5 indicates a test of no diagnostic value. The diagnostic accuracy was calculated by sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV).

The best cutoff points were selected according to the Youden index from the ROC curve to identify the presence and absence of minimal fibrosis or cirrhosis. For this purpose, we selected cutoff points with a 95% certainty, thus assuming a 5% false negative result which is acceptable clinically.

Comparison of available noninvasive biomarkers to evaluate patient's liver biopsy data

Serum samples and data of the characteristics of the patients and liver specimens were collected from each patient for further biochemical analysis. All patients were evaluated for FI, APRI, FIB-4, FCI, age-AST model and AP index. Superiority of GP model was compared with the other selected fibrosis models, and ROC curves and correlation analysis were employed to predict minimal fibrosis or cirrhosis, and deduce the diagnostic accuracy.

RESULTS

Patient data

The demographic and clinical outcomes of the 114 patients with HBV infection are shown in Table 1. The evaluation of inflammatory grade yielded mild chronic hepatitis in 44 patients, moderate chronic hepatitis in 64 patients and severe chronic hepatitis in 6 patients. And 22 patients had liver fibrosis at stage F0, 34 at F1, 28 at F2, 18 at F3, and 12 at F4. Among the patients with HBV DNA < 1000 copies/mL, there were 20 cases of mild chronic hepatitis, 24 moderate chronic hepatitis, and none had severe chronic hepatitis, while among those with HBV DNA > 1000 copies/mL, there were 24 cases of mild chronic hepatitis, 40 moderate chronic hepatitis, and six severe chronic hepatitis. The latter showed a higher inflammatory grade than the former ($\chi^2 = 37.487$, $P < 0.001$). Among the E antigen positive (e+) patients, 12 at stage F0, 15 at F1, 11 at F2, 7 at F3, and 7 at F4. Among E antigen negative (e-) patients, 10 at stage F0, 19 at F1, 17 at F2, 11 at F3 and 5 at F4. There was no significant difference between e+ and e- patients in FS ($\chi^2 = 2.301$, $P = 0.681$).

Correlation between clinical findings and three FS

Three levels of liver fibrosis (F0-F1, F2-F3 and F4) were analyzed. Thirteen demographical, hematological, and biochemical variables were studied. HBV DNA was divided into two groups (≥ 1000 copies/mL and < 1000 copies/mL) or three groups (< 1000 copies/mL, ≥ 1000 copies/mL and < 10^5 copies/mL, ≥ 10^5 copies/mL) and gender was described as categorical data. Of the 13 variables studied by Bivariate Spearman's rank correlation analysis, 6 variables (age, PLT, T.bil, D.bil, GLOB and PT) were correlated significantly with the three FS (cor-

Figure 1 Scores of platelet count (A) and globulin (B) in three fibrosis stages (F0-F1, F2-F3 and F4). The top and bottom of each box are the 25% and the 75% centiles. The line through the box is the median, and the error bars are the 5th and 95th centiles. GLOB: Globulin; PLT: Platelet count.

relation coefficients were 0.23, -0.412, 0.208, 0.220, 0.314 and 0.212; *P* value 0.014, < 0.001, 0.026, 0.018, 0.001 and 0.024).

Selection of variables and construction of a model for predicting FS

Univariate analysis revealed that only PLT and GLOB of the 13 variables were independent predictive factors and significantly different in FS (PLT: $F = 11.772$, $P < 0.001$, GLOB: $F = 6.612$, $P = 0.002$). There was no difference among the other variables (P all > 0.05). These biochemical markers can also be helpful in staging the liver fibrosis. Figure 1 shows the box plots of the two markers with liver histological stages. It is clear from Figure 1 that as the fibrosis progressed, GLOB level increased, while PLT gradually decreased with fibrosis progression. However, it was interesting to note that GLOB and PLT both were in the normal limits, making the diagnosis of fibrosis difficult by using single biochemical markers.

To amplify the opposite relationship between the stage of fibrosis and the two markers, GLOB and PLT, based on their significance not only by the rank correlation analysis but also by univariate analysis, we developed a new fibrosis model named GP model in HBV infection for predicting cirrhosis and minimal fibrosis. It can be represented as GP model = GLOB (g/dL) $\times$ 100/PLT ($\times 10^9$/L).

The GP model distribution for the patients in the respective FS is represented in Figure 2. The median values for GP model in F0-F1, F2-F3 and F4 patients were 1.461, 1.720 and 2.634, respectively. GP model was correlated significantly with the liver FS ($r = 0.466$, $P < 0.001$). The diagnostic values of GP model to predict F0-F1 and F4 patients were evaluated by the AUC (Figure 3).

Step-wise multiple regression analysis, which was used to formulate a suitable multivariable model for the correlation between 11 count variables and the three FS, F0-F1, F2-F3 and F4, revealed that PLT and GLOB were also independently correlated with histological FS, $R^2 = 0.237$. The contribution of PLT and GLOB to R^2 was 0.197 and 0.04, respectively (data not shown). The final multiple regression model incorporating both PLT and GLOB was: FS = 1.683 - 0.008 $\times$ PLT ($\times 10^9$/L) + 0.485 $\times$ GLOB (g/dL). We simplified it as: FS = 1.7 - 0.01 $\times$ PLT ($\times 10^9$/L + 0.5 $\times$ GLOB (g/dL).

The median values for FS model in F0-F1, F2-F3 and F4 patients were 1.218, 1.480 and 2.085, respectively. FS model significantly correlated with the liver FS (Spearman's rank correlation coefficient, $r = 0.47$, $P < 0.001$, data not shown). The diagnostic values of FS to differentiate F0-F1 and F4 patients were assessed using the ROC. Interestingly, AUC was very close between FS model and GP model in F0-F1 (0.760 *vs* 0.762) and F4 (0.781 *vs* 0.783), and in complete coincident sensitivity and specificity for the detection of minimal fibrosis and cirrhosis (data not shown). GP model was simpler than FS model in term of calculation. Therefore, we used GP model to express our findings.

Comparison of GP model with FI, APRI, FIB-4, FCI, age-AST model and AP index

The scores of six indexes (FI, APRI, FIB-4, FCI, age-AST model and AP index) were calculated by the formula shown in Table 2. The relationship between the histological severity of fibrosis (F0-F1, F2-F3 and F4) and six models is shown in Figure 2 and Table 3. There was a significant correlation between F0-F1, F2-F3 and F4 and serum indexes of FIB-4, AP index and GP model ($P < 0.05$), but not in FI, APRI, FCI, age-PLT model ($P > 0.05$). A gradual increase at the level of AP index, FIB-4, GP model was observed in different FS. GP model showed the strongest correlation with the three FS ($r = 0.441$, $P < 0.001$).

The predictive value of the seven noninvasive models for predicting minimal fibrosis (F0-F1) is summarized in Table 4. APRI, FIB-4 and FCI had a high PPV, specificity, but the sensitivity and NPV were low. AP index had a high sensitivity and NPV, but specificity and PPV were low. GP model had not only high PPV and specificity, but also high NPV and sensitivity. AUC of age-AST model was close to 0.5, showing little value for prediction of minimal fibrosis. We compared the AUC of GP model with the other five noninvasive models using the 95% confidence interval (CI) and the standard error (SE) of the mean, and found that although the differences were not statistically

Figure 2 Box plot of fibrosis index, aspartate aminotransferase-platelet ratio, FIB-4, fibrosis-cirrhosis index, age-aspartate aminotransferase model, age-platelet count ratio and globulin/platelet model in relation to F0-F1, F2-F3 and F4 fibrosis stage. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the asterisks represent outliers. The line across the box indicates the median value. FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; AP index: Age-platelet count ratio.

Figure 3 Receiver operating characteristic curves generated by globulin/platelet model for prediction of F0-F1 (A) and F4 (B). The area under the curve of GP model for the discrimination between minimal fibrosis (F0-F1) and significant fibrosis (F2, F3, F4), and between non-cirrhosis (F0, F1, F2, F3) and cirrhosis (F4) was 0.732 [confidence interval (CI): 0.642-0.823] and 0.738 (CI: 0.612-0.864). Using a cutoff value of < 1.68, GP model had a sensitivity of 72.4%, a positive predictive value (PPV) of 71.2%, a specificity of 69.6%, and a negative predictive value (NPV) of 70.8% for the prediction of F0-F1. For the prediction of cirrhosis (F4) at a cutoff value of > 2.53, GP model had a sensitivity of 72.7%, a PPV of 33.4%, a specificity of 84.5%, and a NPV of 96.7%.

significant, the GP model seemed to have a best predictive value (largest AUC) (*P* all > 0.05) (Table 4).

The predictive value of GP model for cirrhosis (F4) is shown in Table 5. All of the models had very good NPV (> 96%), also high sensitivity (> 70%). The AUC of age-AST model was less than 0.5, which cannot be used to predict cirrhosis. We compared the AUC of GP model with the other five noninvasive models as well using the 95% CI and the SE, and found that AUC of GP model was not significantly high (*P* all > 0.05) (Table 5).

DISCUSSION

Although only a small number of patients with chronic inactive HBV infection develop advanced liver disease[20-22], the risk of HCC is higher for HBV infected patients than for those without HBV infection[23]. The cirrhosis in HBsAg carriers often progress insidiously. Chu *et al*[24] concluded that the so-called inactive carrier state cannot be considered generally as an innocuous, persistent condition with good prognosis, suggesting that regular follow-

Table 2 Noninvasive simple fibrosis models composed of routine clinical and laboratory parameters

Fibrosis test	Calculation
FI	8.0-0.01 × PLT (× 10^9/L)-ALB (g/dL)
APRI	AST (IU/L) × 100/PLT (× 10^9/L)
FIB-4	Age (yr) × AST (IU/L)/PLT (× 10^9/L) × ALT (IU/L) 1/2
FCI	ALP (IU/L) × T.bil (g/dL)/ALB (g/dL)/ PLT (× 10^9/L)
Age-AST model	Age (yr)/AST (IU/L)
AP index	Age (yr)/PLT (× 10^9/L)

PLT: Platelet count ; ALB: Albumin; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; T.bil: Total bilirubin; AP index: Age-PLT ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; FCI: Fibrosis-cirrhosis index.

Table 3 Correlation analysis of seven models and different fibrosis stages (F0-F1, F2-F3 and F4)

F0-F1, F2-F3 and F4 fibrosis stages	Bivariate Spearman's rank correlation coefficient	*P* value
FI	0.231	0.06
APRI	0.146	0.237
FIB-4	0.307	0.012
FCI	0.159	0.20
Age-AST model	0.132	0.286
AP index	0.246	0.045
GP model	0.441	0.000

AP index: Age-platelet count ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index.

Table 4 Validity of age-platelet count ratio, aspartate aminotransferase-platelet ratio, age-aspartate aminotransferase model, fibrosis index, FIB-4 and fibrosis-cirrhosis index for prediction of minimal fibrosis (F0-F1) and comparison with globulin/platelet model

Fibrosis test	Cutoff value	Specificity %	Sensitivity %	PPV %	NPV %	AUC (95% CI)	Standard error	*P* value (*vs* GP model)
FI	1.58	41.1	89.7	61.2	79.4	0.695 (0.600-0.791)	0.049	> 0.05
APRI	0.85	87.5	41.4	77.4	59.0	0.635 (0.533-0.738)	0.052	< 0.05
FIB-4	1.7	85.7	51.7	78.9	63.1	0.720 (0.627-0.813)	0.047	> 0.05
FCI	0.17	80.4	55.2	74.5	63.4	0.692 (0.594-0.790)	0.050	> 0.05
Age-AST model	-	19.6	94.8	55.0	78.4	0.524 (0.417-0.632)	0.055	< 0.001
AP index	0.17	46.4	89.7	63.4	81.3	0.726 (0.634-0.818)	0.047	> 0.05
GP model	1.68	69.6	72.4	71.2	70.8	0.762 (0.676-0.848)	0.044	

AP index: Age-platelet count ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index; PPV: Positive predictive values; NPV: Negative predictive values; AUC: Area under the curve; CI: Confidence interval.

Table 5 Validity of age-platelet count ratio, aspartate aminotransferase-platelet ratio, age-aspartate aminotransferase model, fibrosis index, FIB-4, and fibrosis-cirrhosis index for prediction of cirrhosis (F4) and comparison with globulin/platelet model

Fibrosis test	Cutoff value	Specificity %	Sensitivity %	PPV %	NPV %	AUC (95% CI)	Standard error	*P* value (*vs* GP model)
FI	2.16	53.4	81.8	15.8	96.4	0.717 (0.569-0.865)	0.075	> 0.05
APRI	0.77	72.8	72.7	22.2	96.1	0.703 (0.584-0.822)	0.061	> 0.05
FIB-4	2.29	89.3	72.7	42.0	96.8	0.768 (0.050-0.931)	0.083	> 0.05
FCI	0.10	68.0	81.8	21.4	97.2	0.738 (0.612-0.864)	0.064	> 0.05
Age-AST model	-	-	-		-	0.486 (0.365-0.608)	0.062	-
AP index	0.32	74.8	72.7	23.6	96.2	0.735 (0.575-0.895)	0.082	> 0.05
GP model	2.53	84.5	72.7	33.4	96.7	0.781 (0.638-0.924)	0.073	

AP index: Age-platelet count ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index; PPV: Positive predictive values; NPV: Negative predictive values; AUC: Area under the curve; CI: Confidence interval.

up is necessary. Active HBV infection more often caused significant fibrosis and cirrhosis than inactive HBV infection. We attempted to find a simple and common index to evaluate their fibrosis in active and inactive HBV infections. We retrospectively studied the pathological changes about fibrosis in patients with HBV infection from two liver disease centers. We found that the HBV DNA with a higher inflammation grade, was not significantly different among different FS. We also found no significant difference between e+ and e- populations in FS. A study[25] recruited patients with HBV DNA < 10^4 copies/mL, and found the mean liver stiffness in inactive HBsAg carriers was 5.6 ± 2.1 kPa, significantly higher than in normal subjects. In 16.4% (23) of inactive carriers, liver stiffness exceeded 7 kPa (the cutoff for fibrosis F2-F4); and in patients with undetectable viral loads (with a detection limit of 51 copies/mL for HBV DNA), the mean liver stiffness was significantly lower than in those with detectable DNA (< 10^4 copies/mL). Assessing the noninvasive liver stiffness in inactive HBsAg carriers by transient elastography, Fattovich *et al*[26] reported that HBeAg-negative chronic hepatitis patients had a higher risk for progression to cirrhosis (8-10 per 100 person years) than HBeAg-positive chronic hepatitis patients. This may reflect the duration of infection, and a late phase in the natural history of the disease, as opposed to *de novo* infec-

tion with a variant not producing HBeAg. Another study found in chronic HBV carriers with clinical normal liver function tests, inflammation grade and FS had no correlation with the level of HBV DNA or the state of HBeAg positivity[27]. Our study with a detection limit of 1000 copies HBV DNA/mL, enrolled all of HBV-infected patients, including some patients with active inflammation. This may be the reason for the inconsistency with other studies[28].

Many studies on prediction of significant fibrosis (F2-F4) and cirrhosis (F4) in patients with HBV infection have been published in the past few years, such as Fibroscan and FibroTest-ActiTest, galactose and methacetin breath tests, TE, fibrotest, cirrhosis discriminant score, AST/ALT ratio, APRI, FIB-4 and AP index[11,29-33]. However, some of the tests need expensive instruments and some are somewhat difficult to use in clinical practice, since these assay utilizes less common biochemical markers such as α2-macroglobulin, haptoglobin, and apolipoprotein A1, and also requires use of a special computer program to perform calculations.

In our study, we attempted to develop a single model using routinely available laboratory test results to predict minimal fibrosis and cirrhosis in treatment-naive patients with HBV infection. We found by Step-wise multiple regression analysis and correlation analysis, that PLT and GLOB were significantly correlated with different FS. Other variables such as T.bil, D.bil, PT, or GGT may play a role in the discrimination function and have been found useful in patients with minimal fibrosis or cirrhosis. However, compared with PLT and GLOB, other variables were correlated with the histological FS at a much smaller coefficient of determination. Wai *et al*[16] also suggested that two variables can be practically used as a prediction index, and in our model we used PLT and GLOB because of their convenience of application in general practice. Nevertheless, the value of these two parameters was proposed to evaluate minimal fibrosis and cirrhosis. The concept in prediction of minimal fibrosis by a ratio of two important variables is not new. These findings echoed results from many previous studies. The value of PLT as a marker of liver fibrosis has already been reported[6,16,34-37]. Some studies showed that GLOB was correlated with FS and was a predictor of either significant fibrosis or cirrhosis[28,38].

GP model was simple to use and accurate in predicting both minimal fibrosis and cirrhosis in HBV-infected patients (Figure 3 and Tables 3-5). To evaluate its predictive value, we compared GP model with the other available models, because the variables used in these models were also involved in our collected data. GP model showed the highest correlation coefficient with FS. Comparison of GP model with the other six models in AUC, GP model showed the highest value than other models, although there was no significant difference. Using values below the lower cut-off level (1.68), a presence of minimal fibrosis could be predicted in 71% of patients. Similarly, using values below the higher cut-off level (2.53),

a prediction of non-cirrhosis could be made in 96% of patients.

There exist some limitations in our study. Our study is based on the data from liver biopsy, which is considered as the gold standard for assessing hepatic fibrosis, but sampling error as well as intra- and inter-observer variability can complicate the correlations between histology and noninvasive markers of hepatic fibrosis. Arase *et al*[39] found that some patients with a nodular liver surface at laparoscopy were not diagnosed as having liver cirrhosis when only histological samples were used; HBV-positive patients with a nodular liver surface have a tendency of sampling error compared with the HCV-positive patients. On the other hand, there was overlap among patients with different stages of fibrosis. Thus, the value of GP model for the prediction of fibrosis in individual patients with HBV infection must be confirmed in prospective studies. However, we are not yet able to get enough patient data for verification of GP model in a new cohort. The data of patients were derived from two different hospitals, and the sample size is still small. Whether the model can reflect the change of FS in treatment process awaits further studies.

In conclusion, We established a simple model using available laboratory variables. Minimal fibrosis and cirrhosis can be diagnosed accurately using this model, thus reducing the need for liver biopsy in chronic HBV-infected patients.

COMMENTS

Background
Liver fibrosis is known as the major condition causing morbidity and mortality in chronic hepatitis B virus (HBV)-infection patients. Liver biopsy (LB) as an invasive method is used as the gold standard in diagnosing the degree of fibrosis, but because there is a risk of complications causing discomfort, not many patients are willing to undergo a LB. HBV-infected patients are prone to fibrosis, but there have been few studies about the relationship between noninvasive fibrosis biomarker and liver LB among these patients.

Research frontiers
Some noninvasive methods were expected and used in patients with hepatitis C or B virus infection. Simpler and cheaper methods for the prediction of hepatic fibrosis are being studied. Several scoring systems have been proposed, such as age-platelet count (PLT) ratio (AP index), aspartate aminotransferase (AST)-platelet ratio, age-AST model, fibrosis index, FIB-4, and fibrosis-cirrhosis index, using different thresholds to predict presence or absence of fibrosis or cirrhosis in patients infected with hepatitis C virus or HBV.

Innovations and breakthroughs
This study developed a new noninvasive serum model named Globulin/platelet (GP) model, including globulin and PLT, by assessing several clinicopathological features. Comparing with six available models, GP model showed highest correlation coefficient. The sensitivity and positive predictive value at a cutoff value < 1.68 for predicting minimal fibrosis F0-F1 were 72.4% and 71.2%. The specialty and negative predictive value at a cutoff value < 2.53 for the prediction of cirrhosis were 84.5% and 96.7%. The area under the curve of GP model for predicting minimal fibrosis and cirrhosis were 0.762 and 0.781, respectively, which is the largest among the seven models.

Applications
The simple model developed in this study using readily available laboratory results can identify chronic HBV-infected patients with minimal fibrosis and cirrhosis with a high degree of accuracy, and it seems more efficient than frequently used models. This model may decrease the need for liver biopsy in

HBV-infected patients.

Peer review

This study might be interesting and useful for the readers regarding the daily management of patients with viral hepatitis. Application of this model may decrease the need for liver biopsy in HBV infection cases.

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BRIEF ARTICLE

Transepithelial leak in Barrett's esophagus patients: The role of proton pump inhibitors

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Abstract

AIM: To determine if the observed paracellular sucrose leak in Barrett's esophagus patients is due to their proton pump inhibitor (PPI) use.

METHODS: The *in vivo* sucrose permeability test was administered to healthy controls, to Barrett's patients and to non-Barrett's patients on continuous PPI therapy. Degree of leak was tested for correlation with presence of Barrett's, use of PPIs, and length of Barrett's segment and duration of PPI use.

RESULTS: Barrett's patients manifested a near 3-fold greater, upper gastrointestinal sucrose leak than healthy controls. A decrease of sucrose leak was observed in Barrett's patients who ceased PPI use for 7 d. Although initial introduction of PPI use (in a PPI-naïve population) results in dramatic increase in sucrose leak, long-term, continuous PPI use manifested a slow spontaneous decline in leak. The sucrose leak observed in Barrett's patients showed no correlation to the amount of Barrett's tissue present in the esophagus.

CONCLUSION: Although future research is needed to determine the degree of paracellular leak in actual Barrett's mucosa, the relatively high degree of leak observed with *in vivo* sucrose permeability measurement of Barrett's patients reflects their PPI use and not their Barrett's tissue *per se*.

INTRODUCTION

The sucrose permeability test is designed to measure paracellular (non-transcellular) leak of sucrose from the lumen

of the upper gastrointestinal (GI) tract into the vasculature because sucrose is markedly hydrophilic and lacks any significant affinity for any carbohydrate transport proteins on the cell surface[1]. Moreover sucrose is destroyed enzymatically in the duodenum, and ceases to exist as a disaccharide after the early-mid portion of the small bowel. Sucrose leak would therefore be detected only while the probe (sucrose) still exists chemically in the upper GI lumen. This limits potential sites of leak to the esophageal, gastric and early intestinal mucosa. However, considerations of surface area and contact time (after oral consumption of the sucrose) argue that the sucrose permeability test results reflect primarily the gastroduodenal mucosa and less so the esophageal mucosa which has the smallest surface area (no villi or folds-except in Barrett's) and the shortest contact time.

Still, Barrett's patients are believed not to have a histological mucosal defect other than in the esophagus. Therefore when in previous work our group published that patients with a known diagnosis of Barrett's esophagus (BE) manifested a greater magnitude of transepithelial sucrose leak across the upper gastrointestinal tract than that seen in healthy control subjects, the strong difference was ascribed to the presence of Barrett's metaplasia in these patients[2]. In other words, a transepithelial leak was presumed to exist in the Barrett's metaplasia.

In this current work we revisited that conclusion by asking whether considerations other than the simple presence of Barrett's metaplasia were the reason for the increased leak observed in BE patients. Aside from the presence of Barrett's tissue, the foremost distinction about BE patients is their chronic, long-term use of acid suppression medications, most notably proton pump inhibitors (PPIs). We therefore posed the question of whether the leak we observed in BE patients was traceable to their regular use of PPI medications or to the presence of Barrett's metaplasia. Results indicated that the observed leak is due to PPI use by these patients.

MATERIALS AND METHODS

Study population

Patients with a prior known history of Barrett's esophagus or healthy controls with no current or history of upper GI disease were recruited by a gastroenterologist in a tertiary care teaching hospital bordering the suburbs of Philadelphia, PA. Diagnosis of BE was made by endoscopic exam and only after biopsies were documented to possess goblet cell metaplasia. All enrolled subjects gave informed consent and the study was approved by the Main Line Hospitals Institutional Review Board committee.

Test subjects were recruited without regard to gender or ethnicity. Exclusion criteria were: diabetes mellitus, steroid use, prior gastric or esophageal surgery, age < 18 years, current GI bleeding, weight loss, intractable nausea and vomiting or renal insufficiency. PPI use comprised omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole.

Sucrose permeability testing

All test subjects consumed in their homes a chilled solution of 100 g of sucrose in 200 cc of water containing 5 g of a citric acid-based flavoring agent at bedtime. An 8 h (overnight) urine sample was collected in a container with 5 mL of 10% thymol in isopropanol and mixed. For patients undergoing upper endoscopic examination, the sucrose permeability test was performed either before the procedure or at least two weeks later to avoid potential effects of endoscope trauma on epithelial barrier tissue. The total urine volume was measured and recorded. The concentration of sucrose in the urine sample was then measured by an enzymatic/spectrophotometric assay after prior desalting of the urine sample by anion and cation exchange resins[3]. The total amount of sucrose in the urine in mg was determined by multiplying the urine volume in mL by the sucrose concentration in mg/mL. This equates to the amount of sucrose which leaked out of the upper GI lumen.

Test subjects were instructed to refrain from solid food for at least 2 h prior to the sucrose permeability test and 8 h after testing. Specific foods were however not prohibited. Test subjects were also instructed to refrain from alcohol or non-steroidal anti-inflammatory medications for 24 h prior to testing and for 8 h after testing. Brushing teeth or flossing was proscribed before testing and until at least 20 min after testing.

Statistical analysis

Data are reported throughout as the mean $\pm$ SE. Experimental and control groups are compared throughout by unpaired Student's *t* tests, with statistical significance being ascribed when $P < 0.05$.

RESULTS

Sucrose permeability testing was conducted on 19 patients, 9 carrying a diagnosis of BE and 10 being healthy controls. The 9 BE patients were on PPI therapy at the time of their testing while the healthy controls were receiving no acid-suppressive medication. As was shown in previous studies from our group[2], a significantly greater sucrose leak was observed in the BE patients (345.9 $\pm$ 59.6 mg) compared to the healthy controls (124.7 $\pm$ 26.9 mg) (Figure 1). This difference was statistically significant with a $P < 0.003$. Following this confirmation of an increased transepithelial sucrose leak in BE patients, the etiology of the leak (as a result of BE itself or of PPI use) was pursued.

First, a second group of BE patients was studied to evaluate the effect of cessation of their PPI therapy on their observed sucrose leak. Thirty-eight BE patients underwent sucrose permeability testing while on a continuous PPI regimen. They then performed a second sucrose permeability test after having stopped their PPIs for 7 d, in order to look for any change in observed leak. Patients were allowed to consume antacid medications during this period but not PPIs or H-2 receptor antagonists. Any patients reporting very difficult reflux symptoms

Figure 1 Results of sucrose permeability studies showing a significant difference in sucrose leak in patients with Barrett's esophagus (and taking proton pump inhibitors) compared to healthy controls. Data represents the mean ± SE. $P < 0.003$ (Student's *t* test). SE: Standard error.

Figure 2 Sucrose leak among Barrett's esophagus patients after discontinuation of proton pump inhibitors for 7 d. Data represents the mean ± SE. $P = 0.28$ ($n = 38$). SE: Standard error; PPI: Proton pump inhibitor.

were allowed to leave the study. The mean sucrose leak decreased by approximately 30% from 155.4 ± 40.8 mg to 109.5 ± 15.3 mg after PPI medications were temporarily stopped for this 7 d period (Figure 2). These findings were however not statistically significant, with a $P = 0.283$ (paired Student's *t*-test). As before, a high SE was found for patients on PPI therapy, indicating a considerable amount of variability. The sucrose leak observed in the BE patients after their PPIs were discontinued for 7 d decreased to nearly the same level observed in Figure 1 for healthy controls not taking PPIs.

Since BE exists in a wide range of segment lengths, and a (passive diffusion) transepithelial leak due specifically to Barrett's metaplasia should correlate with the surface area of the Barrett's metaplasia, we investigated whether sucrose leak in the Barrett's patients correlated with length of segment of the Barrett's tissue. Endoscopically, characteristic findings of reddish/velvety Barrett's mucosa are defined as short segment BE if < 3 cm and long segment BE if ≥ 3 cm in length. Data from 49 BE patients were examined. Greater leak was not observed in patients with long-segment Barrett's. Mean leak was actually greater in short-segment Barrett's (209 *vs* 148.9). Analysis of sucrose leak between the two segment groups showed similar medians of 130.5 ± 40.4 mg (short) and 121.0 ± 21.4 mg (long) ($P = 0.36$) with no statistically significant difference between them.

Due to the magnitude of sucrose leak variability that we observed among patients (especially among patients on PPIs), other potential confounding sources of variability were explored. First, the issue of potential effect of duration of PPI use on observed transepithelial sucrose leak was investigated. In a non-BE population taking PPIs on a regular, long-term basis (gastroesophageal reflux disease patients not known to have Barrett's), the sucrose leak was found to vary as a function of the duration of PPI use. Specifically, the magnitude of leak correlated inversely with the length of time on PPI medi-

cations (Figure 3A). A statistically significant difference in leak was observed in patients on PPIs for < 3 mo (336.9 ± 53.8 mg) in comparison to those on PPIs for 3 mo to 25 years (135.4 ± 22.8 mg) ($P < 0.0003$; Figure 3B). The PPI-induced leak thus appeared to exhibit tachyphylaxis, and this would lead to wider observed variation in leak data if one did not correct for duration of PPI therapy.

Discontinuation of long-term PPI use followed by resumption of PPIs did not result in re-induction of leak. In a separate group of 14 patients on long-term PPI therapy, PPI use was discontinued for 7 d, and was then reinstituted. Sucrose permeability testing was again utilized to assess upper gastrointestinal leak 7 d after resumption of PPI use. A significant leak did not re-occur in these patients who had previously been on long-term PPI treatment (Table 1). This is in sharp contrast to patients taking PPIs for the first time, where induced sucrose leak is substantial[3].

DISCUSSION

When our clinical studies with Barrett's patients were begun in 2005, we did not anticipate increased barrier leak being induced by PPIs. In fact, the opposite was expected due to PPIs' documented ability to allow for mucosal (both gastric and esophageal) healing from inflammation and micro-ulceration by means of suppression of acid secretion and subsequent elevation of the pH of gastric luminal contents. Therefore when we first noted a transmucosal leak in BE patients we ascribed the observed leak to the presence of Barrett's metaplasia, as BE patients typically do not have other upper GI pathology[2]. When we later observed that Barrett's patients' transmucosal sucrose leak did not correlate with the length of the Barrett's segment and the surface area of the Barrett's metaplasia, we began to question if the source of the leak was in fact the metaplasia. We realized that PPI medications were the other common characteristic-in addition to the metaplasia itself-of all the Barrett's patients studied.

Figure 3 Proton pump inhibitor-induced transepithelial leak as a function of time. A: Scatter plot illustrating a decrease in sucrose leak in reflux disease patients on proton pump inhibitors (PPIs) over time with each data point representing the sucrose leak of one patient (*n* = 41); B: Statistically significant decrease in sucrose leak between patients on PPIs for < 3 mo *vs* 3 mo to 25 years. Data represents the mean ± SE. *P* = 0.003. SE: Standard error.

Table 1 Sucrose leak in Barrett's patients as a function of proton pump inhibitor use

	On PPIs	7 d off PPIs	7 d after PPI resumption
Median	87.8	101.9	63.0
mean ± SE	116.6 ± 16.5	142.7 ± 34.2	101.5 ± 21.0

Sucrose leak in Barrett's esophagus patients (*n* = 14) on proton pump inhibitors (PPIs), following 7 d off PPIs, and after the reintroduction of PPIs for 7 d. The acute leak phenomenon observed in previous studies in PPI naïve patients is not seen with resumption of PPIs after 7 d. A statistical significance in sucrose leak is not demonstrated among the different groups (on PPIs *vs* off PPIs *P* = 0.497, off PPIs *vs* back on PPIs *P* = 0.323, on PPIs *vs* back on PPIs *P* = 0.574). SE: Standard error.

We then began a series of clinical studies to ask whether PPIs in fact could induce upper GI leak in healthy controls free of upper GI disease, and found that PPIs indeed have this effect[3]. We further explored that phenomenon in an animal model (Sprague Dawley rat), observing that exposure of rat gastric corpus to omeprazole can lead to an immediate increase in transmucosal permeability which is bidirectional, concentration-dependent and size-specific[4-6]. This work confirmed the earlier findings by Hopkins *et al*[7] that omeprazole induced leak to ^{14}C-mannitol in rat gastric corpus. These previous findings together with our current observation (that the sucrose leak seen in Barrett's patients decreases when their PPI medications are discontinued) suggests that PPI use, not the presence of BE *per se*, is the cause of the upper GI leak seen in BE patients.

Note however that this still does not address whether Barrett's epithelium is or is not paracellularly leaky. It simply means that the clinical, *in vivo*, sucrose permeability test which is employed here is reflecting the effects of PPIs on upper GI barrier function. To determine whether Barrett's metaplasia is leaky will likely require Ussing chamber-type permeability studies with actual Barrett's

tissue. This could be difficult to perform since the major source of Barrett's tissue that is large enough in surface area for typical Ussing studies is esophagectomy surgery for adenocarcinoma. Here, adjacent Barrett's tissue may be available but it can be considerably modified/eliminated by radiation and chemotherapy prior to surgery, which is now a current, common practice in esophageal adenocarcinoma management. This is therefore clearly a situation where Ussing studies using Barrett's biopsy tissue (readily available through upper endoscopic screening) would be highly useful[8-10].

An unanswered question from our earlier study on sucrose leak in Barrett's patients is that not only was sucrose leak in Barrett's patients significantly greater than sucrose leak in healthy controls, but it was also greater than the leak measured in patients with chronic gastroesophageal reflux disease (GERD)[1]. As both GERD and Barrett's patient groups would be receiving PPI therapy, it is unclear why leak in the Barrett's group would be quantitatively and significantly different if PPIs were the cause of leak in both patient groups. One possible explanation is that our patient population happened to be too small in the earlier study to get a fully accurate representation, and that sucrose permeability testing can carry intrinsically high variability, being affected by certain dietary constituents as well as certain over-the-counter drugs such as aspirin[11-13].

The sucrose leak that we observed in the Barrett's cohort, or any of our patient groups taking PPIs, was not only high in absolute value but was associated with a large variance and high standard error of the mean. This led us to examine whether the PPI-induced leak was stable over time, since the length of the duration on PPI therapy was a frequent variable in our studies. As shown in Figure 3, the effect of PPIs on leak is indeed not stable over time, but in fact decreases over long-term PPI use. In contrast, a similar tachyphylactic aspect to PPI activity has not been observed for PPI-inhibition of acid secretion[14]. The inability of PPIs to induce a major transmucosal, molecular leak (> 200 mg of sucrose in the described test) after long-term PPI use has been interrupted for 7 full days, suggests that long-term PPI use has caused modification of intracellular signaling pathways and/or cell/tissue structural aspects that mitigate against reintroduction of leak.

In summary, sucrose leak observed in Barrett's esophagus patients appears to be due to the use of PPIs by these patients, not due to their Barrett's metaplasia. This PPI-induced upper GI leak appears to diminish during long-term PPI therapy. After long-term use of PPIs, leak is difficult to re-induce even after short interruption of PPI therapy. Finally, variability associated with sucrose permeability testing in a clinical population may necessitate use of relatively large patient groups to support one's conclusions.

ACKNOWLEDGMENTS

COMMENTS

Background

In earlier work, the research group has shown that Barrett's esophagus (BE) patients manifest a barrier leak to the paracellular probe, sucrose, in their upper gastrointestinal tract. This phenomenon could be due to a molecular-level leak in the Barrett's metaplasia, or to some other aspect of the Barrett's patient cohort. This study was an attempt to determine if the common medication of all BE patients, proton pump inhibitors (PPIs), is responsible for the phenomenon.

Innovations and breakthroughs

The phenomenon of PPI-induced gastric barrier leak is certainly an unexpected side-effect of PPI therapy. The mechanism for the phenomenon remains unknown. More importantly, the clinical implications of the phenomenon are as yet poorly understood.

Applications

If one adopts a stance that a PPI-induced gastric barrier leak is clinically benign, it is possible that the phenomenon could be useful in drug delivery. However a greater understanding of the characteristics of the leak are needed (what can actually permeate through the leak), as well as a full realization of the clinical implications (if any). These could range from localized inflammation to altered kinetics of uptake of other (oral) medications into the bloodstream.

Terminology

Barrier function refers here to the ability of the gastric mucosa to separate the stomach luminal compartment from interstitial fluid and bloodstream. Barrier leak can result from either injured/dying/detaching epithelia or altered (and leaky) epithelial tight junctions.

Peer review

This study found that BE manifested a greater magnitude of transepithelial sucrose leak across the upper gastrointestinal tract than that seen in healthy control subjects, previously. In the present study, they determined if the observed paracellular sucrose leak in BE patients is due to their proton pump inhibitors use. This subject is a new topic in the research area and may contribute to the literature.

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BRIEF ARTICLE

Plasma microRNA profiles distinguish lethal injury in acetaminophen toxicity: A research study

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Abstract

AIM: To investigate plasma microRNA (miRNA) profiles indicative of hepatotoxicity in the setting of lethal acetaminophen (APAP) toxicity in mice.

METHODS: Using plasma from APAP poisoned mice, either lethally (500 mg/kg) or sublethally (150 mg/kg) dosed, we screened commercially available murine microRNA libraries (SABiosciences, Qiagen Sciences, MD) to evaluate for unique miRNA profiles between these two dosing parameters.

RESULTS: We distinguished numerous, unique plasma miRNAs both up- and downregulated in lethally compared to sublethally dosed mice. Of note, many of the greatest up- and downregulated miRNAs, namely 574-5p, 466g, 466f-3p, 375, 29c, and 148a, have been shown to be associated with asthma in prior studies. Interestingly, a relationship between APAP and asthma has been previously well described in the literature, with an as yet unknown mechanism of pathology. There was a statistically significant increase in alanine aminotransferase levels in the lethal compared to sublethal APAP dosing groups at the 12 h time point ($P < 0.001$). There was 90% mortality in the lethally compared to sublethally dosed mice at the 48 h time point ($P = 0.011$).

CONCLUSION: We identified unique plasma miRNAs both up- and downregulated in APAP poisoning which are correlated to asthma development.

Ward J, Bala S, Petrasek J, Szabo G. Plasma microRNA profiles distinguish lethal injury in acetaminophen toxicity: A research study. *World J Gastroenterol* 2012; 18(22): 2798-2804 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i22/2798.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i22.2798

INTRODUCTION

Acetaminophen (APAP) continues to be an important cause of acute liver failure in the developed world, second only to infectious etiologies worldwide[1]. It is also the most common cause of death due to analgesic ingestion in the United States[2]. Numerous emergency department

patients in the setting of acetaminophen overdose are admitted to hospital for N-acetylcysteine (NAC) treatment. This necessary treatment modality places these patients at risk for health-care associated disease. It also places likely unnecessary additional financial strain on an already burdened healthcare system. In addition, the estimated United States cost of treating intentional acetaminophen overdose is $86.9 million per year[3]. However, in over 30 years of research it is still unclear how the exact mechanism of acetaminophen toxicity occurs[4].

Current literature implicates N-acetyl-para-benzoquinone-imine, or N-acetyl-p-benzoquinoneimine (NAPQI), as the primary metabolite responsible for hepatotoxicity. An estimated 90% of APAP is metabolized in the liver *via* either glucuronidation or sulfation. Another approximate 5% is urinated unaltered. However, approximately 5% of APAP is metabolized by the cytochrome P450 2E1 pathway into NAPQI. In the scenario of APAP overdose, the normal route of metabolism becomes overburdened, so an overabundance of NAPQI is produced, causing hepatotoxicity. Glutathione can rescue this process, and is the reason NAC is used as a treatment modality. However, it is presumed only a portion of hepatotoxicity occurs *via* this mechanism. Lipid peroxidation *via* free radical formation, and mitochondrial dysfunction *via* increased permeability of the mitochondrial permeability transition, are also postulated as causes of APAP-associated hepatotoxicity[5,6].

Clearly, a better understanding of how APAP specifically causes hepatic toxicity from a pathophysiologic perspective still needs to be determined. In addition, standard clinical laboratory testing may not reveal evidence of hepatic injury for up to 24 h following APAP ingestion. A considerable proportion of APAP-exposed individuals therefore receive unnecessary empiric treatment with an antidote before hepatic injury can be ruled out. To overcome this clinical problem, early diagnostic indicators of hepatic injury have been sought.

MicroRNA fragments (miRNAs), are short, chemically stable biomolecules, noncoding posttranslational regulators that bind to untranslated mRNA sequences to produce gene silencing[7-10]. Moreover, each miRNA targets several different mRNAs; the same target gene may be regulated by several different miRNAs in different biological situations, a process that allows enormous complexity and flexibility in their regulatory potential. miRNAs have been characterized as regulators of protein expression in diverse disease processes, including acute hepatic injury[9,10]. Importantly, miRNAs have already been successfully utilized as early biomarkers for esophageal squamous cell carcinoma detection in serum[7], identifying Parkinson's disease onset and disease progression[8], and diagnosis of hepatocarcinoma[9,10], demonstrating miRNAs as an ideal area of research to determine other early biomarkers for disease states, notably APAP-associated hepatotoxicity[11]. In addition, miRNA fragments do not require the post-translational modifications necessary in protein production; with fewer human miRNAs to evaluate (an estimated 1000 human miRNAs compared to approximately 20 000 proteins), there is an improved likelihood of identifying unique APAP-associated miRNA profiles[11].

Recent work has also shown the medical utility of miRNA[12,13]. Interestingly, literature also supports its association specifically in the setting of acetaminophen toxicity. For instance, Wang *et al*[11] (2009) showed increased levels of miR-122 and miR-192 in the plasma of acetaminophen overdosed mice, yet decreased levels of these miRNAs in the liver tissue. In addition, these determined markers changed with time and dosing corresponding to histologic liver damage. Of note, these profiles were not evaluated at the lethal APAP dosing of 500 mg/kg, a parameter requiring further investigation to specifically compare lethal and sublethal miRNA profiles. Furthermore, additional literature has described miRNA involvement in acetaminophen toxicity, as well as the utility of miRNAs as biomarkers useful in the setting of other hepatotoxic disorders, such as hepatitis B and C, alcoholic liver disease, non-alcoholic fatty liver disease, and primary biliary cirrhosis[14].

However, these miRNAs could be a potential marker for liver toxicity in the human patient as well, specifically in the setting of acetaminophen overdose. The identification of early plasma markers of acetaminophen toxicity is necessary and paramount. Early identification of acetaminophen toxicity would identify those requiring more expeditious treatment, potentially improving morbidity and mortality of these individuals. It would also possibly abrogate the need for patient admission, mitigating the resultant financial system burden and iatrogenic risk of hospital-acquired infections.

MATERIALS AND METHODS

Acetaminophen toxicity

C57Bl/6 wild-type mice were purchased from Jackson Laboratory (Bar Harbor, ME) and received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School.

For all experiments, 6-wk-old female C57/BL6 mice, with food deprivation 12 h prior to experimentation, were used for intraperitoneal (ip) injections. For lethal dosing APAP experiments, 20 C57/BL6 mice were each injected with acetaminophen 500 mg/kg (0.9% normal saline suspension) ip at time zero. For the sublethal dosing APAP experiments, 20 C57/BL6 mice were injected with acetaminophen 150 mg/kg acetaminophen (0.9% normal saline suspension) ip at time zero. At times 0.5 h, 2 h, 12 h, 24 h, and 48 h, 5 mice per group (both lethal and sublethal) were sacrificed *via* cervical dislocation. Just prior to sacrifice, 400 mL of cheek blood was obtained from each mouse. The whole blood was then centrifuged at 14 000 *g* for 10 min at room temperature. The plasma was removed, aliquoted, and stored at -80 ℃. After sacri-

Figure 1 Plasma microRNA both up- and downregulated in lethally (500 mg/kg) compared to sublethally (150 mg/kg) dosed acetaminophen mice at the 12 h time point. A total of 528 microRNAs were screened using the reverse transcriptase[2] miRNA polymerase chain reaction (PCR) array of mouse whole genome, per the manufacturer's protocol (SABioscience, Qiagen Sciences, MD). Quantitative PCR data were analyzed using manufacturer software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

fice, livers were snap frozen in liquid nitrogen for protein, stored in RNA stabilization reagent (RNAlater, Qiagen, Hilden, Germany) for RNA extraction, or fixed in 10% neutral-buffered formalin for histopathologic analysis. Five mice were injected with saline only ip and sacrificed at time 48 h as controls.

Hepatotoxicity verification
Alanine aminotransferase (ALT) was quantified by biochemical assay (D-Tek Analytical Laboratories Inc, San Diego, CA).

Survival studies
Ten mice were injected with APAP 500 mg/mL ip and an additional 10 mice were injected with APAP 150 mg/mL, all at time zero. Mice were monitored for 48 h and the mortality for each recorded and plotted using Kaplan-Meier survival statistics (GraphPad Prism Software, LaJolla, CA). All mice were housed, watered and fed under the same conditions throughout the experimental protocol.

Histopathologic analysis
Sections of formalin-fixed, paraffin-embedded livers after sublethal and lethal APAP dosing were stained with hematoxylin and eosin and assessed for inflammatory infiltrate calculated with Microsuite (Olympus Soft Imaging Solution GmbH, Munster, Germany) image analysis software in 20 X objective.

MicroRNA library screen
MicroRNA was purified from plasma using the MiRNeasy Mini kit (Qiagen Sciences, MD). The cDNA was prepared using (reverse transcriptase[2]) RT[2] First Strand cDNA kit (SABiosciences, Qiagen Sciences, MD). The libraries were screened using pooled plasma samples from the 12 h time point for each APAP dosing parameter using saline injected mice as controls (5 mice/group). The

Figure 2 Increased hepatotoxicity (measured in elevated alanine aminotransferase levels) in lethally dosed acetaminophen mice over time. [a]$P < 0.05$ *vs* 0.5 h time point for the 150 mg/kg and 500 mg/kg treatment groups, respectively. ALT: Alanine aminotransaminase.

screening libraries utilized were the RT[2] miRNA PCR arrays for mouse whole genome, per the manufacturer's protocol (SABiosciences, Qiagen Sciences, MD). Real-time quantitative polymerase chain reaction (QPCR) was performed using RT[2] QPCR SYBR green MasterMix (SABiosciences, Qiagen Sciences, MD) and the iCycler iQ Cycler (Bio-Rad Laboratories, Inc, Hercules, CA).

QPCR data were analyzed using manufacturer software (http://pcrdataanalysis.sabiosciences. com/pcr/arrayanalysis.php).

RESULTS

APAP toxicity confirmation: ALT levels and survival
Numerous plasma miRNAs were both upregulated and downregulated, respectively, for the lethally compared to sublethally dosed APAP mice (Figure 1). Increase in serum levels of ALT is a well characterized marker of liver injury in the clinical setting as well as in animal models. Hepatotoxicity induced by APAP administration in our experiments was confirmed utilizing an ALT assay. An ALT average for the sublethal APAP dosing (150 mg/kg) peaked at 883 IU/L and for the lethal APAP dosing (500 mg/kg) peaked at 7396 IU/L, both at 24 h (Figure 2). There was a statistically significant increase in ALT levels in the lethal dosing compared to the sublethal dosing groups (642 IU/L compared to 6796 IU/L, respectively) starting at the 12 h time point ($P < 0.001$). There remained statistically significant increased ALT levels for both the sublethal and lethal dosed groups at the 48 h time point compared to the 0.5 h and 2 h time points (Figure 2).

With administration of a high dose APAP (500 mg/kg), we found that there was 90% lethality compared to no lethality with the sublethal dosing (150 mg/kg) at the 48 h time point as demonstrated by the nonparametric maximum likelihood estimate of a Kaplan-Meier survival curve (Figure 3). This difference was statistically significant at $P = 0.011$, with ten mice utilized per dosing pa-

Figure 3 Survival of lethally compared to sublethally dosed acetaminophen mice. Kaplan-Meier survival curve over time (h) after sublethal (150 mg/kg) and lethal (500 mg/kg) acetaminophen poisoning ($P = 0.011$).

Figure 4 Histopathologic analysis of liver after lethal compared to sublethal acetaminophen dosing. Hematoxylin and eosin liver tissue staining at 12 h of (A) sublethally (150 mg/kg) acetaminophen poisoned mice, with no signs of centrilobular inflammation or necrosis; and (B) lethally (500 mg/kg) acetaminophen poisoned mice with extensive centrilobular necrosis, enlarged hepatocytes, and highly vacuolated cytoplasm.

rameter analyzed (Graphpad Prism Software; LaJolla, CA).

APAP toxicity confirmation: Histology

For the sublethally dosed mice, the portal and periportal regions appear normal, with no signed of centrilobular necrosis or inflammation (Figure 4A). However, for the lethally dosed mice, obvious extensive centrilobular necrosis and inflammation were present, with distinctively en-

Table 1 Greatest fold plasma microRNA changes in lethally dosed acetaminophen mice

MicroRNA	Fold increase	MicroRNA	Fold decrease
574-5p	203.7	342-3p	0.0005
135a*	173.6	195	0.0041
466g	110.7	375	0.0085
1196	82.7	29c	0.0134
466f-3p	71	148a	0.0152
877	64.4	652	0.0199
139-3p	59.7	202-5p	0.0317
686	48.8	200a	0.039
346	47.5	320	0.0422
149	34.9	374*	0.0508
485	34.5	9*	0.0556
409-3p	30.7	342-5p	0.0629
202-3p	28.1	192	0.0656
298	27.4	412	0.0713
15a	27.1	1	0.0775
341	26.2	199b	0.0775
296-3p	24.3	741	0.0902
466i	22.1	100	0.1145
1186	21.4	18b	0.1604
200a	20.5	122	0.2348

larged hepatocytes and highly vacuolated cytoplasm. The lethal dosed liver also demonstrated pyknotic nuclei with extensive ballooning vacuolar degeneration (Figure 4B).

MicroRNA profile of APAP toxicity

In an attempt to evaluate plasma miRNAs as potential indicators of APAP-induced liver damage, we screened plasma of mice after administration of a lethal or sublethal dose of APAP. Out of the 528 murine miRNAs analyzed, there were more than 40 potential miRNAs that were both greater than 2-fold up- and downregulated in the lethal (500 mg/kg) compared to sublethal (150 mg/kg) dosing (Table 1). The miRNAs listed were effectively detected suggesting the actual fold-change value is as large as the calculated and reported fold-change result (SABiosciences, Qiagen Sciences, MD). The small nucleolar RNA, C/D Box 68 (Snord68), was used as the internal control for each library evaluated.

Finally, we performed an extensive literature search to gather information on the known and putative targets of 12 miRNAs that were uniquely changed in the plasma after administration of the lethal dose of APAP (Table 2). Interestingly, we found 6 out of the top 12 miRNAs with the greatest fold change (both up- and downregulated) in the lethally compared to sublethally dosed APAP mice were associated with asthma. The other miRNAs found both highly up- and downregulated were found to be associated with hypoxia-inducible factor (HIF)-1[15], follicle stimulating hormone[16], type 1 diabetes[17], procollagen type Ⅲ[18], colon cancer[19], gastric carcinoma[20], and elongation factor 2 tumor suppression[21] (Table 2).

DISCUSSION

Our study reveals numerous miRNAs, notably 574-5p, 135a*, 466g, 1196, 466f-3p, and 877, are upregulated

Table 2 Lethal acetaminophen associated plasma microRNAs and potential correlative function

MicroRNA	Function
574-5p	Acute and chronic asthma (Garbacki *et al*[23]); Procollagen type III A1 (Sterling[18])
135a	Hypoxia-inducible factor-1 and nuclear factor-κB production (Gonsalves *et al*[15])
466g	Acute and chronic asthma (Garbacki *et al*[23])
1196	Follicle-stimulating hormone regulation (Yao *et al*[16])
466f-3p	Acute and chronic asthma (Garbacki *et al*[23]); Procollagen type III A1 (Sterling[18])
877	Human type 1 diabetes (Zhou *et al*[17])
139-3p	Colon and rectal cancer (Slattery *et al*[19])
342-3p	HBV infection and HBV-positive hepatocarcinoma biomarker (Li *et al*[10])
195	E2F tumor suppressor (Xu *et al*[21])
375	Acute and chronic asthma (Garbacki *et al*[23]); Pyruvate dehydrogenase kinase inhibition in gastric carcinomas (Tsukamoto *et al*[20])
29c	Acute and chronic asthma (Garbacki *et al*[23])
148a	HLA-G and risk asthma (Tan *et al*[22])

HBV: Hepatitis B virus; E2F: Elongation factor 2; HLA-G: Human leukocyte antigen-G.

in the setting of lethal compared to sublethal APAP-associated hepatotoxicity, whereas miRNAs 342-3p, 195, 375, 29c, 148a and 652 are markedly downregulated. We demonstrate elevated ALT levels as well as histologic evidence supporting worsened hepatotoxicity in the setting of lethally dosed mice compared to non-lethally dosed mice (Figures 2 and 4). With 90% lethality in the lethally dosed mice in relation to no lethality in sublethally dosed mice ($P = 0.0011$), this supports the premise that unique plasma miRNA profiles may correlate with non- *vs* life-threatening APAP dosing (Figure 3). The fold-change of a variety of miRNAs in the setting of lethally dosed mice compared to sublethal doses is of interest. Of note, more than 40 were both up- and downregulated, with the greatest fold miRNA changes reported (Table 1).

A literature search to investigate possible functions of the miRNAs both up- and downregulated was undertaken. Intriguingly, many of those most up- and downregulated in the lethally compared to sublethally dosed mice, namely 574-5p, 466g, 466f-3p, 375, 29c, and 148a, have also been implicated in the development of asthma[22,23]. For instance, 574-5p may be involved with asthma pathogenesis, with decreased miRNA 574-5p in chronic compared to acute asthma in a mouse model sensitized with ovalbumin[23]. In another study, a potential relationship between histocompatibility antigen-G, chronic asthma, and miRNA 29c was determined[22]. This consequently suggests a pathophysiologic relationship between APAP toxicity and asthma[22,23].

Interestingly, prior research has shown an association between APAP use and asthma although the exact association is still unclear[24,25]. For instance, an adult case control study described a relationship between acetaminophen use and asthma[26]. Additional literature revealed an increased risk of wheeze in children whose mothers

used prenatal APAP[27]. The etiology still remains unclear. However, one theory is that decreased glutathione (due to depletion secondary to APAP toxicity) provides the opportunity for unchecked reactive oxygen species to promote asthma development[24]. Additional theories include increased prostaglandin E2 production secondary to elevated cyclooxygenase-2 activity in the presence of APAP promoting a T2 allergic response[25]. A third cause could be direct lung damage from NAPQI, a byproduct of APAP metabolism[28]. Clearly, more information is needed to further elucidate the relationship between APAP and risk of asthma.

The previous literature describing the function of the other miRNAs up- and downregulated in our study is more varied. For example, some literature reveals a miRNA 135a association with HIF 1-α[15]. Interestingly, previous literature has demonstrated HIF 1-α induction prior to APAP toxicity in the setting of lethal APAP dosing, with toxicity prevented by the presence of cyclosporine A, a HIF 1-α inhibitor which prevents mitochondrial permeability transition and oxidative stress[29]. Additional studies have also shown elevation of HIF 1-α in the setting of APAP toxicity, with increased HIF 1-α causing increased glucose transporter-1 expression[30]. Of note, miRNAs 195 and 342-3p have been shown as involved with hepato cytopathology in the setting of tumor suppression in hepatocellular carcinoma models[21] and hepatitis B virus hepatocarcinoma diagnosis[10], respectively.

However, how these miRNAs in total affect hepatotoxicity in the setting of APAP poisoning still needs to be elucidated. Prior studies reveal upregulation of plasma miR-122 in the setting of APAP-associated liver toxicity, while our data suggest downregulation of plasma miR-122 at the 12 h time point. This discrepancy could be potentially explained by examination of upregulation of miR-122 at 1 h, 3 h and 24 h time points, not at a 12 h time point in previous literature. In addition, different APAP dosing levels were used in each study (300 mg/kg compared to 500 mg/kg), again demonstrating the dynamic nature of miRNA regulation across both time and clinical setting[11]. Of note, we found upregulation of plasma miR-298 and miR-370, whereas other researchers found downregulation of these miRNAs in the setting of APAP-associated hepatotoxicity[31]. Again, this may be due to evaluation at different time points (6 h compared to 12 h) and differing APAP dosing parameters (1000 mg/kg compared to 500 mg/kg)[31]. Together, these results demonstrate the need for further identification of additional plasma microRNA profiles at various time points and dosing levels.

Our ultimate goal would be to eventually have a miRNA APAP nomogram to be used for human patient care, similar to the previous effective Rumack-Matthew nomogram[32,33]. The problem with this current nomogram, however, is that it relies on knowing when a patient initially ingested APAP. This is often difficult, if patients are poor historians or have ingested mind-altering substances

such as alcohol at the time of evaluation. In addition, patients may have been taking APAP chronically, not acutely, making use of the Rumack-Matthew nomogram pointless. The importance of our work is to establish a novel miRNA nomogram that would be used for patients who have taken APAP at an unknown time or with chronic ingestions. In turn, this would avoid investigation of patients who have taken it chronically, thus preventing unnecessary treatment and iatrogenic ingestions. Additional studies in this field, with additional time points and dosing levels, however, are clearly still necessary.

The specificity of this profile also requires further improvement. For instance, in a recent report miRNA-122 was shown to be increased in the setting of APAP toxicity, although this increase was under the detection cut-off of our study[11]. Of note, miRNA-122 is also upregulated in hepatitis C settings which, by itself, is evidently not specific enough to uniquely identify APAP toxicity[14]. However, with additional future studies and data analysis, this may possible. This approach may also be used as a model to develop profiles for additional disease processes, namely non-alcoholic fatty liver disease and hepatocellular carcinoma, since microRNA profiles are already being used as early biomarkers for numerous pathologic states[9,10]. Together, this may improve the diagnostic accuracy of hepatopathology, namely early APAP-induced hepatotoxicity. In turn, this may allow clinicians to better and more rapidly distinguish which patients who have ingested APAP will actually mandate therapy. Subsequently, this may result in decreasing the number of patients who receive unnecessary, expensive empiric treatment.

In conclusion, lethal dosing of APAP in a murine model is consistent with hepatotoxicity and up- and downregulation of a unique pattern of circulating plasma miRNAs, which is different from the plasma miRNA profile associated with sublethal APAP dosing. These differences may be useful in the future to distinguish lethal and sublethal APAP toxicity in humans.

COMMENTS

Background
Acetaminophen (APAP) continues to be an important cause of acute liver failure in the developed world, being the most common cause of death due to analgesic ingestion in the United States. The authors report unique microRNA (miRNA) profiles associated with lethal acetaminophen poisoning. Determining which specific miRNAs are associated with lethal acetaminophen toxicity may prove helpful in the future for prognosticating which patients will require N-acetylcysteine treatment.

Innovations and breakthroughs
The importance of our work is to establish a novel miRNA nomogram that would be used for patients who have taken APAP at an unknown time or with chronic ingestions. This would be the first miRNA profile that could prognosticate patients who will require treatment for hepatotoxicity due to acetaminophen poisoning.

Applications
The ultimate goal is to eventually have a miRNA APAP nomogram to be used for human patient care. The limitation of the current Rumack-Matthew nomogram for APAP toxicity is that it relies on knowing the time of APAP ingestion. This is often difficult if patients are poor historians or have ingested mind-altering substances at the time of evaluation, or have been chronically ingesting APAP.

Terminology
Recent work has also shown the medical utility of miRNA. miRNAs, are short, chemically stable biomolecules that produce gene silencing. Furthermore, additional literature has described miRNA involvement in acetaminophen toxicity, as well as the utility of miRNAs as biomarkers.

Peer review
The manuscript provides some interesting information on the potential use of miRNAs for the early diagnosis of APAP associated liver toxicity. The scientific goal of the study has significant clinical application but the interpretation of the data needs to be revised.

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BRIEF ARTICLE

Pre-diagnostic levels of adiponectin and soluble vascular cell adhesion molecule-1 are associated with colorectal cancer risk

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Abstract

AIM: To examine the relationships between pre-diagnostic biomarkers and colorectal cancer risk and assess their relevance in predictive models.

METHODS: A nested case-control study was designed to include all first primary incident colorectal cancer cases diagnosed between inclusion in the SUpplémentation en VItamines et Minéraux AntioXydants cohort in 1994 and the end of follow-up in 2007. Cases (*n* = 50) were matched with two randomly selected controls (*n* = 100). Conditional logistic regression models were used to investigate the associations between pre-diagnostic levels of hs-CRP, adiponectin, leptin, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1, E-selectin, monocyte chemoattractant protein-1 and colorectal cancer risk. Area under the receiver operating curves (AUC) and relative integrated discrimination improvement (RIDI) statistics were used to assess the discriminatory potential of the models.

RESULTS: Plasma adiponectin level was associated with decreased colorectal cancer risk (*P* for linear trend = 0.03). Quartiles of sVCAM-1 were associated with increased colorectal cancer risk (*P* for linear trend = 0.02). No association was observed with any of the other biomarkers. Compared to standard models with known risk factors, those including both adiponectin and sVCAM-1 had substantially improved performance for colorectal cancer risk prediction (*P* for AUC improvement = 0.01, RIDI = 26.5%).

CONCLUSION: These results suggest that pre-diagnostic plasma adiponectin and sVCAM-1 levels are associated with decreased and increased colorectal cancer risk, respectively. These relationships must be confirmed in large validation studies.

Key words: Colorectal cancer; Adiponectin; Soluble vascular cell adhesion molecule-1; Nested case-control study;

Prospective study

INTRODUCTION

Colorectal cancer is the third most frequently diagnosed cancer worldwide, accounting for more than one million cases and 600 000 deaths every year[1]. The identification of pre-diagnostic biomarkers associated with subsequent colorectal cancer risk is a key challenge. Markers of adiposity, endothelial adhesion, and inflammation may be suitable candidates[2-5]. Adipose tissue is an endocrine organ that produces adipokines and plays a critical role in the regulation of inflammatory processes[6]. Leptin reflects body fat storage and acts as a pro-inflammatory adipokine. Conversely, adiponectin production is decreased in obesity and generally has anti-inflammatory properties. Adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the chemokine monocyte chemoattractant protein-1 (MCP-1) are important in cell-cell and cell-basement membrane interactions. They are also intimately involved in inflammatory reactions[7]. C-reactive protein (CRP) is a widely used systemic biomarker for diagnosing acute and chronic inflammation[8].

Previous cross-sectional studies suggest the potential involvement of these biomarkers in colorectal carcinogenesis, with higher blood levels of CRP[9], leptin[10], soluble adhesion molecules[11,12], and lower levels of adiponectin[10,13] observed in patients with colorectal cancer compared to controls. The prognostic value of these markers has also been suggested by research with colorectal cancer patients[10,12]. However, few prospective studies have investigated the association between these biomarkers and colorectal cancer risk, and the current evidence is conflicting[14-19]. In addition, such studies did not evaluate the discriminatory capabilities of these biomarkers regarding colorectal cancer risk by contemporary statistical methods[20,21].

Thus, our objectives were twofold: (1) to prospectively examine the relationships between biomarkers of adiposity, endothelial adhesion, and inflammation and development of colorectal cancer; and (2) to statistically compare the pertinence of models including these bio-markers to standard models with known risk factors of colorectal cancer.

MATERIALS AND METHODS

Study population

The SUpplémentation en VItamines et Minéraux AntioXydants (SU.VI.MAX) study is a population-based, double-blind, placebo-controlled, randomized trial initially designed to assess the effect of a daily antioxidant supplementation on the incidence of cardiovascular disease and cancer[22,23]. A total of 13 017 subjects were enrolled in 1994-1995. The intervention study lasted 8 years, and follow-up of health events was maintained until July 2007. Subjects provided written informed consent and the study was approved by the Ethics Committee for Studies with Human Subjects at the Paris-Cochin Hospital, "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale", No. 706 and the "Commission Nationale de l'Informatique et des Libertés", No. 334641.

Baseline data collection

At enrolment, all participants underwent a clinical examination and anthropometric measurements carried out by study nurses and physicians. The participants also completed questionnaires on socio-demographic data, smoking, alcohol intake and physical activity. A fasting venous blood sample was obtained. Plasma aliquots were immediately prepared and stored frozen in liquid nitrogen.

Case ascertainment

Confirmed or suspected cancer events were self-reported by subjects during the follow-up process. Investigations were conducted for all such events to obtain medical data from participants, physicians and/or hospitals. All information was reviewed by an independent expert committee and cancer cases were validated by pathological report and classified using the International Chronic Diseases Classification, 10th Revision, Clinical Modification.

Nested case-control study

All first primary incident colorectal cancer cases diagnosed between inclusion in the SU.VI.MAX cohort in 1994 and July 2007 were included in the present study. For each cancer case, two controls were randomly selected among the remaining participants with complete follow-up data and without cancer diagnosis by the end of follow-up. Cases and controls were matched for sex, age (by 2-year strata), body mass index (BMI, $<$ *vs* $\geq$ 25 kg/m^2) and intervention group.

Baseline plasma samples of the selected subjects were used to determine the levels of highly-sensitive CRP (hs-CRP), leptin, adiponectin, soluble ICAM-1 (sICAM-1), soluble VCAM-1 (sVCAM-1), soluble E-selectin (sE-selectin) and MCP-1. Biomarker levels were determined with ELISA sandwich technique (R and D Laboratory Systems). Intra-assay (IACV) and inter-assay (IRCV) co-

Table 1 Baseline characteristics of colorectal cancer cases and controls

	Cases (*n* = 50)		Controls (*n* = 100)		*P* value[1]
Age, yr	51.8	± 5.6	52.1	± 5.6	0.8
Gender					1.0
Men	28	56.0%	56	56.0%	
Women	22	44.0%	44	44.0%	
Intervention group					1.0
Yes	27	54.0%	54	54.0%	
No (placebo)	23	46.0%	46	46.0%	
BMI, kg/m²					1.0
< 25	24	48.0%	48	48.0%	
≥ 25	26	52.0%	52	52.0%	
Waist circumference, cm	88.2	± 12.9	82.2	± 12.1	0.01
Height, cm	169.3	± 7.1	167.8	± 8.5	0.3
Smoking status					0.9
Never smoker	23	46.0%	46	46.0%	
Former smoker	21	42.0%	40	40.0%	
Current smoker	6	12.0%	14	14.0%	
Alcohol intake, g/d	24	± 24.4	15.4	± 16.3	0.01
Physical activity					0.4
Low	10	20.0%	26	26.0%	
Moderate	18	36.0%	25	25.0%	
High	22	44.0%	49	49.0%	
Educational level, yr					0.9
< 12	30	60.0%	61	61.0%	
≥ 12	20	40.0%	39	39.0%	
Family history of colorectal cancer[2]					
No	45	90.0%	84	84.0%	0.3
Yes	5	10.0%	16	16.0%	
Plasma levels of biomarkers					
Adiponectin, μg/mL	9.0	± 4.7	10.9	± 7.5	0.2
Leptin, ng/mL	8.5	± 5.3	8.6	± 8.7	0.5
sVCAM-1, ng/mL	750.3	± 316.2	677.6	± 215.3	0.2
sICAM-1, ng/mL	249.7	± 80.3	247.8	± 67.3	0.9
sE-selectin, ng/mL	41.1	± 16.9	39.3	± 16.0	0.7
MCP-1, pg/mL	268.2	± 117.4	249	± 78.2	0.3
hs-CRP, mg/L	2.4	± 4.5	2.2	± 4.4	0.3

[1]*P* value for the comparison of cases and controls by Student *t* test or χ^2 test, as appropriate. Biomarker variables were log-transformed to improve normality. Values are mean ± SD or *n* % as appropriate. [2]In first degree relatives. BMI: Body mass index; hs-CRP: Highly sensitive C-reactive protein; sICAM-1: Soluble intercellular adhesion molecule-1; sVCAM-1: Soluble vascular cell adhesion molecule-1; sE-selectin: Soluble E-selectin; MCP-1: Monocyte chemoattractant protein-1.

efficients of variation were all < 10%. hs-CRP had the lowest (1.6%) and MCP-1 had the highest (6.2%) IACV, and hs-CRP had the lowest (3.6) and sE-selectin had the highest (9.1%) IRCV.

Statistical analyses

The participants' baseline characteristics were compared between colorectal cancer cases and controls using Student's *t*-tests or χ^2 tests. Associations between biomarkers and incident colorectal cancer were examined with conditional logistic regression models and expressed as odds ratios (OR) with 95% confidence intervals (CI). The ORs for sex-specific quartiles and for a 1 standard deviation (SD) increase in the corresponding biomarker were com-

puted in unadjusted and multivariate models. Multivariate models were adjusted for age, sex, BMI, height, intervention group, alcohol intake, physical activity, smoking status, family history of colorectal cancer, waist circumference and educational level.

The improvement in colorectal cancer prediction performance attributed to the biomarkers was assessed with both the area under the receiver operating curves (AUC) and the more recently proposed statistical tool, the Relative Integrated Discrimination Improvement (RIDI)[21]. The latter measures the percentage of increased discrimination upon addition of another variable to the prediction model. The Bootstrap method was used to derive the 95% CI for the RIDI estimates, which were based on 1000 replications. The added prediction performance was determined separately for each biomarker identified as statistically significantly associated with cancer risk (in the logistic regression analyses step), and then for a combination of these biomarkers simultaneously. Tests of significance for AUC improvement were one-sided, as improvement in model fit was expected. All other statistical tests were two-sided, and *P* < 0.05 was considered significant. Analyses were performed with SAS software (v9.1, Cary, NC, United States).

RESULTS

A total of 50 incident colorectal cancer cases were diagnosed during follow-up (30 colon and 20 rectal cancers). Each case was matched with two randomly selected controls; thus, 150 subjects were included in the analyses. Median follow-up was 6.5 years in cases and 13 years in controls. Baseline characteristics of cases and non-cases are presented in Table 1. Compared to controls, cancer cases had a higher waist circumference and a higher alcohol intake.

In multivariate models, a one SD change in plasma adiponectin level was associated with a decreased colorectal cancer risk [OR (95% CI) = 0.45 (0.22-0.91), *P* = 0.03]. This association was also observed when adiponectin was considered as quartiles (OR for Q4 *vs* Q1 = 0.11 (0.01-0.93), *P* for linear trend = 0.03) (Table 2).

Quartiles of plasma sVCAM-1 level were positively associated with increased colorectal cancer risk (*P* for linear trend = 0.02) (Table 2). This association was borderline non-significant when sVCAM-1 was coded as a continuous variable (*P* = 0.07).

Unadjusted models (matching factors only) showed similar results (data not shown). A sensitivity analysis excluding cases that were diagnosed during the first two years of follow-up (7 cases) did not modify the findings, nor did sensitivity analyses excluding subjects with high hs-CRP values (> 15.5 ng/mL, *i.e.*, mean + 3SD, *n* = 3 subjects; data not shown).

Indicators of the predictive potential of colorectal cancer risk models (Table 3) showed improvement when adiponectin alone was included in the multivariate model

Table 2 Odds ratios and 95% confidence intervals for quartiles of each biomarker level and colorectal cancer risk from multivariate conditional logistic regression models[1]

	For a change in 1SD	Quartile1	Quartile2	Quartile3	Quartile4
Adiponectin					
OR	0.45	1 (ref)	0.83	0.42	0.11
95% CI	0.22-0.91		0.12-5.65	0.06-2.93	0.01-0.93
P for linear trend	0.03				0.03
Leptin					
OR	0.55	1 (ref)	0.19	2.22	0.29
95% CI	0.21-1.40		0.02-1.9	0.25-20.09	0.02-3.65
P for linear trend	0.2				0.6
sVCAM-1					
OR	1.69	1 (ref)	6.89	11.59	19.11
95% CI	0.96-2.98		0.72-66.4	0.64-209.81	1.4-261.27
P for linear trend	0.07				0.02
sICAM-1					
OR	0.74	1 (ref)	0.38	0.07	0.13
95% CI	0.40-1.40		0.04-3.23	0.01-0.76	0.01-1.93
P for linear trend	0.4				0.08
sE-selectin					
OR	0.95	1 (ref)	1.23	0.9	1.59
95% CI	0.49-1.81		0.13-11.49	0.1-8.14	0.16-15.62
P for linear trend	0.9				0.9
MCP-1					
OR	1.35	1 (ref)	1.67	1.02	2.02
95% CI	0.73-2.49		0.27-10.24	0.17-6.24	0.26-15.97
P for linear trend	0.3				0.4
hs-CRP					
OR	0.8	1 (ref)	0.73	2.22	1.53
95% CI	0.52-1.24		0.06-9.38	0.25-19.9	0.13-17.84
P for linear trend	0.3				0.6

[1]Adjusted for age, sex, body mass index, intervention group, alcohol intake, physical activity, smoking status, family history of colorectal cancer, waist circumference, height and educational level. *n* = 50 colorectal cancer cases and 100 controls. Cut-offs for sex-specific quartiles were: hs-CRP: 0.6, 1.2, 2.3 in men and 0.5, 0.9, 2.1 in women; sICAM-1: 198.7, 242.0, 287.4 in men and 193.0, 232.5, 286.0 in women; sVCAM-1: 539.0, 653.5, 798.6 in men and 523.0, 651.5, 875.7 in women; sE-selectin: 29.8, 42.5, 51.7 in men and 26.0, 36.3, 44.6 in women; MCP-1: 216.7, 263.5, 316.5 in men and 171.0, 212.0, 238.0 in women; Leptin: 3.1, 5.0, 8.2 in men and 5.4, 9.6, 15.4 in women; Adiponectin: 4.2, 6.6, 10.0 in men and 9.5, 13.9, 16.0 in women. OR: Odds ratio; CI: Confidence interval; hs-CRP: Highly sensitive C-reactive protein; sICAM-1: Soluble intercellular adhesion molecule-1; sVCAM-1: Soluble vascular cell adhesion molecule-1; sE-selectin: Soluble E-selectin; MCP-1: Monocyte chemoattractant protein-1; ref: Reference category.

(*P* for AUC improvement = 0.009). The RIDI statistic indicated a 12.2% (10.9-13.6) improvement. Improvement in the prediction of colorectal cancer risk was limited when sVCAM-1 only was introduced into the multivariate model (*P* for AUC improvement = 0.09), with 9.9% (8.7-11.0) improvement, as indicated by the RIDI statistic. Prediction was substantially improved when adiponectin and sVCAM-1 were simultaneously included in the multivariate model: *P* for AUC improvement was equal to 0.01, and the RIDI reached 26.5% (24.4-28.7).

DISCUSSION

In this prospective study, pre-diagnostic plasma adiponectin level was associated with decreased colorectal cancer risk, independently of other known risk factors. On the contrary, plasma sVCAM-1 level was associated with increased colorectal cancer risk. Models including these two biomarkers showed significantly improved discriminatory capabilities compared to models including only established risk factors.

Lower levels of circulating adiponectin have been observed in prevalent colorectal cancer cases compared to controls[10,13,24-26]. Single nucleotide polymorphism analyses have found that some variants of the adiponectin genes are related to either increased (rs822395, rs1342387) or decreased (rs266729) colorectal cancer risk[27], although no association was detected in a recent study in the United Kingdom[28]. Another study suggested that variants of the adipokine genes may affect colorectal cancer risk in combination with variants in diabetes-related genes[29]. Studies with colorectal cancer patients showed that higher adiponectin levels were associated with a better prognosis[10,13,30]. It has been suggested that adiponectin may be used for estimation of advanced stage of cancer and for estimating risk of cancer recurrence[31]. However, to date, only three nested case-control studies have investigated the prospective association between adiponectin and colorectal cancer risk, showing inconsistent results[16-18]. Two studies did not find any associations; one of them included 381 male colorectal cancer cases[17] and the other included 306 colorectal cancer cases of both genders[16]. Consistent with our findings, the study of Wei *et al*[18], based on 179 male colorectal cancer cases, found an

Table 3 Predictive potential of adiponectin and soluble vascular cell adhesion molecule-1 regarding colorectal cancer risk: Relative integrated discrimination improvement and improvement of area under the curve

	AUC	*P* value for AUC improvement	RIDI (%)	95% CI
Multivariate model[1]	0.89			
+ Adiponectin	0.98	0.009	12.2	10.9-13.6
+ sVCAM-1	0.92	0.09	9.9	8.7-11.0
+ Adiponectin + sVCAM-1	0.98	0.01	26.5	24.4-28.7

[1]Multivariate model was adjusted for age, sex, BMI, intervention group, alcohol intake, physical activity, smoking status, family history of colorectal cancer, waist circumference, height and educational level. Models including adiponectin and/or sVCAM-1 were compared to the multivariate model. *n* = 50 colorectal cancer cases and 100 controls. BMI: Body mass index; RIDI: Relative integrated discrimination improvement; AUC: Area under the receiver operating curve; sVCAM-1: Soluble vascular cell adhesion molecule-1; CI: Confidence interval.

inverse association between pre-diagnostic adiponectin levels and colorectal cancer risk. Circulating levels of adiponectin in those studies were comparable to the levels found in the present study. However, none of those three studies matched cases and controls on BMI. Adiponectin is strongly related to adiposity, which is, in turn, associated with an adverse effect on colorectal cancer development, especially in stathmin-positive patients, as recently shown by Ogino *et al*[32]. Thus, matching on BMI is crucial and is a strength of our study compared to previous reports in the literature. Several mechanisms support the inverse relationship between adiponectin and colorectal cancer risk[33]. Adiponectin suppresses tumorigenesis in Apc(Min)(/+) mice[34] and also suppresses colonic epithelial proliferation *via* inhibition of the mammalian target of the rapamycin (mTOR) pathway under a high-fat diet[35]. It inhibits colorectal cancer cell growth through the AMP-activated protein kinase/mTOR pathway[36] and possibly the PI3K/Akt signal pathway[37]. Adiponectin also attenuates interleukin-6-induced colon carcinoma cell proliferation *via* STAT-3[38].

Several case-control studies have observed higher circulating levels of sVCAM-1 in colorectal cancer cases compared to controls[11,12,39-41]. In addition, it has been suggested that the serum level of sVCAM-1 may be a valuable prognostic marker in colorectal carcinoma[12,42], reflecting both tumour progression and metastasis[39]. For instance, Mantur *et al*[11] observed a significant correlation of serum levels of sVCAM-1 with tumor, node, metastases (TNM) stage and lymph node involvement in colorectal cancer patients. Yamada *et al*[43] observed a positive association between concentrations of sVCAM-1 and risk of postoperative colorectal cancer recurrence. Consequently, investigations have been conducted to test for the chemopreventive potential of some molecules (e.g., celecoxib) *via* down-regulation of VCAM-1 in the colon cancer cell line HT29[44].

However, to the best of our knowledge, our study is the first to investigate the prospective association be-

tween pre-diagnostic levels of sVCAM-1 and colorectal cancer risk. The observed positive association is supported by a mechanistic plausibility. Indeed, it has been demonstrated experimentally that sVCAM-1 stimulates angiogenesis and neovascularization[45,46] and is negatively correlated with the degree of tumour differentiation[41]. Cell adhesion molecule expression has been demonstrated in endothelial cells of small vessels at the invasive margin of tumour cells involved in metastatic spread[47]. The association among immunohistochemical cell adhesion molecule expression, tumour vascularity and leukocyte infiltration suggests an important role for these molecules in host immune response and in tumour progression[48].

Epidemiologic studies usually estimate the strength of the association between a biomarker and disease risk. Assessment of the discriminatory capabilities of a biomarker in predicting risk of the studied pathology is another approach that may lead to slightly different but complementary information[21]. To the best of our knowledge, no study has previously evaluated the discriminatory capabilities of hs-CRP, leptin, adiponectin, sICAM-1, sVCAM-1, sE-selectin and MCP-1 in predicting colorectal cancer risk, using *ad-hoc* statistical methods such as the novel RIDI statistic[21]. Indeed, the use of the traditional AUC method as a comparative measure of prediction between models has certain limitations[49], and the complementary use of the novel RIDI statistic appears to be more sensitive and accurate[21]. Several factors are already known to influence colorectal cancer risk (e.g., age, smoking status, physical activity, *etc.*) and are usually included in predictive models. As shown in Table 3, the RIDI statistic suggests that when quartiles of adiponectin and quartiles of sVCAM-1 plasma levels are added to the model, the ability of the model to predict colorectal cancer risk is improved by 26.5%, compared to a model including only well-established risk factors (age, smoking status, *etc.*). Thus, our results suggest that adiponectin, and possibly sVCAM-1, should not be ignored as predictors of colorectal cancer risk. In addition, the improvement in the predictive potential was substantially increased when both biomarkers were simultaneously added to the model. This might result from the mechanistic interrelations between adiposity and endothelial adhesion, notably though an inflammation pathway[6,50,51]. Large prospective and validation studies are needed to confirm and better quantify the predictive performance of these biomarkers in colorectal carcinogenesis.

Strengths of our study include its prospective design, the simultaneous measurement of seven biomarkers in the same individuals and, to our knowledge, the first assessment of the discriminatory capabilities of these biomarkers for estimating colorectal cancer risk by the novel RIDI statistic.

Some limitations should also be acknowledged. Firstly, the number of cases was limited in this exploratory study. This may explain some of the null results observed; however, it is unlikely to explain the observed relationships between adiponectin, sVCAM-1 and colorectal cancer

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BRIEF ARTICLE

Patatin-like phospholipase domain containing-3 gene I148M polymorphism, steatosis, and liver damage in hereditary hemochromatosis

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Abstract

AIM: To investigate whether the *patatin-like phospholipase domain containing-3* gene (*PNPLA3*) I148M polymorphism is associated with steatosis, fibrosis stage, and cirrhosis in hereditary hemochromatosis (HH).

METHODS: We studied 174 consecutive unrelated homozygous for the C282Y HFE mutation of HH (C282Y+/+ HH) patients from Northern Italy, for whom the presence of cirrhosis could be determined based on histological or clinical criteria, without excessive alcohol intake (< 30/20 g/d in males or females) or hepatitis B virus and hepatitis C virus viral hepatitis. Steatosis was evaluated in 123 patients by histology (n = 100) or ultrasound (n = 23). The PNPLA3 rs738409 single nucleotide polymorphism, encoding for the p.148M protein variant, was genotyped by a Taqman assay (assay on demand, Applied Biosystems). The association of the PNPLA3 I148M protein variant (p.I148M) with steatosis, fibrosis stage, and cirrhosis was evaluated by logistic regression analysis.

RESULTS: PNPLA3 genotype was not associated with metabolic parameters, including body mass index (BMI), the presence of diabetes, and lipid levels, but the presence of the p.148M variant at risk was independently associated with steatosis [odds ratio (OR) 1.84 per p.148M allele, 95% confidence interval (CI): 1.05-3.31; P = 0.037], independently of BMI and alanine aminotransaminase (ALT) levels. The p.148M variant was also associated with higher aspartate aminotransferase (P = 0.0014) and ALT levels (P = 0.017) at diagnosis, independently of BMI and the severity of iron overload. In patients with liver biopsy, the 148M variant was independently associated with the severity (stage) of fibrosis (estimated coefficient 0.56 ± 0.27, P = 0.041). In the overall series of patients, the p.148M variant was associated with cirrhosis in lean (P = 0.049), but not in overweight patients (P = not significant). At logistic regression analysis, cirrhosis was associated with BMI $\geq$ 25 (OR 1.82, 95% CI: 1.02-3.55), ferritin > 1000 ng/mL at diagnosis (OR 19.3, 95% CI: 5.3-125), and with the G allele in patients with BMI < 25 (OR 3.26, 95% CI: 1.3-10.3).

CONCLUSION: The PNPLA3 I148M polymorphism may represent a permissive factor for fibrosis progression in patients with C282Y+/+ HH.

INTRODUCTION

Hereditary hemochromatosis (HH) is a genetic disorder of iron metabolism characterized by defective release or activity of hepcidin, the hepatic hormone that inhibits iron absorption by binding and inactivating ferroportin[1]. HH is most frequently related to hampered hepcidin up-regulation by iron stores as a consequence of homozygosity for the C282Y mutation in the *HFE* gene[2]. The resultant increase in serum iron leads to progressive accumulation in the liver and other parenchymal organs, however, although hepatic iron overload leads to progressive liver fibrosis and cirrhosis in some affected individuals, the phenotypic expression is unpredictable and highly variable[3].

Indeed, liver disease is the most frequent clinical manifestation of homozygous for the C282Y HFE mutation of HH (C282Y+/+ HH), but it is now clear that only a proportion of subjects carrying this genotype will ever develop hepatic fibrosis[4]. Most of C282Y +/+ male subjects develop expanded iron stores during life, whereas due to the physiological iron losses during fertile age, the female gender represents a major protective factor. In population based screening studies it has been shown that between 75% and 94% of C282Y+/+ males develop elevated transferrin saturation, and that 64% to 68% will have an increased serum ferritin[4-8]. However, even in males, the prediction of risk of clinical disease remains uncertain[9].

The recognition of the incomplete penetrance of HH has led to a search for genetic and other modifiers of clinical expression. HH expression may be influenced at different levels[9]: (1) by factors affecting iron loading, including sex and genetic factors (genes regulating hepcidin expression, beta-thalassemia trait[10]); (2) by factors influencing the progression to liver disease, such as hepatic steatosis[11], viral hepatitis, genes regulating pro-inflammatory cytokines and oxidative injury[12,13]; and (3) by those regulating both, such as alcohol intake and hepatitis C virus (HCV) infection[14,15].

There is established evidence that increased body mass (BMI) and the metabolic syndrome[16] are strong risk factors for hepatic steatosis[17] and that steatosis accelerates the progression of liver diseases by favoring oxidative stress and hepatocellular damage. In 214 C282Y +/+ patients[11], a significant association between steatosis and the presence of fibrosis was detected. This relationship remained significant after adjustment for confounding factors such as alcohol intake and iron loading.

Recently, the rs738409 C > G single nucleotide polymorphism (SNP) of *patatin-like phospholipase domain containing-3* gene (*PNPLA3*), encoding for the I148M protein variant (p.I148M), has been identified as a determinant of liver fat content and of the susceptibility to develop steatohepatitis and progressive fibrosis[18-23]. Importantly, PNPLA3 genotype influences liver fat independently of body mass, dyslipidemia, and insulin resistance[24,25].

Since hepatic steatosis has been reported to influence HH expression, the aim of this study was to determine whether the PNPLA3 I148M variant predisposes to the development of steatosis, and to progressive liver damage, as evaluated fibrosis stage and the presence of cirrhosis, in patients with pure C282Y+/+ HH stratified according to the presence of overweight.

MATERIALS AND METHODS

Patients

From 232 consecutive unrelated C282Y+/+ HH patients referred to two centers in the Milan area of Northern Italy, we excluded subjects with alcohol intake > 30/20 g per day in male/female, hepatitis B virus (HBV) and/or HCV infections, and other cofactors of liver disease (n = 30), and those with an uncertain diagnosis of cirrhosis or incomplete clinical data (n = 28), and finally included 174 patients in the analysis (Figure 1). DNA samples were available for all patients.

Diagnosis of cirrhosis was based upon liver histology (n = 100) or clinical evidence (n = 74): in particular, cirrhosis was diagnosed by liver histology in 26 patients, and by clinical criteria in 6 cases (in the presence of hepatic decompensation or of portal hypertension; liver biopsy was not indicated for ethical reasons), whereas it was excluded by liver histology in 74 cases, and by clinical criteria in the remaining 68 cases (when liver biopsy was not indicated and not performed for ethical reasons).

Tissue sections were stained with hematoxylin and eosin, impregnated with silver for reticulin framework, and stained with trichrome for collagen and Perls for iron. Steatosis was considered present when involving at least 5% of hepatocytes and graded according to Kleiner[26]. Tissue iron was graded according to Scheuer[27]. Fibrosis was scored according to Ishak[28]. The minimum biopsy size was 1.7 cm and the number of portal areas was 10. For data analysis, a fibrosis stage of 6 was attributed to patients with a clinical diagnosis of cirrhosis.

Ultrasonographic diagnosis of steatosis at diagnosis

Figure 1 Study flow chart. C282Y+/+ HH: Homozygous for the C282Y HFE mutation of hereditary hemochromatosis; M/F: Male/female; BMI: Body mass index.

Table 1 Demographic, anthropometric, clinical, and histological features, as evaluated at diagnosis, of 174 Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis subdivided according to the PNPLA3 I148M genotype

	All patients	PNPLA3 I148M genotype			P value
		I/I	I/M	M/M	
n (%)	174	82 (47)	70 (40)	22 (13)	
Age (yr)	47 ± 13	46 ± 13	47 ± 14	50 ± 11	0.22
Gender F (%)	49 (28)	24 (30)	19 (27)	6 (27)	0.79
DAI (g)	10 (0-20)	10 (0-20)	5 (0-20)	10 (0-20)	0.98
BMI (kg/m^2)	24 ± 3	24 ± 3	24 ± 3	25 ± 2	0.11
Diabetes (%)	16 (9)	7 (9)	6 (9)	3 (14)	0.57
Total cholesterol (mg/dL)	191 ± 44	197 ± 37	186 ± 50	182 ± 44	0.25
HDL cholesterol (mg/dL)	53 ± 16	53 ± 14	53 ± 17	57 ± 15	0.47
Triglycerides (mg/dL)	120 ± 66	123 ± 64	122 ± 74	99 ± 35	0.30
TS %	80 ± 16	80 ± 16	79 ± 15	81 ± 23	0.94
Ferritin (ng/mL)	1000 (509-1800)	1018 (490-1813)	947 (516-1732)	1053 (504-2101)	0.36
AST (IU/mL)	34 ± 21	31 ± 16	35 ± 24	44 ± 29	0.019
ALT (IU/mL)	46 ± 32	41 ± 26	47 ± 34	55 ± 45	0.06
GGT (IU/mL)	24 (16-37)	24 (17-37)	24 (15-38)	23 (14-30)	0.95
Steatosis[1] (%)	55 (48)	18 (31)	29 (58)	8 (53)	0.014
Advanced fibrosis % (Ishak 4) %	18 (10)	9 (11)	7 (10)	2 (9)	0.95
Cirrhosis (Ishak 5-6) %	32 (18)	13 (16)	14 (20)	5 (23)	0.39

[1]Available in 123 patients. I: Ileucine; M: Methionine; *n*: Number; F: Female; DAI: Daily alcohol intake; BMI: Body mass index; HDL: High density lipoprotein cholesterol; TS: Transferrin saturation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyl transferase.

by an experienced operator (available in 123) was based on evident ultrasonographic contrast between the hepatic and right renal parenchyma of the right intercostal sonogram in the midaxillary line, or abnormally intense, high-level echoes arising from the hepatic parenchyma, and was graded on a three-grade scale as none, mild, or severe in accordance with intensity[29].

Cirrhosis was considered clinically absent only if all these conditions were satisfied: (1) age < 40 years; (2) alanine aminotransaminase (ALT) within normal levels; and (3) ferritin < 1000 ng/mL. These criteria have been shown to rule out not only cirrhosis, but also advanced fibrosis with high specificity in patients with C282Y+/+ HH without viral hepatitis and excessive alcohol intake[30].

Overweight was considered present when BMI > 25 kg/m^2. For each patient we collected data on sex, age, geographical origins, BMI, alcohol consumption, aspartate aminotransferase (AST), ALT and γ-glutamyl transferase (GGT) levels, ferritin, transferrin saturation percentage, total cholesterol, high density lipoprotein cholesterol and triglycerides levels, glucose, and type 2 diabetes[31]. Clinical features of the patients included are shown in Table 1. Informed written consent was obtained from each patient included. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of the Institutions involved.

Genetic analysis

DNA was extracted from peripheral blood by the phenol-chloroform method. Success rate in extracting DNA was 100% for each study group. The PNPLA3 rs738409 SNP was genotyped by a Taqman assay (assay on demand for rs738409, Applied Biosystems, Foster City, CA, United States) by personnel unaware of patients and controls clinical status. Post-polymerase chain reaction allelic discrimination was carried out measuring allele-specific fluorescence on the Opticon2 detection system (MJ Research, Waltham, MA, United States). Random samples

were confirmed by direct genotyping which provided concordant results in all cases[19]. Quality controls were performed to verify the reproducibility of the results. Valid genotypic data were obtained for 100% of subjects analyzed.

Statistical analysis

Values are expressed as mean ± SD or median (interquartile range) according to distribution. Mean values were compared by analysis of variance or Wilcoxon, and frequencies by F test and χ^2 test for trend, when appropriate. The study had a > 85% power to detect a two-fold higher risk of cirrhosis in carriers of the 148M allele, but only 33% to detect a 33% increased risk. Independent predictors of AST and ALT levels were analyzed by generalized linear model. The association of the PNPLA3 p.148M variant with fibrosis was evaluated by ordinal logistic regression analysis, and with the presence of steatosis and cirrhosis was evaluated by multivariate logistic regression analysis. P values were considered significant when < 0.05 (two-tailed). Analyses were carried out with JMP 6.0 statistical analysis software (SAS Institute Inc., Cary, NC, United States).

Figure 2 Frequency distribution of the rs738409 C > G single nucleotide polymorphism, encoding for the I148M protein variant, in 123 patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis subdivided according to the presence of steatosis (*P* = 0.015).

Table 2 Independent predictors of steatosis and cirrhosis in Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis, as evaluated by logistic regression analysis

	OR	95% CI	*P* value
Independent predictors of steatosis *n* = 123			
BMI (per kg/m^2)	1.22	1.06-1.42	0.008
ALT (per IU/mL)	1.01	0.99-1.02	0.353
PNPLA3 genotype (per p allele)	1.84	1.05-3.31	0.037
Independent predictors of cirrhosis *n* = 174			
Ferritin (ng/mL)	1.001	1.000-1.002	< 0.0001
Diabetes	0.54	0.26-1.16	0.101
BMI (>/m^2)	1.83	1.02-3.57	0.055
PNPLA3 p allele present (BMI > 25)	0.76	0.37-1.53	0.453
PNPLA3 p allele present (BMI ⩽ 25)	3.26	1.28-10.30	0.024

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; ALT: Alanine aminotransaminase; p.148M: PNPLA3 rs738409 148Met protein variant.

RESULTS

Association of PNPLA3 gene genotype with steatosis

We first sought to confirm the association of the G allele encoding for the p.148M variant with liver fat in C282Y+/+ HH. The frequency distribution of the rs738409 PNPLA3 SNP (*P* = not significant for Hardy-Weinberg equilibrium testing) in patients subdivided according to the presence of steatosis is shown in Figure 2 (*P* = 0.014). The frequency of the G allele was 0.41 in patients with and 0.26 in those without steatosis (*P* = 0.011), and did not change after the exclusion of patients with ultrasonographic evaluation of the presence of steatosis. Independent predictors of steatosis at logistic regression analysis, considered as independent variables selected by a stepwise mixed regression model, are shown in Table 2. Steatosis was independently associated with BMI (*P* = 0.008) and PNPLA3 genotype [odds ratio (OR) 1.84 per G allele, 95% confidence interval (CI): 1.05-3.31; *P* = 0.037].

Association of PNPLA3 gene genotype with liver enzymes

As expected, PNPLA3 genotype was not significantly asso-

ciated with demographic or anthropometric features, daily alcohol intake, metabolic parameters, including the presence of diabetes, and the severity of iron overload (Table 1). However, we observed an association between PNPLA3 and transaminases, which was significant for AST levels [*P* for trend (i.e., for increasing levels with increasing number of 148M alleles) = 0.019 for AST and *P* for trend = 0.06 for ALT], whereas GGT levels were not affected. Independent predictors of AST and ALT levels in the generalized linear model are shown in Table 3; variables included were selected by a stepwise mixed regression model. Both AST and ALT levels were significantly and independently correlated with younger age, higher iron parameters (TS% and ferritin levels), GGT levels, BMI, and the number (0-2) of 148M PNPLA3 alleles (*P* = 0.0014 and *P* = 0.017 for AST and ALT levels, respectively).

Association of PNPLA3 gene genotype with severity of fibrosis and cirrhosis

We next evaluated whether PNPLA3 genotype influences fibrosis stage. At ordinal regression analysis conducted in patients with liver biopsy or clinical diagnosis of cirrhosis (*n* = 106; shown in Table 4), fibrosis stage (0-6) was independently associated with gender, ALT and GGT values, and PNPLA3 p.148M alleles (estimated coefficient of correlation 0.56 ± 0.27, *P* = 0.04). Possibly due to the relatively low number of patients studied, PNPLA3 genotype was not significantly associated with cirrhosis in the whole cohort (Table 1), although the presence of the 148M allele was nominally significantly associated with cirrhosis in patients with BMI < 25 (*P* = 0.05, *P* = 0.1 after Bonferroni correction; Figure 3). Importantly, positivity for the PNPLA3 148M variant was associated with an increase in the prevalence of steatosis in subjects with BMI < 25, which reached levels similar to those of overweight patients (BMI < 25: 17/37, 46% *vs* 7/32, 22%, *P* = 0.036; BMI ⩾ 25: 22/31, 71% *vs* 10/26, 38%; *P* = 0.017 for patients positive and negative for the 148M variant, respectively). Independent predictors of cirrhosis are shown in Table 2. At logistic regression analysis, cirrhosis was associated with BMI ⩾ 25 (OR 1.82, 95% CI:

Table 3 Independent predictors of aspartate aminotransferase and alanine aminotransaminase levels in 174 Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis, in the multivariate generalized linear model

	AST			ALT		
	Estimate	95% CI	*P* value	Estimate	95% CI	*P* value
Age (yr)	-0.27 ± 0.09	-0.45 - -0.09	0.0034	-0.55 ± 0.14	-0.81 - -0.27	0.0001
TS (%)	0.14 ± 0.07	-0.01 - 0.29	0.063	0.28 ± 0.11	0.05 - 0.50	0.016
Ferritin (ng/mL)	0.01 ± 0.001	0.008 - 0.012	< 0.0001	0.01 ± 0.001	0.009 - 0.014	< 0.0001
GGT (IU/mL)	0.10 ± 0.03	0.03 - 0.16	0.003	0.20 ± 0.05	0.10 - 0.29	< 0.0001
BMI (kg/m²)	0.86 ± 0.42	0.03 - 1.69	0.042	3.36 ± 0.63	2.12 - 4.60	< 0.0001
PNPLA3 genotype (per p.148M allele)	5.46 ± 1.68	2.15 - 8.67	0.0014	6.05 ± 2.51	1.09 - 11.00	0.017

AST: Aspartate aminotransferase; ALT: Alanine aminotransaminase; CI: Confidence interval; TS: Transferrin saturation; GGT: γ-glutamyl transferase; BMI: Body mass index; p.148M: PNPLA3 rs738409 148Met protein variant.

Table 4 Independent predictors of fibrosis stage in 106 Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis, at ordinal logistic regression analysis

	Estimate	*P* value
Age (yr)	-0.03 ± 0.02	0.077
Gender (female)	-0.57 ± 0.26	0.029
BMI (kg/m²)	-0.08 ± 0.08	0.311
Diabetes	0.57 ± 0.34	0.09
ALT	-0.02 ± 0.008	0.013
GGT (IU/mL)	0.02 ± 0.01	0.035
Ferritin (ng/mL)	0.0001 ± 0.00	0.421
PNPLA3 genotype (per p.148M allele)	0.56 ± 0.27	0.041

BMI: Body mass index; ALT: Alanine aminotransaminase; GGT: γ-glutamyl transferase; p.148M: PNPLA3 rs738409 148Met protein variant.

1.02-3.55), ferritin > 1000 ng/mL at diagnosis (OR 19.3, 95% CI: 5.3-125), and with the G allele in patients with BMI < 25 (OR 3.26, 95% CI: 1.3-10.3).

Figure 3 Effect of the rs738409 G allele, encoding for the 148M PNPLA3 variant, on liver cirrhosis in 174 patients with HH subdivided according to the presence of overweight. NS: Not significant; BMI: Body mass index.

DISCUSSION

In this study, we evaluated the effect of the PNPLA3 rs738409, encoding for the p.I148M variant, on steatosis and liver damage in patients affected by C282Y+/+ HH without other causes of liver damage. Our results confirm the association of the PNPLA3 148M allele with the presence of steatosis and liver enzymes, independently of iron overload, which represents the major cause of progressive liver damage in HH patients. Furthermore, PNPLA3 rs738409 was also associated with fibrosis stage, and with the presence of cirrhosis, albeit only in the presence of normal BMI.

Although data on steatosis were not available in the whole series of patients evaluated, the association of PNPLA3 with steatosis in HH was expected based on data obtained in the general population, in patients with non-alcoholic fatty liver disease (NAFLD), and in other liver diseases. In addition, the magnitude of the observed association was in line with previous reports[18,19,32]. Due to the retrospective design of the study, insulin resistance evaluation was not available for all patients, however, previous studies have excluded a major effect of PNPLA3

genotype on insulin resistance, and the 148M variant was not associated with diabetes in this study.

As steatosis has been reported to influence fibrosis progression in C282Y +/+ patients, independently of alcohol intake and iron loading[11], the main aim of the present study was to evaluate whether PNPLA3 genotype influences liver damage progression in HH. We found that the PNPLA3 148M allele was a strong predictor of transaminase levels, and in particular of AST levels, which are generally more strongly linked with chronic liver damage (fibrosis stage) than ALT[11,33]. PNPLA3 polymorphism has been reported to represent a major determinant of transaminase levels in the general population, in patients with NAFLD, and in obese subjects at risk of steatosis[18,19,34,35]. Our findings indicate, that this is also true for patients with C282Y+/+ HH. It is likely that this association reflects the predisposing effect of the 148M PNPLA3 allele on steatosis. Interestingly, BMI, which was the other determinant of steatosis in our series of patients, was also associated with transaminases. Furthermore, we demonstrated that PNPLA3 genotype was associated with fibrosis stage in patients with HH, consistent with the hypothesis that the 148M allele of PNPLA3 influences the progression of liver damage in

HH, even if this has to be confirmed in a larger series of patients.

The association of the 148M variant with the risk of cirrhosis, a turning point in the natural history of patients with HH due to the frequent progression to hepatocellular carcinoma[36,37], is also in line with this hypothesis. Interestingly, data obtained by our and other groups indicate that the 148M variant is significantly associated with cirrhosis and hepatocellular carcinoma in patients with HCV-related chronic hepatitis, thus suggesting that this can be true also for liver diseases of different etiology[32,38].

Based on the previously reported interaction between the effect of the PNPLA3 I148M mutation and body mass[35], the other major determinant of steatosis in our series of patients, we analyzed the effect of PNPLA3 rs738409 on cirrhosis risk in patients stratified according to the presence of overweight. We found an association between PNPLA3 genotype and cirrhosis, but this was restricted to subjects with normal BMI. There are several possible explanations for this finding. The first is that the result is due to chance. Because of the relatively limited number of subjects included, the study power to detect an association with cirrhosis was relatively low, i.e., > 85% to detect a two-fold higher risk of cirrhosis in carriers of the 148M allele, but only 33% to detect a 33% increased risk. Secondly, this was a retrospective study with data evaluation at the time of diagnosis, therefore we could not exclude the fact that some patients "normalized" their body mass after the development of cirrhosis due to the malnutrition typical of this condition. However, increased BMI was independently associated with steatosis and tended to be associated also with cirrhosis risk, and the shift in body weight should have equally affected patients with and without the 148M allele. Lastly, we cannot exclude that the PNPLA3 148M variant, by favoring steatosis development in subjects with normal BMI, may play a permissive role in the progression of liver damage in lean patients with C282Y+/+ HH, whereas in overweight subjects steatosis is mainly related to metabolic factors, reducing the role played by PNPLA3. Additional studies are required to clarify this issue, and to evaluate whether the 148M variant may predispose to hepatocellular carcinoma development also in HH and in patients carrying HFE mutations[39-41].

In conclusion, we showed that in Italian patients with C282Y+/+ HH the PNPLA3 I148M polymorphism is associated with the risk of steatosis, increased liver enzymes, higher stage of fibrosis, and possibly with an increased risk of cirrhosis at diagnosis, particularly in subjects with normal body mass. Future studies should evaluate whether PNPLA3 I148M genotype might be clinically useful for selecting HH patients for biopsy, or to determine screening intervals for hepatocellular carcinoma in cirrhotics.

COMMENTS

Background

Hereditary hemochromatosis, characterized by progressive accumulation of iron in tissues, is a very frequent genetic disease in individuals of European descent. The most frequent clinical manifestation is liver disease, which may lead to liver cancer. However, disease expression is highly variable. Previous work has led to hypothesize that genetic factors and liver fat accumulation (i.e., "steatosis") are implicated in this process. Recently, the common I148M patatin-like phospholipase domain containing-3 genetic polymorphism has been recognized together with obesity as a key factor regulating fat accumulation in the liver, contributing significantly to the liver disease burden in the general population.

Research frontiers

The identification of genetic factors involved in the penetrance and expression of hereditary hemochromatosis is a very active area of research, as such markers would be helpful to identify subjects at risk during screening and to personalize treatment and follow-up in patients presenting with liver disease.

Innovations and breakthroughs

The key findings of the study are that the PNPLA3 genetic variant, present in 40% of patients, was a key determinant, together with overweight, of hepatic fat accumulation and of alterations of biochemical indices of liver damage. Furthermore, this marker was also associated with chronic fibrotic damage detected by liver biopsy. Importantly, the PNPLA3 variant put at risk of steatosis also normal weight patients, who would be normally protected, allowing the development of progressive liver damage and cirrhosis, with potential clinical complications.

Applications

These results raise new hope to offer better, personalized treatment to patients with hemochromatosis, which will be tested in future studies. In particular, intensified follow-up and preventive treatments could be proposed to subjects at risk of developing liver cancer, the leading cause of death in patients with clinically overt hemochromatosis, which is also favored by steatosis.

Terminology

Hereditary hemochromatosis is a genetic disorder of iron metabolism characterized by defective release or activity of hepcidin, the hepatic hormone that inhibits iron absorption, leading to progressive accumulation in the liver and other parenchymal organs; PNPLA3 is an enzymes with phospholipase activity, which is expressed in the liver, and likely involved in the breakdown triglycerides; genetic polymorphisms: inherited variant of the DNA, which is detected in > 1% of the population and is not associated *per se* with a pathologic phenotype.

Peer review

This is an interesting clinical study, which provides evidence for association between the PNPLA3 148M polymorphism and progression of liver fibrosis. The study is well designed and the data are novel.

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BRIEF ARTICLE

An epidemiological study of collagenous colitis in southern Sweden from 2001-2010

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Abstract

AIM: To estimate the incidence of collagenous colitis (CC) in southern Sweden during 2001-2010.

METHODS: Cases were identified by searching for CC in the diagnostic registers at the Pathology Departments in the county of Skåne. The catchment area comprised the south-west part of the county (394 307 inhabitants in 2010) and is a mixed urban and rural type with limited migration. CC patients that had undergone colonoscopy during the defined period and were living in this area were included in the study regardless of where in Skåne they had been diagnosed. Medical records were scrutinized and uncertain cases were reassessed to ensure that only newly diagnosed CC cases were included. The diagnosis of CC was based on both clinical and histopathological criteria. The clinical criterion was non-bloody watery diarrhoea. The histopathological criteria were a chronic inflammatory infiltrate in the lamina propria, a thickened subepithelial collagen layer $\geq$ 10 micrometers (μm) and epithelial damage such as flattening and detachment.

RESULTS: During the ten year period from 2001-2010, 198 CC patients in the south-west part of the county of Skåne in southern Sweden were newly diagnosed. Of these, 146 were women and 52 were men, i.e., a female: male ratio of 2.8:1. The median age at diagnosis was 71 years (range 28-95/inter-quartile range 59-81); for women median age was 71 (range 28-95) years and was 73 (range 48-92) years for men. The mean annual incidence was $5.4/10^5$ inhabitants. During the time periods 2001-2005 and 2006-2010, the mean annual incidence rates were $5.4/10^5$ for both periods [95% confidence interval (CI): 4.3-6.5 in 2001-2005 and 4.4-6.4 in 2006-2010, respectively, and 4.7-6.2 for the whole period]. Although the incidence varied over the years (minimum 3.7 to maximum $6.7/10^5$) no increase or decrease in the incidence could be identified. The odds ratio (OR) for CC in women compared to men was estimated to be 2.8 (95% CI: 2.0-3.7). The OR for women 65 years of age or above compared to below 65 years of age was 6.9 (95% CI: 5.0-9.7), and for women 65 years of age or above compared to the whole group the OR was 4.7 (95% CI: 3.6-6.0). The OR for age in general, i.e., above or 65 years of age compared to those younger than 65 was 8.3 (95% CI: 6.2-11.1). During the last decade incidence figures for CC have also been reported from Calgary, Canada during 2002-2004 ($4.6/10^5$) and from Terrassa, Spain during 2004-2008 ($2.6/10^5$). Our incidence figures from southern Sweden during 2001-2010 ($5.4/10^5$) as well as the incidence figures presented in the studies during the 1990s (Terrassa, Spain during 1993-1997 ($2.3/10^5$), Olmsted, United States during 1985-2001 ($3.1/10^5$), Örebro, Sweden during 1993-1998 ($4.9/10^5$), and Iceland during 1995-1999 ($5.2/10^5$) are all in line with a north-south gradient, something that has been suggested before both for CC and inflammatory bowel disease.

CONCLUSION: The observed incidence of CC is comparable with previous reports from northern Europe and America. The incidence is stable but the female: male ratio seems to be decreasing.

INTRODUCTION

In 1976, a pathology colleague at our hospital, Clas G Lindström, received rectal biopsies for a second opinion. The patient was a 48-year-old woman with chronic non-bloody watery diarrhoea and based on his observations he described the first ever case of collagenous colitis (CC)[1]. In his case report, Lindström actually described a typical case of CC, i.e., a middle-aged woman with chronic non-bloody watery diarrhoea. Independently, a group from Canada also reported this entity[2]. Other common CC symptoms include abdominal pain, weight loss and faecal incontinence[3-5]. The diagnosis of CC can only be established by microscopic examination of colonic mucosal biopsies. Endoscopy usually reveals a macroscopically normal mucosa, although some changes may be seen with special staining methods, e.g. altered vessel formation and disturbed pattern of the mucous membrane[6,7]. Histopathologically, CC is characterised by a thickened subepithelial collagen layer, combined with a chronic inflammatory infiltrate in the lamina propria and surface epithelial damage. In 1989, Lazenby[8] described lymphocytic colitis (LC), a similar condition clinically and histopathologically, but without a thickened subepithelial collagen layer. Collagenous colitis and LC are included in the umbrella term microscopic colitis (MC).

About 10% of patients investigated for non-bloody diarrhoea and with a macroscopically normal colonic mucosa are diagnosed with MC[8-11] even though an incidence as high as 29% has been observed[12]. These figures indicate that the condition could be prevalent. Incidence data on CC is available from the mid 1990s[13]. Initially, CC was considered to be a rare disease, but with time it has become evident that the incidence of CC is higher than was first anticipated.

The aim of this study was to estimate the incidence of CC in a well-defined population in southern Sweden during the ten year period from 2001-2010.

MATERIALS AND METHODS

Catchment area

The catchment area in this study comprised the south-west part of the county of Skåne (the county had 1 243 329 inhabitants on December 31st, 2010). The catchment area (including the cities of Malmö and Trelleborg, and the villages Vellinge and Svedala) is a mixed urban and rural type with limited migration (from 2001 to 2010 the population increased by 13%, from 349 693 to 394 307 inhabitants). The town of Malmö has the third largest population in Sweden, while Trelleborg is a small town. Vellinge and Svedala are smaller communities. In the catchment area there are two hospitals, one in Malmö and one in Trelleborg. Colonoscopy is carried out at both hospitals as well as by some private practitioners. There is only one Pathology Department in the catchment area (in Malmö) and all mucosal colonic biopsy specimens from the hospitals in Malmö and Trelleborg as well as from the private practitioners are sent to this Pathology Department.

Patients

Patients living in the catchment area who underwent colonoscopy due to watery diarrhoea and were diagnosed with CC from 2001-2010, were included in the study. Cases were identified by searching for the diagnosis of CC in the diagnostic register at the Pathology Department in Malmö. The medical records for the identified CC patients were subsequently scrutinized for clinical data to ensure that only newly diagnosed cases were included.

Histopathologically uncertain cases as well as cases not diagnosed by an experienced gastrointestinal pathologist were reassessed by a pathologist specialised in gastrointestinal pathology (Martin Olesen). Patients not fulfilling the histopathological criteria for CC were excluded. Patients with a histopathological diagnosis of "unspecific chronic inflammation" and with watery diarrhoea were also re-evaluated to identify putative missed CC cases.

All CC patients living in the catchment area were included regardless of where in Skåne they were diagnosed. To eliminate the risk of missing patients living in the catchment area during the study period in 2001-2010, but having had a diagnostic colonoscopy with biopsies in adjacent areas outside the catchment area (i.e., in the remaining area of Skåne), the diagnostic registers at the other three Pathology Departments in Skåne were scrutinized for cases with CC. In accordance with this, patients not living in the catchment area from 2001-2010, but diagnosed at our Pathology Department were excluded.

All information regarding the size of the population as well as age and sex distribution was obtained from Statistics Sweden, the central bureau for national socioeconomic information.

Figure 1 Age- and sex-specific annual incidence of collagenous colitis in southern Sweden.

Figure 2 Annual incidence of collagenous colitis in southern Sweden.

Diagnostic criteria

The diagnosis of CC was based on both clinical and histopathological criteria. The clinical criterion was non-bloody watery diarrhoea. The histopathological criteria were as follows: (1) A chronic inflammatory infiltrate in the lamina propria; (2) A thickened subepithelial collagen layer $\geq$ 10 micrometers (μm); and (3) Epithelial damage such as flattening and detachment.

The subepithelial collagen layer was measured with an ocular micrometer in a well orientated section of the mucosa. Measurement of the subepithelial collagen layer was obtained using special stains for collagen fibres (Masson's trichrome or van Gieson) and/or reticulin fibres (Sirius red).

Statistical analysis

For the purpose of calculating the incidence rate, it was assumed that the entire population in the catchment area was at risk. The incidence calculations were based on the date of diagnosis and 95% confidence interval (CI): were included. The calculation of mean annual incidence/100 000 as well as age-related incidence was based on the number of inhabitants on December 31st of each year. Data on the studied population are presented as

median and range/inter-quartile range (IQ) (25th-75th percentiles). Comparisons between groups were carried out using odds ratios (OR) and corresponding 95% CI.

Ethics

The study was approved by the Committee of Research Ethics at Lund University.

RESULTS

Patients

In the ten year period from 2001-2010, 198 CC patients were newly diagnosed. Of these, 146 were women and 52 were men (female:male ratio 2.8:1). The median age at diagnosis was 71 years (range 28-95/IQ 59-81); for women median age was 71 (range 28-95) years and was 73 (range 48-92) years for men (Figure 1).

Incidence

During the time periods 2001-2005 and 2006-2010, the mean annual incidence rates were $5.4/10^5$ for both periods (95% CI: 4.3-6.5 in 2001-2005 and 4.4-6.4 in 2006-2010, respectively, and 4.7-6.2 for the whole period). Conse-

Table 1 Mean annual incidence of collagenous colitis year by year during 2001-2010

Year	Annual incidence/10^5 (total)	Annual incidence/10^5 (women)
2001	6.6	7.8
2002	5.1	6.1
2003	4.5	4.9
2004	6.7	8.7
2005	3.9	6.5
2006	4.4	6.9
2007	3.7	6.3
2008	5.8	8.3
2009	6.4	9.1
2010	6.6	12.5

Table 2 Mean annual incidence and female:male ratio of collagenous colitis in different countries/100 000 inhabitants

Ref.	Time period	N	Mean annual incidence[1]	Female:male ratio
Raclot *et al*[32]	1987-1992		0.6	
Bohr *et al*[13]	1984-1993	30	1.8	9
Pardi *et al*[14]	1985-2001	46	3.1	4.4
Fernández-Bañares *et al*[10]	1993-1997	23	2.3	4.8
Olesen *et al*[11]	1993-1998	51	4.9	7.5
Agnarsdottir *et al*[15]	1995-1999	71	5.2	7.9
Williams *et al*[16]	2002-2004	75	4.6	
Fernández-Bañares *et al*[17]	2004-2008	40	2.6	3.4
Present study	2001-2010	198	5.4	2.8

[1]Southern (left) and northern (right) latitudes. N: Number of new collagenous colitis patients.

quently, although the incidence rates varied over the years (minimum 3.7 to maximum 6.7/10^5) no increase or decrease could be identified (Figure 2, Tables 1 and 2).

The OR for CC in women compared to men was estimated to be 2.8 (95% CI: 2.0-3.7). The OR for women 65 years of age or above compared to below 65 years of age was 6.9 (95% CI: 5.0-9.7), and for women 65 years of age or above compared to the whole group the OR was 4.7 (95% CI: 3.6-6.0). The OR for age in general, *i.e.*, above or 65 years of age compared to those younger than 65 was 8.3 (95% CI: 6.2-11.1).

DISCUSSION

Information on the incidence of CC is available from one centre in the United States, one in Canada and a few in Europe (including Örebro in the central part of Sweden). Our study has added information on the epidemiology of CC in the southern part of Sweden. We report a mean annual incidence of CC of 5.4/10^5 for the period 2001-2010, which is in line with previous data from Örebro in central Sweden during 1993-1998 (4.9/10^5)[11], and is in accordance with the reported figures from Olmsted County, Minnesota, United States from 1997-2001 (6.2/10^5)[14], Iceland from 1995-1999 (5.2/10^5)[15] and Calgary, Alberta, Canada from 2002-2004 (4.6/10^5)[15,16]. However, these findings are in contrast to the incidence reported from Terrassa, Spain during 2004-2008 (2.6/10^5)[17] (Table 2). Although the findings are not contradictory or novel in comparison with, for example, the report from Örebro[11], the number of cases is large and the epidemiological information is updated in a new region that has not been studied before.

Most relevant from the Swedish perspective, are the extensive investigations from Örebro, in the central part of Sweden, where the incidence rates were calculated over a 15-year period from 1984-1998. An increased incidence of CC from 1.8/10^5 in 1984-1993 to 3.7/10^5 in 1993-1995 and 6.1/10^5 in 1996-1998 was reported[11,12]. In Spain, the incidence increased from 1.1/10^5 in 1993-1997 to 2.6/10^5 in 2004-2008[10,17]. In accordance with these reported increases, the Minnesota data from 1985 to 2001 show the same phenomenon. In 1985-1997, the incidence was 1.6/10^5 compared to 7.1/10^5 in 1998-2001[14]. An explana-

tion for these reported increases in incidence rates could be an increased awareness of CC and more frequent use of colonoscopies with biopsies in the diagnostic procedure, although there was probably a true increase in incidence as well. The incidence of other gastrointestinal disorders such as ulcerative colitis, Crohn's disease and coeliac disease has increased in the Western world during the last few decades[18-21] and the influence of a common environmental factor (or several) cannot be ruled out. Interestingly, coeliac disease- similar to CC-has also increased over the last few decades and these diseases are related to each other[22]. Lansoprazole and other proton pump inhibitors are, in addition to nonsteroidal anti-inflammatory drugs, associated with a higher risk of CC[22-30]. The possibility that other factors such as infectious agents and components in our food could contribute to the increased incidence has also been contemplated.

The time period of ten years in our study is fairly long and provides information on possible fluctuations in the CC incidence rate in our catchment area during the period 2001-2010. Despite some variation in the annual incidence we did not observe any significant increase or decreases in the incidence during the study period. This is in contrast with the reported increases mentioned above. The stable incidence in our study might be due to the fact that CC was described at our hospital in Malmö by Lindström as early as 1976, *i.e.*, 35 years ago. Accordingly, CC has been known among medical doctors in this area for more than 30 years, and as a consequence regular use of colonoscopies, with multiple biopsies throughout the entire length of the colon, has been standard in the diagnostic procedure of chronic watery diarrhoea for a long time. Furthermore, there has been a close clinical and scientific collaboration between gastroenterologists and pathologists within the field of CC.

During the last decade incidence figures for CC have been reported from Canada during 2002-2004 (4.6/10^5) and from Spain during 2004-2008 (2.6/10^5)[16,17]. There was a considerable difference in the incidence of CC in these studies despite the fact that the study periods were similar and relatively recent. Fernandez-Banares highlighted the possibility of a north-south gradient with a

higher incidence further north compared to those closer to the equator[17]. This possibility is further strengthened by our study. A larger number of studies have been listed in Table 2 to illustrate this phenomenon. This is also in line with the north-south gradient suggested for inflammatory bowel disease[31].

The female: male ratio in the present study (2.8:1) was significantly lower than that in previous reports (Table 2). The previous Swedish studies from Örebro reported a female: male ratio as high as 9 and 7.5 in 1984-1993 and 1993-1998, respectively. Interestingly, it might very well be that the ratio may have decreased over the years as indicated in Table 2, which to the best of our knowledge, has not been reported before.

The OR calculated for age in general, women compared to men, older women compared to younger women, and older women compared to the whole group indicated that the risk of acquiring CC is higher in older persons, especially women, which is in line with observations from several other studies. Based on the levels for the different OR it could be speculated that age (OR 8.3 for age in general and 6.9 for older women) contributes more than sex (OR 2.8) to the risk of CC.

The geographical area studied, the south-west part of Skåne, a county in southern Sweden, has several advantages for carrying out epidemiological studies. The organisation for medical care is well defined, with two hospitals and one Pathology Department. The catchment area *per se* is also well defined. In addition, the migration rate in the area is limited. Furthermore, the use of personal identity numbers in Sweden that follow the individual throughout her or his entire life span makes it possible to identify every individual with CC in the region and to determine whether she or he lives within the catchment area. Accordingly, the conditions for conducting an epidemiologic CC study in Sweden are favourable. Another strength of this CC study was the precautions taken to identify patients who belonged to the catchment area but who had been diagnosed in another part of the county, by scrutinizing residents with CC at all Pathology Departments in Skåne. This study is also fairly large with 198 identified CC cases during the period (Table 2).

In conclusion, we observed a mean annual incidence of CC in southern Sweden of $5.4/10^5$, in line with previous data. In contrast to previously reported increases in the incidence rate, we report a stable incidence during the ten year study period from 2001-2010. The female: male ratio (2.8:1) was lower than previously reported. Based on data from available studies it seems that the female: male ratio is decreasing. In accordance with previously presented data it also seems that CC is more common in northern countries.

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COMMENTS

Background
Collagenous colitis (CC) predominantly affects middle-aged women and results in chronic watery diarrhoea. It has become evident that the incidence of CC is much higher than was first anticipated.

Research frontiers
Information on the incidence of CC is available from one centre in the United States, one in Canada and a few in Europe. The incidence of CC in Örebro, in central Sweden, was $4.9/10^5$ from 1993-1998. This level is in accordance with the reported figures from Olmsted County, Minnesota, United States during 1997-2001 ($6.2/10^5$), Iceland during 1995-1999 ($5.2/10^5$) and Calgary, Alberta, Canada during 2002-2004 ($4.6/10^5$). However, these findings are in contrast to the incidence reported from Terrassa, Spain in 2004-2008 ($2.6/10^5$), which is much lower.

Innovations and breakthroughs
The study added information on the epidemiology of CC in the southern part of Sweden. The authors observed a mean annual incidence of CC in southern Sweden of $5.4/10^5$, which is in line with previous data. In contrast to previously reported increases in the incidence rate, the authors report a stable incidence in the ten year study period from 2001-2010. The female: male ratio (2.8:1) was lower than previously reported. Based on data from available studies it seems that the female:male ratio is decreasing. In accordance with previously presented data, it also seems that CC is more common in northern countries.

Applications
During the 25 years since the first epidemiological study, the incidence of CC increased from 1.8 to 5.4/100 000 inhabitants. It now seems to have levelled out. At the same time the female: male ratio has decreased. Consequently, more men (in relation to women) have been affected in recent years. Despite this, age is associated with a higher OR for disease than sex. The risk of disease is higher in northern than in southern Europe. The following remain to be clarified: Why the incidence is no longer increasing, why the female: male ratio has decreased and why people in northern Europe have a higher risk of disease. Environmental factors could have a substantial impact on CC; smoking, nonsteroidal anti-inflammatory drugs and proton pump inhibitors are known triggers but it can not be ruled out that several other factors are responsible for the high incidence of CC.

Terminology
Incidence represents how many persons are affected by a certain disease/year in each group of 100 000 inhabitants within a defined area. Collagenous colitis mean inflammation in the large intestine that is predominantly visible *via* examination with a microscope, where the collagen layer can be observed in the epithelium. The disease results in chronic watery diarrhoea.

Peer review
The manuscript is relatively well written, and the results are moderately interesting.

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BRIEF ARTICLE

Evaluation of SNPs in miR-196-a2, miR-27a and miR-146a as risk factors of colorectal cancer

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Abstract

AIM: To investigate whether selected single nucleotide polymorphisms (SNPs) in miR-196a2, miR-27a and miR-146a genes are associated with sporadic colorectal cancer (CRC).

METHODS: In order to investigate the effect of these SNPs in CRC, we performed a case-control study of 197 cases of sporadic CRC and 212 cancer-free controls originating from the Central-European Caucasian population using TaqMan Real-Time polymerase chain reaction and allelic discrimination analysis.

RESULTS: The genotype and allele frequencies of SNPs were compared between the cases and the controls. None of the performed analysis showed any statistically significant results.

CONCLUSION: Our data suggest a lack of association between rs11614913, rs895819 and rs2910164 and colorectal cancer risk in the Central-European Caucasian population, a population with an extremely high incidence of sporadic colorectal cancer.

Hezova R, Kovarikova A, Bienertova-Vasku J, Sachlova M, Redova M, Vasku A, Svoboda M, Radova L, Kiss I, Vyzula R, Slaby O. Evaluation of SNPs in miR-196-a2, miR-27a and miR-146a as risk factors of colorectal cancer. *World J Gastroenterol* 2012; 18(22): 2827-2831 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i22/2827.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i22.2827

INTRODUCTION

Sporadic colorectal cancer represents a typical multifacto-

rial disease with an intense crosstalk of the genetic background with the environment, including lifestyle habits and diet. Certain populations present higher rates of sporadic colorectal cancer, independently of diet and lifestyle habits than others[1], which supports the hypothesis that individual genetic background is involved in the etiopathogenesis of the disease. An extremely high incidence of colorectal cancer[1] has been repeatedly reported for the Central-European Caucasian population, significantly exceeding the peak incidence observed in the United States and other developed countries[2]. This population is, therefore, highly likely to carry a strong genetic predisposition to sporadic colorectal cancer and could be a good model population for sporadic colorectal cancer.

MicroRNAs (miRNAs) are short non-coding RNAs, 18 to 25 nucleotides in length, which regulate gene expression[3]. Single nucleotide polymorphisms (SNPs) may occur at the level of the miRNA biogenesis pathway genes, pri-miRNA, pre-miRNA or mature miRNA sequences. Such polymorphisms may be functional with regard to the biogenesis and actions of the mature miRNA. Specific SNPs are located at predicted miRNA target sites within 3' of untranslated regions of mRNAs. These SNPs have the potential to affect the efficiency of miRNA binding at their target sites as well as to create or disrupt binding sites. Resulting gene dysregulation may involve changes in phenotype and may eventually prove critical for the susceptibility to and the onset of cancer, as well as for prognosis and therapy response prediction[1].

The most frequently studied miRNA-associated SNP in cancer is rs11614913 in the pre-miRNA region of miR-196-a2. Hu *et al*[4] observed the association of the rs11614913: T > C variant genotype with a significantly increased risk of breast cancer [odds ratio (OR) 1.23; 95% confidence interval (CI): 1.02-1.48]. A number of case-control studies were consequently performed in breast[5,6], lung[7,8], gastric[9], esophageal[10], hepatocellular[11] and head and neck cancer[12]. More recently, two contradictory studies were published evaluating rs11614913 as a potential risk factor for colorectal cancer in the Chinese population (T *vs* C allele-OR 1.320; CI: 1.056-1.649, *P* = 0.014[13] *vs* OR 1.065; CI: 0.803-1.414, *P* = 0.665[14]). SNP rs895819, located in the terminal loop of a pre-miR-27a oncogene, was initially evaluated in familial breast cancer, whereas the G allele was associated with reduced familial breast cancer risk (*P* = 0.0215). The opposite of this association was observed by Sun *et al*[15] in a gastric cancer case-control study where subjects with variant genotypes (AG + GG) showed a significantly increased risk of gastric cancer relative to AA carriers (OR 1.48; 95% CI: 1.06-2.05; *P* = 0.019). AG to C SNP (rs2910164) located within the sequence of the miR-146a precursor was first studied by Shen *et al*[16] due to the fact that predicted miR-146a target genes include BRCA1 and BRCA2, i.e., key breast and ovarian cancer susceptibility genes. Breast and ovarian cancer patients who had at least one miR-146a variant allele were diagnosed at an earlier age. Subsequently, the distribution of the miR-146a polymorphism

rs2910164 was evaluated in breast[6], esophageal[17], hepatocellular[18] and thyroid cancer[19].

Thus, a significant association with the risk of various types of solid cancers, with the exception of colorectal cancer, has been repeatedly reported for SNPs: rs11614913 in miR-196-a2, rs895819 in hsa-miR-27a and rs2910164 in miR-146a; consequently, we decided to perform a case-control study evaluating these three SNPs and the risk of sporadic colorectal cancer in a Central-European Caucasian population.

MATERIALS AND METHODS

Patients and controls
The study included patients with newly diagnosed sporadic colorectal cancer treated at the Masaryk Memorial Cancer Institute, Czech Republic between January 2008 and December 2010. The patient cohort consisted of 197 subjects [105 men, 92 women; age (mean ± SD): 63 ± 9 years] with histologically confirmed colorectal adenocarcinomas, whereas the control cohort included a total of 202 cancer-free blood donor volunteers recruited from the same institute with a similar age distribution (93 men, 109 women; mean age: 65 ± 14 years) and no previous history of any type of cancer. Due to its invasiveness, colonoscopy was not performed to exclude colorectal cancer (CRC) in the control cohort; however, all subjects were symptom free and no anemia was present. All study subjects were Caucasian. The hospital ethical committee approved the study and all study subjects supplied a written informed consent which was subsequently archived.

DNA isolation and genotyping
Genomic DNA was isolated from the full peripheral blood using the MagNA Pure DNA Isolator (Roche). DNA concentration was measured on the Nanodrop ND-1000 (NanoDrop Technologies, Inc.). For analysis of rs11614913 in miR-196-a2, rs895819 in hsa-miR-27a and rs2910164 in miR-146a, Real-Time polymerase chain reaction (PCR) allelic discrimination was performed on Step-One Real-Time PCR (Applied Biosystems, United States) using standard TaqMan genotyping assays according to the manufacturer's instructions. In brief, probes, primers and TaqMan universal PCR Master Mix were obtained from Applied Biosystems. A reaction solution of 10 μL contained 0.5 μL TaqMan Genotyping Assay mix (consisting of 20X Mix of unlabeled PCR primers and TaqMan minor groove binder probe, 6-carboxyfluorescein and VIC dye-labeled), 8 μL of PCR mixture reagent and 10 ng of genomic DNA. Reactions were run according to the manufacturer's instructions. The PCR consisted of pre-PCR read at 60 ℃ for 30 s, holding stage at 95 ℃ for 10 min, 50 cycles of denaturing at 92 ℃ for 15 s, annealing 60 ℃ for 1 min 30 s and post-PCR read at 60 ℃ for 30 s.

Statistical analysis
The Hardy-Weinberg equilibrium was tested for each

Table 1 Logistic regression analysis of genotype frequencies of single nucleotide polymorphisms rs11614913, rs895819 and rs2910164 in colorectal cancer cases and controls in the Czech population

		Control		CRC		OR[1]	95% CI	*P* value
		n	%	*n*	%			
miR-27a	A/A	93	43.87	88	44.67	1		0.996[a]
	A/G	94	44.34	86	43.65	0.98	(0.64-1.49)	0.950
	G/G	25	11.79	23	11.68	1.04	(0.54-1.98)	0.970
	AG + GG *vs* AA					1.01	(0.68-1.51)	0.954
	[G] *vs* [A]					0.999	(0.71-1.39)	0.995
	Trend	212		197		0.99	(0.8-1.22)	0.9118[a]
miR-146a	G/G	124	58.49	115	58.38	1		0.761[a]
	C/G	79	37.26	70	35.53	0.93	(0.61-1.41)	0.740
	C/C	9	4.25	12	6.09	1.31	(0.52-3.27)	0.556
	CG + CC *vs* GG					1.03	(0.69-1.54)	0.879
	[C] *vs* [G]					1.37	(0.56-3.33)	0.494
	Trend	212		197		0.97	(0.79-1.19)	0.7558[a]
miR-196-a2	C/C	87	41.04	82	41.62	1		0.6098[a]
	C/T	103	48.58	89	45.18	0.95	(0.62-1.45)	0.794
	T/T	22	10.38	26	13.2	1.32	(0.69-2.54)	0.415
	CT + TT *vs* CC					1.01	(0.68-1.51)	0.951
	[T] *vs* [C]					1.04	(0.75-1.45)	0.811
	Trend	212		197		1.08	(0.8-1.46)	0.5987[a]

P-values are calculated according to Wald's test. [a]*P*-values according to likelihood ratio-test; [1]Age and sex adjusted; CRC: Colorectal cancer; OR: Odds ratio; CI: Confidence interval.

polymorphism using the χ^2 test in patients and controls separately. Allelic frequencies were estimated by the "counting method" and differences in allele frequencies between case and control subjects were tested using the likelihood ratio χ^2 tests for 2 £ 2 tables (two alleles, case *vs* control subjects). The homozygote of the most frequent allele was used as a reference for calculating the OR. For an OR and 95% confidence interval, logistical regression was used based on a model for sex and age of the patients. Data analysis was performed using the Statistica v. 9.0 (Statsoft Inc., Tulsa, OK, United States) program package. Values of $P < 0.05$ were considered statistically significant.

RESULTS

All polymorphisms met the criteria of the Hardy-Weinberg equilibrium in the individual patient and control groups. Logistic regression modeling was used to estimate the odds ratios of the investigated genotypes and alleles of SNPs rs2910164, rs11614913 and rs3746444 in CRC cases as well as in the controls (Table 1). All of the examined polymorphisms displayed a clear lack of statistically significant associations with colorectal cancer risk.

DISCUSSION

Sporadic colorectal cancer is a multifactorial disease with multiple genetic determinants of varied significance. Numerous SNP analyses of sporadic colorectal cancer were conducted in order to clarify the genetic background. It has been hypothesized that polymorphic genetic variants involved in metabolism, DNA repair and apoptosis are linked to susceptibility to colorectal cancer[20]. Although

alterations in miRNA function have been detected in a broad spectrum of hematological malignancies and solid tumors[21-23], including CRC[24], only two studies performed to date have focused on miRNA-associated SNPs in CRC; although these studies were carried out in the Chinese population[13,14], their results were contradictory. Although it has been hypothesized that SNPs in miRNA genetic regions may affect the transcription of pri-miRNA transcripts, processing of miRNA precursors to mature miRNAs or miRNA target interactions, genetic variants in pre-miRNA regions are rare and unlikely to be functionally important, mainly due to the serious pressure imposed by natural selection on the evolutionary conserved pre-miRNA sequences[3].

In our study, we performed a case-control study of the three most frequently studied SNPs in miRNA genes (rs11614913 in miR-196-a2, rs895819 in miR-27a and rs2910164 in miR-146a), to investigate the degree of risk of CRC in the Central-European Caucasian population.

As it has been experimentally validated that the rs11614913 polymorphism located in the miR-196-a2 mature sequence affects the maturation and effect of target mRNA possibility, it is biologically plausible that genetic variation of hsa-miR-196a2 could modulate cancer susceptibility. In accordance with this finding, rs11614913 is one of the most frequently studied SNPs associated with miRNAs in case-control studies of a wide range of solid cancers[5-14]. For example, Hu *et al*[8] reported that the CC homozygous genotype of rs11614913 located in miR-196a2 was associated with a statistically significant increase in the mature miR-196a and a worse prognosis in non-small-cell lung cancer (NSCLC), proposing that this SNP could serve as a prognostic marker of NSCLC. Another Chinese study reported a clear association between

CC and CC/CT genotypes of rs11614913 and increased risk of breast cancer (OR 1.23; 95% CI: 1.02-1.48)[4]. When reviewed together, the majority of these studies described significant associations of the rs11614913-C allele with susceptibility and/or poor prognosis of lung cancer[7,8], gastric cancer[9], esophageal cancer[10], hepatocellular carcinoma[11] and head and neck cancer[12]. More recently, two Chinese studies focusing on an association between this SNP and susceptibility to CRC and its progression were performed[13,14].

Although the frequency of CC homozygotes of rs11614913 was higher in CRC patients than in healthy controls (41.62% *vs* 41.04%) in our study, the genotypes carrying the C allele (CT and CC) expressed the opposite trend in frequencies (64.21% in CRC *vs* 65.33% in controls). Moreover, the frequency of the C allele in CRC patients (64.21%) was not significantly lower than in healthy controls (65.33%). Furthermore, no significant association between the miR-196a2 polymorphism and the risk of CRC was observed in our study. These results are in agreement with the findings by Chen *et al*[14]. On the other hand, Zhan's group described the C allele as a risk factor for CRC in the Chinese population. Neither of the Chinese studies reported any associations between the rs11614913 polymorphism and CRC progression, including tumor grade, stage, lymph node and distant metastasis[13,14]. The discrepancy in the potential significance of rs11614913 in CRC reported by the above-mentioned independent studies may be due to different molecular pathogenetic mechanisms as different contributors to cancer or population-specific factors such as the different genetic backgrounds of the studied cohorts.

MiR-27a, in general, is a very important miRNA involved in the development of chemoresistance in solid cancer[15]. This study presents the first case-control investigation of the role of the A/G polymorphism (rs895819) in miR-27a in CRC; however, no significant associations were observed. Although the frequency of AA homozygotes was higher in CRC patients than in healthy controls (44.67% *vs* 43.87%), the frequencies of the genotypes carrying the A allele (AA and AG) did not show significant differences between the study cohorts (66.50% in CRC patients *vs* 66.04% in healthy controls). In gastric cancer, it has been reported that the variant genotypes of rs895819 located at miR-27a conferred a 48% increased risk of developing gastric cancer in the Chinese population; moreover, this trend tended to be age-specific. The authors concluded that elevated levels of miR-27a, through regulating the Zinc finger and BTB domain containing 10 (ZBTB10), result in the over-expression of Sp proteins and Sp-dependent genes, which play important roles in gastric cancer cell survival and angiogenesis[15,25]. Furthermore, Yang *et al*[26] found that the G-allele of rs895819, located in the terminal loop of the pre-miR-27a oncogene, is associated with reduced familial breast cancer risk (OR = 0.88; 95% CI: 0.78-0.99; *P* = 0.0287).

MiRNA-146a and its G to C common polymorphism, rs2910164, located within the sequence or the miR-146a precursor represent another miRNA hotspot evaluated in CRC for the first time in the present study. This SNP leads to change from a G:U pair to a C:U mismatch, and consequently, to reduced levels of pre- and mature miR-146a[17,19]. As BRCA1 and BRCA2, key breast and ovarian cancer susceptibility genes, are predicted targets of miR-146a, the majority of studies have been focused on breast and ovarian cancer. The results of Chen *et al*[16], who primarily studied rs2910164 in breast cancer and postulated that breast and ovarian cancer patients who had at least one variant allele were diagnosed at an earlier age (*P* = 0.029, *P* = 0.014, respectively), were not confirmed by further and larger independent case-control studies performed by Hu *et al*[4] and Catucci *et al*[6]. Garcia *et al*[27] concluded that the rs2910164: G > C SNP in the miR-146a gene is not associated with breast cancer risk in BRCA1 and BRCA2 mutation carriers. This case-control study, i.e., the first study to investigate the role of the miR-146a polymorphism, rs2910164, in CRC risk, found no significant association. Although our results did not indicate any significant relationship between the above-mentioned, miRNA-associated, SNPs and risk of CRC, we believe that a more detailed and comprehensive characterization of miRNA SNPs will improve our understanding of the miRNAs involved in CRC onset and progression which is necessary for the development of novel diagnostic and therapeutic strategies and approaches to this deadly disease.

COMMENTS

Background
The Central-European Caucasian population displays an extraordinarily high incidence of sporadic colorectal cancer and although there is a long-term tendency towards a decrease in mortality rates attributed to colorectal cancer, it still represents a major cause of death in the population. Susceptibility to colorectal cancer is typically multifactorial and can be characterized by intensive crosstalk between the genetic background of an individual and environmental factors. Susceptibility genes are involved in metabolic pathways controlled by epigenetic mechanisms where microRNA-associated regulations may play an important role.

Research frontiers
MicroRNAs (miRNAs) are small non-coding RNAs regulating gene expression. It has been recently suggested that single nucleotide polymorphisms (SNPs) in genes encoding mir196-a2, miR-27a and mir146-a may be associated with increased risk of various types of solid cancer. However, no such study of colorectal cancer has been conducted so far. This study investigated three SNPs (rs11614913 in miR-196-a2, rs895819 in hsa-miR-27a and rs2910164 in miR-146a) which were previously reported to be significantly associated with various types of solid cancer.

Innovations and breakthroughs
To the best of our knowledge, this is the first study focusing on the significance of rs11614913 in miR-196-a2, rs895819 in hsa-miR-27 and rs2910164 in miR-146a in sporadic colorectal cancer. The study was conducted using a highly homogenous Central-European Caucasian population with extremely high rates of sporadic colorectal cancer.

Applications
The significance of SNPs in genes encoding miRNAs remains controversial. Positive associations of the investigated polymorphisms were reported in various types of solid cancer. Based on the results, however, the investigated SNPs in miRNA genes do not seem to be major genetic determinants of genetic susceptibility to sporadic colorectal cancer in the Central-European population.

Terminology
Genetic susceptibility refers to inherited predisposition to increased risk of developing a certain disease, typically a multifactorial disease. mRNAs mean short non-coding RNAs, 17-22 nucleotides in length, which regulate gene expression and thereby play significant roles in cancer.

Peer review
The manuscript is well presented and supported by data.

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BRIEF ARTICLE

Progression of remnant gastric cancer is associated with duration of follow-up following distal gastrectomy

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Abstract

AIM: To re-evaluate the recent clinicopathological features of remnant gastric cancer (RGC) and to develop desirable surveillance programs.

METHODS: Between 1997 and 2008, 1149 patients underwent gastrectomy for gastric cancer at the Department of Digestive Surgery, Kyoto Prefectural University of Medicine, Japan. Of these, 33 patients underwent gastrectomy with lymphadenectomy for RGC. Regarding the initial gastric disease, there were 19 patients with benign disease and 14 patients with gastric cancer. The hospital records of these patients were reviewed retrospectively.

RESULTS: Concerning the initial gastric disease, the RGC group following gastric cancer had a shorter interval [$P < 0.05$; gastric cancer *vs* benign disease: 12 (2-22) *vs* 30 (4-51) years] and were more frequently reconstructed by Billroth-I procedure than those following benign lesions ($P < 0.001$). Regarding reconstruction, RGC following Billroth-II reconstruction showed a longer interval between surgical procedures [$P < 0.001$; Billroth-II *vs* Billroth-I: 32 (5-51) *vs* 12 (2-36) years] and tumors were more frequently associated with benign disease ($P < 0.001$) than those following Billroth-I reconstruction. In tumor location of RGC, after Billroth-I reconstruction, RGC occurred more frequently near the suture line and remnant gastric wall. After Billroth-II reconstruction, RGC occurred more frequently at the anastomotic site. The duration of follow-up was significantly associated with the stage of RGC ($P < 0.05$). Patients diagnosed with early stage RGC such as stage I-II tended to have been followed up almost every second year.

CONCLUSION: Meticulous follow-up examination and early detection of RGC might lead to a better prognosis. Based on the initial gastric disease and the procedure of reconstruction, an appropriate follow-up interval and programs might enable early detection of RGC.

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Key words: Remnant gastric cancer; Surveillance; Follow-up; Reconstruction; Distal gastrectomy

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INTRODUCTION

The incidence of remnant gastric cancer (RGC) following distal gastrectomy has been reported to account for 1%-2% of all gastric cancers in Japan[1,2]. Previously, RGC was reported to be caused by multiple factors, and the incidence, pathological features, and potential mechanisms have been extensively investigated[3-5]. Specifically, RGC is commonly found at an advanced stage, resulting in low rates of curative resection (38%-40%) and a consequently poor prognosis[6,7]. However, recently, the incidence and etiology of RGC have been changing[8] because of the long latency periods, decreasing prevalence of gastrectomy for benign disease[6,9], early detection and improved outcomes in patients with gastric cancers[10,11]. Moreover, recent advances in diagnostic and treatment techniques have led to a higher detection rate of early RGC following distal gastrectomy[12]. Consequently, endoscopic therapy such as endoscopic mucosal resection or endoscopic submucosal dissection is applicable for treatment of early-stage RGC[13,14]. Indeed, more than half of the RGC patients were treated for T1 or T2, node-negative and early stage cancer at our institution and almost 80% of patients with RGC were curatively resected. Therefore, it is necessary to re-evaluate the risk factors of RGC to develop an optimal new surveillance program and treatment guide. However, there is limited information available to help guide the treatment of patients with RGC. This study was designed to re-evaluate the clinicopathological characteristics and surgical outcomes of RGC and to develop desirable surveillance programs.

MATERIALS AND METHODS

Patients

Between 1997 and 2008, 1149 patients underwent gastrectomy for gastric cancer. Of these, 33 consecutive patients with primary RGC were treated in the Department of Digestive Surgery, Kyoto Prefectural University of Medicine. All patients underwent gastrectomy with lymphadenectomy for RGC. The clinicopathologic findings of these patients were determined retrospectively based on their hospital records. Macroscopic, microscopic and histopathological classifications of gastric cancers were based on the Japanese Classification of Gastric Carcinomas[15] and tumor-node-metastasis staging system[16].

Histologic types were classified as differentiated (papillary, moderately or well-differentiated adenocarcinoma) and undifferentiated (poorly or undifferentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous adenocarcinoma).

Follow-up program after initial gastrectomy

The follow-up program after initial gastrectomy at our institution is comprised of a regular physical examination and laboratory blood tests, chest X rays, an upper gastrointestinal series or endoscopy and ultrasonography or computer tomography for the first 5 years, and yearly endoscopy thereafter, if possible.

Evaluation of clinical associations between remnant gastric cancer and various clinical factors

The correlations between clinical factors and an initial factor such as previous disease or method of reconstruction in initial surgery were examined. Moreover, the follow-up interval is very important for screening recurrence and second primary gastric cancers. Therefore, correlation between follow-up periods and progression was evaluated in RGC.

Statistical analysis

The patient was included as a cause-specific death when the cause of death was specified as recurrent RGC. χ^2 test and Fisher's exact probability test were performed for categorical variables, while Student's *t*-test and Mann-Whitney *U*-test for unpaired data with continuous variables were performed to compare the clinicopathological characteristics between two groups. Kruskal-Wallis *H* test was used as a nonparametric procedure that can be used to compare more than two groups for analyses of follow-up interval. A *P* value less than 0.05 was considered significant.

RESULTS

Clinicopathologic characteristics of patients with primary remnant gastric cancer

The mean patient age was 68 years, and the male:female ratio was 2.7:1. Regarding the initial gastric disease, there were 19 patients with benign disease and 14 patients with gastric cancer. The median interval between the 1st and 2nd surgery was 20 years. Reconstruction during the 1st surgery was mainly Billroth-Ⅰ or Billroth-Ⅱ. En bloc resection of the tumor by total remnant gastrectomy was performed with jejunal mesentery and D2 lymphadenectomy and concomitant organ resection. Eighteen patients additionally received splenectomy, four patients received distal pancreatectomy, two patients received partial colon resection and two patients received liver resection. Reconstructions were performed in 16 patients by Billroth-Ⅰ, 16 patients by Billroth-Ⅱ and one by Roux-en-Y procedure for all resected RGC tumors. Tumors were located at the anastomotic site in 16 (61%) patients, corpus and/or cardia in nine (34%), and throughout the

Table 1 Association between clinicopathologic characteristics and initial disease *n* (%)

Variables	*n*	Benign (*n* = 19)	Cancer (*n* = 14)	*P* value
			Initial disease	
Age (yr) (mean)		70 (51)	66 (49)	0.26
Gender				
Male	24	15 (63)	9 (38)	
Female	9	4 (44)	5 (56)	0.35
Interval from initial surgery				
Year (median)		30 (4-51)	12 (2-22)	< 0.05
Reconstruction of 1st surgery				
Billroth- I	16	4 (25)	12 (75)	
Billroth- II	16	15 (94)	1 (6)	
R-Y	1	0 (0)	1 (100)	< 0.001
Location of RGC				
Anastomotic site	11	9 (82)	2 (18)	
Suture line	7	2 (29)	5 (71)	
Others	15	8 (53)	7 (47)	0.08
Histological type				
Differentiated	13	8 (62)	5 (38)	
Undifferentiated	20	11 (55)	9 (45)	0.71
Lymphatic invasion				
Negative	16	8 (50)	8 (50)	
Positive	17	11 (65)	6 (35)	0.39
Venous invasion				
Negative	16	8 (50)	8 (50)	
Positive	17	11 (65)	6 (35)	0.39
Tumor size				
cm (mean)		51 (46)	61 (54)	0.40
Depth of tumor				
T1	10	4 (40)	6 (60)	
T2, 3, 4	23	15 (65)	8 (35)	0.18
Lymph node metastasis				
Negative	20	10 (50)	10 (50)	
Positive	13	9 (69)	4 (31)	0.27
Stage				
I	17	8 (47)	9 (53)	
II, III, IV	16	11 (69)	5 (31)	0.21

Significant values are shown in boldface type. *P* values were derived from χ^2 or Fisher's exact test and were considered significant at < 0.05. R-Y: Roux-en Y; RGC: Remnant gastric cancer.

Table 2 Association between clinicopathologic characteristics and reconstruction of 1st surgery *n* (%)

Variables	*n*	Billroth- I (*n* = 16)	Billroth- II (*n* = 16)	*P* value
		Reconstruction at first surgery		
Age (yr) (mean)		68 (50)	69 (50)	0.64
Gender				
Male	24	13 (54)	11 (46)	
Female	8	3 (38)	5 (63)	0.69
Interval from initial surgery				
Year (median)		12 (2-36)	32 (5-51)	< 0.001
Initial gastric disease				
Benign	19	4 (21)	15 (79)	
Cancer	13	12 (92)	1 (8)	< 0.001
Location of RGC				
Anastomotic site	11	2 (18)	9 (82)	
Suture line	7	5 (71)	2 (29)	
Others	14	9 (64)	5 (36)	0.11
Histological type				
Differentiated	13	8 (62)	5 (38)	
Undifferentiated	19	8 (42)	11 (58)	0.47
Lymphatic invasion				
Negative	15	6 (40)	9 (60)	
Positive	17	10 (59)	7 (41)	0.48
Venous invasion				
Negative	16	8 (50)	8 (50)	
Positive	16	8 (50)	8 (50)	0.72
Tumor size				
mm (mean)		51	56	0.67
Depth of tumor				
T1	10	6 (60)	4 (40)	
T2, 3, 4	22	10 (45)	12 (55)	0.76
Lymph node metastasis				
Negative	14	9 (47)	10 (53)	
Positive	13	7 (54)	6 (46)	1
Stage				
I	17	9 (53)	8 (47)	
II, III, IV	15	7 (47)	8 (53)	1

Significant values are shown in boldface type. *P* values were derived from χ^2 or Fisher's exact test and were considered significant at < 0.05. RGC: Remnant gastric cancer.

whole remnant in one (4%) patient. Consequently, more than half of the RGC patients demonstrated T1 or T2, undifferentiated, node-negative and early stage cancer. In 78.8% (26/33) of patients, resections were performed with curative intent.

Association between clinicopathologic characteristics and initial disease

Clinicopathologic findings of 33 patients with primary RGC are listed in Table 1 according to the nature of the primary disease. Patients with RGC following gastric cancer showed a significantly shorter interval between the 1st and 2nd surgery [*P* < 0.05, gastric cancer *vs* benign disease: 12 (2-22) *vs* 30 (4-51) years] and were more frequently reconstructed by the Billroth- I method than those following benign disease (*P* < 0.005). Other factors did not significantly differ between the two groups.

Association between clinicopathologic characteristics and reconstruction of 1st surgery

Table 2 shows details of 33 RGC patients according to

the method of reconstruction. RGC following Billroth- II reconstruction showed a longer interval between surgical procedures (*P* < 0.001) and tumors were more frequently associated with benign disease (*P* < 0.001) than those following Billroth- I reconstruction. Figure 1 shows the tumor location of 32 RGC following distal gastrectomy according to the method of reconstruction. After Billroth- I reconstruction, RGC occurred more frequently near the suture line and remnant gastric wall. After Billroth- II reconstruction, RGC occurred more frequently at the anastomotic site.

The duration of follow-up after distal gastrectomy

The duration of follow-up was significantly associated with the stage of progression in RGC (*P* < 0.05). Patients diagnosed with early stage RGC such as stage I - II tended to have been followed up almost every second year (Figure 2).

DISCUSSION

Gastric cancer is the second leading cause of cancer-

Figure 1 Location of 32 remnant gastric cancer tumors following distal gastrectomy according to the method of reconstruction. After Billroth-Ⅰ reconstruction, remnant gastric cancer (RGC) occurred more frequently near the suture line and remnant gastric wall; whereas, RGC after Billroth-Ⅱ reconstruction occurred more frequently at the anastomotic site.

| Mean interval (yr) | ESD[1] 1.58 | Stage I 2.04 | Stage Ⅱ 2.50 | Stage Ⅲ 3.12 | Stage Ⅳ 4.50 |

Kruskal Wallis *H*-test $P < 0.05$

Figure 2 Association of endoscopic follow-up intervals and the stage of progression. The follow-up interval was significantly associated with the stage of progression in remnant gastric cancer ($P < 0.05$). [1]Patients treated with endoscopic submucosal dissection (ESD), who were not included in this study, are presented for the purpose of comparison.

related death in the world[17]. However, recent advances in diagnostic methods, less invasive treatment techniques and better peri-operative management have increased the early detection of gastric cancer and decreased the mortality and morbidity rates[18-20]. Consequently, the number of cured patients has been increasing and some of these patients are at risk of acquiring second primary cancer in the remnant stomach. This implies that more cases of RGC will be encountered in the future.

In previous reports, RGC was commonly found at an advanced stage, resulting in low rates of curative resection (38%-40%) and a consequent poor prognosis[6,7]. However, recently, the incidence and etiology of RGC following distal gastrectomy may be changing due to diagnostic and technological advances. In our study, more than half of the RGC patients were treated for T1 or T2, node-negative and early stage cancer, contrary to that in previous series (Table 1). Almost 80% of patients were curatively resected with intensive lymphadenectomy. Thereby, the survival curves of primary proximal gastric cancer (PGC) and RGC were similar and without a significant difference, although patients with RGC tended to have a higher incidence of undifferentiated cancer, vascular invasion, and T4 component than patients with PGC (data not shown). Therefore, RGC is not always advanced at diagnosis and if so, intensive surgery for RGC does not necessarily mean a poor prognosis in comparison to that for primary gastric cancer. Therefore, it is necessary to re-evaluate the risk factors of RGC to develop an optimal new endoscopic surveillance program and treatment guide.

Regarding surveillance systems for early detection and curative treatment of RGC, periodic endoscopic examinations of the gastric remnant are shown to be extremely important in our study (Figure 1). However, a follow-up program that is too intensive may not be beneficial to the patient. The initial gastric disease and the interval between the 1st and 2nd surgery could affect the incidence of RGC. In our study, RGC following gastric cancer had a significantly shorter interval between 1st and 2nd surgery than that following benign disease [*P* <

0.05; gastric cancer *vs* benign disease: 12 (2-22) years *vs* 30 (4-51) years]. However, surveillance systems for gastric cancer should be especially considered because of decreasing gastrectomy for benign disease. Furthermore, 86% of all initial gastric cancer patients underwent Billroth-Ⅰ reconstruction at our institution and their median interval between 1st and 2nd surgery was 12 (2-36) years. Moreover, the duration of follow-up was significantly associated with the stage of RGC progression and an early detection of RGC led to better prognosis (Figure 2). Taken together, annual surveillance endoscopic screening should be required for at least 12 years following distal gastrectomy. Furthermore, after 12 years of follow-up, surveillance endoscopy should be recommended every second year because we found that patients diagnosed with early stage RGC such as stage Ⅰ-Ⅱ tended to have been followed almost every second year. In particular, meticulous endoscopy examination should be performed near the suture line and remnant gastric wall after Billroth-Ⅰ reconstruction and also should be performed at the anastomotic site after Billroth-Ⅱ reconstruction.

In conclusion, due to recent advances in diagnostic and treatment technologies, the etiology of RGC has been changing. Meticulous follow-up examination and early detection of RGC might lead to a better prognosis. Considering both the initial gastric disease and the procedure of reconstruction, an appropriate follow-up interval and programs should facilitate the detection of early RGC.

COMMENTS

Background
Recently, the incidence and etiology of remnant gastric cancer (RGC) have been changing because of the long latency periods, decreasing prevalence of gastrectomy for benign disease, early detection and improved outcomes in patients with gastric cancers. Moreover, recent advances in diagnostic and treatment technique have led to a higher detection rate of early RGC following distal gastrectomy.

Research frontiers

It is necessary to re-evaluate the risk factors of RGC and develop an optimal new surveillance program and treatment guide. However, there is limited information available to help guide the treatment of patients with RGC. In this study, the authors re-evaluated the clinicopathological characteristics and surgical outcomes of RGC and developed desirable surveillance programs.

Innovations and breakthroughs

In this study, more than half of the RGC patients were demonstrated to have T1 or T2, undifferentiated, node-negative and early stage cancer. The duration of follow-up was significantly associated with the stage of progression in RGC. Patients diagnosed with early stage RGC such as stage Ⅰ-Ⅱ tended to have been followed almost every second year. After Billroth-Ⅰ reconstruction, RGC occurred more frequently near the suture line and remnant gastric wall. After Billroth-Ⅱ reconstruction, RGC occurred more frequently at the anastomotic site.

Applications

RGC following gastric cancer had a significantly shorter interval between 1st and 2nd surgery than those following benign disease. Annual surveillance endoscopic screening should be required for at least 12 years following distal gastrectomy. Furthermore, after 12 years of follow-up, surveillance endoscopy should be recommended every second year.

Terminology

The incidence of RGC following distal gastrectomy has been reported to account for 1%-2% of all gastric cancers in Japan. In previous reports, RGC was commonly found at an advanced stage, resulting in low rates of curative resection (38%-40%) and a consequent poor prognosis.

Peer review

Authors have given new thoughts while designing this study. The paper is nicely written.

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BRIEF ARTICLE

Role of ascites adenosine deaminase in differentiating between tuberculous peritonitis and peritoneal carcinomatosis

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Abstract

AIM: To investigate the usefulness of tumor markers and adenosine deaminase in differentiating between tuberculous peritonitis (TBP) and peritoneal carcinomatosis (PC).

METHODS: A retrospective analysis of data was performed on consecutive patients who underwent peritoneoscopic and abdominal computed tomography (CT) evaluations. Among 75 patients at the Seoul National University Hospital from January 2000 to June 2010 who underwent both tests, 27 patients (36.0%) and 25 patients (33.3%) were diagnosed with TBP and PC, respectively. Diagnosis was confirmed by peritoneoscopic biopsy.

RESULTS: Serum c-reactive protein (7.88 ± 6.62 mg/dL *vs* 3.12 ± 2.69 mg/dL, *P* = 0.01), ascites adenosine deaminase (66.76 ± 32.09 IU/L *vs* 13.89 ± 8.95 IU/L, *P* < 0.01), ascites lymphocyte proportion (67.77 ± 23.41% *vs* 48.36 ± 18.78%, *P* < 0.01), and serum-ascites albumin gradient (0.72 ± 0.49 g/dL *vs* 1.05 ± 0.50 g/dL, *P* = 0.03) were significantly different between the two groups. Among tumor markers, serum and ascites carcinoembryonic antigen, serum carbohydrate antigen 19-9 showed significant difference between two groups. Abdominal CT examinations showed that smooth involvement of the parietal peritoneum was more common in the TBP group (77.8% *vs* 40.7%) whereas nodular involvement was more common in the PC group (14.8% *vs* 40.7%, *P* = 0.04). From receiver operating characteristic (ROC) curves ascites adenosines deaminase (ADA) showed better discriminative capability than tumor markers. An ADA cut-off level of 21 IU/L was found to yield the best results of differential diagnosis; sensitivity, specificity, positive predictive value, and negative predictive value were 92.0%, 85.0%, 88.5% and 89.5%, respectively.

CONCLUSION: Besides clinical and radiologic findings, ascitic fluid ADA measurement is helpful in the differential diagnosis of TBP and PC.

INTRODUCTION

Tuberculous peritonitis (TBP) and peritoneal carcinomatosis (PC) are two of the most common causes of exudative ascites in South Korea and both diseases require rapid recognition for the appropriate therapeutic management[1-3]. In a clinical situation, etiological diagnosis of the two diseases is very difficult because of the lack of specific differential clinical, radiological, or laboratory findings. Peritoneoscopy is thought to be the method of choice in the diagnosis of the two diseases[4-6]. However, the diagnostic failure rate of peritoneoscopy can reach as high as approximately 14%; the main reason for failure is interference from adhesions due to tumor, tuberculosis or previous surgery[4].

The purpose of this study was to clarify the differences in clinical, radiological, laboratory and peritoneoscopic findings between TBP and PC and to evaluate the diagnostic capacity of ascites adenosine deaminase (ADA) and tumor markers for the differentiating the two diseases.

MATERIALS AND METHODS

Patients

Between January 2000 and June 2010, patients over 18 years of age with exudative ascites of unknown etiology who underwent abdominal computed tomography (CT) scan and peritoneoscopy for diagnosis were enrolled in this study. All patients had laboratory tests such as complete blood count, serum biochemical tests, tumor markers from blood and ascites fluid, ascites cytology, ascites cell count and biochemical tests, and ascites ADA. Diagnosis of TBP was made if one of the following criteria was met: (1) ascites was positive for acid-fast bacilli stain and culture; (2) tuberculosis polymerase chain reaction test from ascites or peritoneoscopic biopsy specimen was positive; or (3) caseating granuloma was noted in the peritoneal biopsy specimen. PC was diagnosed if cancer cells from ascites cytology were detected or cancer cells were documented from the peritoneoscopic biopsy specimen. This study protocol was approved by the Ethics Committee at Seoul National University Hospital.

Peritoneoscopy

Patients underwent peritoneoscopy under local anesthesia with systemic analgesics. A lidocaine injection was done from skin to fascia at about 1 cm left side from umbilicus. After local anesthesia, a Veress needle was inserted, and then air insufflation was performed through the needle. After removal of the Veress needle, a trochar was inserted into peritoneum and peritoneoscope (Olympus; Tokyo, Japan) was inserted through the trochar into the peritoneum. After ascites fluid was drained for examination, a detailed observation of the peritoneum and intra-abdominal organs was performed. Experienced endoscopists performed all peritoneoscopic procedures and observation. Pictures of all important peritoneoscopic findings were taken and stored in a picture archiving and communication system. Two endoscopists (Kang SJ, Kim JW) reviewed the peritoneoscopic images to assess the nature of the ascites fluid and look for abnormalities such as nodules, patches, and adhesions. Nodules were classified according to size as $\leq$ 1 cm or > 1 cm. Patches were classified according to their location (parietal or visceral peritoneum). Membranous patches were defined as plaques.

Radiologic examination

All patients had abdominal CT scan examination. The following CT scanners were used in this study: Hi Speed/RP single channel CT scanner (GE Healthcare, Milwaukee, Wisconsin, United States) ($n = 14$), MX 8000 four-channel CT scanner (Marconi Medical Systems, Cleveland, Ohio, United States) ($n = 32$), LightSpeed eight-channel CT scanner (GE Healthcare) ($n = 12$), Sensation 16 16-channel CT scanner (Siemens Medical Solutions, Erlangen, Germany) ($n = 13$), and Brilliance 64 64-channel CT scanner (Philips Healthcare, Cleveland, Ohio, United States) ($n = 4$). Section thickness and reconstruction interval were both 7mm for the single channel CT scanner and 5mm for the four-channel and eight-channel CT scanners, for the 16- and 64-channel CT scanners, section thickness and reconstruction interval were 3mm and 2.5 mm, respectively. Scanning was performed from the dome of the diaphragm through the pubic symphysis. Contrast-enhanced CT scan was performed after injection of nonionic contrast material (iopromide, Ultravist 370; Bayer Healthcare, Germany).

All scans were obtained on a GE 9800 (General Electric, Milwaukee, Wisconsin, United States) or a Somatom DR3 (Siemens, Erlangen, Germany) scanner with a slice thickness of 10 mm at 10- to 13-mm intervals from the dome of the diaphragm to the pubic symphysis. Two radiologists (Baek JH, Kim SH) reviewed abdominal CT scans of patients and looked at ascites (presence of loculation), parietal involvement patterns (smooth thickening, irregular or nodular thickening, seeding nodules), mesenteric changes, mesenteric thickening, mesenteric nodules (micronodule, macronodule), omental thickness, omental changes (smudged, nodular, omental cake), and intestinal involvement.

Statistical analysis

Values for continuous variables were presented as mean ± SD or median with ranges and as the number of individuals (and the percentage in each group) for the cat-

Table 1 Demographics and clinical characteristics of the patients *n* (%)

	Tuberculous peritonitis (*n* = 27)	Peritoneal carcinomatosis (*n* = 25)	*P* value
Age (yr)	58.04 ± 12.61	61.12 ± 11.67	0.37
Gender (M:F)	11:16	16:9	0.09
Duration of symptoms			0.01
< 1 mo	11 (40.7)	2 (8.0)	
≥ 1 mo	16 (59.3)	23 (92.0)	
Symptoms			
Abdominal pain	12 (44.4)	15 (60.0)	0.26
Abdominal distension	25 (92.6)	21 (84.0)	0.33
Weight loss	8 (29.6)	11 (44.0)	0.28
Loss of appetite	12 (44.4)	8 (32.0)	0.36
Night sweating	3 (11.1)	0 (0.0)	0.09
Fever	16 (59.3)	0 (0.0)	< 0.01
Diarrhea	2 (7.4)	3 (12.0)	0.58

M/F: Male/female.

Table 2 Laboratory features including tumor markers in serum and ascites of the patients

	Tuberculous peritonitis (*n* = 27)	Peritoneal carcinomatosis (*n* = 25)	*P* value
Serum lab			
Serum WBC (/mm^3)	6083.7 ± 3115.9	6429.6 ± 2466.6	NS
Serum lymphocyte (%)	16.70 ± 8.39	22.01 ± 7.19	0.02
Serum CRP (mg/dL)	7.88 ± 6.62	3.12 ± 2.69	0.03
Serum CEA (ng/mL)	1.79 ± 1.09	10.26 ± 25.38	0.02
Serum CA 19-9 (U/mL)	10.63 ± 8.52	2283.56 ± 6211.99	0.05
Serum CA 125 (U/mL)	591.36 ± 440.95	676.73 ± 1088.28	NS
Ascites lab			
Ascites WBC (/mm^3)	1325.9 ± 955.1	951.1 ± 773.1	NS
Ascites lymphocyte (%)	67.77 ± 23.41	48.36 ± 18.78	< 0.01
Ascites albumin (g/dL)	2.30 ± 0.75	2.32 ± 0.76	NS
SAAG	0.72 ± 0.49	1.05 ± 0.50	0.03
Ascites ADA (IU/L)	66.76 ± 32.09	13.89 ± 8.95	< 0.01
Ascites CEA (ng/mL)	1.36 ± 0.83	682.77 ± 1955.34	0.01
Ascites CA 19-9 (U/mL)	17.53 ± 24.15	12344.10 ± 33569.78	NS
Ascites CA 125 (U/mL)	1069.20 ± 578.74	1188.56 ± 1439.06	NS

WBC: White blood cells; CRP: C-reactive protein; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 125: Carbohydrate antigen 125; SAAG: Serum-ascites albumin gradient; ADA: Adenosine deaminase; NS: Not significant. All statistical significance tests were performed by Mann-Whitney *U*-test.

egorical variables. Nominal data were compared by using the Fisher exact test or Pearson χ^2 test, and continuous variables were compared by using the Student *t* test or Mann-Whitney *U*-test. A receiver-operating characteristic (ROC) curve was plotted and the area under the curve (AUC) was calculated to determine the predictive ability of different ascites ADA and tumor markers level cutoff values for differentiation. For all analyses, a *P* value of < 0.05 (two-tailed) was taken as statistically significant. All statistical analyses were performed with SPSS 15.0K for Windows (SPSS South Korea, Seoul, South Korea).

RESULTS

Clinical characteristics of patients

A total of 75 patients underwent abdominal CT scan and diagnostic peritoneoscopy. Of that population, 27 patients were diagnosed with TBP and 25 patients were diagnosed with PC according to the definition stated in the methods section. Other patients were diagnosed with various diseases such as pelvic inflammatory disease, continuous ambulatory peritoneal dialysis peritonitis, systemic lupus erythematosus (SLE), or peritoneal lymphomatosis. PC group included only adenocarcinomas from the various origins (6 pancreatic cancers, 4 ovary cancers, 5 malignancies of unknown origin, 4 colorectal cancers, 3 advanced gastric cancers and 3 other cancers). Clinical characteristics of patients are shown in Table 1. In the TBP group, there were 11 men (40.7%) and 16 women (59.3%), ranging from 28 to 83 years of age (mean ± SD = 58.04 ± 12.61 years). The PC group had more males (16 men and 9 women), but no significant difference in gender was found between the two groups (*P* = 0.09). There were 11 patients (40.7%) with duration of symptoms < 1 mo in the TBP group, while most patients in the PC group (*n* = 23, 92.0%) developed symptoms > 1 mo before they were diagnosed (*P* = 0.01). In the TBP group, 3 patients had night sweats, whereas no patients in the PC group complained of that symptom (*P* = 0.09). Fever

was the predominant manifestation in the TBP group (16, 59.3%), whereas no patient in the PC group developed fever (*P* < 0.01).

Laboratory findings and tumor markers in serum and ascites of patients

Results of blood and ascites laboratory tests including tumor markers are summarized in Table 2. Serum c-reactive protein (CRP) was significantly higher in the TBP group. Among ascites laboratory findings, TBP group showed severer lymphocytosis, lower serum ascites albumin gradient, and higher ascites ADA.

Tumor markers [cercinoembryonic antigen (CEA), CA 19-9, CA 125] in serum and ascites in both groups are also presented in Table 2 and theirs scatter plots are presented in Figure 1. In PC group, serum and ascites CEA and serum CA 19-9 were higher than TBP group. Serum and ascites CA 125 were elevated in both groups and showed no significant differences.

Radiological characteristics of patients

Ascites was found in the abdominal CT scan of all patients. The parietal involvement pattern was significantly different between the PC and TBP groups as shown in Table 3. In the TBP group, 21 patients (77.8%) showed smooth thickening of the parietal peritoneum, whereas smooth thickening was found in 10 patients (40.0%) in the PC group. Irregular and nodular parietal involvement was noted in 11 patients (44.0%) in the PC group whereas only 4 patients (14.8%) showed irregular or nodular involvement in the TBP group (*P* = 0.04). Thickening of mesentery was found in 15 (55.6%) and 8 (32.0%) patients in TBP and PC group, respectively (*P* = 0.09).

Figure 1 Scatter plots shows the distributions of tumor markers and adenosine deaminase in serum and ascites between peritoneal carcinomatosis group and tuberculous peritonitis group. All tests were performed by Mann-Whitney *U*-test. PC: Peritoneal carcinomatosis; TBP: Tuberculous peritonitis; ADA: Adenosine deaminase; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 125: Carbohydrate antigen 125.

Table 3 Abdominal computed tomography characteristics of the patients *n* (%)			
	Tuberculous peritonitis (*n* = 27)	Peritoneal carcinomatosis (*n* = 25)	*P* value
Ascites loculation	11 (40.7)	10 (40.0)	0.96
Parietal involvement			0.04
No	2 (7.4)	3 (12.0)	
Smooth thickening	21 (77.8)	10 (40.0)	
Irregular or nodular	4 (14.8)	11 (44.0)	
Seeding nodule	0 (0.0)	1 (4.0)	
Mesenteric change	23 (85.2)	17 (68.0)	0.14
Thickening of mesentery	15 (55.6)	8 (32.0)	0.09
Mesenteric nodule			0.31
No	15 (55.6)	12 (48.0)	
Micronodule	12 (44.4)	10 (40.0)	
Macronodule	0 (0.0)	2 (8.0)	
Omental thickness (mm)	20.48 ± 11.03	23.00 ± 15.77	0.51
Omental change			0.56
No	2 (7.4)	2 (8.0)	
Smudged	8 (29.6)	4 (16.0)	
Nodular	3 (11.1)	2 (8.0)	
Omental cake	13 (48.1)	17 (68.0)	
Intestinal involvement	3 (11.1)	5 (20.0)	0.53

In the TBP group, mesenteric nodularities were seen in 12 patients (44.4%) and all nodules were micronodules. Twelve patients (48.0%) in the PC group showed mesenteric nodules, which were composed of 10 micronodules and 2 macronodules. There was no discriminative difference in omental thickness and patterns of change between the two groups.

Peritoneoscopic findings of patients

Peritoneoscopic images were reviewed by 2 experienced endoscopists (Kang SJ and Kim JW), and findings are summarized in Table 4. Twenty patients (74.1%) in the TBP group were found to have micronodules on the peritoneum (example of micronodule was shown in Figure 2A), and this number was greater than the number of TBP patients found to have micronodules on CT scan. Macronodules were not observed in TBP group. In the PC group, micronodules were detected in 8 patients, and macronodules were observed in 9 patients (*P* = 0.01). The distribution of whitish patches and presence of plaques were not significantly different (*P* = 0.41) between the two groups (example of patch lesion in Figure 2B). Adhesions between the peritoneum and abdominal organs were seen in 14 patients in the TBP group and 10 patients in the PC group (*P* = 0.41).

Laboratory parameters for differentiation between TBP and PC groups

Among the evaluated laboratory parameters, parameters that showed significant difference between two groups were serum CRP, CEA, CA 19-9 and ascites ADA, CEA. AUC was calculated using ROC curves and these results

Table 4 Peritoneoscopic findings of the patients *n* (%)

	Tuberculous peritonitis (*n* = 27)	Peritoneal carcinomatosis (*n* = 25)	*P* value
Nodules on peritoneum			< 0.01
No	7 (25.9)	7 (28.0)	
< 1 cm micronodule	20 (74.1)	8 (32.0)	
> 1 cm macronodule	0 (0.0)	9 (36.0)	
Peritoneum			0.41
Multiple whitish patches on parietal peritoneum	2 (7.4)	5 (20.0)	
Multiple whitish patches on parietal and visceral peritoneum	0 (0.0)	1 (4.0)	
No patches	19 (70.4)	15 (60.0)	
Whitish plaques	4 (14.8)	3 (12.0)	
Adhesion	14 (51.9)	10 (40.0)	0.41

Table 5 Discriminative capability of tumor markers and adenosine deaminase between tuberculosis peritonitis and peritoneal carcinomatosis using receiver operating characteristics curve

	AUC	95% CI	*P* value
Serum CRP	0.705	0.537-0.872	0.033
Serum CEA	0.721	0.562-0.880	0.017
Serum CA 19-9	0.693	0.527-0.860	0.044
Ascites ADA	0.966	0.916-1.000	< 0.001
Ascites CEA	0.823	0.686-0.960	0.002

ADA: Adenosine deaminase; CI: Confidence interval; CRP: C-reactive protein; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; AUC: Area under the curve.

Figure 3 **Receiver operating characteristic curve of ascites adenosine deaminase for differentiating between tuberculous peritonitis and peritoneal carcinomatosis.** AUC of this receiver operating characteristic curve is 0.97 (95% CI: 0.92-1.00, *P* < 0.01). CI: Confidence interval; AUC: Area under the curve.

Figure 2 **Peritoneoscopic pictures of tuberculous peritonitis and peritoneal carcinomatosis.** A: Peritoneoscopic picture of tuberculous peritonitis of female patient. Multitudinous miliary nodules are seen on the parietal peritoneum. Biopsy revealed caseous granulomatous inflammation and the biopsy specimen stained positive for acid-fast bacilli; B: Colon cancer with peritoneal seeding. Multiple irregular whitish patch lesions were found on the parietal peritoneum. Poorly differentiated adenocarcinoma was documented on biopsy.

were presented in Table 5. Among these markers, ascites ADA level was the strongest factor that differentiated TBP and PC patients. The ability to consider ascites ADA levels as a biomarker for differentiating TBP and PC patients was evaluated using ROC curve analysis. The AUC for TBP was 0.966 [95% confidence interval (CI): 0.916-1.00; *P* < 0.01] (Figure 3). The sensitivity, specificity and positive and negative predictive values of ascites ADA were 92.0%, 94.4%, 95.8% and 89.5% at cut-off

level of 21.0 IU/L and 88.0%, 94.4%, 95.7% and 85.0% at 32.0 IU/L. When compared to cut-off level of 32.0, cut-off level of 21.0 shows higher sensitivity and negative predictive value and same specificity and positive predictive value.

DISCUSSION

TBP and PC are two of the most common causes of exudative ascites[1]. Differentiating between the two disease entities is difficult, and laparoscopy is thought to be the gold standard for diagnosis. Laparoscopy with biopsy has shown impressive sensitivity and specificity rates of 93% and 98% respectively[7]. Yet even with laparoscopy, some patients cannot be diagnosed, because the biopsy findings can be insufficient for diagnosis. Furthermore, some patients cannot be evaluated by laparoscopy because of their poor general condition or technical failure. In those cases, clinical and laboratory findings can be helpful for differentiating between TBP and PC. This study demonstrates that ascites ADA is valuable laboratory finding in differentiating between TBP and PC. To the best of our knowledge, this is the first comprehensive series to date that compares tumor markers, abdominal CT scan, and

laparoscopic findings between tuberculous peritonitis and PC patients.

It is well known that ascites ADA has high sensitivity and specificity in the early diagnosis of TBP[8-10]. When used for diagnosing exudative ascites, at the cut-off level of 30 IU/L, ADA has demonstrated sensitivity of approximatley 94%[4]. One study about pediatric patients report 100% sensitivity and 97% specificity for diagnosis of TBP[11]. Of interest, in our study ADA also had the high diagnostic value in differentiating tuberculosis peritonitis and PC and showed highest differentiating value (largest AUC) at the cut-off level of 21 IU/L. This suggests that when two diseases, TBP and PC, are strongly suspected from clinical, laboratory and radiological findings, and other diseases causing exudative ascites are reliably excluded, an ADA cut-off level of 21 IU/L that is lower than the usual cut-off level used for diagnosis could be used for discrimination. But this study only includes 27 TBP patients and 25 PC patients, about the precise cut-off level of ascites ADA for discrimination of two diseases, further verification with large numbers of patients are required.

Ascites ADA is accurate but insensitive for detecting TBP in liver cirrhosis patients living in the United States where the prevalence of tuberculosis is low. In the United States, which has a low tuberculous burden, 59% of TBP patients have liver cirrhosis[12]. This means that in developed country where tuberculosis burden is low, TBP is mainly developed in immune compromised patients such as liver cirrhosis. In South Korea, the tuberculous burden is intermediate, so in our study only 14.8% (4 out of 27) of TBP patients had liver cirrhosis[13]. Low portion of cirrhotic patients partly explains high ascites ADA performance. Furthermore, 75% of TBP patients with liver cirrhosis (3 out of 4 patients) had a higher ascites ADA level than normal. So even cirrhotic patients, ascites of TBP patients shows high ADA. This also explains the sensitivity of ascites ADA for diagnosing TBP in our study.

Increased serum and ascites CA -125 levels, detected in up to 80% of women with late-stage ovarian cancer, have demonstrated great value in treatment monitoring and recurrence detection of ovary cancer[14-16]. In TBP, serum CA 125 levels are as high as ovarian cancer associated with peritoneal infiltration, and, by the end of the fourth month of anti-tuberculous therapy in a patient with TBP, serum CA 125 levels have returned to normal[17-19]. Similar to previous reports, this study demonstrated that both TBP and PC patients have elevated serum and ascites CA 125 levels[20-22]. As a results, ascites and serum CA 125 are cautiously interpreted in differentiating between TBP and PC.

Radiological findings of TBP are similar to PC. With a model of multivariate logistic analysis using mesenteric macronodules, omental lining, irregularity of omentum, and splenic abnormalities, the sensitivity for predicting tuberculous peritonitis was 69%, whereas the sensitivity for PC was 91%[23]. In our study, only the parietal involvement pattern was significantly different between the TBP and PC groups. A thickened mesentery and loss of normal mesenteric configuration are known to be characteristics of TBP and helpful for diagnosis, but our results did not show these findings to be helpful in differentiating between two disease[24]. When parietal involvement patterns were used as marker for differentiation, the sensitivity and specificity for diagnosing TBP in this study were 60.5% and 75.0%, respectively. This characteristics is insufficient as a marker for differentiation of diseases but can be used in adjunct to other examinations.

Peritoneoscopy is the diagnostic tool of choice in patients with exudative ascites of unknown origin[2,4]. Peritoneal tubercles and ascites are the main features of TBP and appear in more than 90% of TBP cases[25-27]. In our study, micronodules on the peritoneum and ascites were seen in 20 of 27 patients (74.1%). The low micronodule detection rate compared to previous studies may be due to the discovery of patch and plaque lesions in 6 patients (2 patch lesions and 4 plaque lesion, 22.2%) of the TBP group during peritoneoscopy, because nearly all suspicious patch lesions were biopsied whenever possible. For diagnosis of PC, peritoneoscopy has higher sensitivity and specificity than helical CT scan with 5-mm slice thickness, and similar results were found in this study[28]. The diagnostic failure rate of peritoneoscopy is substantially high, however, reaching about 14%; the main reason for diagnostic failure is interference from adhesions from previous surgery or tumor adhesion[7].

Interpretation of our findings requires careful consideration of several aspects. First, the positive and negative predictive values are usually affected by pretest probability, so those values of the ascites ADA in this study may be variable in countries of low tuberculosis prevalence. Second, PC group is composed of various cancers. So discriminative ability of each tumor markers such as CEA for specific cancer could not fully tested in this study, so larger numbers of patients are needed to assess the predicting ability of each ascites tumor marker for each type of cancer.

In conclusion, clinical findings such as duration of symptom less than 1 mo and fever are helpful for differentiating TBP and PC of various causes. Among laboratory findings, ascites ADA was the most valuable discriminative marker. For differentiation of two diseases, in addition to clinical findings and radiologic characteristics, ascites ADA should also be considered.

COMMENTS

Background
Tuberculous peritonitis (TBP) and peritoneal carcinomatosis (PC) are two of the most common causes of exudative ascites but in a clinical situation, etiological diagnosis of the two diseases is very difficult. Peritoneoscopy with biopsy is thought to be the method of choice in the differential diagnosis of the two diseases but the morbidity related with peritoneoscopic procedure and diagnostic failure rate reaches as high as approximately 8% and 14%, respectively. So we investigate the diagnostic capacities of adenosines deaminase (ADA) and tumor markers for differentiating the two diseases.

Research frontiers
Diagnostic abilities of serum ADA and tumor markers in differentiating two

diseases are hotspots in recent studies. Serum c-reactive protein, serum cercinoembryonic antigen (CEA), serum CA 19-9, ascites ADA and ascites CEA were significantly different between two disease groups. Among them, ascites ADA showed largest area under the curves (AUC) in receiver operating characteristic curves (AUC = 0.966; 95% confidence interval: 0.916-1.00; $P < 0.01$). In addition to ascites ADA, clinical findings such as symptom duration less than 1 mo and fever was more frequent finding in tuberculosis peritonitis. In abdomen computed tomography findings, smooth thickening was the most common in TBP whereas in PC group nodular pattern was the most common finding.

Innovations and breakthroughs
This study showed that among laboratory findings, ascites ADA was the most valuable marker for discriminating TBP and PC.

Applications
This analysis for the diagnostic capabilities of ADA implicates that ascites ADA may be helpful for differentiation of two diseases.

Peer review
It is a very nice paper with an excellent statistic work and very interesting findings.

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BRIEF ARTICLE

XAF1 is frequently methylated in human esophageal cancer

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Abstract

AIM: To explore epigenetic changes in the gene encoding X chromosome-linked inhibitor of apoptosis-associated factor 1 (XAF1) during esophageal carcinogenesis.

METHODS: Methylation status of XAF1 was detected by methylation-specific polymerase chain reaction (MSP) in four esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and partially methylated in TE3 cell lines), nine cases of normal mucosa, 72 cases of primary esophageal cancer and matched adjacent tissue. XAF1 expression was examined by semi-quantitative reverse transcriptional polymerase chain reaction and Western blotting before and after treatment with 5-aza-deoxycytidine (5-aza-dc)**,** a demethylating agent. To investigate the correlation of XAF1 expression and methylation status in primary esophageal cancer, immunohistochemistry for XAF1 expression was performed in 32 cases of esophageal cancer and matched adjacent tissue. The association of methylation status and clinicopathological data was analyzed by logistic regression.

RESULTS: MSP results were as follows: loss of XAF1 expression was found in three of four esophageal cell lines with promoter region hypermethylation (completely methylated in KYSE30, KYSE70 and BIC1 cell lines and partially in TE3 cells); all nine cases of normal esophageal mucosa were unmethylated; and 54/72 (75.00%) samples from patients with esophageal cancer were methylated, and 25/72 (34.70%) matched adjacent tissues were methylated (75.00% *vs* 34.70%, χ^2 = 23.5840, *P* = 0.000). mRNA level of XAF1 measured with semi-quantitative reverse transcription polymerase chain reaction was detectable only in TE3 cells, and no expression was detected in KYSE30, KYSE70 or BIC1 cells. Protein expression was not observed in KYSE30 cells by Western blotting before treatment with 5-aza-dc. After treatment, mRNA level of XAF1 was detectable in KYSE30, KYSE70 and BIC1 cells. Protein expression was detected in KYSE30 after treatment with 5-aza-dc. Immunohistochemistry was performed on 32 cases of esophageal cancer and adjacent tissue, and demonstrated XAF1 in the nucleus and cytoplasm. XAF1 staining was found in 20/32 samples of adjacent normal tissue but was present in only 8/32 samples of esophageal cancer tissue (χ^2 = 9.143, *P* = 0.002). XAF1 expression was decreased in cancer samples compared with adjacent tissues. In 32 cases of esophageal cancer, 24/32 samples were methylated, and 8/32 esophageal cancer tissues were unmethylated. XAF1 staining was found in 6/8 samples of unmethylated esophageal cancer and 2/24 samples of methylated esophageal cancer tissue. XAF1 staining was inversely correlated with XAF1 promoter region methylation (Fisher's exact test, *P* = 0.004). Regarding methylation status and clinicopathological data, no significant differences were found in sex, age, tumor size, tumor stage, or metastasis with respect to methylation of XAF1 for the 72 tissue samples from patients with esophageal cancer.

CONCLUSION: XAF1 is frequently methylated in esophageal cancer, and XAF1 expression is regulated by promoter region hypermethylation.

INTRODUCTION

Esophageal cancer is the eighth most common cancer worldwide. The incidence of esophageal cancer has increased rapidly in the past 20 years, and about 380 000 patients die each year from the disease[1-3]. Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer. It is highly invasive, rapidly metastatic, and results in a poor postoperative quality of life[4,5]. The mechanisms contributing to ESCC carcinogenesis are poorly understood. Recent studies have shown that aberrant promoter DNA methylation contributes to gene silencing and may participate in the carcinogenesis of human cancer. In-depth investigation of the relationship between DNA methylation and gene expression in ESCC will facilitate research in tumor pathogenesis and guide clinical practice.

Inhibitors of apoptosis (IAPs) are antiapoptotic factors in cancer cells that render cells resistant to apoptosis by inhibition of core death executioners, the caspases, or by neutralizing antagonists[6]. In the IAP family, X chromosome-linked IAP (XIAP) has been recognized as the most versatile caspase inhibitor[7,8]. In many models of cancer, XIAP is overexpressed[9]. XIAP-associated factor 1 (XAF1) is one of the antagonists that has been identified as a mediator of XIAP by rescuing XIAP-suppressed caspase activity[10]. XAF1 is also a new candidate tumor suppressor. Recent studies have suggested that loss of XAF1 expression may occur in different human cancers because of aberrant DNA methylation[11-17]. Zou *et al*[15] found that loss of XAF1 expression is associated with tumor progression in human gastric and colon cancers. Lee *et al*[14] also discovered that downregulation of XAF1 expression is correlated with human urogenital malignancies. However, the relationship between the expression level of XAF1 and the methylation status of XAF1 in esophageal cancer has not been demonstrated.

In this study, we investigated whether promoter region methylation was associated with the progression of esophageal cancer and analyzed the relationship between XAF1 expression and promoter region methylation. We identified XAF1 as a potential esophageal cancer biomarker for prognosis and a target for future therapeutic agents. Detection of the methylation status of XAF1 appears to be promising as a predictive factor in primary ESCC.

MATERIALS AND METHODS

Human tissue samples and cell lines

Tissue samples taken from 72 cases of ESCC and 72 matched adjacent normal tissues were used in this study. Nine cases of normal esophageal epithelia were removed during endoscopy biopsy and then snap frozen. All samples were collected from the Chinese PLA General Hospital under the guidelines approved by the Institutional Review Board of the Chinese PLA General Hospital.

Four esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and TE3) were examined in this study. All esophageal cancer cell lines were previously established from primary esophageal cancer and were maintained in 90% RPMI 1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum. Cells were passaged once at a ratio of 1:3. Cells were then allowed to grow to total confluence (about 10^6 cells) on a 75-cm^2 culture flask (NEST Biotechnology, Jiangsu, China).

Esophageal cancer cell lines were split to low density (30% confluence) 12 h before treatment. Cells were treated with 2 mol/L 5-aza-2'-deoxycytidine (5-aza-dc) (Sigma, St. Louis, MO, United States), a demethylating agent, which was added fresh every 24 h for a total of 96 h. At the end of the treatment, RNA and protein were extracted from the cells (see below).

RNA isolation and reverse transcriptional polymerase chain reaction

Total RNA was isolated with the Trizol reagent (Life Technologies, Gaithersburg, MD, United States). Agarose gel (1%) electrophoresis and spectrophotometric analysis ($A_{260 nm}/A_{280 nm}$ ratio) were used to evaluate RNA quality and quantity. RNA was stored at -80 ℃ prior to use. First-strand cDNA was synthesized from 5 μg total RNA with random 6-mer primers and a Superscript Ⅱ reverse transcriptional kit (Invitrogen). The reaction mixture was then diluted to 100 μL with water. Subsequently, 2.5 μL of this diluted cDNA mixture was used for polymerase chain reaction (PCR) amplification in a 25 μL reaction (final volume). PCR amplification of XAF1 was carried out using primers 5'-GAGCATGCAGAAGTCCTCGCT-3' (forward) and 5'-CCTGTTCACTGCGACAGACATCT-3' (reverse). The primer set for XAF1 was designed to span intronic sequences between exons to exclude amplification of genomic DNA. A total of 32 cycles of amplification was performed for each reverse transcriptional polymerase chain reaction (RT-PCR) experiment. As an internal control, glyceraldehyde-3-phosphate dehydrogenase was amplified with 25 cycles to ensure cDNA quality and quantity for each RT-PCR reaction. Amplified products were analyzed on 1.5% agarose gels.

Figure 1 X chromosome-linked inhibitor of apoptosis-associated factor 1 expression was silenced by DNA methylation. A: X chromosome-linked inhibitor of apoptosis-associated factor 1 (XAF1) expression was analyzed by semi-quantitative reverse transcriptional polymerase chain reaction before and after 5-aza-dc treatment (2 mol/L, 96 h) of the esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and TE3). Methylation status of XAF1 CpG islands in esophageal cancer cell lines, esophageal normal mucosa, esophageal cancer tissue, and matched adjacent normal tissue. Primer efficiency was verified with a positive control (*in vitro* methylated DNA, IVD) and a negative control (normal blood lymphocyte DNA, NL). "U" indicates the presence of unmethylated alleles; "M" indicates the presence of methylated alleles; B1: Methylation of XAF1 in esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and TE3); B2: Methylation of XAF1 in normal esophageal mucosa (NE1, NE2, NE3, NE4 and NE5); B3: Representative methylation-specific polymerase chain reaction (MSP) results for XAF1 in esophageal primary cancer tissue samples (EC); B4: Representative MSP results for XAF1 in esophageal matched adjacent normal tissue (EA). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Methylation-specific PCR

Genomic DNA from all types of samples was prepared using the proteinase K method. After phenol/chloroform extraction, DNA was precipitated in ethanol, dissolved in low TE buffer, and stored at -20 ℃. Genomic DNA from esophageal cancer, adjacent tissues, and cell lines was bisulfite modified as described before[18]. Methylation-specific PCR (MSP) was carried out using primers XAF1-ML: 5'-TTTGTAAGAAACGAAATTTAATCGA-3' and XAF1-MR: 5'-CCTACCCTTAAAACCCACGAT-3' and XAF1-UL: 5'-TTTGTAAGAAATGAAATTTAATT-GA-3' and XAF1-UR: 5'-CTCCTACCCTTAAAACCCA-CAAT-3'[10]. Each MSP reaction included about 200 ng bisulfite-treated DNA, 25 pmol each primer, 100 pmol dNTPs, 2.5 μL 10 × PCR buffer, and 1 U Taq Polymerase (Invitrogen) in a final reaction volume of 25 μL. Cycle conditions were as follows: 95 ℃ for 10 min; 35 cycles of 94 ℃ for 30 s, 57 ℃ for 30 s, and 72 ℃ for 30 s; and 72 ℃ for 10 min. MSP products were analyzed using 2% agarose gel electrophoresis.

Immunohistochemistry

Immunohistochemistry was performed on 4-μm-thick serial sections cut from paraffin blocks containing formaldehyde-fixed esophageal cancer tissue and paired adjacent tissue. After deparaffinization and rehydration, endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% hydrogen peroxide. Antigen retrieval was performed in target retrieval solution for 45 min at 96 ℃, which was followed by a cooling-off period of 20 min. The primary rabbit antibody (anti-XAF1, 1:200; OriGene Technologies, MD, United States) was then incubated overnight at 4 ℃. Then, the catalyzed sig-

nal amplification system (ZSGB Biotech., Beijing, China) was used to detect XAF1 staining.

Protein preparation and Western blotting

KYSE30 cells were treated with 5-aza-dc (as described above), harvested, and lysed in ice-cold Tris buffer (20 mmol/L Tris, pH 7.5) containing 137 mmol/L NaCl, 2 mmol/L ethylene diamine tetraacetic acid, 1% Triton X-100, 10% glycerol, 50 mmol/L NaF, 1 mmol/L dithio-threitol, and a protease inhibitor cocktail (Roche Applied Science). Cell lysate (35 μg) was loaded into each lane, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroblotted onto PVDF membranes (Hybond-P; Amersham, United States). After being blocked with 5% nonfat milk and 0.1% Tween-20 in TBS, the membranes were incubated with primary rabbit anti-XAF1 (1:1000; OriGene Technologies). Rabbit anti-actin (Beyotime Biotech., China) was used as a loading control. The blots were visualized using enhanced chemilumines-cence (Pierce Bioscience, Rockford, IL, United States).

Statistical analysis

Statistical analysis was carried out using the χ^2 test and Fisher's exact test. The relationship between methylation status and clinicopathological data was carried out using multiple logistic regression. $P < 0.05$ was considered statistically significant.

RESULTS

XAF1 promoter methylation status in esophageal cancer

To ascertain whether the XAF1 promoter methylation status was associated with esophageal carcinogenesis,

KYSE30-   KYSE30+

XAF1

β-actin

Figure 2 Western blotting analysis of X chromosome-linked inhibitor of apoptosis-associated factor 1 protein expression in the KYSE30 cell line before and after treatment with 2 mol/L 5-aza-deoxycytidine (+) for 96 h. XAF1: X chromosome-linked inhibitor of apoptosis-associated factor 1.

MSP was performed on nine cases of normal esophageal mucosa, 72 samples taken from patients with esophageal cancer, and 72 paired adjacent normal tissue samples. All nine cases of normal esophageal mucosa were unmethylated; 54 of 72 (75%) samples taken from patients with esophageal cancer were methylated; and 25 of 72 (34.7%) matched adjacent normal tissues were methylated (χ^2 = 23.5840, P = 0.000; Figure 1B). These results suggest that methylation of the XAF1 promoter region is a potential early detection marker of esophageal cancer.

XAF1 promoter methylation and XAF1 expression in esophageal cancer cell lines

To determine whether XAF1 is a tumor suppressor in esophageal cancer tumorigenesis, XAF1 expression levels were detected with semi-quantitative RT-PCR and Western blotting. The mRNA level of XAF1 was detectable only in the TE3 cell line, and no expression was detected in the KYSE30, KYSE70 or BIC1 cell lines (Figure 1A). Protein expression was not observed in the KYSE30 cell line before treatment with 5-aza-dc (Figure 2). To examine if these findings were due to promoter region methylation of XAF1, the methylation status of XAF1 was analyzed in these cells lines with MSP. XAF1 was completely methylated in the KYSE30, KYSE70 and BIC1 cell lines and partially methylated in TE3 cells (Figure 1B).

To demonstrate further whether XAF1 expression was restored with promoter region methylation, these cell lines were treated with 5-aza-dc. As shown in Figure 1, XAF1 was expressed in KYSE30, KYSE70 and BIC1 cells after 5-aza-dc treatment, and we detected protein expression in KYSE30 after treatment with 5-aza-dc (Figure 2), suggesting that XAF1 expression was regulated by promoter region methylation in esophageal cancer.

Correlation of XAF1 expression and methylation status in esophageal cancer tissue

To explore the expression of XAF1, immunohistochemistry was performed on 32 cases of esophageal cancer and adjacent tissue paraffin samples. XAF1 expression was found in the nucleus and cytoplasm as previously reported[19,20]. XAF1 staining was found in 50% (16/32) of adjacent tissue samples and only in 25% (8/32) of cancer tissue samples (χ^2 = 9.143, P = 0.002). Reduced expression of XAF1 was found in cancer tissues as compared

with the adjacent tissue samples (Figure 3).

We then analyzed the relationship between the methylation status and expression of XAF1 in 32 esophageal cancer samples. Twenty-four esophageal cancer samples (24/32, 75.00%) were methylated, and eight esophageal cancer tissue (8/32, 25.00%) were unmethylated. XAF1 staining was found in six samples (6/8, 75.00%) of unmethylated esophageal cancer tissue and two samples (2/24, 8.33%) of methylated esophageal cancer tissue. XAF1 staining was inversely correlated with XAF1 promoter region methylation (Fisher's exact test, P = 0.004).

Correlation of XAF1 methylation status with clinicopathological factors

Regarding methylation status and clinicopathological data, we found no significant differences in sex, age, tumor size, tumor stage, or metastasis with respect to the methylation status of XAF1 for the 72 primary esophageal cancer patients (Table 1).

DISCUSSION

The development of cancer is influenced by many factors and many genes and progresses through many stages[21]. In some ways, cancer is considered an epigenetic disease as well as a genetic disease. Epigenetics is the inheritance of information based on gene expression levels, whereas genetics refers to information transmitted according to the gene sequence. Epigenetic changes may lead to chromosomal instability, activation of parasitic endogenous sequences, loss of imprinting, illegitimate expression, aneuploidy, and mutations, and it may contribute to the transcriptional silencing of tumor suppressor genes[22]. In the human genome, CpG dinucleotides are inconsonantly distributed, resulting in CpG-rich regions[23]. The main epigenetic modification is aberrant CpG island methylation, which is tissue-specific but not species-specific. Methylation affects many pathways in cellular networks, such as the cell cycle and apoptosis[22].

In life and death decisions at the cellular level, there is a balance between pro- and antiapoptotic factors, and a variety of pathological conditions such as cancer and autoimmune and neurodegenerative diseases can result from disruption of this balance[6]. Apoptosis is crucial for eliminating defective or potentially dangerous cells and provides a defense against malignant transformation and autoimmunity[11]. IAPs are a new family of intrinsic cell death proteins that work as endogenous caspase inhibitors and participate in cell cycle regulation and modulation of receptor-mediated signal transduction. A recent study has reported that the level of XIAP mRNA is relatively high in many human cancers, suggesting that XIAP is one of the most potent and versatile inhibitors of caspases and apoptosis[15].

XAF1 has been previously identified as a binding partner of XIAP. In contrast to Smac/DIABLO and HtrA2, which promote caspase activation, XAF1 reverses XIAP-mediated inhibition of caspase-3 activity. Furthermore, XAF1 induces cell cycle arrest during G2/M phase and

Figure 3 Immunohistochemistry analysis of X chromosome-linked inhibitor of apoptosis-associated factor 1 in esophageal cancer tissue and adjacent tissue. Esophageal cancer and adjacent normal tissue samples were immunohistologically analyzed with anti-X chromosome-linked inhibitor of apoptosis-associated factor 1 (XAF1) (1:200 dilution; × 400). A: XAF1 was not detected in esophageal cancer tissue; B: XAF1 was localized in the nucleus and cytoplasm in adjacent normal esophageal tissue.

mitotic catastrophe, and the restoration of XAF1 expression induces cancer cell apoptosis and inhibits tumor growth in many types of human cancers[24]. XAF1 is ubiquitously expressed in all normal adult and fetal tissues but is drastically decreased in many cancer cell lines[11]. Loss of XAF1 expression is associated with methylation in its promoter region in many cancers. For example, XAF1 is present at very low or undetectable levels in gastric cancer, colorectal cancer[15], and cervical carcinoma[25].

In this study, we found that XAF1 was frequently methylated in esophageal cancer. Moreover, expression of XAF1 was inversely correlated with its methylation status. XAF1 was methylated in three esophageal cancer cell lines and 54 samples of esophageal cancer tissue. XAF1 methylation resulted in loss of expression in esophageal cancer cell lines, and the expression of XAF1 was restored in KYSE30, KYSE70 and BIC1 cells after treatment with 5-aza-dc. Furthermore, we observed the expression of XAF1 in the methylated cell line KYSE30 after treatment with 5-aza-dc. The results indicated that promoter region methylation regulated the expression of XAF1. XAF1 was frequently methylated in esophageal cancer tissue (75%) but was methylated only in 34.7% of matched adjacent normal tissues and not at all in normal esophageal mucosa, indicating that promoter region methylation of XAF1 was likely to be related to

Table 1 Clinicopathological characteristics and XAF1 methylation status of 40 patients with esophageal cancer *n* (%)

Clinical parameter	*n*	XAF1 methylation status		*P* value
		Methylated *n* = 30 (75%)	Unmethylated *n* = 10 (25%)	(χ^2 test)
Age (yr)				
< 65	40	30 (75)	10 (25)	1.0
⩾ 65	32	24 (75)	8 (25)	
Gender				
Male	53	40 (75.5)	13 (24.5)	0.8773
Female	19	14 (73.7)	5 (26.3)	
Tumor size (cm)				
< 5	48	33 (68.8)	15 (31.2)	
⩾ 5	24	21 (87.5)	3 (12.5)	0.0833
Tumor stage				
Ⅰ	12	6 (50.0)	6 (50.0)	0.081
Ⅱ	21	17 (81.0)	4 (19.0)	
Ⅲ	6	6 (100.0)	0 (0)	
Ⅳ	1	1 (100)	0 (0)	
Metastasis				
Negative	45	33 (73.3)	12 (26.7)	0.6733
Positive	27	21 (77.8)	6 (22.2)	

In this 72 patients, only 32 cases with tumor stage history.

esophageal carcinogenesis. In addition, XAF1 protein expression was decreased in cancer tissues as compared with adjacent normal samples, and low expression of XAF1 was significantly correlated with promoter region methylation. XAF1 expression and promoter region methylation status have been reported to be useful for identifying poorly differentiated cancer or patients with a poor disease outcome[16,17,26].

XAF1 is frequently methylated in esophageal cancer, and XAF1 expression is regulated by promoter region methylation. The loss of XAF1 expression may play an important role in tumor growth, and methylation of XAF1 may serve as an early detection marker for esophageal cancer.

COMMENTS

Background

Esophageal cancer is the eighth most common cancer worldwide. The incidence of esophageal cancer has increased rapidly in the past 20 years. Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer. It is highly invasive, rapidly metastatic, and results in a poor postoperative quality of life. The mechanisms contributing to ESCC carcinogenesis are poorly understood. Recent studies showed that aberrant promoter DNA methylation contributes to gene silencing and may participate in the carcinogenesis of human cancer. X chromosome-linked inhibitor of apoptosis (XIAP)-associated factor 1 (XAF1) is a new candidate tumor suppressor gene. Recent studies have suggested that loss of XAF1 expression may occur in different human cancers because of aberrant DNA methylation. However, the relationship between the expression level of XAF1 and the methylation status of XAF1 in esophageal cancer has not been demonstrated.

Research frontiers

XAF1 is a new candidate tumor suppressor gene. Recent studies have suggested that loss of XAF1 expression may occur in human gastric cancer, colon cancer and urogenital malignancies because of aberrant DNA methylation. However, the relationship between expression level of XAF1 and methylation status of XAF1 in esophageal cancer has not been demonstrated. In this study, the authors demonstrated that expression level of XAF1 was inversely correlated with methylation status of XAF1, and XAF1 expression was regulated by

promoter region methylation.

Innovations and breakthroughs

Recent reports have highlighted that the loss of XAF1 expression or downregulation of XAF1 expression may occur in different human cancers because of aberrant DNA methylation. This is the first study to report that XAF1 is also loss of expression because of aberrant DNA methylation in esophageal cancer.

Applications

By understanding the relationship between loss of XAF1 expression and methylation status of XAF1 in esophageal cancer, and by inducing its expression with 5-aza-deoxycytidine, this study may represent a future strategy for therapeutic intervention in the treatment of patients with esophageal cancer.

Terminology

Inhibiters of apoptosis (IAPs) are antiapoptotic factors in cancer cells that render cells resistant to apoptosis by inhibition of core death executioners, the caspases, or by neutralizing antagonists. In the IAP family, XIAP has been recognized as the most versatile caspase inhibitor. In many models of cancer, XIAP is overexpressed. XAF1 is one of the antagonists that has been identified as a mediator of XIAP by rescuing XIAP-suppressed caspase activity. XAF1 is a new candidate tumor suppressor gene. Recent studies have suggested that loss of XAF1 expression may occur in different human cancers because of aberrant DNA methylation.

Peer review

The study is very interesting and throws light on future studies and confirms increased methylation of XAF1 in squamous cell carcinoma.

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DNA-dependent activator of interferon-regulatory factors inhibits hepatitis B virus replication

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Abstract

AIM: To investigate whether DNA-dependent activator of interferon-regulatory factors (DAI) inhibits hepatitis B virus (HBV) replication and what the mechanism is.

METHODS: After the human hepatoma cell line Huh7 was cotransfected with DAI and HBV expressing plasmid, viral protein (HBV surface antigen and HBV e antigen) secretion was detected by enzyme-linked immunosorbent assay, and HBV RNA was analyzed by real-time polymerase chain reaction and Northern blotting, and viral DNA replicative intermediates were examined by Southern blotting. Interferon regulatory factor 3 (IRF3) phosphorylation and nuclear translocation were analyzed *via* Western blotting and immunofluorescence staining respectively. Nuclear factor-κB (NF-κB) activity induced by DAI was detected by immunofluorescence staining of P65 and dual luciferase reporter assay. Transwell co-culture experiment was performed in order to investigate whether the antiviral effects of DAI were dependent on the secreted cytokines.

RESULTS: Viral protein secretion was significantly reduced by 57% ($P < 0.05$), and the level of total HBV RNA was reduced by 67% ($P < 0.05$). The viral core particle-associated DNA was also dramatically downregulated in DAI-expressing Huh7 cells. Analysis of involved signaling pathways revealed that activation of NF-κB signaling was essential for DAI to elicit antiviral response in Huh7 cells. When the NF-κB signaling pathway was blocked by a NF-κB signaling suppressor (IκBα-SR), the anti-HBV activity of DAI was remarkably abrogated. The inhibitory effect of DAI was independent of IRF3 signaling and secreted cytokines.

CONCLUSION: This study demonstrates that DAI can inhibit HBV replication and the inhibitory effect is associated with activation of NF-κB but independent of IRF3 and secreted cytokines.

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INTRODUCTION

Hepatitis B virus (HBV) is a noncytopathic DNA virus which belongs to the *Hepadnaviridae* family. Infection of HBV results in acute or chronic hepatitis, liver failure, and hepatocellular carcinoma[1-2]. HBV clearance is usually associated with a multispecific CD4^{+} and CD8^{+} T-cell response coordinated with an effective humoral immune component[3-5]. However, a growing body of evidence suggests that the innate immune response is important for limiting viral replication. Expression of key proteins in pattern recognition system, such as RNA sensor melanoma differentiation-associated gene-5, the caspase recruitment domain of retinoic acid inducible gene I and the adaptor protein, myeloid differentiation primary response protein 88 (MyD88), and interferon-β promoter stimulator 1 (IPS-1) can activate innate immune response and inhibit HBV replication in human hepatocyte-derived cells[6,7].

DNA-dependent activator of interferon-regulatory factor (DAI/DLM-1/ZBP1) is the first identified sensor of cytosolic dsDNA. Recent studies have demonstrated that DAI can initiate innate immune responses, including the induction of type I interferon (*IFN*) genes, independently of Toll-like receptor 9[8-10]. It was reported that herpes simplex virus 1 production was notably higher in DAI blocked L929 cells[10]. As DAI is highly expressed in the differentiated hepatocyte after interferon treatment[11], it is hypothesized that DAI could be a protein possessing antiviral activity against HBV in human hepatocytes.

The aim of the present study was to determine whether DAI can inhibit HBV replication and what the underlying molecular mechanism is. We found that expression of DAI could inhibit HBV gene expression and replication noncytopathically in Huh7 cells. Further study revealed that activation of nuclear factor-κB (NF-κB) signaling was essential for DAI to elicit antiviral responses, but this inhibitory effect is independent of cytokines' secretion.

MATERIALS AND METHODS

Cell culture and transfection

Huh7 and HEK 293T cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Gibco). For plasmid transfections, we used Fugene reagent (Roche), according to the manufacturer's protocol. The transfection efficiency was normalized by detecting the expression of green fluorescent protein (GFP) after transfection of equal amount of plasmid GFP.

Transwell plate (Cat. No. 3450) was purchased from Corning Company (New York, the United States). The transwell chamber contains 0.4 μm pore polyester membrane which is optimal for cell attachment and growth and is permeable for flow of liquids. The cytokines can transfer through the membrane freely while the cells are blocked.

Plasmids and chemicals

pCAGGS-hemagglutinin (HA)-DAI encoding the whole transcript of DAI was kindly provided by Professor Tadatsugu Taniguchi[9]. pHBV1.3 containing a 1.3-copy of the HBV genome was described previously. The plasmid pCMV-IκBα-SR expresses a repressor form of IκBα in which serines 32 and 36 were mutated to alanine[12]. The NF-κB-dependent luciferase reporter plasmid pNF-κB-Luc was obtained from Stratagene Corporation (La Jolla, CA, the United States). pIRF-3 and pIRF-3ΔN were provided by John Hiscott[13]. The cytokines IFN-α and tumor growth factor (TGF)-α were purchased from R and D Company (Lorton, VA, the United States).

Quantitative real-time polymerase chain reaction analysis of hepatitis B viral RNA

Total RNA was extracted directly using TRIzol reagent (Invitrogen), and reversely transcribed to cDNA using a complementary DNA (cDNA) synthesis kit (Fermentas) according to the manufacturer's instructions. The cDNA was mixed with SYBR Green polymerase chain reaction (PCR) Master Mix (Toyoba) and subjected to real-time PCR using the ABI PRISM 7500 (Applied Biosystems). Cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA from the same cDNA was used as an internal control. The primers specific for HBV and GAPDH are available upon request. Forty cycles of PCR were performed with cycling conditions of 15 s at 95 ℃, 20 s at 55 ℃, 25 s at 72 ℃，and 35 s at 79 ℃ to detect signal.

Southern blotting analysis of viral DNA replicative intermediates

HBV DNA replicative intermediates in HBV core particles isolated from transfected Huh7 cells were analyzed using Southern blotting. The intracellular viral DNA was extracted as previously reported[14].

The normalized viral DNA replicative intermediates were electrophoresed onto 1% agarose gel. Then DNA was blotted onto a positive nylon membrane (Roche) in 20 × SSC. After fixing at 120 ℃ for 30 min, the membrane was prehybridized for 1 h at 42 ℃ in ULTRAhyb hybridization solution, and then hybridized with full-length HBV DNA probes labeled with (α-^{32}P) deoxycytidine triphosphate (dCTP) by hexamer random labeling kit (Roche) under the same condition of prehybridization at 42 ℃ for 16 h. After stringent washing at 68 ℃, signals were detected by autoradiography.

Northern blotting analysis of total viral RNA

Total RNA was extracted directly from transfected cells using TRIzol reagent. Ten μg total cytoplasmic RNA was electrophoresed on 1% formaldehyde-agarose gel and then transferred to nylon membranes (Roche). Hybridization was undertaken as described above using (α-^{32}P) dCTP-labeled full-length HBV DNA probes. To normalize the total quantity of RNA loaded on each gel, blots

were stripped and rehybridized with $(\alpha\text{-}^{32}P)$ dCTP-labeled GAPDH probes.

Western blotting analysis

Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted electrophoretically onto a nitrocellulose membrane (Whatman). The membrane was blocked with phosphate-buffered saline (PBS) containing 5% skim milk and then incubated overnight with 1:1000 anti-HA (Roche), 1:2000 anti-GFP (Sigma), 1:1000 anti-phospho-IRF3 (Cell signaling), 1:1000 anti-IRF3 (Santa Cruz), 1:10000 anti-β actin antibody (Sigma), then washed three times in PBST (0.05% Tween 20 in PBS), and incubated with peroxidase conjugated secondary antibody (1:2000) for 1 h. After further washing with PBST, chemiluminescence detection was carried out using enhanced chemiluminescence detection reagents.

Enzyme-linked immunosorbent assay

The HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) levels in the supernatants obtained from HBV transfected Huh7 cells were detected using a standard enzyme-linked immunosorbent assay (ELISA) (Sino-American Biotech). The assay was performed according to the manufacture's protocol. All experiments were performed at least three times.

Immunofluorescence staining

Huh7 cells were fixed with 3.5% paraformaldehyde for 10 min. The cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature and incubated in blocking buffer supplemented with 3% bovine serum albumin (Sigma) for 1 h at room temperature. Interferon regulatory factor 3 (IRF3) was detected by staining with rabbit anti-human IRF3 (1:200 dilution, Santa Cruz), followed by Cy3-coupled goat anti-rabbit IgG (1:500 dilution, Jackson Immunologicals). Flag-tagged IPS1 was detected by staining with mouse anti-Flag antibody (1:2000, Sigma), followed by Alexa488-coupled goat anti-mouse IgG (1:200, Jackson Immunologicals). P65 was detected by staining with diluted (1:100) rabbit anti-human P65 (cell signaling) and Cy3-coupled goat anti-rabbit IgG (1:500). The nuclei were counterstained with 10 μg/mL 4',6'-diamidino-2-phenylindole (DAPI) (Sigma). After incubation with the secondary antibodies, the cells were visualized under a confocal laser scanning microscope.

Dual-luciferase reporter assay

To measure report gene activation, 293T cells seeded into 24-well plates at density of 1×10^5 cells/well were transiently transfected with NF-κB dependent luciferase reporter plasmid 100 ng pNF-κB-Luc, 10 ng renilla luciferase-HSV thymidine kinase promoter (expressing Renilla luciferase, Promega) together with pCAGGS-HA-DAI or the empty vector pCAGGS-HA at the indicated amount. The cells were lysed and analyzed for firefly luciferase and renilla luciferase activity (Promega). The results were

reported as the normalized mean ± SD.

Statistical analysis

Results were reported as means ± SD. *T* tests were applied for comparisons between groups; and $P < 0.05$ was considered statistically significant.

RESULTS

DAI inhibits HBV replication in the human hepatoma Huh7 cells

To investigate the antiviral activity of DAI against HBV, we firstly examined the effect of DAI on the synthesis of HBV proteins. HBV-replicating plasmid HBV1.3 was co-transfected with either empty vector or HA-DAI into Huh7 cells. Supernatants were collected and HBsAg and HBeAg were analyzed by standard ELISA immunoassay. Compared with the control, the secretion of HBsAg was reduced by 17%, 33% and 57% and secretion of HBeAg was reduced by 25%, 34% and 57% when the increasing amount of DAI was transfected (Figure 1A). In order to study the inhibitory effect of DAI on HBV RNA transcription, the HBV RNA level was examined by quantitative real-time PCR. Results showed that HBV RNA level was also decreased by 44%, 51%, and 67% with an increased level of DAI expression. Expression of DAI in Huh7 cells was monitored by Western blotting (Figure 1B). To further investigate the effect of DAI on HBV viral RNA transcription, Northern blotting analysis was employed. As MyD88 has been reported as interferon inducible protein which can inhibit HBV replication[6,7], MyD88 and 1000 IU/mL IFN-α treatment were included as positive controls. As shown in Figure 1C, expression of DAI dramatically reduced HBV RNA level. To investigate the influence of DAI on HBV replication, Southern blotting was performed to analyze the viral DNA replicative intermediates which were extracted from core particles. As shown in Figure 1D, the HBV core particle-associated DNA was significantly reduced. These results suggested that viral genome replication, viral RNA transcription and viral protein expression were all downregulated by DAI.

To exclude the possibility that the reduction of HBV RNA and DNA in Huh7 cells was due to cell death induced by DAI, the growth of DAI-expressing Huh7 cells was examined by cell counting assay for 6 d. Results demonstrated that DAI did not obviously affect cell growth (Figure 1E). Taken together, DAI can inhibit HBV gene expression and replication noncytopathically in Huh7 cells.

IRF3 signaling pathway is not required for inhibition of HBV by DAI

The activation of innate immune system by DAI was through IRF3 or NF-κB mediated signaling pathways[10]. To investigate the possible effect of the pathways DAI on it, we firstly examined the activation of IRF3 after over-expression of DAI. IPS1, which can activate IRF-3 sig-

Figure 1 Expression of DNA-dependent activator of interferon-regulatory factors in Huh7 cells can suppress hepatitis B virus replication. A: ELISA analysis of HBV protein synthesis. GFP was transfected to monitor transfection efficiency; B: Real-time PCR analysis of HBV RNA. Huh7 cells were cotransfected with pHBV1.3 and different doses of hemagglutinin (HA)-DAI. Total RNA was extracted 48 h after transfection and HBV RNA was examined by real-time PCR; C: Northern blottings analysis of HBV RNA; Huh7 cells were cotransfected with pHBV1.3 and control DNA or MyD88 and HA-DAI. 1000 IU/mL IFN-α was added 12 h after transfection, and 48 h later, total RNA was extracted for Northern blotting hybridization. The positions of the HBV 3.5-, 2.4- and 2.1-kb RNA were indicated; D: Southern blotting analysis of HBV core particle associated DNA. Huh7 cells were treated as in C. HBV core particle associated DNA was analyzed 48 h later. Southern blotting was performed to detect HBV DNA as described. The positions of relaxed circular (RC), double stranded (DS) and single stranded (SS) DNAs were indicated; E: Effect of DAI on cell growth. Cell number was counted by adding cell counting kit-8 at 1, 2, 3, 4, 5, 6 d after transfection. DAI: DNA-dependent activator of interferon-regulatory factors; HBV: Hepatitis B virus; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; IFN: Interferon; GFP: Green fluorescent protein; HBsAg: Hepatitis B virus surface antigen; HBeAg: Hepatitis B virus e antigen; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MyD88: Myeloid differentiation primary response protein 88.

Figure 2 Interferon regulatory factor 3 signaling pathway is not required for inhibition of hepatitis B virus by DNA-dependent activator of interferon-regulatory factors. A: DAI did not induce IRF-3 phosphorylation. Control DNA, hemagglutinin (HA)-DAI or Flag-interferon-β promoter stimulator 1 (IPS1) was transfected into Huh7 cells. Forty-eight hours later, the phosphorylated form of IRF-3 was analyzed by Western blotting; B: Blockage of IRF-3 signaling did not affect inhibitory effect of DAI on HBV replication. Northern blotting assay was performed as shown in Figure 1C; C: Expression of DAI could not induce IRF-3 nuclear translocation. Cells were harvested 48 h after transfection and IRF-3 was stained as described. DAI: DNA-dependent activator of interferon-regulatory factors; IRF: Interferon regulatory factor; IRF-3ΔN: IRF-3 dominant negative plasmid; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4',6'-diamidino-2-phenylindole; YFP: Yellow fluorescent protein.

naling pathway, was set as positive control[15]. The results showed that DAI cannot induce the phosphorylation of IRF-3 (Figure 2A). Furthermore, as shown in Figure 2C, nuclear translocation of IRF-3 was not observed after DAI expression. These results indicated that DAI cannot activate IRF-3. To further confirm that DAI-mediated inhibition of HBV replication is not associated with IRF-3,

an IRF-3 dominant negative plasmid (IRF-3ΔN), in which the DNA binding domain was removed to express the repressor form of IRF-3, was used[13]. Results suggested that when the IRF-3 pathway was blocked by IRF-3ΔN, the inhibitory effect of DAI on HBV replication was not affected (Figure 2B). Taken together, inhibition of HBV replication by DAI was not associated with acti-

Figure 3 Inhibition of hepatitis B virus by DNA-dependent activator of interferon-regulatory factors depends on activation of nuclear factor-κB. A: NF-κB activity was induced by dependent activator of interferon-regulatory factors (DAI). Forty-eight hours after transfection, NF-κB (p65) was stained as described. NF-κB dependent luciferase reporter plasmid pNF-κB-Luc was co-transfected with control DNA or different doses of hemagglutinin (HA)-DAI into 293T cells. Renilla luciferase-herpes simplex virus thymidine kinase promoter was transfected to monitor the transfection efficiency; B: Blockage of NF-κB activation abolished DAI-mediated suppression of hepatitis B virus (HBV) replication. Forty-eight hours after transfection, the levels of hepatitis B e antigen and hepatitis B surface antigen were examined by enzyme-linked immunosorbent assay. HBV RNA was determined by Northern blotting hybridization. NF-κB: Nuclear factor-κB; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4',6'-diamidino-2-phenylindole; YFP: Yellow fluorescent protein.

vation of IRF3 pathway.

Inhibition of HBV replication by DAI depends on activation of NF-κB

In addition to IRF-3 signaling pathway, NF-κB is another key pathway induced by DAI to activate antiviral innate immunity. In most cells, NF-κB signaling pathway complexes are inactive, residing primarily in the cytoplasm in a complex with the inhibitory IκB proteins. Once activated, NF-κB is released from the IκBα and translocated

to nucleus, where it binds to specific κB sequences in the promoter or enhancer regions to induce the expression of multiple target genes[14,16]. To investigate if NF-κB was activated by DAI, we firstly examined the translocation of NF-κB p65. Yellow fluorescent protein-DAI and control DNA was transfected into Huh7 cells and TNF-α treatment was included as positive control. As expected, nuclear translocation of p65 was observed in both DAI-expressing and TNF-α-treated cells. Furthermore, a NF-κB-dependent luciferase reporter plasmid (pNF-κB-Luc)

Figure 4 Inhibiting hepatitis B virus replication by DNA-dependent activator of interferon-regulatory factors is an intracellular event. Transwell co-culture experiment was performed: Huh7 cells were seeded in both 6-well plates (below) and transwells (top). In transwell co-culture group, pHBV1.3 was transfected into the cells in 6-well plates while hemagglutinin (HA)-DNA-dependent activator of interferon-regulatory factors (DAI) was transfected into the cells in transwells. Twenty-four hours after the transfection, the cells in 6-well plates and transwells were co-cultured; in the direct cotransfection groups, pHBV1.3 and control DNA or HA-DAI were cotransfected into cells in 6-well plates; in interferon (IFN)-α treatment group, pHBV1.3 was transfected into the cells in 6-well plates, 1000 IU/mL IFN-α was added 12 h later. Seventy-two hours after transfection, all the cells were harvested and hepatitis B virus (HBV) RNA was determined by real-time polymerase chain reaction. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

was used to detect NF-κB activity after overexpression of DAI. Results showed that DAI increased the NF-κB-dependent luciferase activity in a dose dependent manner (Figure 3A), suggesting that DAI can induce the activation of NF-κB signaling pathway.

To further confirm that suppression of HBV replication by DAI was NF-κB-dependent, we used a NF-κB signaling suppressor IκBα-SR, in which Ser32 and Ser36 residues critical for phosphorylation are replaced by alanine[12]. As shown in Figure 3B, the inhibition of HBV RNA by DAI was reversed in the presence of IκBα-SR.

In conclusion，these data demonstrated that the activation of NF-κB signaling pathway played an indispensable role in DAI-mediated suppression of HBV replication.

Inhibition of HBV by DAI is independent of secreted cytokines

After NF-κB was activated, IFNs, chemokines and other pro-inflammatory cytokines could be induced, which might be directly involved in inhibiting viral infection[17]. To investigate whether the observed antiviral effects of DAI were dependent on the secreted cytokines, transwell co-culture experiments were conducted. The IFN-α treatment was set as positive control. We found that the

obvious inhibitory effects of DAI on HBV replication could be observed in the HBV and HA-DAI directly co-transfected cells, but not in transwell co-cultured Huh7 cells (Figure 4), indicating a secreted cytokine-independent mechanism of inhibiting HBV replication by DAI.

The above results suggested that the observed inhibition of HBV replication by DAI was most likely due to some inducible intracellular factor(s), rather than secreted cytokines.

DISCUSSION

After viral infection, innate immune receptors can detect the invading virus and subsequently initiate the synthesis of IFN and protective cellular genes to directly limit viral replication[18,19]. DAI is the first identified sensor of cytosolic dsDNA, which elicits innate immune responses and induces type I IFN to control viral replication. In this study, we found that DAI could inhibit HBV replication in Huh7 cells, and further study revealed that NF-κB signaling pathway was essential for this inhibition.

NF-κB signaling pathway plays pivotal roles in mediating inflammation, immune responses to pathogen infections, proliferation, apoptosis, and other cellular activities. The activation of NF-κB presents different results for different viruses. Some viruses activate NF-κB pathway to improve their transcription and replication[20]. Under other conditions, activation of NF-κB can repress viral replication. For example, NF-κB activation can mediate inhibition of human cytomegalovirus replication[21]. Rotavirus could antagonize cellular antiviral responses by inhibiting the nuclear accumulation of NF-κB[22]. As far as HBV is concerned, on one hand, viral replication itself can activate NF-κB, and on the other hand, upregulation of NF-κB by some host cytokines' stimulation has shown to be an inhibitory factor. For example, TNF-α could inhibit HBV replication by activating NF-κB signaling[23]. Besides, activation of NF-κB is also required for MyD88, IPS-1 and TRIF to elicit antiviral response to limit HBV replication[23]. In this study, we also found that DAI inhibited HBV replication *via* activating NF-κB signaling. Therefore, we speculated that the NF-κB signaling might be a common pathway for host to inhibit the replication of HBV and other DNA viruses.

The activation of NF-κB is associated with increased transcription of genes encoding chemokines, such as IL-8, MCP-1, cytokines such as IL-6, TNF-α, IFNs, adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule-1), enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis[24-26]. These molecules are important components of the innate immune response to invading microorganisms. When further exploring the mechanisms responsible for suppression of viral replication after NF-κB activation, we speculated that cytokines, especially type I interferon, may be the direct effector to inhibit HBV replication. However, we did not detect the induction of IFN in DAI-

expressing cells. By transwell experiment, we found that the secreted cytokines were not required for the inhibition of HBV replication by DAI (Figure 4). Interestingly, some components of pattern-recognition receptor system, such as MyD88, IPS-1 and TRIF，could also control HBV replication in a cytokine independent manner[24]. It is possible that there is a common strategy to inhibit HBV by these functional proteins. In addition, the antiviral factor downstream of NF-κB induced by DAI is worthy to be further explored.

In summary, this study demonstrates that DAI is a cellular antiviral protein. When expressed in Huh7 cells, DAI activated NF-κB but not IRF-3 signaling to suppress HBV replication. This inhibitory effect is independent of secreted cytokines. The findings could potentially lead to the development of novel therapies that induce the host cytoplasmic antiviral protein to control HBV infections.

COMMENTS

Background
The hepatitis B virus (HBV) is a DNA virus that replicates its genome *via* an RNA intermediate using reverse transcription. Chronic infection with this virus can result in cirrhosis and hepatocellular carcinoma. Nowadays, more and more evidence suggests that the innate immune response is important for limiting viral replication. Pattern recognition receptors play a pivotal role in host innate immune responses against microbial infection. DNA-dependent activator of interferon-regulatory factor (DAI/DLM-1/ZBP1) is a potent activator of immune responses during infection or tissue damage.

Research frontiers
Expression of key proteins in pattern recognition system, such as RNA sensor melanoma differentiation-associated gene-5, the caspase recruitment domain of retinoic acid inducible gene Ⅰ and the adaptor protein, and myeloid differentiation primary response protein88 can activate innate immune response and inhibit HBV replication in human hepatocyte-derived cells. DAI is the first identified sensor of cytosolic dsDNA. Recent studies have demonstrated that DAI can initiate innate immune responses, including the induction of type Ⅰ interferon genes, independently of Toll-like receptor 9. The authors hypothesize that DAI could be a protein possessing antiviral activity against HBV replication.

Innovations and breakthroughs
The aim of the present study was to determine whether DAI can inhibit HBV replication and what the underlying molecular mechanism is. The authors found that expression of DAI could inhibit HBV gene expression and replication non-cytopathically in Huh7 cells. Further study revealed that activation of Nuclear factor-κB signaling was essential for DAI to elicit antiviral responses, but this inhibitory effect was independent of cytokines' secretion.

Applications
The study could potentially lead to the development of novel therapies that induce the host cytoplasmic antiviral protein to control HBV infections.

Peer review
The study describing the inhibitory role of DAI on HBV replication is generally well-performed and the results support the conclusions reached. The manuscript is well-written.

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BRIEF ARTICLE

Double balloon enteroscopy in the old: Experience from China

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Abstract

AIM: To evaluate the safety, efficacy and management of double balloon enteroscopy (DBE) carried out in those aged individuals with suspicious small intestine diseases.

METHODS: DBE is a wonderful invention of the past decade and is widely used as an examination tool for the gastrointestinal tract. From January 2003 to July 2011, data from patients who were $\geq$ 65 years old and underwent DBE examination in the Nanfang Hospital were included in a retrospective analysis.

RESULTS: Fifty-nine individuals were found and sub-
sequently analyzed. The mean age was 69.63 ± 3.89 years (range 65-84), 34 were males. Indications for DBE were melena/hematochezia (36 cases), abdominal pain (15 cases), diarrhea (3 cases), stool change (1 case), weight loss (1 case), vomiting (2 cases), and debilitation (1 case). The average duration of symptoms was 33.34 ± 64.24 mo. Twenty-seven patients suffered from age-related diseases. Severe complications were not found during and after DBE. Comparison between systolic and diastolic blood pressure before and after DBE was statistically significant (mean ± SD, $P < 0.01$, $P < 0.05$, respectively). Small bowel pathologies were found by DBE in 35 patients, definite diagnoses were made in 31 cases, and detection rate and diagnostic yield for DBE were 68.6% and 60.8%, respectively.

CONCLUSION: DBE is a safe and effective method for gastrointestinal examination in the aged population. Aging alone is not a risk factor for elderly patients with suspicious gastrointestinal diseases and thorough preparation prior to the DBE procedure should be made for individuals with multiple diseases especially cardiopulmonary disorders.

INTRODUCTION

As the world population is aging, age-related diseases continue to increase and utilization of endoscopic techniques is rising[1], but the feasibility, safety, and effectiveness of endoscopic use for the elderly are still unknown, and there are still concerns about the use of clinical endoscopy in the elderly. Double balloon enteroscopy (DBE) is a wonderful invention in the past decade[2] and widely used as an examination tool in the gastrointestinal tract, which has the double advantages of being useful for diagnosis and interventional management. Use of DBE has mainly been in the small bowel in the past few years[3-10].

Compared to esophagogastroduodenoscopy (EGD), colonoscopy, and push endoscopy, clinical endoscopists are usually uncertain or perplexed about whether elderly individuals who are suspect for small bowel disorders could endure and be investigated by DBE with prolonged duration examination. There are a number of clinical studies regarding DBE performed in adults[11-15] and clinical trials using DBE performed in children[16-18], but little data concerning DBE exclusively used in the aged population. Therefore, the safety, efficacy, diagnosis and therapeutic management of DBE carried out in those aged subjects were assessed.

MATERIALS AND METHODS

Inclusion criteria were senile patients who were $\geq$ 65 years old and underwent DBE from January 2003 to July 2011 at Department of Gastroenterology, Nanfang Hospital (a tertiary center and university hospital), Southern Medical University, Guangzhou, China. The records of all patients were included in a retrospective analysis. This work was approved by Institutional Review Broad of Nangfang hospital, Southern Medical University.

All procedures were performed by DBE with no obvious absolute contraindications and carried out after written informed consent from patients and/or their guardians. A low residue and liquid diet were required and colored foods were avoided as far as possible at least one day prior to the test. All patients finished a bowel cleansing preparation by ingesting a 1.8-2 L polyethyleneglycol solution followed by overnight fasting (if the test was carried out in the morning), at least 6 h prior to the start of the procedure.

The Fujinon system (EN-450P5/20, Fuji Photo Optical Incorporated Company, Fujinon Inc, Japan) was used. This system consists of an endoscope at a working length of 200 cm with an outer diameter of 8.5 mm, and a flexible overtube 140 cm in length and 12 mm in outer diameter. By inflating the overtube balloon enough to grip the intestinal wall, the endoscope can be inserted further without forming redundant loops in the small bowel, and then the overtube can in turn be inserted while the endoscope balloon is inflated. DBE could be moved back and forth in a controlled manner by experienced endoscopists and an assistant to produce observations of the bowel.

Preparations and evaluation before and during the DBE procedure were as follows: (1) comprehensive assessment was carried out combining medical history and physical examination of the patients, individuals with suspected small bowel lesions and without absolute contraindications were included in the DBE investigation; (2) all subjects without other systematic diseases were evaluated prior to DBE operation, including vital signs (body temperature, blood pressure, pulse, respiration); hemoglobin (if this was abnormally low, it had to be corrected to a value > 80 g/L); electrolytes in serum (these also had to be corrected to normal range if abnormal levels were detected); electrocardiograph, chest X-ray, abdominal ultrasound were performed as regular examinations. Combined advice from other medical professionals was considered if unusual manifestations were found and the trans-oral or anal route or a combination of the two approaches was taken into account; (3) patients receiving pharmacotherapy for other known diseases were asked to discontinue administration the day before starting the test (steroid hormones, nonsteroidal anti-inflammatory drugs, and anticoagulant drugs had to be discontinued for at least a week). Experts in other professional departments, including anesthesiologists, were invited to participate in diagnosis and treatment of patients who suffered from concomitant diseases. The risk of DBE manipulation was decided in combination with endoscopists. Cases who were at extremely high risk were required to be treated by medical means in stable conditions if DBE was necessary, according to the suggestions of other professionals; (4) endoscopists, clinicians, anesthesiologists, and endoscopic nurses jointly participated in the DBE process. Concurrently, real-time monitoring equipment, resuscitative devices and necessary drugs were always ready for use in case of emergency. DBE was implemented in the operating room with full equipped medical measures being used when it was necessary; (5) antegrade, retrograde or a combination of the two approaches was performed with or without intervention under conscious or deep sedation, or general anesthesia (antegrade approaches generally included mechanical ventilation); and (6) specific management was individualized on the basis of different conditions in distinct patients before and during DBE procedure.

Observations were followed after DBE exploration, as described above, related laboratory parameters, and serious complications were monitored and managed accordingly.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 for Windows software. Continuous data were represented as means, mean ± SD or range and categorical variables were expressed as frequency or percentages. Student's test was used to compare continuous variables. The χ^2 test or Fisher exact probability test were used to compare differences in categorical variables examined.

Table 1 Characteristics of included elderly patients (*n* = 59)

Features	
Gender (M/F)	34/25
Age (yr, mean ± SD, range)	69.63 ± 3.89 (65-84)
Age group (yr)	
≥ 65	58
≥ 80	1
Complaints	
Melena/hematochezia	36
Abdominal pain	15
Diarrhea	3
Vomiting	2
Weight loss	1
Stool change	1
Debilitation	1
Duration of symptoms	33.34 ± 64.24 (0.10- 324.00)
(mo, mean ± SD, range)	
Other medical examination	
Esophagogastroduodenoscopy	52
Colonoscopy	50
Computed tomography	14
Barium study	8
Digital subtraction angiography	4
Magnetic resonance imaging	3
Mekel's scan	3
Bone marrow aspiration	3
Position-emission tomography	1
Capsule endoscopy	20
Other diseases	27
Hypertension	19
Coronary diseases	2
Hypertension+ coronary diseases	1
Hypertension+ chronic bronchitis	1
Diabetes mellitus	2
Chronic bronchitis	1
Blood systemic diseases	1
Blood transfusion (Y/N)	29/30
Hemoglobin level (g/L)	96.00 ± 26.40 (39.00-160.00)
(mean ± SD, range)	
Prior surgery (Y/N)	19/40
Abdominal operation	15
Thoracic operation	1
Other operation	3

M/F: Male/female; Y/N: Yes/no.

McNemar's χ^2 test was used in comparison of diagnosis between capsule endoscopy (CE) and DBE. Agreement analysis between CE and DBE was assessed by the kappa statistic. $P < 0.05$ (two-sided) was considered statistically significant.

RESULTS

Characteristics of patients

Fifty-nine individuals who were aged ≥ 65 years were found and subsequently analyzed; only one patient was ≥ 80 years. The mean age was 69.63 ± 3.89 years (range 65-84), 34 were males. Indications for DBE were melena/hematochezia (36 cases), abdominal pain (15 cases), diarrhea (3 cases), stool change (1 case), weight loss (1 case), vomiting (2 cases), and debilitation (1 case). The average duration of symptoms was 33.34 ± 64.24 mo (range 0.10-324.00 mo). Prior blood transfusion had been

Table 2 Capsule endoscopy *vs* double balloon enteroscopy for examination of gastrointestinal tract in this study[a] (*n* = 19)

CE Findings	DBE Findings		Total
	Positive	Negative	
Positive	5	1	6
Negative	9	4	13
Total	14	5	19

CE: Capsule endoscopy; DBE: Double balloon enteroscopy. [a]P = 0.021; κ = 0.10.

performed at least once in 29 subjects. Almost half the patients (27 cases) suffered from age-related diseases, including cardiovascular diseases, respiratory diseases, cardiopulmonary sickness, endocrine illnesses, *etc*. Hypertension and coronary disease were the main cardiovascular diseases and the most common respiratory illness was chronic bronchitis. Anticoagulant drugs were used in 1 case and 19 individuals had a prior surgical procedure. The mean hemoglobin level in plasma at initial examination was 96.00 ± 26.40 g/L (range 39.00-160.00 g/L). The demographic information was listed in Table 1.

Twenty individuals had prior CE investigation. Inspection time between CE and DBE was within 1-13 d of the DBE procedure. One patient did not complete the entire CE process because the CE battery ran out. CE was successfully discharged through the anus. The remaining patients accomplished examinations without any complications. Abnormities were seen in 17 patients, clear diagnoses were established in 6. Comparison between CE and DBE was given in Table 2.

Safety and efficacy of DBE

In this review, the mean levels of systolic and diastolic blood pressure in patients before the DBE procedure were 130.49 ± 17.19 mmHg (range 98-171 mmHg), 76.56 ± 10.70 mmHg (range 55-105 mmHg), respectively. Low levels of hemoglobin and abnormal levels of electrolytes were all corrected prior to DBE; heart rate remained in the normal range. The mean level of oxygen saturation before the test was 99.15% ± 1.54 % (range 92%-100%).

All patients received atropine prior to the DBE procedure. Administration of benzodiazepines such as diazepam or midazolam, meperidine or fentanyl, rocuronium, and propofol were used for sedation, induction and maintenance of narcosis through injection; real-time blood pressure control for patients undergoing DBE was maintained according to the distinct conditions of different patients; drug use was under real-time monitoring by electrocardiography, and measurement of transdermal oxygen saturation during the intervention process and carried out by professional anesthetists.

All patients completed the DBE procedures whether a peroral, peranal or combination approach was chosen. Severe complications were not found during and after DBE. Only a few patients complained of slight discomfort after DBE, and the symptoms soon disappeared

Table 3 Aged patients with small bowel pathologies examined by double balloon enteroscopy in this study (*n* = 51)

Findings	*n* = 51
Location	
Duodenum	9
Jejunum	14
Ileum	7
Cecum	1
Multiple segments of small bowel	4
Final diagnoses	
Primary or metastatic tumors	15
Diverticula	7
Single ulcer	5
Angiectasis	4
Erosions	2
Angioma	1
Lymphangiectasis	1
Lymphangioma	1
Single stenosis	1
Crohn's disease	1
Functional gastrointestinal diseases	13

without medical treatment. The average levels of systolic and diastolic blood pressure in patients after DBE were 124.15 ± 17.18 mmHg (range 88-170 mmHg) and 72.34 ± 9.88 mmHg (range 50-92 mmHg), respectively. The mean level of oxygen saturation after the test was 99.76% ± 0.47% (range 98%-100%). There was a statistically significant change in systolic and diastolic blood pressure before and after DBE examination.

Diagnosis of gastrointestinal pathologies via DBE

Fifty-nine cases underwent 81 DBE procedures, including 27 performed by the antegrade approach, 10 by the retrograde approach, and 22 by combining the two approaches. Total enteroscopy combining the 2 approaches which could scrutinise the whole small bowel was achieved in 12 patients. The mean insertion depth was 278.37 ± 102.68 cm (from the pylorus to the furthest distance, performed by antegrade DBE), and 305.00 ± 97.72 cm (from the ileum valve to the furthest distance, performed by retrograde DBE) respectively; the mean total procedure time was 112.61 ± 39.32 min (antegrade DBE) and 119.50 ± 37.52 min, respectively. Twenty subjects received endoscopic biopsy and definite positive findings were made in 3 individuals. Lesions detected in the gastrointestinal tract were found in 42 patients and the diagnosis yield was 64.4% (38/59). Twenty-three individuals underwent surgical procedures and one person underwent intra-operative enteroscopy. All lesions in final diagnoses were found in the stomach, small intestine and other organs.

Gastric lesions in 8 patients diagnosed by DBE were excluded from having any small bowel pathology. Pathologies were found by DBE in 35 cases, definite diagnoses were made in 31, and the detection rate and diagnostic yield were 68.63% and 60.78%, respectively. Lesions examined were in the duodenum (9 cases), jejunum (14 cases), ileum (7 cases), cecum (1 case) and multiple sections

of small intestine (4 cases). Final diagnoses with lesions in gastrointestinal tract were found to be in the stomach of 8 patients, duodenum of 8 patients, jejunum of 14 patients, ileum of 7 patients, small bowel as multiple segmental lesions of 4 patients, the cecum of 1 patient, and other locations in 3 patients. Negative diagnoses were determined by DBE in 20 patients whose symptoms were melena/hematochezia in 10 individuals, abdominal pain (7 cases), diarrhea (2 cases), and weight loss (1 case). Twenty-seven DBE procedures incorporated 7 oral routines, 6 anal routines and 7 combined routines. Lesions were found in 4 subjects, final diagnoses were 1 case with metastatic lung cancer, 1 with metastatic liver cell carcinoma, 1 with gastrointestinal stromal tumor, 2 with pancreatic carcinoma, 2 with intestinal adenocarcinoma and others with gastrointestinal functional diseases (Table 3; Figures 1-3).

DISCUSSION

With advances in society and development of medical science, aging of the world's population is an inevitable trend. According to the latest demographic data, released on August 16, 2011 in Beijing, the China Committee on Aging's Office "2010 Annual Statistical Bulletin of China Aging Development" showed that in developing countries as China, 180 million of the population were aged ≥ 60 years and 120 million were aged ≥ 65 years. An increasingly aging population is bound to be accompanied by rises in age-related disorders. Gastrointestinal diseases are very common in children, adults and the elderly; consequently, the frequency of use of gastrointestinal endoscopy which can visualize the digestive tract is rising sharply.

Traditional and conventional upper gastrointestinal endoscopy and colonoscopy could scrutinise only the proximal small bowel and distal ileum owing to their limitations of length; the mid gut which spans the stomach and the colon is the longest part of the intestinal tract and could not be directly observed for examination, diagnosis and even intervention. Even if traditional techniques such as push enteroscopy, barium meal and advanced methods such as computed tomography (CT), magnetic resonance imaging, positron emission tomography could make correct diagnoses of gastrointestinal diseases, their limitations, such as length, and the difficulty of smaller lesions, make therapeutic management impossible, especially in the small bowel. That fact, coupled with unspecific clinical symptoms presented by small bowel diseases, misdiagnosis, missed lesions, delayed diagnosis and treatment usually promote poor prognosis and increase mortality. Over the past decade, there have been two significant inventions, namely CE and DBE, which have been applied in practice and revolutionized gastrointestinal diseases, particularly in the small intestine. Screening and/or diagnosis of patients with intestinal illnesses have been greatly improved by CE and DBE[19-22].

With aging, the deterioration of physiological function in various organs also gradually becomes clear and

Figure 1 Gastrointestinal stromal tumor in ileum was diagnosed by retrograde double balloon enteroscopy in a 73 year-old male patient (A), and confirmed by histopathology (B, hematoxylin and eosin) and immunohistochemistry (C:CD117; D:CD34).

Figure 2 A 74 year-old male with adenocarcinoma was diagnosed using trans-oral double balloon enteroscopy. Tumor and stricture of the small bowel were found (A) and verified through pathology (B, HE). HE: Hematoxylin and eosin.

increases in age-related systemic diseases such as cardiovascular diseases, lung diseases, malignant tumors, *etc*. A number of clinical studies showed that EGD, colonoscopy, and endoscopic retrograde cholangiopancreatography were safe and effective methods for use in the aged[1,23-26]. CE was considered a non-invasive method for screening the gastrointestinal tract[6,27,28]; nevertheless, the lower specificity[29,30] and inability of its use for therapeutic management[31] were regarded as its shortcomings.

Plentiful studies confirmed excellent safety, effectiveness and appreciable benefits for adults investigated by DBE, and these results were reported in published literature. Diagnosis, treatment and safety of DBE for children who were aged ≥ 3 years were also demonstrated in clinical trials[16,17,32]. In contrast to other endoscopic methods, DBE with prolonged performance which is used in the elderly is usually performed more meticulously by clinicians and endoscopists due to safety concerns. This is partly because intervention costs significantly rise and, if necessary, anesthesia itself may also have moderate or severe side effects[1]. Clinicians are likely to choose non-invasive and relatively safe methods to investigate gastrointestinal pathogenesis that have given rise to various manifestations; patients are also likely to undergo a number of medical examinations as well but unclear diagnosis is obtained.

Patients with heart- or lung-related diseases for DBE must be taken into detailed consideration; insufficient preparations for those with poor health status are hazardous during and after DBE investigation. In this ret-

Figure 3 A 66 year-old individual with diverticulum accompanied by bleeding in the jejunum was diagnosed using antegrade double balloon enteroscopy (A) and affirmed by histopathology (B).

rospective review, all patients completed the entire DBE procedure and serious adverse effects were not reported. Degraded function of aged organs makes the body have higher sensitivity and poorer tolerance for drug administration, if coexisting diseases are present; sedatives and anesthetic drugs were used in the process of DBE much less in adults. It is important to highlight that close attention must be paid during anesthesia, because intervention by DBE and/or combining with drug use may result in serious consequences or even death. Frequent anesthesia-related complications such as hypotension, desaturation and apnea were not found in our group. A slight decrease in blood pressure after DBE exploration was found and could be attributed to after effects of drug use during the procedure on account of lowering blood pressure and poor metabolism of the elderly. Complications such as perforation[33], pancreatitis[11,34,35], and intussusception resulting from the use of DBE described in previous studies[36] were not found in our study.

The results in this study suggested that the average age of the participants was approximately 70 years old which was considered a high-risk age associated with various diseases. Age-related disorders, the majority of which were cardiopulmonary diseases, were also found in our series. All patients complained of obvious discomfort and the most frequent symptoms were gastrointestinal bleeding and abdominal pain. A higher diagnostic yield for gastrointestinal diseases than usual was achieved and the diagnostic yield of small bowel diseases was 60.78%. Missed lesions are unavoidable by total enteroscopy in patients with prior abdominal surgery and where single routine insertion by DBE is not readily advanced. Final diagnoses of tumor, gastrointestinal functional disorders and intestinal diverticula were common in our study and this is different from results in western countries. Based on the above results, we maintain that DBE is a safe and effective method for gastrointestinal examination in the aged population. Aging alone is not a risk factor for elderly patients with suspicious gastrointestinal diseases and thorough preparations prior to the DBE procedure should be made for individuals with multiple systematic diseases, especially cardiopulmonary disorders.

A problem was revealed in this study in that the non-normative examination flow of DBE was used for small bowel evaluation at an early stage since DBE was first introduced in our unit in 2003. DBE was used in a few patients followed by combining negative EGD or negative colonoscopy with other medical examinations such as barium enema or abdominal CT rather than combining negative EGD with negative colonoscopy. The practical problems were corrected at a later stage and a series of normative flow for balloon-assisted enteroscopy have been established and all DBE procedures are carried out in patients with negative EGD and colonoscopy in our unit. Our study is not novel but has clinical significance and interest. The limitations of small sample size, retrospective analysis, non-randomized selection of elderly patients, and few patients aged ≥ 80 years were found in our study. Prospective, large-scale and blinded randomized trials are expected to be used in future clinical studies.

COMMENTS

Background
As the world population is aging, age-related diseases continue to increase and utilization of endoscopic techniques is rising, but few studies about the feasibility, safety, and effectiveness of double balloon enteroscopy (DBE) for the elderly have been performed, and there is still concern about its use in the elderly.

Research frontiers
The invention of balloon assisted enteroscopy was first reported in 2001 by Yamamoto. A growing number of studies were chiefly focused on research of whole small bowel diseases, in order to facilitate earlier diagnoses and intervention in disorders of the mid gut. This study investigated the value of DBE for examination of an older population with suspected small bowel disease.

Innovations and breakthroughs
Although the prevalence of small intestine diseases is not as high as that of colon disease, its manifestations are always unspecific and can even be fatal because of delayed diagnosis. The present study suggests that a higher proportion of the Chinese elderly population has tumors in the small bowel or functional gastrointestinal diseases. The results show that DBE is a safe and effective method in the older population.

Applications
DBE-based examination can be used safely and effectively in older patients for diagnosis and treatment of small bowel diseases.

Peer review
This retrospective study of DBE in the old is of significant interest in clinical practice. The safety and efficacy of DBE confirmed in this study may result in

wider use of balloon-assisted enteroscopy for exploration of the small intestine when older patients are suspected of having small bowel disorders.

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BRIEF ARTICLE

Intervention of Mirtazapine on gemcitabine-induced mild cachexia in nude mice with pancreatic carcinoma xenografts

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Abstract

AIM: To investigate the effect of Mirtazapine on tumor growth, food intake, body weight, and nutritional status in gemcitabine-induced mild cachexia.

METHODS: Fourteen mice with subcutaneous xenografts of a pancreatic cancer cell line (SW1990) were randomly divided into Mirtazapine and control groups. Either Mirtazapine (10 mg/kg) or saline solution was orally fed to the mice every day after tumor implantation. A model of mild cachexia was then established in both groups by intraperitoneal injection of Gemcitabine (50 mg/kg) 10 d, 13 d, and 16 d after tumor implantation. Tumor size, food intake, body weight, and nutritional status were measured during the experiment. All mice were sacrificed at day 28.

RESULTS: (1) After 7 d of gemcitabine administration, body-weight losses of 5%-7% which suggested mild cachexia were measured; (2) No significant difference in tumor size was detected between the Mirtazapine and control groups ($P > 0.05$); and (3) During the entire experimental period, food intake and body weight were slightly greater for the Mirtazapine group compared with controls (although these differences were not statistically significant). After 21 d, mice in the Mirtazapine group consumed significantly more food than control mice (3.95 ± 0.14 g *vs* 3.54 ± 0.10 g, $P = 0.004$). After 25 d, mice in the Mirtazapine group were also significantly heavier than control mice (17.24 ± 0.53 g *vs* 18.05 ± 0.68 g, $P = 0.014$).

CONCLUSION: Mild cachexia model was successfully established by gemcitabine in pancreatic tumor-bearing mice. Mirtazapine can improve gemcitabine-induced mild cachexia in pancreatic tumor-bearing mice. It was believed to provide a potential therapeutic perspective for further studies on cachexia.

Jiang SM, Wu JH, Jia L. Intervention of Mirtazapine on gemcitabine-induced mild cachexia in nude mice with pancreatic carcinoma xenografts. *World J Gastroenterol* 2012; 18(22): 2867-2871 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i22/2867.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i22.2867

INTRODUCTION

Pancreatic cancer is one of the most lethal malignancies as the overall survival rate remains 4% for all stages and

races[1]. Clinical studies have established gemcitabine as the standard treatment for advanced pancreatic cancer. These studies have demonstrated significant clinical benefits (including improved survival) from gemcitabine[2-4]. Unfortunately, there are serious side effects associated with this anti-cancer drug that can adversely affect the patient's quality of life. These include nausea, vomiting, dyspepsia, weight loss, and cachexia.

Cachexia is characterized by major metabolic abnormalities and maladaptations. Often, food/energy intake is reduced, resting energy expenditure is increased, and catabolism is accelerated[5]. Cachexia is associated with anorexia, fat- and muscle-tissue wasting, and a progressive deterioration in the quality of life[6]. As many as 80% of all patients with cancer develop cachexia before death, and in over 20% of these patients, cachexia is the primary cause of death[7-9]. For patients with advanced-stage cancer, 80% suffer from cancer-associated anorexia/cachexia syndrome[10].

Mirtazapine represents a new class of antidepressant drugs. It is a noradrenergic and specific serotonergic antidepressant, which stimulates 5-hydroxytryptamine (HT) 1 receptors, but blocks serotonin 5-HT2/3 and histamine H1 receptors[11]. We have previously shown that 78% of patients with pancreatic cancer also suffer from clinical depression[12]. This figure jumps to 92.3% for patients undergoing chemotherapy. Mirtazapine has the potential to increase appetite and stimulate body-weight gains in these patients[13-15]. Using a mouse model for pancreatic cancer, we previously demonstrated that Mirtazapine increases food intake, enhances body weight, and improves the nutritional status of mice with cancer[16].

A 5% loss of body mass suggests an advanced to mild form of cachexia, whereas losses > 10% suggest severe cachexia[17,18]. Early interventions that treat mild cachexia often lead to substantial quality-of-life benefits for cancer patients. For the study reported herein, we therefore established a mouse model of chemotherapy-induced mild cachexia using gemcitabine and measured the effects of Mirtazapine monotherapy on tumor growth, food intake, body weight, and general nutritional status. These experiments were performed using nude mice implanted with human pancreatic cancer cells.

MATERIALS AND METHODS

Drugs and reagents

Mirtazapine was kindly provided by Organon (Oss, The Netherlands). Gemcitabine was purchased from Eli Lilly and Co. (Indianapolis, IN, United States). RPMI1640 and fetal bovine serum were purchased from Gibco (Grand Island, NY, United States).

Animals

BALB/*c nu*/*nu* mice were purchased from the Experimental Animal Center, Guangzhou University of Chinese Medicine. Approval for these studies was acquired from the animal-care committee (license number: SCXK 2008-0020). Mice were bred and maintained under patho-

gen-free conditions in the Animal Center of Sun Yat-Sen University. Mice were housed 4-5 per cage under standard conditions, i.e., 22 ± 1 ℃, ad libitum access to water and standard rat chow, and a 12-h light/dark cycle. Experiments were performed using mice that were 4-6 wk old and 20-22 g.

Pancreatic cancer cell lines and culture conditions

The human pancreatic cancer cell line SW1990 was the kind gift of the Second Affiliated Hospital of Sun Yat-Sen University. Cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS). Monolayer cultures were maintained in culture flasks and incubated under 50 mL/L CO_2 and 950 mL/L O_2 at 37 ℃. Trypsinization was stopped with medium that contained 10% FBS. Cells were then washed once in serum-free medium and resuspended in Hanks' balanced salt solution. Only single-cell suspensions that displayed greater than 90% viability were used for injections.

Establishment of a chemotherapy-induced cachexia model using gemcitabine

The subcutaneous pancreatic cancer model was established using the methods of Jia *et al*[19] with slight modifications. To produce the SW1990 tumor, 3×10^6 cells (in 0.2 mL) were inoculated subcutaneously into the right flank of each nude mouse. Tumor sizes were measured *via* callipering. When the subcutaneous solid tumors reached approximately 1 cm in diameter, they were aseptically removed from the donor animals. Macroscopically visible necrotic tissue was cut away, and the remaining healthy tumor tissue was cut with scissors into pieces that were approximately 1 mm^3. The tumor pieces were placed into Hanks' balanced salt solution that contained 100 units/mL penicillin and 100 mg/mL streptomycin. A small incision was then made through the right dorsal flank of each nude mouse and a piece of tumor was implanted beneath its skin. Chemotherapy-induced mild cachexia was established by intraperitoneal injection of 50 mg/kg gemcitabine on days 10, 13, and 16 after tumor implantation.

Mirtazapine administration

Fourteen mice were randomly assigned to either the Mirtazapine or the control group (7 mice per group). After tumor implantation, Mirtazapine (10 mg/kg) was orally fed to the Mirtazapine group once per day. Normal saline solution was fed to the control group. Chemotherapy-induced mild cachexia was then established in both groups, as described above. Animals were sacrificed after 28 d.

Measurements

The transplanted tumor sizes were measured using a Vernier caliper every fourth day, and tumor volume (V) was calculated as: $V = w^2 \times l/2$, where w is the width and l is the length of the tumor[20]. Mice were sacrificed on day 28 after tumor transplantation, and the tumors were then removed. Both the tumors and the mice carcasses were then weighed. During the experiment, body weight was

Table 1 Effect of Mirtazapine on body weight and nutritional status

Nutritional status	Group	Baseline	5 d	9 d	13 d	17 d	21 d	25 d	28 d
Body weight (g)	Control	21.64 ± 0.96	21.51 ± 0.89	21.20 ± 0.73	20.86 ± 0.71	20.15 ± 0.67	18.70 ± 0.58	17.24 ± 0.53	16.04 ± 0.66
	Mirtazapine	21.75 ± 0.75	21.69 ± 0.79	21.59 ± 0.75	21.14 ± 0.52	20.50 ± 0.50	19.13 ± 0.55	18.05 ± 0.68	16.89 ± 0.73
	P value	0.783	0.654	0.282	0.386	0.266	0.151	0.014	0.017
Subcutaneous fat (mm)	Control	0.74 ± 0.14	0.72 ± 0.14	0.70 ± 0.13	0.69 ± 0.09	0.66 ± 0.09	0.60 ± 0.10	0.50 ± 0.10	0.41 ± 0.07
	Mirtazapine	0.71 ± 0.12	0.70 ± 0.11	0.70 ± 0.10	0.69 ± 0.10	0.68 ± 0.10	0.63 ± 0.10	0.52 ± 0.09	0.44 ± 0.08
	P value	0.691	0.773	0.928	0.938	0.845	0.697	0.708	0.548
Arm circumference (mm)	Control	19.45 ± 1.21	19.03 ± 0.81	18.29 ± 0.88	17.24 ± 0.52	16.00 ± 0.62	15.13 ± 0.68	13.76 ± 0.65	13.06 ± 0.52
	Mirtazapine	19.52 ± 0.88	19.20 ± 0.73	18.51 ± 0.65	17.58 ± 0.67	16.27 ± 0.67	15.31 ± 0.68	14.04 ± 0.54	13.25 ± 0.53
	P value	0.901	0.694	0.619	0.323	0.482	0.621	0.424	0.566

Table 2 Effects of Mirtazapine on tumor size, pancreatic tumor weight and food intake

Effects on tumor size (mL)

Group	5 d	9 d	13 d	17 d	21 d	25 d	28 d
Control group	37.67 ± 7.57	62.59 ± 24.06	105.39 ± 19.92	174.77 ± 15.16	258.54 ± 20.98	365.58 ± 17.63	472.62 ± 13.03
Mirtazapine group	37.12 ± 10.55	58.09 ± 26.00	100.21 ± 21.87	171.57 ± 16.94	246.88 ± 25.64	357.80 ± 15.75	466.95 ± 14.21
P value	0.926	0.748	0.638	0.726	0.376	0.411	0.454

Effects on pancreatic tumor weight (g)

Group	Mice body weight (Tumor-bearing) (g)	Mice body weight (Tumor removed) (g)	Tumor weight (g)	*P* value
Control group	16.04 ± 0.66	15.61 ± 0.59	0.42 ± 0.09	0.35
Mirtazapine group	16.89 ± 0.73	16.51 ± 0.63	0.37 ± 0.11	

Effects on food intake

Group	Basic line	7 d	14 d	21 d	28 d
Control group	5.15 ± 0.12	5.13 ± 0.13	4.21 ± 0.10	3.54 ± 0.10	3.02 ± 0.16
Mirtazapine group	5.17 ± 0.15	5.19 ± 0.14	4.41 ± 0.16	3.95 ± 0.14	3.57 ± 0.11
P value	0.917	0.544	0.054	0.004	0.003

measured every forth day. Food intake was expressed as daily consumption in grams per animal weekly. The rate of weight loss was calculated as: weight loss (%) = (1-body weight$_{timepoint}$/body weight$_{base-line}$) × 100%. A weight loss > 5% suggested the development of mild cachexia[17,18]. Abdominal skin-fold and arm diameter were measured using the caliper every third day. Subcutaneous fat was calculated as: subcutaneous fat (mm) = skin-fold thickness × 0.5. Arm circumference was calculated as: arm circumference (mm) = diameter × 3.14.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 for Windows. Data were expressed as mean ± SD, and were compared using one-way analysis of variance and the Student-Newman-Keuls test for multiple comparisons between groups. Tumor inhibition rates were compared using the χ^2 test. Differences were considered statistically significant for $P < 0.05$ using two-tailed tests.

RESULTS

Establishment of a model for chemotherapy-induced mild cachexia using gemcitabine

Seven days after the first gemcitabine injection, body weight in the control group had declined from 21.64 ± 0.96 g to 20.15 ± 0.67 g (a 6.89% decrease) (Table 1). A similar decline was measured for the Mirtazapine group: 21.75 ± 0.75 g to 20.50 ± 0.50 g (a 5.75% decrease). A mild form of cachexia had therefore been established, indicating that the administration of gemcitabine (50

mg/kg) for 1 wk could induce mild cachexia in mice that carried a pancreatic tumor.

Effect of Mirtazapine on tumor growth

As the experiment progressed, small increases in the sizes of the tumors were regularly measured (Table 2). Similar rates of tumor growth were evident in both the Mirtazapine and control groups, however, statistically significant differences in tumor size were never detected between the two groups.

Tumor weight was measured at 28 d, immediately after the mice were sacrificed. Again, a statistically significant difference between the Mirtazapine and control groups concerning tumor weight was not detected (0.37 ± 0.11 g *vs* 0.42 ± 0.09 g, $P > 0.05$). It was indicated that gemcitabine had inhibitory effect on pancreatic cancer growth, which could not be apparently strengthened by Mirtazapine.

Effect of Mirtazapine on daily food intake

Following the administration of gemcitabine, daily food intake gradual declined in both groups (Table 2). By day 21 of the experiment, however, mice given Mirtazapine were eating more food than did the controls (3.54 ± 0.10 g *vs* 3.95 ± 0.14 g, $P < 0.01$). This effect was also seen at the end of the experiment (day 28) (3.02 ± 0.16 g *vs* 3.57 ± 0.11 g, $P < 0.01$), demonstrating that Mirtazapine can slow the reduction in food intake caused by chemotherapy.

Effect of Mirtazapine on body weight and nutritional status

At the beginning of the study, mice from the two groups

had similar average body weights ($P > 0.05$). Throughout the course of the experiment, mice in both groups exhibited a gradual decrease in body weight (Table 1). During initial stages of the experiment, the control group seemed to lose slightly more weight than did the Mirtazapine group, although these differences were not statistically significant ($P > 0.05$). At day 25, however, mice fed Mirtazapine were significantly heavier than control mice (18.05 ± 0.68 g *vs* 17.24 ± 0.53 g, $P = 0.014$). This phenomenon was also seen at 28 d suggesting that early Mirtazapine interventions can ameliorate the weight loss that is typically associated with chemotherapy (e.g., gemcitabine).

Subcutaneous fat and arm circumference were also measured for the two groups of mice (Table 1). For both groups these measured parameters gradually decreased during the course of the experiment. The data suggest that slower reductions were taking place in the Mirtazapine group (compared with the control group), but statistically significant differences were not detected ($P > 0.05$).

DISCUSSION

Cachexia is a disease process that develops in numerous chronic and end-stage pathologies. Clinical manifestations of cachexia include weight loss, anorexia, fatigue, muscle wasting, aesthesia, anemia, and edema. Particularly strong correlations between cachexia and solid tumors of the upper gastrointestinal tract have been described. It is estimated that 83% of pancreatic cancer patients suffer from cachexia during the course of their disease[17]. In addition, patients with pancreatic cancer have the highest incidence of weight loss (83%-87%), with about 30% reporting a weight loss of > 10%[21,22].

To experimentally dissect cachexia, a variety of cachexia models have been established. Murine colon-26 adenocarcinoma cells, Yoshida ascites hepatoma (AH-130) ascites hepatoma cells, and several other cachexigenic cell lines (JHU012, JHU022, and MAC1) have been used to establish different cachexia models[23-25]. The administration of chemotherapy drugs, however, has only rarely been used to generate mild cachexia. Gemcitabine is an extremely effective chemotherapy agent that inhibits the growth of cancerous tumors. Unfortunately, this drug is also associated with a number of adverse side effects, which include nausea, vomiting, loss of appetite, weight loss, and cachexia[26,27]. For the study reported herein, we injected nude mice with Gemcitabine to induce cachexia. Significant weight loss was observed in these animals, suggesting that a model for mild cachexia had been established.

Potential treatments for cancer cachexia (e.g., megestrol acetate, testosterone, growth hormone, or ghrelin) have been the subject of intense research recently. One study suggested that progestogens (e.g., megestrol acetate, or medroxyprogesterone) should be preferentially used to treat anorexia in patients with cancer because of toxic side effects associated with corticosteroids[17]. Several studies have demonstrated that ghrelin, which is a peptide found in both the brain and gut and stimulates food intake, may ameliorate cancer cachexia[9,28]. To date, however, there are no consistent opinions or guidelines that support the use of one therapeutic agent over another.

Mirtazapine represents a novel antidepressant and has been shown to cure insomnia, depression, and anxiety, but it is unclear if it also stimulates appetite and improves the nutritional status of patients[29-32]. We have previously shown that a 6-wk administration of Mirtazapine in a murine model of pancreatic cancer increased food intake and body weight by 16.39% and 8.39%, respectively. These nutritional improvements were significantly better than was seen with other antidepressants[16]. Our previous work had also shown that gemcitabine induces cachexia. In the current study, therefore, we investigated whether an early Mirtazapine intervention could ameliorate the mild cachexia associated with gemcitabine (50 mg/kg) administration in mice that bear pancreatic tumors. Mirtazapine significantly improved both food intake and body weight of these mice, although losses in subcutaneous fat and skeletal muscle were not slowed. The reason for these fat and muscle losses may be that Mirtazapine does not affect lipolysis or the adenosine triphosphate/ubiquitin/proteasome system, both of which are upregulated in cachexia. In the future, therefore, it will be important to determine the dose-effect relationship between Mirtazapine and chemotherapy-induced cachexia. We will also test whether enhanced nutritional improvements can be obtained *via* combinatorial treatments that include Mirtazapine and megestrol acetate or ghrelin.

In summary, for the first time, chemotherapy-induced mild cachexia has been established in a murine model of pancreatic cancer using gemcitabine. In addition, our results demonstrate that early administration of Mirtazapine may represent an effective treatment for both chemotherapy- and cancer-related cachexia. Future experiments that use larger groups of animals and long-term courses of treatment will be necessary to confirm these findings. Clinical tests in cancer patients are also needed.

COMMENTS

Background

Gemcitabine is commonly used to treat pancreatic cancer. This anti-cancer drug, however, is often associated with side effects (e.g., nausea, vomiting, dyspepsia, weight loss, and cachexia) that adversely affect the patient's quality of life. Mirtazapine may increase appetite and weight gain, thereby ameliorating cachexia in this context.

Research frontiers

Mirtazapine can significantly increase the food intake, enhance the body weight and improve the nutritional state in a pancreatic cancer mouse model in the authors' previous researches. In this study, the authors do the advanced research to find whether Mirtazapine could also show positive effects on improvement of appetite loss and weight loss which were the main features of cachexia.

Innovations and breakthroughs

The research shows that Mirtazapine improved gemcitabine-induced mild cachexia in mice that bear pancreatic tumors.

Applications

Mirtazapine represents a new class of antidepressant that may also positively affect appetite and weight gain. These results suggest novel therapeutic applications for Mirtazapine, and will help direct future studies concerning cachexia.

Peer review

This is an interesting research that the authors utilized gemcitabine to induce

mild cachexia in mice with pancreatic tumors and found that Mirtazapine improved the symptoms associated with mild cachexia. These findings identify a novel means of treating cachexia, although additional experiments, both in the lab and in the clinic, are necessary before this strategy can be widely applied to cancer patients.

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CASE REPORT

Bleeding duodenal hemangioma: Morphological changes and endoscopic mucosal resection

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Abstract

Recently, the development of endoscopic procedures has increased the availability of minimally invasive treatments; however, there have been few case reports of duodenal hemangioma treated by endoscopic mucosal resection. The present report describes a case of duodenal hemangioma that showed various endoscopic changes over time and was treated by endoscopic mucosal resection. An 80-year-old woman presented with tarry stools and a loss of appetite. An examination of her blood revealed severe anemia, and her hemoglobin level was 4.2 g/dL. An emergency upper gastrointestinal endoscopy was performed. A red, protrusive, semipedunculated tumor (approximately 20 mm in diameter) with spontaneous bleeding on its surface was found in the superior duodenal angle. Given the semipedunculated appearance of the tumor, it was suspected to be an epithelial tumor with a differential diagnosis of hyperplastic polyp. The biopsy results suggested a telangiectatic hemangioma. Because this lesion was considered to be responsible for her anemia, endoscopic mucosal resection was performed for diagnostic and treatment purposes after informed consent was obtained. A histopathological examination of the resected specimen revealed dilated and proliferated capillary lumens of various sizes, which confirmed the final diagnosis of duodenal hemangioma. Neither anemia nor tumor recurrence has been observed since the endoscopic mucosal resection (approximately 1 year). Duodenal hemangiomas can be treated endoscopically provided that sufficient consideration is given to all of the possible treatment strategies. Interestingly, duodenal hemangiomas show morphological changes that are influenced by various factors, such as mechanical stimuli.

Nishiyama N, Mori H, Kobara H, Fujihara S, Nomura T, Kobayashi M, Masaki T. Bleeding duodenal hemangioma: Morphological changes and endoscopic mucosal resection. *World J Gastroenterol* 2012; 18(22): 2872-2876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i22/2872.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i22.2872

INTRODUCTION

Vascular lesions of the duodenum, including hemangiomas, are rare causes of gastrointestinal bleeding. Indeed,

there have been very few reports of hemangioma in the duodenum. Endoscopically, hemangiomas have various morphological features. The present case is the first report of a duodenal hemangioma that showed various morphological features at the time of endoscopy. Although duodenal hemangioma is treated by surgical duodenectomy, the use of less-invasive procedures, such as endoscopic mucosal resection (EMR), has increased. The present study found that EMR is an effective treatment for hemorrhagic duodenal hemangioma. In addition, the present study summarized previous case reports of duodenal hemangioma.

CASE REPORT

An 80-year-old woman presented with tarry stools and a loss of appetite. She had undergone laparoscopic cholecystectomy for acute cholecystitis when she was in her seventies. She had experienced a loss of appetite and anemic symptoms since approximately March 10, 2008, but she did not seek medical attention. She noticed a tarry stool on March 15 and experienced pallor of the face and astasia on March 18, which was the day she presented at the emergency department of Sakaide municipal hospital. The physical findings upon admission included a body height of 148 cm, a body weight of 45 kg, clear sensorium, a body temperature of 36.6 ℃, a blood pressure of 116/56 mmHg, a pulse rate of 84 bpm, and an oxygen saturation of 100%. The palpebral conjunctiva was noted to be anemic. The abdomen was flat and soft with no spontaneous pain or tenderness. An examination of the blood at the time of admission revealed significant anemia with a hemoglobin level of 4.2 g/dL. In addition, her albumin level was decreased to 3.3 g/dL. Platelet counts, coagulation tests, and renal/hepatic function tests were all normal. An emergency upper endoscopy was performed on admission, but no abnormalities were found in the esophagus or the stomach. A red, semipedunculated tumor (20 mm in diameter) with spontaneous bleeding was found in the superior duodenal angle (SDA) (Figure 1). This patient had received an upper endoscopy at another hospital 9 mo earlier and had undergone biopsy after a blue, nonpedunculated, submucosal tumor-like lesion was found in SDA (Figure 2). Because there was no active bleeding during the endoscopic observation, biopsies were obtained from the basal and apical portions of the protruding lesion for diagnostic purposes. Although the pathological examination of the biopsy samples only identified necrotic material in the basal sample, we observed a dense proliferation of capillaries in the apical sample, which resulted in a diagnosis of suspected capillary hemangioma. Contrast-enhanced computed tomography (CT) revealed a slightly enhanced tumor (approximately 20 mm in diameter) in the SDA. Interestingly, we did not observe the presence of thick supplying blood vessels or an extramural extension of the tumor.

Based on the upper endoscopic findings and the

Figure 1 A red protruding lesion (20 mm in diameter) with spontaneous bleeding was found in the superior duodenal angle.

Figure 2 A blue, nonpedunculated, submucosal tumor-like lesion. The tumor is almost entirely covered by mucosa.

results of the pathological examination, a duodenal hemangioma was considered to be the source of the bleeding. With conservative therapy, the hemoglobin level increased to 7 g/dL, and hemostasis was achieved.

Because of the possibility of recurrent bleeding, EMR was performed to remove the hemangioma (Figure 3A-D). The top of the tumor was semipedunculated and irregular. First, the endothelial tumor was distinguished, but the biopsy showed the presence of a hemangioma, and the CT revealed an absence of thick supplying blood vessels. Additionally, the patient was of advanced aged and suffered from dementia. Also, the patient's family did not want her to undergo surgery. Moreover, the duodenal mucosa was sufficiently elevated following a local injection of salin solution; thus, EMR was performed. If histology of the resected specimen had revealed malignant potential, then surgery would have been performed.

We performed another upper endoscopy 3 d after the EMR, and hemostasis was confirmed. The patient had a favorable postoperative course and was discharged from the hospital without complications 1 wk after the EMR. Histopathological examination revealed the proliferation of endothelial cells lining the lumens of capillaries of various sizes and confirmed the diagnosis of capillary hemangioma (Figure 4A and B).

Figure 3 Duodenal hemangioma and endoscopic mucosal resection. A: EMR was performed after the mucosa was sufficiently elevated by local injection; B: The mucosal defect; C: The mucosal defect was closed using the clipping technique, and the procedure was completed; D: A resected specimen obtained by EMR. EMR: Endoscopic mucosal resection.

Figure 4 Pathological specimen. A: A magnified pathological image of the resected specimen (10 ×); B: A magnified pathological image of the central part of the 20 mm × 12 mm tumor, which shows proliferation of various sized capillary lumens (100 ×).

DISCUSSION

Hemangiomas only account for approximately 0.3% of all gastrointestinal tumors[1]. A search for Japanese and foreign case reports of duodenal hemangioma between 1978 and 2008 through the Japana Centra Revuo Medicina and MEDLINE only identified 22 reports, including the present case, which indicated a very low incidence of the disease (Table 1)[2-12]. We conducted a clinicopathological review of the identified Japanese case reports, including the present case. The mean age of the patients was 55 years, and the ages ranged from 2 years to 85 years. The chief complaints in the Japanese cases included melena/anemic symptoms in 82% (14 of 17) of the cases and obstructive symptoms in 1 case. Interestingly, there were only 2 asymptomatic cases. Nader et al. reported that only 30% of hemangioma cases were asymptomatic. Gastrointestinal hemorrhage/anemia was observed in 73.2% of the hemangioma cases, and obstructive symptoms were observed in 12.8% of the cases[13]. Tumors were slightly more commonly in the descending portion (8 of 17 cases, 48%) of the duodenum compared with the other portions of the duodenum. In terms of size, cavernous hemangiomas tend to be larger than capillary hemangiomas (11-80 mm *vs* 8-20 mm, respectively). Interestingly, cavernous hemangiomas accounted for the majority of the reported hemangioma cases. Indeed, cavernous hemangioma was found in 11 cases, capillary hemangioma was observed in 4 cases, and mixed capillary hemangioma and multiple

Table 1 Summary of the case reports

	Author	Year	Patient age	Chief complaint	Location in duodenum	size (mm)	Morphology	Pathology	Treatment
1	Koga	1978	65	Anemia	1-2nd	1 × 1	Unknown	Multiple hemangiomas	Surgical resection
2	Ikeda *et al*[6]	1980	33	Tarry stool, shock	2nd	Unknown	SMT-like	Cavernous	Surgical resection
3	Kawamura	1982	77	Anemia	3rd	80 × 30	SMT-like	Cavernous	Surgical resection
4	Furuya	1987	72	Tarry stool, anemia	2nd	19 × 14	SMT-like	Cavernous	Endoscopic polypectomy
5	Tadokoro	1991	55	Massive melena	2nd	10 × 14	SMT-like	Capillary	Endoscopic polypectomy
6	Amau	1991	20	Melena	2nd	Several lesions	SMT-like	Capillary	Local ethanol injection alone
7	Hata	1992	69	Hematemesis	1st	14 × 9	SMT-like	Cavernous	Surgical resection
8	Fujikawa *et al*[7]	1996	52	Anemia	4th	20 × 10	SMT-like	Cavernous	Surgical resection
9	Maeda *et al*[2]	2000	85	Anemia	3rd	11 × 8	SMT-like	Capillary/ venous	Surgical resection
10	Terui	2002	7	Intestinal obstruction	3rd	11 × 7 × 3	SMT-like	Cavernous	Surgical resection
11	Inoue	2002	40	Health check-up	3rd	Unknown	SMT-like	Cavernous	Surgical resection
12	Oikawa	2005	53	Melena	3rd	30	SMT-like	Cavernous	Surgical resection
13	Taniguchi	2006	53	Melena	3rd	30	SMT-like	Cavernous	Surgical resection
14	Kakinuma *et al*[3]	2007	63	Melena	4th	50	SMT-like	Cavernous	Surgical resection
15	Yamashita *et al*[4]	2008	70	Tarry stool	3rd	20	SMT-like	Cavernous	Unknown
16	Sakamoto *et al*[5]	2008	54	Health check-up	3rd	15	SMT-like	Unknown	Follow-up
17	Nishiyama (present case)	2008	83	Tarry stool, shock	1st	20 × 12	Polypous	Capillary	Endoscopic mucosal resection
18	Bibao *et al*[8]	1989	61	Anemia	2nd	Unknown	SMT-like	Unknown	Embolization (catheter)
19	Lee *et al*[10]	1993	31	Melena	2nd	40	Unknown	Cavernous	Surgical resection
20	Chattopadhyay *et al*[11]	2002	2	Vomiting	4th	100	SMT-like	Unknown	Surgical resection
21	Devadason *et al*[12]	2007	4	None	1-2nd	Unknown	SMT-like	Capillary	Follow-up

SMT: Submucosal tumor.

phlebectasia were observed in 1 case.

Characteristic endoscopic findings included color ranging from blue to dark red and a structure that was easily deformed by compression. Although duodenal hemangiomas are morphologically classified as submucosal tumorlike, diffusely infiltrating, or polypous lesions, all of the reported cases except for the present case have been classified as submucosal tumor-like lesions. Interestingly, the present case had a polypous appearance.

Because hemangiomas show variable endoscopic findings in terms of size, shape, and color, examinations and pretreatment information are required for the diagnosis. These examinations include contrast-enhanced CT and magnetic resonance imaging to determine the location of the tumor and the blood stream, endoscopic ultrasonography to determine the extent of the lesion, selective angiography, and duodenal X-rays. The final diagnosis is generally made based on the results of the pathological examination of the resected specimens.

Kaijer *et al*[14] pathologically classified duodenal hemangiomas into 4 types: type Ⅰ, multiple phlebectasia; type Ⅱ, cavernous hemangioma of diffuse and localized polypous types; type Ⅲ, simple capillary hemangioma; and type Ⅳ, angiomatosis with a gastrointestinal lesion. In terms of frequency, type Ⅱ hemangiomas account for the majority (55%) of all cases, but type Ⅱ and a mixture of types Ⅱ and Ⅲ are almost comparable in frequency. The present case was classified as a type Ⅱ lesion of the localized polypous type.

Although duodenal hemangiomas are conventionally treated surgically (i.e., partial intestinal resection), less invasive treatment strategies have recently been reported, including endoscopic polypectomy, EMR, local ethanol injection therapy, catheter embolization therapy, and endoscopic sclerotherapy. Among the reported cases, surgery was performed in 11 cases, polypectomy was performed in 2 cases, and EMR was performed in 1 case (i.e., the present case). Although no formal criteria have been established for the indications of EMR, patients meeting the following three criteria are considered eligible for EMR: (1) patients with a lesion in the endoscopically accessible SDA or descending portion of the duodenum; (2) patients with a 25-mm or smaller polypous lesion classified as type Ⅱ, Ⅲ, or Ⅳ according to Yamada's classification[15]; and (3) patients without a large blood vessel in the lesion (determined using contrast-enhanced abdominal CT or abdominal angiography).

We chose EMR in the present case because the patient had a 20-mm polypous lesion in the SDA, which is an endoscopically accessible region, and we did not detect a large supplying vessel on the contrast-enhanced abdominal CT. Surgical treatment was likely selected in many of the previous cases because the lesions were typically in the descending portion of the duodenum. Because the surgical resection of a duodenal lesion tends to cause excessive surgical stress, endoscopic or catheter-based treatments may be considered as an option after careful consideration. It should be noted, however, that

duodenal blood vessels have thinner walls compared with blood vessels in other parts of the intestine. Furthermore, it is difficult to manipulate an endoscope in the duodenum, which has 2 curved sections. In addition, if a resected specimen falls into the anal side of the intestine, it may be difficult to retrieve. Thus, the indication for treatment should be carefully considered.

When the patient from the present study presented at our hospital, the tumor was exposed on the mucosal surface and had increased in size. Hemangiomas can grow into various shapes according to the influence of several factors, such as thrombi, fibrosis, calcification, infection, peristalsis, and mechanical stimuli. Although the literature search did not identify any case reports of duodenal hemangiomas characterized by increasing size, the lesion in the present case appeared to have initially developed as a submucosal tumor. Interestingly, mucosal bleeding and ulceration caused by mechanical stimuli, such as biopsy and contact with food, led to the tumor being exposed to the lumen and resulted in its deformation into a polypous lesion.

We experienced a rare case of duodenal hemangioma in the present patient, and duodenal hemangioma in general can be treated by an appropriately selected, less invasive treatment strategy. The present case provided a valuable experience that enabled us to understand the morphological changes that hemangiomas can display over time.

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CASE REPORT

Duodenal variceal bleeding after balloon-occluded retrograde transverse obliteration: Treatment with transjugular intrahepatic portosystemic shunt

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Abstract

We report a case of duodenal varix bleeding as a long term complication of balloon occluded retrograde transvenous obliteration (BRTO), which was successfully treated with a transjugular intrahepatic portosystemic shunt (TIPS). A 57-year-old man was admitted to the emergency room suffering from melena. He had undergone BRTO to treat gastric varix bleeding 5 mo before admission. Endoscopy and a computed tomography (CT) scan showed complete obliteration of the gastric varix, but the nodular varices in the second portion of the duodenum expanded after BRTO, and spurting blood was seen. TIPS was performed for treatment of duodenal variceal bleeding, because attempts at endoscopic varix ligation were unsuccessful. The postoperative course was uneventful and the patient was discharged without complications. A follow up CT scan obtained 21 mo after TIPS revealed a patent TIPS tract and complete obliteration of duodenal varices, but multinodular hepatocellular carcinoma had developed. He died of hepatic failure 28 mo after TIPS.

Kim MJ, Jang BK, Chung WJ, Hwang JS, Kim YH. Duodenal variceal bleeding after balloon-occluded retrograde transverse obliteration: Treatment with transjugular intrahepatic portosystemic shunt. *World J Gastroenterol* 2012; 18(22): 2877-2880 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i22/2877.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i22.2877

INTRODUCTION

Balloon-occluded retrograde transvenous obliteration (BRTO) is minimally invasive and effective in patients with gastric variceal bleeding[1-5]. Although BRTO achieves excellent prevention of recurrent bleeding with few complications, its long-term efficacy and safety have not been full evaluated. In particular, aggravation of esophageal varices and portal hypertension have been proposed as serious complications of BRTO. There have also been reports that BRTO may aggravate esophageal varices[2,6-8]. However, there are no reports concerning the deterioration of pre-existing duodenal varices by BRTO. Here, we describe our clinical experience of successfully treating bleeding duodenal varices with a transjugular-intrahepatic portosys-

Figure 1 Endoscopic variceal ligation was performed to control variceal bleeding. A: Tiny nodular varices with no bleeding were found at the duodenum second portion; B: Enhanced computed tomography scan showing large gastric varices (arrow); C: Balloon occluded retrograde transvenous obliteration was successfully performed.

Figure 2 An emergency endoscopic examination revealed a small, linear esophageal varices without evidence of recent bleeding. A: In the second portion of the duodenum, nodular varices expanded before balloon occluded retrograde transvenous obliteration and presented with hematocystic spots; B, C: Enhanced computed tomography scan obtained 5 mo after balloon occluded retrograde transvenous obliteration revealing complete obliteration of gastric varices (black arrow); however, duodenal varices were aggravated (white arrow).

temic shunt (TIPS) and transcatheter embolization.

CASE REPORT

A 57-year-old man was admitted with a history of melena and intermittent hematochezia. He was hepatitis B virus-positive and had liver cirrhosis. Five mo before admission, an emergency endoscopic examination revealed gastric varices with active bleeding. Endoscopic variceal ligation (EVL) was performed to control variceal bleeding. A tiny nodular varix without bleeding was found in the second portion of the duodenum at that time (Figure 1A). Computed tomography (CT) showed dilated gastric varices with a gastrorenal shunt (Figure 1B). BRTO was performed to prevent recurrent gastric variceal bleeding (Figure 1C). The patient did not have any further bleeding events until our examination. At the time of admission, his blood pressure was 112/56 mmHg, heart rate was 115/min, and respiratory rate was 24/min with symptomatic orthostatic hypotension. No abnormalities were noticed on cardiac and respiratory examinations, except tachycardia. Laboratory tests performed on admission revealed a hemoglobin of 5.9 g/dL, a hematocrit of 17.4%, and a thrombocyte count of 1.6×10^4/mL. The

total serum protein and albumin level were 5.5 g/dL and 2.7 g/dL, respectively. Other laboratory data included total bilirubin (0.7 mg/dL) and aspartate aminotransferase [AST: 321 IU/L (normal < 40 IU)]. An emergency endoscopic examination revealed a small, linear esophageal varix without evidence of recent bleeding. In the second portion of the duodenum, the nodular varices had expanded after BRTO and hematocystic spots with fresh blood were seen (Figure 2A). A CT scan showed complete obliteration of gastric varices, but the duodenal varices were aggravated (Figure 2B and C). EVL was done at that site, but the bleeding continued. Therefore, a TIPS was created according to standard procedures. Portography during the TIPS operation revealed a large duodenal varix originating from the superior mesenteric vein and draining into the gonadal vein (Figure 3A). The TIPS tract was created and the vein feeding the duodenal varix was embolized using three metallic coils. The portosystemic gradient was 30 mmHg, which decreased to 12 mmHg after TIPS, and portography showed the disappearance of duodenal variceal flow (Figure 3B). The postoperative course was uneventful. The patient was discharged without complications, and he remained in a stable condition. A follow-up CT scan obtained 21

Figure 3 Transjugular intrahepatic portosystemic shunt was created according to standard procedures. A: Portogram showing duodenal variceal flow; B: After transjugular intrahepatic portosystemic shunt and coil embolization of duodenal varices, variceal flow disappeared.

Figure 4 A follow-up computed tomography scan was obtained 21 mo after transjugular intrahepatic portosystemic shunt. A, B: Enhanced computed tomography scan shows a still patent transjugular portosystemic shunt tract and complete improvement of duodenal varices. Note the appearance of multinodular hepatocellular carcinomas in the liver.

mo after TIPS revealed a patent TIPS tract and complete obliteration of duodenal varices; however, multinodular hepatocellular carcinoma had developed (Figure 4A and B). He died of hepatic failure 28 mo after TIPS.

DISCUSSION

Interventional treatments for variceal bleeding can be roughly classified into two types: Shunt occlusion and shunt creation. BRTO is representative of shunt occlusion therapies, and has been widely performed in Korea and Japan since it was introduced by Kanakawa in the mid-1990s[4]. It is an interventional radiological technique designed specifically for the treatment of gastric fundal varices with gastrorenal shunt. The principle of BRTO is to eradicate the gastric fundal varices by injecting sclerosant into the varices retrogradely with the balloon inflated in the draining veins, after insertion *via* the systemic circulation from the femoral or jugular vein. The sclerosant for BRTO is 5% ethanolamine oleate with iopamidol, which injures the endothelial cells of varicose veins and induces thrombosis in the varices. This technique controls gastric variceal bleeding effectively, with success rates of 76% to 92%[3]. Satisfactory results have been reported for managing variceal bleeding with improved liver function and hepatic encephalopathy[1,3,5,9,10]. Theoretically, shunt-obliterating therapies such as BRTO could increase portal blood flow and pressure, and thereafter

increase the bleeding risk from other varices, except sclerosed varices. Therefore, aggravation of esophageal varices is a serious complication of BRTO[2,6-8]. A long-term consequence of BRTO is the development or worsening of esophageal varices (EV) resulting from increased portal pressure, which has been reported in 10% to 66% of procedures. In a recent study, worsening of EV was observed after BRTO, with aggravation rates of 27%, 58%, and 66% at 1 years, 3 years, and 5 years, respectively[2]. However, shunt creation therapy, such as TIPS, can decompress the portal pressure, and therefore can be used in the treatment of most complications of portal hypertension, except hepatic encephalopathy. These include acute esophageal variceal bleeding refractory to medical therapy, recurrent esophageal variceal bleeding, gastric variceal bleeding, ectopic variceal bleeding, portal hypertensive gastropathy, Budd-Chiari syndrome, refractory ascites, refractory hepatic hydrothorax, hepatorenal syndrome, hypersplenism, and pancreatic arteriovenous malformation[11]. Nevertheless, TIPS has two main drawbacks. One is aggravation of hepatic encephalopathy and the other is deterioration of hepatic function. Therefore, doing both BRTO and TIPS should compensate for each other's weak points during treatment of variceal bleeding, because the two therapies are complementary.

Duodenal varices can develop in patients diagnosed with portal hypertension secondary to liver cirrhosis,

portal vein thrombosis, schistosomiasis, chronic pancreatitis, and, rarely, pancreatic cancer. Although the bleeding is often severe and fatal, no definitive treatments or guidelines for bleeding duodenal varices have been established[12,13]. Several studies of endoscopic therapies, such as band ligation (BL) or sclerotherapy, have reported variable success rates[13]. However, BL cannot ligate duodenal varices larger than 15 mm[12]. In addition, BL does not completely disrupt the blood flow within varices, and the potential for recurrent bleeding remains[12]. Sclerotherapy has technical difficulties, including a non-ideal approach to the vessels when varices are located in the second portion of the duodenum[13].

The use of radiological interventions provides an alternative method for treating patients presenting with bleeding duodenal varices (DV). Several cases concerning the use of TIPS or BRTO for controlling duodenal variceal bleeding have been reported. Akazawa *et al*[12] reported a case of duodenal variceal bleeding managed with BRTO. In our patient, the duodenal variceal bleeding caused by BRTO could not be managed endoscopically. As a result, we performed TIPS and transcatheter embolization. In conclusion, BRTO is believed to be effective for controlling gastric variceal bleeding. However, it may increase the risk of coexisting EV, DV, and ectopic variceal bleeding. Using TIPS in combination with embolization, we successfully treated a patient with DV bleeding.

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CASE REPORT

Adenocarcinoma arising from intrahepatic heterotopic pancreas: A case report and literature review

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Abstract

Heterotopic pancreas is mostly found incidentally, and adenocarcinoma arising from heterotopic pancreas appears to be extremely rare. A case of a 46-year-old woman with adenocarcinoma arising from intrahepatic heterotopic pancreas is reported herein. Computed tomography demonstrated a mass located in the bile duct of the left hepatic lobe. Pathological examination revealed a moderately differentiated adenocarcinoma arising from intrahepatic heterotopic pancreas with nerve infiltration. This may be the first reported case of adenocarcinoma arising from intrahepatic heterotopic pancreas.

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INTRODUCTION

The heterotopic pancreas is defined as aberrant pancreatic tissue without vascular, neural or anatomic continuity with the normal pancreas[1]. The incidence of heterotopic pancreas in autopsy studies is approximately 0.5%-13.7%[2]. Heterotopic pancreatic tissues can be found anywhere in the gastrointestinal tract and the predilection site is the stomach, duodenum and jejunum[3]. Unusual sites include the biliary system and liver[4]. Carcinoma within heterotopic pancreatic tissues is rare, and about 31 well-documented cases have been reported in the literature[5]. We present a case with malignant transformation in heterotopic pancreas of the intra-hepatic biliary tract. To our knowledge, no case of adenocarcinoma arising in the intrahepatic heterotopic pancreas has been described previously.

CASE REPORT

A 45-year-old woman who had been healthy underwent a routine medical check-up in our hospital. The physical examination was normal. Laboratory tests revealed elevated values of γ glutamyl transpeptidase, but normal aspartatate aminotransferase and alanine aminotransferase. The tumor markers including α fetoprotein, carcinoembryonic antigen, CA125 and CA-19-9 were all within normal ranges. Hepatitis virus markers were negative. Ultrasound showed the dilatation of the bile ducts of left

Figure 1 Computed tomography scan shows the dilatation of the bile ducts of left hepatic lobe and a mass located in the bile duct of left hepatic lobe. A-D: Computed tomography scan of abdomen showing dilatation of the bile ducts of left hepatic lobe and a mass located in the bile duct of left hepatic lobe near the hepatic hilus.

hepatic lobe. Abdominal computed tomography (CT) scan revealed a mass located in the bile duct of left hepatic lobe near the hepatic hilus, which had no enhancement in arterial and portal venous, but clear enchancement in delayed phase (Figure 1). Dilatation of the bile ducts of left hepatic lobe was observed up to the periphery of the liver. CT scan and ultrasound failed to demonstrate any pancreatic lesion. A radical resection was performed under the clinical impression of cholangiocarcinoma. She underwent left hepatic and caudate lobectomy and resection of the gallbladder. Postoperative recovery was uneventful.

Gross examination revealed a firm, gray, nodular mass measuring 3.0 cm × 2.0 cm × 2.0 cm, which was located in the bile duct of left hepatic lobe near the hepatic hilus and had a uniform cut surface without hemorrhage or necrosis. The bile duct of left hepatic lobe was also dilated with a maximum circumference of 0.9 cm. Histologically, the pancreatic tissue consisted of acinar cells and centroacinar cells, and ductal elements were often situated around the acini (Figure 2A). The acinar cells contained eosinophilic granules (Figure 2B). The histological diagnosis was consistent with a moderately differentiated adenocarcinoma arising from intrahepatic heterotopic pancreas (Figure 2C and D).

DISCUSSION

Heterotopic pancreas was firstly described in 1727 by Jean Schultz and lacked anatomic or vascular connection with the main body of the pancreas. Although pancreatic heterotopia can be found virtually anywhere along the gastrointestinal tract, the most frequent sites are the stomach (27.5%), duodenum (25.5%), and jejunum (15.9%)[3]. It is also observed less frequently in the esophagus, spleen, gallbladder, biliary tract, liver and lung. Up to now, only eight cases of intrahepatic heterotopic pancreas have been reported, including a case of insular carcinoma probably arising from intrahepatic heterotopic pancreas[6], a case of hepatic and extrahepatic choledochal cysts[7], a case presenting as hepatic mass[8], a case of primary cholesterol hepatolithiasis[9,10], a case found in autopsy liver specimens[11], a case of cirrhosis[12], and a case of Caroli's disease[13].

In 1909, Heinrich *et al*[14] classified heterotopic pancreas into three types. Type I is defined by the presence of ducts, acini and endocrine islets similar to those seen in normal pancreatic tissues. Type II contains a large number of acini, a few ducts and no islets. Type III is characterized by the presence of numerous ducts, a few acini, and no islets. The histological classification of the ectopic pancreatic tissue in the present case is type II, consisting of acini and a few ductal components.

Heterotopic pancreas is often found incidentally during surgery or endoscopy or clinically silent and benign. Malignant transformation in heterotopic pancreas is extremely rare. Thirty-one well-documented cases of carcinoma arising in a heterotopic pancreas were reviewed by

Figure 2 Histopathology shows the heterotopic pancreas and ductal adenocarcinoma arising from heterotopic pancreas. A: Histopathology showing the pancreatic tissue consisting of acinar cells and ductal elements [hematoxylin and eosin (HE), × 4]; B: Histopathology showing the acinar cells containing eosinophilic granules (HE, × 10); C: The solid area showing ductal adenocarcinoma arising from heterotopic pancreas with nerve infiltration (HE, × 4); D: The solid area showing ductal adenocarcinoma arising from heterotopic pancreas with nerve infiltration (HE, × 20).

Goodarzi[5]. The results showed that most tumors in the heterotopic pancreatic tissues occurred in the stomach and most of them were adenocarcinomas. The present case is the first case of adenocarcinoma arising from the intrahepatic heterotopic pancreas. Guillou *et al*[15] suggested that three criteria should be met for a carcinoma to be diagnosed as arising from the heterotypic pancreas. First, the tumor must be within or near the ectopic pancreatic tissue. Second, transition between pancreatic tissue and carcinoma must be observed. Third, the nonneoplastic pancreatic tissue should comprise acini, epithelial and ductal structures. The present case satisfied these three criteria.

A potential confusion is a primary intrahepatic cholangiocarcinoma with unrelated pancreatic heterotopia of the liver, which may happen if the heterotopic pancreas is completely replaced by the adenocarcinoma or if the presence of pancreatic tissue can not be confirmed due to sampling. Furthermore, primary intrahepatic cholangiocarcinoma is associated with intrahepatic bile duct stones and chronic infection in most cases. Zhang *et al*[16] also demonstrated that the adenocarcinoma can arise from heterotopic pancreas. Extensive sampling of the specimen and a high index of suspicion for carcinoma arising from heterotopic pancreas can avoid this diagnostic confusion.

In conclusion, malignant transformation in heterotopic pancreas is extremely rare. Extensive sampling of the specimen and a high index of suspicion for carcinoma arising from heterotopic pancreas can provide accurate diagnosis.

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LETTERS TO THE EDITOR

Colorectal cancer screening behavior and willingness

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Abstract

The outpatient-based study by Deng *et al* [*World J Gastroenterol* 2011 July 14; 17(26): 3133-3139] on the factors that may influence the colorectal cancer (CRC) screening feasibility, encouraged our curiosity. Establishing a simple method for quickly assessing the educational level of patients and modulating a questionnaire for each type of patient, may be an effective protocol to increase the people participation, mainly in countries where sufficient medical resources and financial support are lacking. In fact, the knowledge directly affects the feasibility when screening is offered. Patient educational level influences the understanding of the knowledge and the screening method. This factor may affect patient's priority level on the study participation, the understanding of questions, and the motivation to complete the questionnaire and, consequently, the screening success. Recent studies have found a relationship between high educational level and CRC screening participation, and emphasized the questionnaire ineffectiveness in the illiterate people. Although the questionnaire is an excellent method for this kind of evaluation, physician's contribution could be the most important factor associated with the screening method. Thus, further studies should be conducted to explore the compliance of patients with low educational level and to look for the best solutions for their enrollment.

TO THE EDITOR

We read with great interest the study by Deng *et al*[1] on the factors that may influence the colorectal cancer (CRC) screening feasibility [*World J Gastroenterol* 2011 July 14; 17(26): 3133-3139]. We strongly agree with the authors about the importance of CRC screening knowledge. Indeed, this study shows that a better knowledge leads patients to choose the most accurate test. However, although the questionnaire is an excellent method for this kind of evaluation, bias could affect the results. Thus, the educational level plays a key role in the understanding of questions and the response accuracy. On this regard, we noted that only 6.9% of patients had a low educational level. This factor may affect the patient's priority level on the study participation, the understanding of questions, the motivation to complete the questionnaire and, consequently, the screening success. In order to get a true picture of the screening results, the percentage of patients with low educational levels should be similar to the other two educational classes of patients. Thus, it would be interesting to know the educational class distribution in sections of "not responding" (14.25%) and "unfilled" (28 patients). In a recent study, Garcia *et al*[2] found a relationship between high educational level and CRC screening participation. In the illiterate people, the questionnaire had a reduced efficacy. In addition, von Wagner *et al*[3] demonstrated that lower

health literacy had a direct impact on information-seeking. For these reasons, a variation of the questionnaire design would be useful based on the literacy level[4].

On the other hand, the study of Deng *et al*[1] is associated to a series of studies that emphasize the impact of socioeconomic and cultural factors on the CRC screening feasibility[5,6]. Sung *et al*[7]'s study, which refers to an "ostrich" strategy of the Chinese population, asserts that in a society as the Chinese one, where the public knowledge of CRC is poor, a physician's recommendation is the most important factor associated with the acceptance of CRC screening. Even though in this study[7], the percentage of respondents with a low educational level is significantly low (17.5%). In my opinion, the physician's recommendation, becomes more significant, especially in the population that requires more assurances to be convinced. Thus, this finding is true for various communities worldwide[8-10]. In fact, people with low educational level are less willing to the interview participation.

In conclusion, the protocol used in the study by Deng *et al*[1], is correct to get a greater adherence to the CRC screening. In this regard, it would be appropriate to study, even in multicenters, the difference in terms of adherence to the CRC screening among people who would accept physician's recommendation or other kinds of communications.

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MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
United States

April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

World Journal of Gastroenterology

INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

Instructions to authors

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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (n). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Key words
Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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1   **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224   DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2   **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press
3   **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author
4   **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462   PMCID:2516377   DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author
5   **Vallancien G**, Emberton M, Harving N, van Moorse-laar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given
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]

Volume with supplement
7   **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatrip-tan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume
8   **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue
9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books
Personal author(s)
10   **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)
11   **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)
12   **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings
13   **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper
14   **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)
15   Morse SS. Factors in the emergence of infectious dis-eases. 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

Statistical data
Write as mean ± SD or mean ± SE.

Statistical expression
Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as υ (in Greek), sample number as n (in italics), and probability as P (in italics).

Units
Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics
Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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Contents

Contents

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EDITORIAL

New perspectives in occult hepatitis C virus infection

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Abstract

Occult hepatitis C virus (HCV) infection, defined as the presence of HCV RNA in liver and in peripheral blood mononuclear cells (PBMCs) in the absence of detectable viral RNA in serum by standard assays, can be found in anti-HCV positive patients with normal serum levels of liver enzymes and in anti-HCV negative patients with persistently elevated liver enzymes of unknown etiology. Occult HCV infection is distributed worldwide and all HCV genotypes seem to be involved in this infection. Occult hepatitis C has been found not only in anti-HCV positive subjects with normal values of liver enzymes or in chronic hepatitis of unknown origin but also in several groups at risk for HCV infection such as hemodialysis patients or family members of patients with occult HCV. This occult infection has been reported also in healthy populations without evidence of liver disease. Occult HCV infection seems to be less aggressive than chronic hepatitis C although patients affected by occult HCV may develop liver cirrhosis and even hepatocellular carcinoma. Thus, anti-HCV negative patients with occult HCV may benefit from antiviral therapy with pegylated-interferon plus ribavirin. The persistence of very low levels of HCV RNA in serum and in PBMCs, along with the maintenance of specific T-cell responses against HCV-antigens observed during a long-term follow-up of patients with occult hepatitis C, indicate that occult HCV is a persistent infection that is not spontaneously eradicated. This is an updated report on diagnosis, epidemiology and clinical implications of occult HCV with special emphasis on anti-HCV negative cases.

Key words: Occult hepatitis C virus; Hepatitis C virus RNA; Liver; Peripheral blood mononuclear cells; T-cell response

INTRODUCTION

The hepatitis C virus (HCV), an enveloped single-stranded RNA virus, was identified in 1989 and it was classified within the Flaviviridae family as a separate genus (Hepacivirus)[1]. The virus replicates by the synthesis of the complementary RNA strand (the so-called negative or antigenomic strand)[2]. So far, six major genotypes (HCV-1 to HCV-6) have been described, each containing multiple subtypes[3], with significant differences in their global distribution and prevalence[4]. It is estimated that about 170 million people, 3% of the world's population, are infected with HCV[5] and it is a leading cause of chronic liver disease worldwide including cirrhosis and hepatocellular carcinoma[6,7]. The diagnosis of HCV infection is made by the detection of antibodies against HCV (anti-HCV) and/or by detecting the presence of the HCV RNA in serum[7,8].

However, a new entity of HCV infection was first described in 2004 in patients with persistently elevated liver function tests and who were anti-HCV and serum HCV RNA negative[9]. Despite the absence of conventional HCV markers, 57% of these patients had HCV RNA in the liver and so this clinical situation was termed "occult HCV infection". Moreover, it was proven that the antigenomic HCV RNA strand could be detected also in the hepatocytes of a high proportion of those patients with occult HCV infection, this indicating an active viral replication. Occult HCV infection has also been described in two other different clinical settings. One of these is in anti-HCV positive, serum HCV-RNA negative subjects with persistent normal values of liver enzymes (asymptomatic HCV carriers), of whom nearly 90% have detectable viral RNA in liver and in peripheral blood mononuclear cells (PBMCs)[10-12]. The second one is in anti-HCV positive individuals who resolved HCV infection either spontaneously or after antiviral treatment[13-16]. In these patients, HCV RNA is detected in liver and in PBMCs years after apparent recovery from the disease (normalization of liver enzyme values and loss of serum HCV RNA). This occult HCV infection is related to the persistence of necroinflammation activity in the liver of the sustained responders. Thus, there are two types of occult HCV infection: one can be found among anti-HCV seropositive individuals with normal values of liver enzymes and the other is found among anti-HCV seronegative patients with abnormal levels of liver enzymes. The present review focuses on the latest studies of occult HCV infection performed in anti-HCV negative and serum HCV RNA negative patients.

IDENTIFICATION OF OCCULT HCV INFECTION

Occult HCV infection was first identified in liver of anti-HCV and serum HCV RNA negative patients with abnormal liver function tests and it was also found that viral RNA could be present in the PBMCs of nearly 70% of these patients[9]. Furthermore, it was demonstrated that occult HCV replicates in these cells[17]. By detecting HCV RNA in liver biopsies or in PBMCs, other groups in Japan, Italy, Egypt, Colombia, Pakistan and Iran[18-23] have confirmed the existence of occult HCV infection in patients with elevated liver enzymes and without conventional HCV markers (Table 1). Occult HCV infection has also been found in hemodialysis patients who were persistently anti-HCV and serum HCV RNA negative but with abnormal values of liver enzymes[24], in the family setting of patients with occult hepatitis C[25] and even in healthy subjects with normal alanine aminotransferase (ALT) levels and no clinical evidence of liver disease[26].

Since HCV was replicating in the liver and PBMCs of patients with occult HCV infection, it was speculated that it should exist as circulating viral particles but at such low levels that the virions could not be detected even using the most sensitive reverse-transcription polymerase chain reaction (RT-PCR) techniques. This hypothesis was tested by concentrating HCV virions by ultracentrifugation of 2 mL of serum from patients with occult HCV prior to HCV RNA detection by RT-PCR[27]. In this way, serum viral RNA was found in nearly 60% of the patients. In addition, it was found that the density of the viral particles isolated from patients with occult HCV infection was similar to the highly-infectious lipoviral particles present in the serum of patients with classical chronic hepatitis C[27,28], suggesting that serum from patients with occult HCV is potentially infectious.

HCV-SPECIFIC T-CELL RESPONSES AND THE OCCULT INFECTION

Functional virus-specific memory CD4^{+} and CD8^{+} T-cells have been documented in the circulation of patients with HCV RNA persistence in the liver and so assaying cellular immunity has been proposed as a surrogate marker of occult HCV infection[29,30]. To test HCV-specific T-cell responses, PBMCs isolated from fresh heparinized venous blood by gradient centrifugation are washed twice in phosphate-buffered saline and resuspended in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 20 mmol/L HEPES, 2 mmol/L glutamine and antibiotics. PBMCs are cultured in triplicate (1.0×10^5 viable cells/100 μL) in flat-bottomed 96-well culture plates at 37 ℃, 5% CO_2 and humidity in the presence or absence of 1 μg/mL HCV proteins core, NS3 and NS4; *Staphylococcus aureus* enterotoxin B (10 μg/mL) is used as positive control. On day 6, cultures are pulsed with 1 μCi/well of 3H-thymidine for 16 h and then harvested and transferred to filters and the incorporated radioactivity measured[31].

T-cell responses found in occult HCV infection are similar to those described in anti-HCV-positive patients following spontaneous or treatment-induced recovery[32-34]. HCV-specific T-cell responses have been detected often among occult HCV-infected hemodialysis patients[35], family members of patients with occult or overt HCV infection[36] and among HCV-seronegative sexual partners of patients with chronic hepatitis C (Aguilar-Reina J, personal communication), supporting exposure to trace amounts of HCV RNA. The maintenance of such immune responses may require only a low level productive infection. In fact, sporadic reappearance of minute amounts of HCV RNA stimulates cellular immunity[37]. However, persistence of occult HCV in face of adaptive cellular responses indicates that the latter do not ultimately result in sterilising immunity. The actual impact on the natural history of the occult infection is still a matter of debate. Indeed, HCV-specific T-cell responses have been described in apparently healthy persons, as well as in those who likely have resolved the infection or who have been exposed to, but who apparently did not became infected by, HCV[30]. To summarize, T-cell responses are more frequent and stronger compared with chronic hepatitis C patients[29], contributing to control the

Table 1 Prevalence and hepatitis C virus genotype distribution of occult hepatitis C virus infection

Type	Country	Prevalence[1]	Genotype	Cohort/setting	Ref.
Anti-HCV negative					
	Spain	57/100 (57%)	1b	Cryptogenic chronic hepatitis	[9]
	Japan	Not applicable	Not reported	Cryptogenic cirrhosis with HCC	[18]
	Italy	2/5 (40%)	Not reported	Cryptogenic cirrhosis with HCC	[19]
	Egypt	4/40 (10%)	Not reported	Cryptogenic chronic hepatitis	[20]
	Colombia	Not applicable	1a	Liver retransplantation	[21]
	Pakistan	23/31 (74%)	1a, 3a, 3b	Cryptogenic chronic hepatitis	[22]
	Iran	7/69 (10%)	1a, 1b, 3a	Cryptogenic chronic hepatitis	[23]
	Italy	9/276 (3.3%)	1a, 1b, 2a	General population without liver disease	[26]
Anti-HCV positive					
	United States/Poland	11/11 (100%)[2]	1a, 1b	Asymptomatic anti-HCV carrier	[10]
	Cuba/Mexico	17/18 (94%)	Not reported	Asymptomatic HCV carrier and therapy response	[11]
	Spain	10/12 (83%)	1b	Asymptomatic anti-HCV carrier	[12]
	Canada	16/16 (100%)[2]	1a, 1b, 2a	Spontaneous recovery and therapy response	[13]
	United States/Poland	15/17 (88%)[2]	1a, 1b, 2a, 3a	Therapy response	[14]
	Spain	19/20 (95%)	1b, 2, 3	Therapy response	[15]
	Canada	24/24 (100%)[2]	1, 1a, 3a	Therapy response	[16]
	Egypt	7/62 (11%)	Not reported	Therapy response	[20]

[1]Hepatitis C virus (HCV) RNA detection in liver and/or peripheral blood mononuclear cells; [2]Including HCV RNA detection in serum with nested reverse-transcription polymerase chain reaction-nucleic acid hybridization assay. HCC: Hepatocellular carcinoma.

extent of the infection and thus prevent HCV RNA detection in serum.

HUMORAL IMMUNITY TO HCV DURING THE OCCULT INFECTION

It is unknown how HCV persists in persons who remain anti-HCV non-reactive by currently available antibody screening tests[38]. Antibodies to HCV proteins usually develop within 4-12 wk following exposure to the virus, those directed to the core and non-structural-3 region being the earliest and more frequently detected. Anti-HCV continues to be detectable throughout the duration of the infection although antibody reactivity declines over time after apparent clinical recovery of chronic hepatitis C[39-41]. In immunocompetent individuals, the etiology of the primary occult HCV infection is most likely explained by the sporadic exposure to low infective virus doses resulting in a latent seronegative infection. Thus, anti-HCV reactivity remains undetectable due to prolonged very low antibody titres[38], excluding persons with immunodeficiencies, immunosuppressed or suffering from a concomitant chronic infection.

Isolated reactivity to single proteins or peptides has been reported on supplemental anti-HCV assays in blood donors in samples which are either HCV RNA-positive or -negative[42,43]. Such pattern of anti-HCV indeterminate results resembles the profile of antibody reactivity documented in some international seroconversion panels when tested on supplemental anti-HCV assays. These panels are composed of sequential samples from a single-source donor obtained throughout the antibody development which frequently show single-antigen reactivity at the initial stages of anti-HCV seroconversion. In addition, reactivity recorded as "faint band(s)" has been shown among at risk persons but

seronegative by screening anti-HCV tests[44]. At this point the criteria recommended by the supplier of the supplemental assay to validate the testing as reactive or anti-HCV-positive *vs* indeterminate result should be discussed. But this issue would require a thorough comparison of the HCV antigens employed by the licensed tests and the interpretation of their reactivity in particular populations, which is out of the scope of this review and deserves future investigation.

The majority of persons exposed to HCV who become infected and seroconvert to anti-HCV remain asymptomatic. Up to 80% of seropositive infections are not diagnosed because persons belong to low, or supposedly null, risk groups. Screening programs in the general population would promote awareness and prevention of HCV spread because seropositive persons may be identified and offered appropriate counselling. However, this strategy still will not identify the seronegative infections using the current screening anti-HCV tests, as evidenced by the existence of the primary occult HCV infection.

In an attempt to overcome this, an anti-HCV assay based on a well-conserved core-derived epitope has been reported recently[45]. Briefly, wells of a microtitre plate are coated overnight with HCV-core 5-19-peptide. Wells are washed and non-specific sites are blocked with phosphate buffer saline containing Tween-20 plus heat-inactivated fetal bovine serum. Diluted serum samples are added to the HCV-core coated wells and after incubation for 1 h, wells are washed five times and incubated with horseradish peroxidase-conjugated rabbit polyclonal anti-human IgG for 1 h. After five washings wells are reacted in the dark with 2, 20-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt. Absorbance is measured at 405 nm with a reference at 620 nm. In contrast to the NS3 sequence which shows considerable inter-genotypic heterogeneity[46], the core sequence is largely conserved

among genotypes 1 through 6[47]. Antibody to HCV core was tested in a cohort of 145 anti-HCV screening-negative patients with occult HCV infection of whom 40% were found to be anti-HCV core-positive, including 10% of individuals who were antibody non-reactive at the time of the first sample testing. Also, the anti-HCV core was detected in 99% of chronic hepatitis C patients but in none of the patients with HCV-unrelated liver disease. Thus, anti-HCV core testing allowed serological identification of up to 40% of the anti-HCV screening-negative infections on repeated testing[45]. The finding that a number of patients with occult infection who were initially nonreactive for anti-HCV core antibodies became positive upon subsequent testing underscores the necessity of screening serial samples as proposed by other authors[32,48].

In addition, the anti-HCV core assay has been able to track HCV exposure among relatives of patients with occult HCV. Intrafamilial spread of occult HCV infection seems to occur as often as that of chronic hepatitis C[25]. So, anti-HCV core was detected in 23% anti-HCV-screening-negative relatives of patients with occult HCV infection. Thus, antibody testing to HCV core detected frequent exposure to and possible transmission of HCV among family members of HCV-infected patients compared with screening anti-HCV tests[36]. On the other hand, because patients undergoing hemodialysis are at risk of occult HCV infection[24,35], testing for anti-HCV core has been evaluated in repeatedly anti-HCV screening-negative and serum HCV RNA-negative hemodialysis patients with abnormal liver enzymes. Anti-HCV core antibodies were detectable in 34% hemodialysis patients who have been exposed to HCV and who might have developed occult HCV infection (unpublished results).

Therefore, anti-HCV fails to be detected by screening tests available in some populations of at-risk patients[49], including those individuals multi-exposed such as intravenous drug users or prison inmates[44,50-52]. The ultimate utility of the anti-HCV core-based antibody assay in those cases and other settings such as blood donors warrants further investigation.

MAY OCCULT HCV INFECTION BE DIAGNOSED WITHOUT A LIVER BIOPSY?

Detection of HCV RNA in the liver biopsy is the gold-standard method for the diagnosis of an occult HCV infection. However, as commented before, viral RNA is detectable in the PBMCs and in ultracentrifuged serum of patients with occult HCV[9,27] and anti-core HCV tested by a non-commercial enzyme-linked immunosorbent assay (ELISA) is also found in a substantial proportion of these patients[45]. Therefore, in a recent report it was determined whether all cases of occult HCV infection could be diagnosed without performing a liver biopsy by combining these methods[53]. A total of 122 patients, who were diagnosed of an occult HCV infection by the presence of viral RNA in a liver biopsy and with avail-

able serum samples and PBMCs were included in the study. Anti-core HCV (tested with the non-commercial ELISA) was found positive in 44/122 (36%) of the patients. After ultracentrifugation of serum samples, HCV RNA was found in 70/122 (57%) of the patients, while 74/122 (61%) had viral RNA in PBMCs. When combining the detection of anti-core HCV and the detection of HCV RNA in ultracentrifuged serum and in PBMCs, 91% of the patients (111/122) were positive for at least one of these markers. So, in the light of these results, occult HCV infection may be properly diagnosed in up to 91% of the patients without the need to perform a liver biopsy by testing for anti-core HCV and for HCV RNA in ultracentrifuged serum and in PBMCs.

In summary, when occult HCV infection is suspected and a liver biopsy is not available for HCV RNA detection, the diagnosis can be made by testing, with a highly sensitive real-time PCR technique, for the presence of viral RNA in PBMCs (that identifies between 60%-70% of the cases)[9,53] or in ultracentrifuged serum (that allows identification of occult HCV in around 60% of the patients)[27,53]. The combination of these two approaches along with the detection of anti-core HCV improves the diagnosis of occult HCV infection in more than 90% of the cases. Nevertheless, in order to increase the percentage of patients diagnosed of occult HCV infection with non-invasive methods, more studies should be done in the future to improve the sensitivity of the above mentioned techniques.

CHARACTERISTICS OF OCCULT HCV INFECTION AND RESPONSE TO ANTIVIRAL TREATMENT

Clinical characteristics of patients with occult HCV infection have been compared to those of patients with chronic hepatitis C matched with respect to age, gender and known duration of the disease[54]. In the study it was found that patients with occult HCV presented significantly lower values of iron, alanine aminotransferase, γ-glutamyl transpeptidase and α-fetoprotein whereas triglycerides and cholesterol levels were significantly higher than those of patients with chronic hepatitis C. In the liver biopsies, patients with chronic hepatitis C frequently had more necroinflammation activity (96%) and fibrosis (75%) than patients with occult HCV infection (31% and 15%, respectively), but liver cirrhosis was diagnosed with a similar frequency in both groups (4.4% in occult HCV *vs* 7.2% in chronic hepatitis C). Although cholesterol and triglyceride levels were significantly higher in patients with occult HCV infection than in patients with chronic hepatitis C, the percentage of cases with liver steatosis did not differ significantly between these two groups, suggesting that dyslipidemic disorders did not play a predominant role in the development of steatosis in patients with occult HCV. So, occult HCV infection seems to be a milder form of the disease caused by HCV with less liver damage. However, it is important to point out that

occult HCV may lead to liver cirrhosis and therefore to the development of hepatocellular carcinoma. Regarding this issue, the presence of viral RNA in the tumour and non-tumour tissue of anti-HCV and serum HCV RNA negative patients with liver cancer has been reported[18,19]. Nevertheless, the number of patients analyzed in these reports was low and further studies are needed to ascertain the role of occult HCV in causing hepatocellular carcinoma.

Taking into account that patients with occult HCV present with abnormal liver function tests and may have histological damage, a study was conducted to determine whether these patients could respond to antiviral treatment with pegylated-interferon (PEG-IFN) plus ribavirin[55]. A total of 10 patients with occult HCV genotype 1b infection (anti-HCV and serum HCV-RNA negative but HCV RNA positive in liver) who were HCV RNA positive in PBMCs, had abnormal values of alanine aminotransferase for at least 12 mo and had necroinflammatory activity in a liver biopsy performed within one year before the study entry, were treated with standard doses of PEG-IFN plus ribavirin for 24 wk. The patients received a 24-wk treatment course instead of the recommended 48-wk course for HCV genotype 1 because they were serum HCV RNA negative[56-58]. After treatment, patients were followed for 24 wk. At the end of therapy, 80% of the patients had normalized ALT values and were HCV RNA negative in PBMCs, but at the end of the post-treatment follow-up, only 3 cases remained with normal ALT values and without HCV RNA in PBMCs (complete responders). Five of the patients (2 of them with a complete response) underwent a second liver biopsy at the end of the follow-up period. Necroinflammatory activity and fibrosis scores had decreased in the post-treatment liver biopsy of 3 patients, while scores in the other 2 cases remained unchanged. Viral RNA persisted in the liver of the 5 patients but HCV RNA load was significantly lower in the post-treatment biopsy than in the basal one. Thus, treatment with PEG-IFN plus ribavirin may be beneficial in patients with occult HCV because intrahepatic HCV RNA load decreases and histological liver damage may improve but, as it has been described for chronic hepatitis C[13-16], occult HCV infection is not eradicated.

In conclusion, although occult HCV infection appears to be milder than "classical" chronic hepatitis C, liver fibrosis is present in up to 5% of the patients[4,42]. In addition, necroinflammatory activity is detected in the liver of nearly 35% of the cases[9,54]. This suggests that occult HCV infection may progress to a more serious chronic liver injury. Supporting this notion is the fact that occult HCV infection has been identified in patients with liver cirrhosis and even in hepatocellular carcinoma[18,19]. Thus, the treatment of patients with occult HCV infection with PEG-IFN plus ribavirin is a reasonable option, as it is proven for chronic hepatitis C[59] because the histological liver damage may improve with treatment. However, the infection is not completely eradicated, as described for patients with chronic hepatitis C who respond to antiviral treatment[13-16].

IS OCCULT HCV A TRANSIENT OR A PERSISTENT INFECTION?

In order to determine whether occult HCV infection remains over time, we have performed a study including 37 patients who were anti-HCV and serum HCV RNA negative but with viral RNA in the liver biopsy[60]. Patients were followed for a mean time of 55 mo and serum and PBMCs samples were collected periodically for HCV RNA testing. Evidence of viral persistence in patients with occult HCV infection was found by the detection (over the observational period) of intermittent or persistent HCV RNA positivity in the ultracentrifuged serum or in PBMCs in all but one patient. These results suggest that anti-HVC negative occult HCV is a permanent infection as it has been reported in anti-HCV positive patients who resolved HCV infection[16,61]. Nevertheless, in order to extend the knowledge in the natural history and the pathogenesis of occult HCV infection, a more prolonged follow-up is needed.

GENOTYPES OF OCCULT HCV INFECTION

In the initial studies of occult HCV infection the only HCV genotype detected was 1b[4,19,22]. This result was predictable because HCV genotype 1b is the most prevalent genotype in Spain[62,63]. However, later studies performed worldwide (Table 1) have reported occult HCV infection belonging to HCV genotypes 1a, 2a, 3a and 3b[21-23,26]. So, it can be assumed that occult HCV infection is a universal phenomenon and all genotypes may be involved in this infection. This hypothesis should be proven with several studies performed in countries with different prevalence of HCV genotypes.

ROLE OF OCCULT HCV INFECTION IN LIVER TRANSPLANTATION

In anti-HCV positive patients with occult HCV, the reactivation of HCV infection (with reappearance of serum HCV-RNA) is well documented in special clinical situations, such as immunocompromised patients, patients on long term chemotherapy for cancer or patients receiving immunosuppressive therapy (including those who have undergone liver, kidney or bone marrow transplant)[64-69]. By contrast, there are no studies of the serologically silent (anti-HCV negative) occult HCV infection in these settings, except for a reported case of occult HCV infection as the cause of a liver transplantation and retransplantation[21]. The patient was a 29-year-old man who was transplanted due to liver cirrhosis of unknown etiology. Ten months after the first liver transplant, the patient was retransplanted because of liver failure secondary to severe chronic cholestasis of unknown origin. Liver

samples of both explants were available for study. The patient has remained anti-HCV and HCV RNA negative in serum and plasma since the initial diagnosis, but viral RNA was detected in the liver tissue samples from the two explants. Furthermore, a phylogenetic analysis demonstrated that the HCV RNA isolated from the two liver samples belonged to genotype 1a. Although HCV RNA was undetectable in the PBMCs of the patient, he had an occult HCV infection and probably, the liver graft was infected by PBMCs.

This case suggests that occult HCV infection may play a role as an etiological agent of liver failure in transplanted patients. Also, this report strongly supports the need for performing studies not only in liver transplant, but also in other immunocompromised patients with unexplained elevation of liver enzymes to determine the real magnitude, clinical significance and long-term consequences of occult HCV infection.

OCCULT HCV INFECTION IN HEALTHY POPULATION WITH NORMAL LIVER ENZYMES

Although occult HCV infection was identified in patients with abnormal values of liver function tests, a recent work by De Marco *et al*[26] describes the existence of occult HCV infection among healthy people with normal alanine aminotransferase and normal aminotransferase values. These healthy subjects were enrolled in the frame of three different epidemiological studies: the Italy cohort series of the European Prospective Investigation into Cancer and Nutrition, the Turin Case-Control Bladder Cancer Study and the Italian project in Cervical Cancer Screening. Subjects were tested for anti-HCV and for HCV RNA in plasma and in PBMCs. All of them were anti-HCV and serum HCV RNA negative, but viral RNA was detected in the PBMCs of 9/276 (3.3%) healthy controls with normal liver enzymes. Interestingly, in the studied population, blood donors were over-sampled but the authors do not indicate if any of these blood donors had an occult HCV infection.

So, this work potentially has important implications. Thus, the frequency of occult HCV infection may be underestimated since until now this infection has been exclusively studied among patients with abnormal values of liver enzymes. In this sense, in the study of De Marco *et al*[26] the frequency of occult HCV infection in their health population was 3.3% *vs* the 2.7% prevalence of anti-HCV positivity detected in the general Italian population. Although the number of participants in the former study (276 subjects) should be increased, there is a potential risk for HCV spread from an occult HCV healthy population. Thus in blood donations, despite approaches to reduce the risk of leukocyte-related disease, such as leukodepletion, the efficacy in reducing the risk of transmitting viruses is still under debate[70]. Furthermore, as HCV RNA may be detected after ultracentrifugation of serum samples in more than 50% of patients with occult HCV

infection[27] and this test was not performed in the Italian study, it may be possible that healthy blood donors have HCV RNA in serum undetectable by conventional PCR assays. If this is the case, blood donors with occult HCV infection may potentially transmit this occult infection as it is undetectable by the current applied screening tests for HCV in blood banks. However, more studies should be performed in healthy subjects with normal liver enzymes and especially among blood donors to definitively establish the possible magnitude of this infection.

CONCLUSION

Occult HCV infection has been found in two different settings: in anti-HCV positive, serum HCV RNA negative patients with normal levels of liver enzymes and in anti-HCV negative, serum HCV RNA negative patients with abnormal liver function tests of unknown etiology. Occult HCV is distributed worldwide and all viral genotypes may be involved. Although seronegative occult HCV infection seems to be less aggressive than classical chronic hepatitis C, it has been detected in patients with liver cirrhosis and even in hepatocellular carcinoma. Occult HCV infection has been described also in a healthy population with no evidence of liver disease, indicating that this infection may be present in a wide spectrum of clinical situations. Further studies on the natural history and the clinical significance of occult HCV infection are needed to determine its global prevalence, infectivity, implication in causing extrahepatic diseases and its long-term complications in special circumstances such as immunocompromised patients.

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EDITORIAL

Potential prospects of nanomedicine for targeted therapeutics in inflammatory bowel diseases

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Abstract

Inflammatory bowel diseases (IBDs) such as Crohn's disease are highly debilitating. There are inconsistencies in response to and side effects in the current conventional medications, failures in adequate drug delivery, and the lack of therapeutics to offer complete remission in the presently available treatments of IBD. This suggests the need to explore beyond the horizons of conventional approaches in IBD therapeutics. This review examines the arena of the evolving IBD nanomedicine, studied so far in animal and *in vitro* models, before comprehensive clinical testing in humans. The investigations carried out so far in IBD models have provided substantial evidence of the nanotherapeutic approach as having the potential to overcome some of the current drawbacks to conventional IBD therapy. We analyze the pros and cons of nanotechnology in IBD therapies studied in different models, aimed at different targets and mechanisms of IBD pathogenesis, in an attempt to predict its possible impact in humans.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Tumor necrosis factor-α; Nanomedicine

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) constitute the two principal components of inflammatory bowel diseases (IBDs), which occur as a result of dysregulated immune responses in genetically predisposed individuals due to various environmental conditions[1]. There are sufficient similarities in the pathological conditions in CD and UC that cause about 10% of IBD cases to be diagnosed as indeterminate IBD[2]. Nevertheless, CD and UC show discrete risk factors and dissimilar gene and protein expressions, which manifest distinctive pathophysiological mechanisms. CD exhibits a transmural inflammatory response and can be associated with granulomas, whereas UC usually shows mucosa-confined inflammation[2-8]. Genomic technologies are now being used to separate the effects of different susceptibility genes in the two diseases. For example, Wu *et al*[6] have studied 36 expression profiles of colonoscopic pinch biopsies from CD and UC patients. Affected genes, mostly related to interferon (IFN)-γ inducible T helper cell 1 (TH1) process and antigen presentation in CD patients, were differentially regulated, with the upregulation of 47 genes and downregulation of 30 genes. In contrast, the expression of genes from UC biopsies showed upregulation of 51 genes and downregulation of 81 other genes,

associated with biosynthesis, metabolism and electrolyte transport[6].

The common conventional medications currently in use to treat both CD and UC involve 5-aminosalicylic acid drugs, corticosteroids, immunosuppressive agents, biologic therapies and antibiotics[9], with a customary "step up" approach of starting with aminosalicylates and rising to corticosteroids and immunosuppressive agents in response to the persisting conditions of the disease. The more effective biological therapies are usually considered as a last option and only in case of refractory diseases, because their systemic action in the host often leads to adverse effects[10,11].

Nanomedicines are precise therapeutics established with the aid of nanotechnology to treat diseases at the molecular level[12]. The application of nanotechnology in medicine can be termed as nanomedicine. It is an evolving face of medicine that uses nanoparticulate carriers to deliver therapeutics targeted to specific cells, or constituents of cells or tissues. Studies have shown nanomedicines to be more beneficial than conventional medications, because their size leads to more effecting targeting, better availability at diseased tissues, and decreased adverse effects. Moreover, nanomedicines have been found to have similar or even better therapeutic impacts at lower drug concentrations than their conventional counterparts[12]. However, although the arena of nanomedicine appears to be encouraging for IBD therapy, concerns related to the impact of the nature of nanoparticles due to their size, shape, aggregation potential, and surface chemistry on the IBD gut need to be scrutinized[12,13], and investigations on the impact of nanomedicine in IBD therapy is currently in early stages. As targeted drug or biological delivery to sites of inflammation remains a crucial challenge in the current treatment of IBD[14], nanostrategies involving short interfering RNAs (siR-NAs), antisense oligonucleotides, nanomedicines delivered to the sites of malfunction in IBD can be a valuable therapeutic approach.

The RNA interference technique, notable for specificity, can be speculated to regulate the expression of proinflammatory cytokines and genes related to IBD at the mRNA level[15]. As the impact of noncoding RNAs and RNA silencing in gene modulation is known to be great[16], the use of siRNAs as drugs to silence proinflammatory genes is being scrutinized in various animal models of IBD. This strategy also reduces the chances of immune reactions usually associated with viral vectors[15,17,18]. Due to the potential importance of targeted therapy in IBD, this review is presented to explore the advancements in the prospects of nanomedicine in the modulation of gene expression and targeted therapeutics in IBD (Figure 1).

GENE AND PROTEIN MODULATIONS WITH NANO PROSPECTS

Amongst the key genes involved in IBD pathogenesis,

Figure 1 Graphical representation of nanoinvestigations in inflammatory bowel diseases models. IBD: Inflammatory bowel disease.

the function of tumor necrosis factor (TNF)-α in the mediation of inflammation in IBD is extensively acknowledged. Therefore, many biological therapies comprising monoclonal antibodies or soluble receptors are intended to reduce TNF-α activity, and have been extensively tested in many clinical trials[19-24]. However, there are adverse side effects due to the systemic depletion of TNF-α. These adverse effects involve amplified infusion reactions, immunosuppression, opportunistic infections and decreased efficacy of the biologics due to antibody formation against them[24-26].

The gene silencing nanostrategy, in which orally delivered TNF-α siRNA is encapsulated in thioketal nanoparticles (TKNs) made from the polymer poly-PPADT (1, 4-phenyleneacetone dimethylene thioketal), effectively decreases the levels of TNF-α mRNA levels at sites of intestinal inflammation in dextran sulfate sodium (DSS)-induced mouse models of UC. In this study, the site specific delivery of siRNA was made possible due to the ability of TKNs to degrade in the presence of higher levels of reactive oxygen species (ROS) present in regions of inflammation in the intestinal tissue[27]. In another study, TNF-α siRNA/polyethyleneimine (PEI) nanocomplex was shown to inhibit TNF-α secretion by macrophages *in vitro*, whereas the oral administration of TNF-α siRNA/PEI nanocomplexes in lipopolysaccharides (LPS)-treated mice models was found to reduce specifically the synthesis and secretion of TNF-α in the colon[28]. Nanoparticles in a microsphere oral system (Ni-MOS), comprised of TNF-α siRNA entrapped in type B gelatin enclosed in poly(ε-caprolactone) (PCL) microspheres, were found to exhibit favorable gene silencing in the colon tissues of DSS-treated murine models of UC. This treatment results in the suppression of proinflammatory cytokines such as interleukin (IL)-1β, IFN-γ, chemokine monocyte chemoattractant protein (MCP)-1, permitting an increase in body weight and diminished action of tissue myeloperoxidase in mouse models[29]. A protein modulation nanostrategy involving monovalent and bivalent murine TNF-α neutralizing nanobody proteins has been investigated in DSS-induced murine chronic colitis models. *Lactococcus lactis* engineered to produce the therapeutic nanobodies was orally administered, which

Figure 2 Nanomodulations whose efficacy has been validated in animal models of inflammatory bowel diseases. Genes regulated therapeutically by nano gene silencing in intestinal tissues and macrophages and protein nanobodies that have been investigated to have therapeutic impacts to help control inflammation and tissue destruction in animal models relevant to inflammatory bowel diseases (IBDs). TNF: Tumor necrosis factor; Map4k4: Mitogen-activated protein kinase kinase kinase kinase 4; MMP: Matrix metalloproteinase; NF-κB: Nuclear factor kappa B.

resulted in a significant reduction in the TNF-α driven inflammation in the mucosa of the colon in mouse models, without affecting considerable TNF-α levels in the systemic circulation[30].

Increased TNF-α suppresses the expression of the anti-inflammatory protein prohibitin (PHB) in IBD[31,32], therefore, a study by Theiss *et al*[33] considered the oral delivery of PHB entrapped in poly (lactic acid) nanoparticles in mouse models of DSS-induced colitis. This strategy inhibited the TNF-α-induced nuclear factor (NF)-κB activation; consequently curtailing inflammatory reactions and reducing the severity of colitis. Double-stranded decoy oligonucleotides (ODNs) against the proinflammatory NF-κB gene were enclosed in chitosan-modified poly (D,L-lactide-co-glycolide) nanospheres (CS-PLGA NSs) and delivered orally to DSS-induced murine colitis models. This study showed the absorption of the ODN-CS-PLGA NSs in inflamed mucosal regions, producing considerable curative effects on DSS-induced diarrhea, bloody feces, shortening of colon length, and myeloperoxidase activity[34].

Besides directly inhibiting the TNF-α gene in macrophages, macrophages more generally play a role in inducing the pathogenic inflammatory reactions[35]. This study has revealed the importance of mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4) gene in macrophages in mediating the production of inflammatory cytokines. Map4k4 siRNA encapsulated in β1,3-D-glucan shells silenced Map4k4 expression *in vivo* in mice treated with LPS, protecting them from LPS-induced systemic inflammation by suppressing the production of TNF-α and IL-1β[35].

Matrix metalloproteinases (MMPs) play a vital role in tissue remodeling by regulating the intestinal tissue architecture during the inflammatory reactions and wound healing in IBD[36,37]. Studies have indicated the increased expression of MMP-3 (stromelysin-1) and MMP-10 (stromelysin-2) in causing enhanced tissue injury in DSS-induced murine colitis[38,39]. Furthermore, IBD patients have shown increased MMP-3 and MMP-10 expression in the gut and intestinal ulcer tissues[39-42]. Polymorphisms in various MMP genes may be susceptibility factors for IBD risk, at least in some populations[43]. A study by Kobayashi *et al*[39] demonstrated the specific inhibition of MMP-3 and MMP-10 by siRNA targeted against MMP-3 and MMP-10, having a therapeutic benefit in protecting the colon tissue and reducing the severity of colitis in DSS-treated murine models, which could therefore be a valuable gene silencing option to prevent intestinal damage in IBD (Figure 2).

Cyclin D1 (CyD1) is a cell cycle regulatory protein that is upregulated in IBD in both epithelial and immune cells[44]. A leukocyte-directed siRNA against CyD1 mRNA inhibits the intestinal inflammatory responses in murine models of DSS-induced colitis. Silencing the CyD1 gene decreases the induction of TH1 cell inflammatory cytokines TNF-α and IL-12, but has no impact on the production of TH2 cell cytokine IL-10[45]. Therapeutic efforts to enhance the action of the anti-inflammatory cytokine IL-10, which is known to be critically involved in maintaining mucosal immune balance due to its potent impact on immunosuppression[46] and involvement in CD pathogenesis[47,48], have been largely unsuccessful to date. This is thought to be due to the adverse side effects caused by systemic action of the IL-10 therapies, and the low concentrations of IL-10 delivered to the intestinal tissues[49]. Therefore, biologics intend-

ing to enhance cytokine IL-10 action have been dropped from the current IBD therapies[50]. However, because the involvement of IL-10 and its genetic variations in IBD is great[47,48,51], a consideration of the targeted study by Bhavsar *et al*[52], which involved the nanodelivery of IL-10-producing plasmid to the mucosa in murine models mimicking IBD intestinal epithelial pathogenesis[53] can be scrutinized. According to this study, trinitrobenzene sulfonic acid (TNBS)-induced acute colitis models in Balb/c mice were treated with NiMOS intended for oral gene therapy. This comprised the pORF5-mIL-10 plasmid DNA encapsulated in type B gelatin nanoparticles in PCL. This strategy directed the local transfection of IL-10 plasmid in inflamed intestinal tissues and caused its enhanced expression, which led to suppression of predominant proinflammatory cytokines such as IFN-γ, TNF-α, IL-1α, IL-1β and IL-12, consequently causing the therapeutic benefits of restored colon length and weight, increased body weight, and beneficial clinical activity score[52].

IMPLICATIONS OF IBD DRUGS GOING NANO

Nanomedicines comprising IBD drugs loaded onto nanoparticles, designed to cope and act in accordance with the pathophysiological changes in the intestinal tissues of IBD, can be an intelligent mode of targeted drug delivery. This approach offers the possibility of eliminating undesirable side effects usually caused by systemic action of the drugs[14]. The usual pathophysiological conditions related to inflamed intestinal tissues in IBD predominantly involve abnormal intestinal permeability, increased presence of immune cells, and higher levels of mucus production[54-56].

Cellular interaction of nanoparticles in the IBD gut

Nanomedicines in IBD can potentially be more efficient in their mechanism due to the cellular intake of the nanoparticles by the cells at the targeted sites of delivery. This means that they are not eliminated from the intestinal tract by diarrhea, as are many current conventional medications. This is an important IBD symptom[57,58]. Nanoparticles in the gastrointestinal tract are usually found to be adsorbed either by paracellular transport or endocytosis by regular epithelial cells[59]. Specialized differentiated epithelial cells called M cells, which form major populations of Peyer's patches are involved in the predominant uptake of nanoparticles through transcytosis[60,61]. Predominant CD mutations such as R702W, G908R and 3020insC have been associated with ileal-specific disease[62,63], which show an enhanced presence in Peyer's patches and M cells, which may cause an increase in the uptake of dietary and nanoparticulate substances[13]. In addition to these, translocation of nanoparticles in the intestinal tract can also occur by persorption through gaps or holes at the villous tips[64,65]. Cells involve the autophagic mechanism to cause the clearance

of nanoparticles[66], and Powell *et al*[13] have indicated that mutations in the autophagy gene *Atg16L1* in IBD subjects can be susceptible to possible alterations in the clearance of nanoparticles.

Investigations of IBD drugs in nanomodes

Furthermore, IBD drugs delivered in nanomodes have been shown to have greater therapeutic impacts as compared to their conventional delivery studied in animal models, For example, the anti-inflammatory IBD drug mesalamine (5-ASA) covalently linked to the PCL nanoparticles was found to be 60 times more efficient as a nanomedicine at much lower doses (0.5 mg/kg) than the free solution of 5-ASA (30 mg/kg) in treating TNBS-induced colitis in BALB/c murine models[67]. Moulari *et al*[68] have established that silicon nanoparticles have a sixfold increased ability to adhere to inflamed tissues when compared to tissues in healthy controls. In this study, 5-ASA loaded in its methylated form in silicon nanoparticles was shown to collect in inflamed regions in TNBS-induced murine colitis models, inducing a positive impact on clinical activity score and myeloperoxidase activity at reduced drug doses, as compared to conventional delivery[68]. An immunosuppressive drug tacrolimus, used to treat UC, was encapsulated in polylactic-co-glycolic acid (PLGA) nanoparticles and used to treat murine models of TNBS- and oxazolone-induced colitis. This study showed that nanomedicine had an augmented and specific action with a threefold increased penetration in inflamed tissues when compared to healthy tissues[69]. Also, tacrolimus-loaded PLGA nanoparticles and tacrolimus-loaded pH-sensitive Eudragit P-4135F nanoparticles showed diminished side effects in DSS-induced murine colitis models when compared to the free tacrolimus which causes nephrotoxicity in traditional delivery[70]. An anti-inflammatory tripeptide Lys-Pro-Val (KPV) loaded into polylactide (PLA) nanoparticles delivered in combination with a polysaccharide hydrogel had a similar anti-inflammatory effect at 12 000-fold lower doses (25.2 ng/d) to that of KPV in free solution (200 μg/d), thus demonstrating the greater therapeutic efficiency of the nanomode of the drug in treating DSS-induced colitis in murine models[71]. Nakase *et al*[72] demonstrated that dexamethasone, encapsulated in poly-DL-lactic acid (PDLLA) microspheres was more effective in ameliorating DSS-induced murine colitis when compared to the free solution of the same drug, because the microsphere form was engulfed by the immune cells in the inflamed colonic tissue, which resulted in increased efficiency of the drug in mouse models. An *ex vivo* study by Serpe *et al*[73] showed solid lipid nanoparticles (SLNs) comprising the anti-inflammatory molecule cholesteryl butyrate (chol-but) showed a greater impact than butyrate alone in significantly reducing proinflammatory cytokines such as IL-1β and TNF-α and increasing IL-10 production in whole blood *ex vivo* models of peripheral blood mononuclear cells (PBMCs) obtained from IBD patients taking no anti-inflammatory medications. Furthermore, this study demonstrated SLNs, consisting of the immunosuppres-

Table 1 Comparison of therapeutic parameters

Experimental system	Drug in nano mode	Comparison of differences in certain distinct therapeutic parameters		Ref.
		Nano mode	Controls	
In vivo TNBS-induced murine colitis	5-ASA covalently linked to PCL nanoparticles	Myeloperoxidase (MPO) activity of 5-ASA-NP at 0.5 mg/kg: 15.2 ± 5.6 U/mg	MPO activity of 5-ASA free solution at 30 mg/kg: 16.2 ± 3.6 U/mg	[67]
In vivo TNBS-induced murine colitis	5-ASA in silicon nanoparticles	MPO activity of 5-ASA-Si NP at 25 mg/kg: 5.2 ± 2.4 U/mg	MPO activity of 5-ASA-free solution at 100 mg/kg: 8.2 ± 3.4 U/mg	[68]
In vivo TNBS-induced murine colitis/oxazolone-induced murine colitis	Tacrolimus in PLGA NPs	Enhanced penetration into the inflamed tissue-FK506-NP, 105 ± 24 nmol/cm^2	Healthy tissue penetration-FK506-NP, 51 ± 13 nmol/cm^2	[69]
In vivo DSS-induced murine colitis	Tacrolimus in PLGA/or pH sensitive Eudragit P-4135F NPs	Diminished side effects	Increased susceptibility to nephrotoxicity	[70]
In vivo DSS-induced murine colitis	Anti-inflammatory tripeptide KPV in PLA NPs	Nanomode with lowered doses at 25.2 ng/d	Free solution has the similar anti-inflammatory impact at 200 μg/d	[71]
Whole blood *ex vivo* models of PBMCs	Dexamethasone in SLNs	90% reduction in proinflammatory cytokines IL-1β and TNF-α	25% reduction in TNF-α by the free solution	[73]
Whole blood *ex vivo* models of PBMCs	chol-but in SLNs	Significant decrease in IL-1β, and TNF-α increase in IL-10	-	[73]

NP: Nanoparticle; TNBS: Trinitrobenzene sulfonic acid; DSS: Dextran sulfate sodium; PBMCs: Peripheral blood mononuclear cells; 5-ASA: Mesalamine; KPV: Lys-Pro-Val; PLA: Polylactide; SLNs: Solid lipid nanoparticles; IL: Interleukin; TNF: Tumor necrosis factor; MPO: Myeloperoxidase; PLGA: Polylactic-co-glycolic acid.

sive corticosteroid dexamethasone, suppressed TNF-α by 90% when the free solution of dexamethasone showed a TNF-α suppression of 25% at the highest concentrations in similar whole blood IBD *ex vivo* models. These studies provide preliminary support for the effects of SLNs chol-but and SLN dexamethasone in inducing an enhanced anti-inflammatory activity, due to the more effective cellular intake of the nanodrug forms, as compared to the free drugs in solution (Table 1).

LIKELY CONCERNS OF GOING NANO

The general concern associated with the nano approach is due to the fact that nano-sized materials display altered physicochemical properties[74] as compared to their larger counterparts, with chances of causing possible toxicity, since nonbiological nanoparticulate carriers above a particle size of 100-200 nm can alter normal cellular activity, because they can invoke cell membrane ruffling, cytoskeletal rearrangement and stimulate endocytic machinery causing their ingression in phagocytic cells[75]. However, reliable data on the adverse impacts of nanomedicine in IBD is unavailable, whereas the impact of nanoparticles themselves in the gastrointestinal tract might vary according to the nanoparticle polymer material and nanoparticle size, as surface interactions and surface chemistry differ for different nanoparticle sizes. Also, different nanoparticle sizes can cause different mechanisms of cellular uptake, due to which, nanoparticle sizes can be modulated to cause different intracellular effects[13,76,77]. The studies on the effects of nanoparticles themselves in the human gastrointestinal tract in IBD have been limited and need to be explored further.

Although nanostrategies for IBD therapeutics inves-

tigated in animal and *in vitro* models of IBD have shown promise, it is still only the dawn of the era of interest in IBD nanomedicine, and there is a definite need for further extensive investigations on many issues related to the safety and uptake of the different nanomedical therapeutics acting on various pathways and phases in the human gastrointestinal tract. It is essential to be confident of their consequent impact on immune responses and therapeutic effects in the different genotypic populations, before recommending the clinical use of nanomedicines to treat IBD in humans.

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TOPIC HIGHLIGHT

Luis Bujanda, PhD, Professor, *Series Editor*

Spontaneous regression of pancreatic cancer: Real or a misdiagnosis?

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Abstract

Spontaneous tumor regression has been subject of numerous studies and speculations for many years. This phenomenon is exceptional, but well reported, in some types of tumors, but not in pancreatic cancer. Pancreatic cancer has the worst five-year survival rate of any cancer. Despite numerous molecular studies and clinical approaches, using several mouse models, this cancer responds poorly to the existing chemotherapeutic agents and progress on treatment remains elusive. Although pancreatic cancer tumors seldom undergo spontaneous regression, and some authors take that with skepticism, there are some cases reported in the literature. However, the variability in the description of the reports and technical details could make this process susceptible to misdiagnosis. Distinguishing between different types of pancreatic carcinoma should be taken with caution as they have wide differences in malignant potential. Diseases such as pancreatic benign tumors, insulinomas, or autoimmune pancreatitis could be responsible for this misdiagnosis as a pancreatic cancer. Here we review different cases reported, their clinical characteristics, and possible mechanisms leading to spontaneous regression of pancreatic cancer. We also discuss the possibilities of misdiagnosis.

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INTRODUCTION

Spontaneous tumor regression has been the subject of great interest and speculation for many years. It is an exceptional and well-documented biological event in some types of tumors, but not in pancreatic cancer.

Pancreatic cancer is a special form of cancer with the worst five-year survival rate of any cancer[1]. Despite numerous molecular studies and clinical approaches, using several mouse models[2], this cancer responds poorly to the existing chemotherapeutic agents and progress on

treatment remains elusive[3].

Pancreatic cancer is seldom described as undergoing spontaneous regression, but there are some cases reported in the literature.

In this review, the historical background, clinical features, and possible mechanisms are discussed for spontaneous regression of pancreatic cancer. In addition, we discuss whether it is a real phenomenon or a misdiagnosis.

Further understanding of this process and harnessing of the mechanisms involved will have significant diagnostic, preventative, and therapeutic implications.

HISTORICAL BACKGROUND AND CASES REPORTED FOR PANCREATIC CANCER

Spontaneous regression of cancer (SRC) is defined as the partial or complete disappearance of a malignant tumor in the absence of therapy that is capable of inducing anti-neoplastic effects. Although SRC has often been questioned, the literature reveals different cases showing this phenomenon. In 1966, Everson *et al*[4] published a classical monograph review describing 176 cases of SRC published from 1900 to 1964. In 1990, Challis *et al*[5] reported cases from 1900 to 1987, the majority of which occurred in renal cell carcinoma, choriocarcinoma, neuroblastoma, melanoma, breast cancer, and leukemia and lymphomas. Later, in 1993, O'Regan *et al*[6] agreed that the five most common tumors to undergo spontaneous regression are renal cell carcinoma, leukemia and lymphoma, neuroblastoma, carcinoma of breast and melanoma.

In these three main reviews, only three cases of pancreatic cancer were cited, and none of them were described in detail. Here, we review the most important cases of spontaneous regression of pancreatic cancer that have been reported in the literature. The first reported case was described in 1934 and published in 1967, describing a patient admitted to hospital presenting jaundice, severe pain, nausea, chills, and a high fever[7]. Laparotomy and biopsy confirmed pancreatic carcinoma. The patient's recovery spanned two months, after which she could return to work. She remained in good health, dying seven and a half years later of a pulmonary embolism. An autopsy did not find any tumors.

The second case was reported in 1973 by Lokich *et al*[8] and described a 42-year-old man with progressive diarrhea and weight loss. An upper image suggested a mass in the head of the pancreas. The patient underwent total pancreatectomy and microscopic examination reveled a moderately well differentiated ductal adenocarcinoma arising in the head of the pancreas. Adenocarcinoma was also found in the body of the pancreas, but the tail had only pancreatitis with fibrosis.

Although the patient was stabilized with insulin treatment, one year later he presented with rectal carcinomatosis consistent with adenocarcinoma of pancreatic cancer. Postoperatively, the patient received a combined chemotherapeutic program based on 5-fluorouracil (5-FU)

and carmustine or bis-chloroethylnitrosourea, experiencing a gradual regression. Twenty-six months following onset of therapy treatment, there was no evidence of tumor recurrence.

The third case[9] was a male with a two-month history of ulcer pain and diarrhea. At exploration, he was diagnosed with a large tumor of the pancreatic head, extending into the liver with involved lymph nodes. The disease was confirmed by biopsy but no further manipulation was performed. By the fourth month following surgery, he was asymptomatic. Examined six years later, the patient remained symptom-free and a gastrointestinal exam demonstrated healing of the ulcer.

The fourth case, published in 1974, reported one case in 1962 of a 21-year-old male who presented with jaundice, anorexia, and fever of three months duration[10]. A liver biopsy was followed by abdominal pain, fever, tachycardia, and a decrease in blood pressure. Exploratory surgery to repair bile peritonitis revealed acute cholangitis and pericholangitis. When he was re-operated on seven weeks later, and was diagnosed with pancreatic adenocarcinoma. A T tube placed in the common duct improved symptoms and he made a slow recovery with no recurrence at the time of reporting. Unfortunately, details on the duration and intensity of fever or infection over the course of the illness in most of these cases were not provided.

In 2003, Hoption Cann *et al*[11] reported a case of a 50-year-old man with a three-month history of weight loss, anorexia, and discomfort after meals. By ultrasound and computed tomography (CT) identified a hypoechoic mass of 6.5 cm × 4 cm × 4 cm in the body of the pancreas. The posterior CT-guided biopsy was positive for pancreatic adenocarcinoma (T2N1M0, stage Ⅲ b). A subsequent CT scan revealed a further 50%-60% increase in tumor volume and the tumor was considered inoperable. The patient received chemotherapy based on gemcitabine, mytomycin, and radiotherapy. As CA19-9 levels increased from 38 to 140 U/mL and the patient's health declined, the treatment was considered a failure. Some days later, the patient developed acute abdominal pain and fever and, after surgery, he had a perforated duodenal ulcer with contamination of the abdomen. Recovery was considered doubtful. However 90 d later, the patient's recuperation and weight gain were surprisingly rapid, while the CA19-9 level was normal and a positron emission tomography (PET) scan was negative for any focal disease. An ultrasound, however, confirmed residual tumor, although it had regressed by approximately 70%. However, five months later, an elevated CA19-9 and subsequent PET scan confirmed a relapse. Although the patient was treated with chemotherapy based on oxaliplatin and 5-FU initially, then gemcitabine, his health progressively deteriorated and he died one year later, almost two years following his febrile infection.

Apart from the infection, the authors suggested other factors could be relevant to this tumor regression. Some of them are the vegetarian diet based on Chinese herbs,

high-dose vitamin C and other antioxidant vitamins, hydrogen peroxide, and ginseng, followed by this patient. However, regression presented in this case appeared mostly to coincide with a prolonged febrile infection, similar to that often observed in many other cases of SRC[11,12].

BENIGN TUMORS

Some special types of pancreatic tumors are considered benign or their malignant potential is not well determined. Specifically, solid-pseudopapillary tumors are classified in this category and were often previously described in the literature as being related to spontaneous regression.

In 2008, Nakahara *et al*[13] described a 18-year-old healthy woman who was admitted to hospital for evaluation of a pancreatic mass. A solid-pseudopapillary tumor was suspected from the findings of diagnostic images, and surgery was recommended. However, the patient refused surgery and a later ultrasound-guided transcutaneous biopsy revealed proliferation of tumoral cells with small nuclei showing a pseudopapillary arrangement. periodic acid-Schiff positive granules and alpha-1-antitrypsin positive cells were proven, which led to confirmed diagnosis of pseudopapillary pancreatic tumor. The maximum diameter of the tumor gradually decreased over 10 years from 45 mm to 15 mm. This was the first report describing marked spontaneous shrinkage of this particular type of pancreatic tumor. The authors did not report details of whether the patient took any medication, antioxidant agent, or vitamins.

In this case, the authors showed histological findings and CT images suggesting that the shrinkage of the tumor may be attributable to continued degenerative change, including minor hemorrhage, necrosis, and absorption as the tumor was classified hypovascular.

In 2010, Suzuki *et al*[14] described a 13-year-old boy showing a demarcated hypovascular round mass of 50 mm in diameter in the head of the pancreas, presenting abdominal pain, nauseas, and elevated serum amylase and serum lipase. CT demonstrated a partially enhanced encapsulated tumoral mass with cystic components and calcification, without evidence of invasion to the surrounding organs, which was diagnosed as solid pseudopapillary tumor (SPT) and treated for acute pancreatitis. Six weeks later, the mass had decreased to 43 mm in diameter, and nine weeks after admission, concentrations of tumor markers, such as alpha-fetoprotein, carcinoembryonic antigen, carbohydrate antigen-199, and elastase-Ⅰ, were not elevated, although the level of neuron-specific enolase (NSE) was slightly increased. Follow-up included routine laboratory tests and CT demonstrated that the size of the tumor slowly decreased to non-measurable size. After 4 years, the patient's NSE level was within the normal range. The authors presented CT images indicating that the tumor was highly likely to be SPT, based on the typical CT finding of a tumor bulging from the contour of the pancreas with eggshell-like calcification, the existence of both solid and cystic components with hypovascularity, and the patient's age. The rapid shrinkage of the tumor may be attributable to continued degenerative changes, including minor hemorrhage due to trauma, necrosis, and absorption. The authors did not report the administration of any medication or different agents to the patient. The authors suggest spontaneous tumor shrinkage, although they were unable to obtain pathology for the tumor and are conscious that there have been reports of recurrence and metastasis that developed more than 10 years after tumor resection in this type of neoplasm.

Considering all of the cases reported above, we can distinguish a wide variation in the description of the data presented. The first cases reported, until 1980, do not show images or acute laboratory test results that could verify the real entity of spontaneous regression of pancreatic carcinoma. In some cases, they do not specify the type of pancreatic tumor or give details about the biopsy, making it difficult to determine if it would be classified as a different entity, based in current diagnostic criteria.

However, considering the difference in the availability of medical technology more than 30 years ago compared with the present, this data should be taken with caution, and it is difficult to conclude whether these cases would be considered as genuine spontaneous regression of pancreatic adenocarcinoma today or would be considered as misdiagnoses.

The most recent cases, published since 2000, are more accurate in the presentation of images and blood test values, although they also show variability.

Finally, the most recent report concludes a diagnosis of SPT and not adenocarcinoma of pancreas. All of these data show that there are no recent cases reported of spontaneous regression of adenocarcinoma of the pancreas, unlike pseudopapillary tumors, which represent 1% of primary pancreatic tumors and are characterized by low malignant potential[13,14].

Some other types of pancreatic tumors with spontaneous regression, rather than adenocarcinomas and peudopapillary tumors, have been described. The most common neuroendocrine tumor with spontaneous regression is an insulinoma.

Insulinoma is a rare endocrine tumor developed from pancreatic beta cells. Eighty-seven percent are benign tumors, seven percent belong to multiendocrine neoplasia syndrome, and only six percent are considered malignant, as defined by the presence of metastasis[15].

The diagnosis of insulinoma is established by demonstrating inappropriately high serum insulin concentrations during a spontaneous or induced episode of hypoglycemia. Imaging techniques are then used to localize the tumor[16]. In 2008, Groselj *et al*[17] described a 64-year-old patient presenting with paroxysmal episodes. Electroencephalography finding suggested metabolic encephalopathy and laboratory tests showed hypoglycemia, and high insulin and C-peptide. Finally, ultrasonography and gagnetic resonance imaging (MRI) confirmed an

insulinoma in the head of the pancreas. The authors pointed out that the patient had a spontaneous recovery of the pancreatic tumor.

The overall survival rate of patients with benign insulinoma do not differ from that expected in the general population, and a misdiagnosis could be a reasonable justification for reporting SRC, even if it is not considered a malignant phenotype. Malignant insulinomas are rare, and patients have prolonged survival, even in the presence of liver or lymph node metastasis. It has been reported that some patients with malignant insulinoma who developed metastatic disease 4 years to 12 years after initial diagnosis, remained alive for up to 25 years[18]. This better outcome compared to the acinar or ductal adenocarcinoma could be a reason for a misdiagnosis, or it could also be reported as a spontaneous regression of pancreatic cancer.

AUTOIMMUNE PANCREATITIS MIMICKING PANCREATIC CANCER

Autoimmune pancreatitis (AIP) was described by Sarles *et al*[19] in 1961 and then proposed by Yoshida *et al*[20] in 1995 as a type of chronic pancreatitis occurring secondary to an autoimmune process, which may cause permanent structural and functional damage of the pancreas.

AIP represents approximately 6% of the patients with chronic pancreatitis[21,22] and is a heterogeneous manifestation associated with elevated serum levels of the immunoglobulin G subtype 4 (IgG4), which decreases with corticosteroid therapy. The most common site of extrapancreatic involvement is the bile duct, where distal biliary or mass-forming AIP mimics pancreatic cancer and proximal biliary involvement[23]. Recently, two types of AIP have been described, type 1 (or lymphoplasmacytic sclerosing pancreatitis) and type 2 (idiopathic duct centric pancreatitis or granulocyte epithelial lesion). Although clinically these two entities have comparable presentations, they differ significantly in their demography, serological characteristics, other organ involvement, and relapse rate[24].

While type 1 is associated with elevation of nonspecific autoantibodies and serum IgG4 levels, type 2 does not have definitive serologic autoimmune markers. In addition, high serum IgG4 may also be found in patients with pancreatic cancer[25], and tumoral markers such as CA19-9, SPAN-1, and DUPAN-2 may also be elevated in patients with AIP[26]. These findings can make the diagnosis of AIP confusing. AIP, in contrast to other benign chronic pancreatic diseases, can be cured with immunosuppressant drugs[27]; therefore, the differentiation of AIP from pancreatic cancer is of particular interest in clinical practice[28]. Two studies have also pointed out the possibility that some patients with AIP may develop pancreatic cancer[29,30], and this contributes to increasing misdiagnosis. However, the synchronous presence of adenocarcinoma and AIP can not be excluded, as some cases have been reported[31] and pancreatic cancer can develop after histologically confirmed AIP diagnosis[32].

Attempting to establish applicable diagnostic guidelines, the Japan Pancreas Society[33], the Korean Society[34], and more recently American criteria by Chari *et al*[24] in the Mayo Clinic at the Honolulu consensus proposed specific criteria to distinguished the two histological types of AIP and pancreatic cancer. The five important diagnostic criteria include imaging, histology, serology, other organ involvement and response to therapy, leading to an improvement in the diagnostic yield for AIP and avoidance of misdiagnosis of pancreatic cancer[35]. Nevertheless, several cases have been reported that suspect pancreatic cancer rather than AIP[36-38].

In 2005, Ozden *et al*[39] described a 58-year-old woman with jaundice referred for pancreatic head carcinoma and diagnosed by MRI. By laparotomy, a pancreatic head mass involving the mesocolon, pancreatic body, and tail was found. Pancreatic biopsies revealed cholecystitis and pancreatitis with lymphoplasmacytic infiltration. Two months after the surgery there was no parenchymal lesion on MRI. Serum immunoglobulin G, G4, and E levels were increased.

The authors report this as a spontaneous regression of a pancreatic head mass and biliary obstruction because of autoimmune pancreatitis. In this and other cases of a patient with autoimmune pancreatitis that were initially misdiagnosed as pancreatic cancer, the response to steroid therapy could appear to be a spontaneous resolution of a malignant pancreatic tumor. Patients operated on for pancreatic adenocarcinoma could represent a false spontaneous regression of pancreatic cancer instead of lack of malignancy.

MECHANISMS LEADING TO SPONTANEOUS REGRESSION

Most of the SRC cases reported do not provide a discussion regarding possible explanatory mechanisms. In pancreatic cancer reports, only the most recent publications show data in detail (images and laboratory test) and finally suggest some possible biological mechanisms leading to the spontaneous regression.

The prevalent hypotheses regarding mechanisms leading to spontaneous regression include the immunological response in the host as the most important factor[40-43]. Other mechanisms causing spontaneous regression include increased apoptosis and necrosis, epigenetic modifications, hormonal responses, role of oncogenes and tumoral suppressors, cytokines and growth factors, and psychological mechanisms (Table 1). All of these mechanisms were reviewed in 1996 by Papac[44], who specifically described some cancers, but not pancreatic tumors.

Today, the activation of these mechanisms in spontaneous regression of cancer occurs infrequently[5,40,45,46] and remains not well documented in pancreatic cancer. In other related tumors, like hepatocellular carcinoma, several mechanisms leading to the spontaneous regression of these tumors have been described, such as absti-

Table 1 Possible mechanisms for spontaneous regression in pancreatic cancer

Immunological response
Hormonal response
Induction of spontaneous differentiation
Elimination of the carcinogen
Modification in expression of oncogenes and tumoral suppressor genes
Angiogenesis inhibition
Apoptosis
Necrosis
Epigenetic mechanisms
Psychological mechanisms

nence from alcohol[47], persistent fever[48], withdrawal of androgen[49], blood transfusion[50], massive bleeding[51], and use of herbal medicine[52]. However, in the small number of reported pancreatic cancer cases, no evidence of clear and specific events was observed during the period of spontaneous regression.

Renal cell carcinoma accounts for the largest number of patients with spontaneous regression with acceptable histology and radiological confirmation[53,54]; therefore, this disease offers the best system to study the immunological response in spontaneous regression. The increased incidence of some tumors in immunosuppressed individuals, and regression following reduction of immunosupressive agents, suggests an important role for immunological factors[55,56]. Cytokines, interferon, and interleukin 2 (IL-2), IL-6 and IL-8[57,58], exert antitumor effects. While cytokines could activate T-lymphocytes, natural killer, lymphocyte-activated killer cells, and tumor infiltrating lymphocyte cells as a mechanism of action, interferons are capable of multiple immunomodulatory effects, involving monocytes, macrophages, and B-cells, as well as induction of IL-2 receptors[59,60].

Regression often occurs in the setting of febrile illness (bacterial or viral), other, different, cytokines associated with the host response to infections could mediate the regression as tumor necrosis factors[61]. Patients diagnosed with pancreatic cancer frequently suffer infections and all of these cytokines could play an important role in the spontaneous regression of pancreatic cancer; however, there is no evidence of this phenomena in the literature. Angiogenesis, as an essential component of the malignant process, has also been investigated as a mechanism contributing to regression. Several cytokines are known to inhibit this process, such as tumor necrosis factor-alpha and transforming growth factor beta, which could play a role in spontaneous regression[62]. Regarding hormonal mechanisms that could exert a role in pancreatic cancer, there is no data suggesting specific effects in spontaneous regression, although studies should be made because the endocrine pancreas could be exerting an important role.

A mechanism that has received more attention for spontaneous regression in the literature is the apoptotic process inside the tumor. The activation of this programmed cell death was proposed as the basis for sponta-

neous regression, especially in neuroblastoma[63] and renal cell carcinoma. Several authors suggest that the neoplastic cells, in response to different stimuli, such as T-cell mediated survival signals or cytokine regulation, could undergo apoptosis, followed by clinical remission.

As several data have demonstrated, vascular endothelial growth factor receptor blockade leads to rapid, robust, and progressive regression of tumor vasculature, increased intratumoral hypoxia, and apoptosis, and reduced tumor invasiveness and metastasis in pancreatic islet cancer[64]. Thus, this process could also be implicated in tumor regression. This apoptotic process is driven by oncogenes and tumoral suppressor gene expression and, although there is no specific, documented examples on the role of changes in the expression of regulator genes, this possibility has been cited in leukemia[65].

The expression of these oncogenes or tumoral suppressors could be switched by mutations and by epigenetic mechanisms, leading to apoptosis inside the tumor. The cited epigenetic changes have been demonstrated in retinoblastoma tumors[66] by abnormalities in methylation levels[67]. Some authors have suggested that loss of hypermethylation may be involved in the spontaneous regression of some retinoblastomas, but there is no confirmed evidence. In addition, repression of telomerase activity has been proposed as a possible mechanism for regression[68-70]. Some studies showed that patients whose tumors do not show telomerase activity underwent spontaneous regression, suggesting repression of telomerase activity as a possible mechanism for regression, although it has not yet been demonstrated in any type of pancreatic cancer.

Differentiation, a mechanism by which malignant cells develop a non-malignant phenotype, has been shown to occur in several types of cancer, such as retinoblastoma, neuroblastoma, choriocarcinoma, teratocarcinoma, and leukemias[71,72], where differentiation is possibly the major factor contributing to spontaneous regression, but this is still unknown in pancreatic cancer. Finally, related to the immunological response in tumors, psychological mechanisms have been proposed as a possible phenomenon in some cancers, but this is still regarded with skepticism. Although authors have reported psychological reasons, corroborating biological studies are lacking[73,74] and are not approved by most investigators.

This lack of information forces us to conclude that spontaneous regression in pancreatic cancer is not a well-documented phenomenon, the mechanisms leading to the regression remains unknown, and only hypotheses can be made based on some other types of tumors. In recent reports, where the radiological and histological confirmation of pancreatic disease are more precise, only an immunological response has been suggested as the most probable mechanism leading to regression of pancreatic cancer. Most of the causative factors leading to this phenomenon remain speculative.

In conclusion, it is very difficult to determine the characteristics of pancreatic cancer patients who experi-

ence spontaneous regression and the mechanisms leading to such spontaneous regression.

Currently, the existence of spontaneous regression of pancreatic cancer is a matter of debate. The small number of cases cited in the literature as a possible spontaneous regression could represent a nonmalignant disease, such as AIP or specific pseudopapillary tumor of the pancreas. In the cases described many years ago, the data presented make it difficult evaluate the diagnosis because of the lack of advanced images techniques or laboratory tests to distinguish pancreatic cancer from other diseases. In addition, many cases are not completely well documented, the presence of metastasis is questionable, therapy may have played a role, or the temporary or permanent regression of tumor growth was not defined.

Therefore, cases of spontaneous regression of pancreatic cancer described in the literature should be taken caution. Biological and molecular findings cannot provide a complete explanation of the underlying mechanisms and accumulation of such cases and further investigations of regression will contribute to better understanding of this intriguing phenomenon.

Elucidation of the mechanism could lead to better understanding and replication of the process, and to improved therapies for pancreatic cancer treatment.

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Animal models for the study of hepatitis C virus infection and replication

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Abstract

Hepatitis C virus (HCV) hepatitis, initially termed non-A, non-B hepatitis, has become one of the leading causes of cirrhosis and hepatocellular carcinoma worldwide. With the help of animal models, our understanding of the virus has grown substantially from the time of initial discovery. There is a paucity of available animal models for the study of HCV, mainly because of the selective susceptibility limited to humans and primates. Recent work has focused modification of animals to permit HCV entry, replication and transmission. In this review, we highlight the currently available models for the study of HCV including chimpanzees, tupaia, mouse and rat models. Discussion will include methods of model design as well as the advantages and disadvantages of each model. Particular focus is dedicated to knowledge of pathophysiologic mechanisms of HCV infection that have been elucidated through animal studies. Research within animal models is critically important to establish a complete understanding of HCV infection, which will ultimately form the basis for future treatments and prevention of disease.

INTRODUCTION

The hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus of the family Flaviviridae. There are six distinct genotypes with several subgenotypes that have been identified. The World Health Organization estimates that the virus affects approximately 3% of people worldwide. Approximately 170 million people are chronic carriers of the virus. There is higher prevalence in the Far East, Mediterranean countries and in certain African and eastern European areas. HCV infects primarily liver cells causing an acute hepatitis. Of those acutely infected, roughly 15% will clear the virus without medical intervention while the remainder will develop chronic hepatitis C. Approximately 30% of chronic hepatitis C will progress to cirrhosis, of which 20% will develop hepatocellular carcinoma. Thus, HCV infection remains a global health problem requiring continued research efforts to understand viral infection and to develop improved drug therapies.

Efforts to obtain information regarding the intricacies of hepatitis C viral entry, lifecycle and replication have been limited by the lack of model organisms available to serve as experimental hosts to the virus. Hu-

mans and other primates are the only known organisms naturally permissive to HCV infection. Costs and ethical concerns, thus, limit the study of the disease in chimpanzees. Recently, novel animal systems have permitted detailed examination of HCV infection, replication, and host responses. In this review, the status of HCV animal models is summarized, and the advantages as well as disadvantages, are discussed in terms of the potential impact on the development of future novel anti-HCV therapies.

ANIMAL MODELS

Chimpanzees and immune responses to HCV infection

The path leading to the discovery of HCV began in 1975 when some patients with viral hepatitis were found to lack markers for hepatitis A virus or hepatitis B virus (HBV) in serum. This led to the recognition that a separate unrelated hepatitis virus was responsible. This entity was initially termed non-A non-B hepatitis (NANBH). Thus began a wide body of research to isolate and identify the virus using chimpanzees as hosts for infection with human NANBH-infected serum. In 1989, Choo *et al*[1] and Houghton *et al*[2] created a complementary DNA clone of NANBH (clone 5-1-1) derived from positive-strand RNA, and confirmed that it encoded an antigen specific to NANBH infections. The cDNA was used to create antigens (c100-3) subsequently used as substrates to detect circulating serum antibodies *via* enzyme immunoassay. Through these discoveries in chimpanzees, antibody testing enabled screening of blood for the presence of the agent, now named HCV.

The study of HCV in chimpanzees has provided a wealth of knowledge regarding the mechanism of infection, replication, and both innate and humoral antiviral immune responses. Chimpanzees infected with HCV display elevations of aminotransferases and liver biopsies show necroinflammatory changes after acute infection. However, chimpanzees differ from humans in that their course of infection is milder; chronic carriers do not develop cirrhosis or fibrosis and only one chimpanzee has been reported to have developed HCV-related hepatocellular carcinoma[3]. Other differences include lack of efficacy of interferon (IFN) treatment as evidenced by constant viral loads despite administration of this agent. Alternative studies of direct antiviral agents are currently being studied in chimpanzees. For example, Olsen *et al*[4] showed that administration of a nucleoside analogue and protease inhibitor resulted in viral load decline in HCV-infected chimpanzees. Together with recent clinical trials and use of novel HCV protease inhibitors, success in the treatment of HCV-infected chimpanzees has potential to spark new human clinical trials using antiviral agents without concurrent use of pegylated-IFN and ribavirin.

Chimpanzees offer a valuable animal model for active immunization studies as well as for investigating mechanisms of innate and cell-mediated antiviral activity. Through studies on chimpanzees that have naturally cleared infection, Nascimbeni *et al*[5] have described the role of memory T-cell (both CD4 and CD8) responses that may help prevent infection upon re-challenge with virus. The varying quantity and quality of this cell mediated response helps explain differing responses to reinfection among individual chimpanzees. Barth *et al*[6] recently highlighted the importance of neutralizing antibodies to prevent early viral replication. They also showed that heightened CD8+ and natural killer (NK) cell activity increased production of IFN stimulating genes and IFN I / II, thus further supporting the role of adaptive immunity in limiting viral re-infection. Results of vaccination studies in HCV-infected chimpanzees have proven difficult to interpret for a variety of reasons including heterogeneity of genotypes, the error-prone RNA polymerase that creates mutations resistant to neutralizing antibodies, and downregulation of NK and T-cell responses *via* gpE2 interaction with CD81. Important information can nonetheless be gathered from both therapeutic and prophylactic vaccination studies[7]. Meta-analyses of HCV therapeutic vaccination studies in chimpanzees by Dahari *et al*[8] concluded that vaccinations that included non-structural HCV proteins were less effective in achieving HCV clearance in comparison to inclusion of structural proteins in vaccines, which were hypothesized to heighten T-cell responses. However, successful vaccination data should be interpreted carefully, because most studies use endpoints as reduction in clinical disease rather than sustained virological response. The search for a prophylactic vaccination for HCV has been challenging. The mechanism of protective vaccination is usually the generation of neutralizing antibodies. In HCV, neutralizing antibodies have been observed to coexist with high HCV titers, thus suggesting their presence does not limit HCV entry into cells *in vivo*. Neutralizing antibody levels also tend to decrease after infection resolves, indicating a lack of a memory response or capability to prevent re-infection. More recent work has subsequently focused on generating a reliable T-cell (CD4+ and CD8+) response in attempts to protect against the development of infection with exposure to the virus. Folgori *et al*[9] developed a prophylactic vaccination strategy in chimpanzees using adenoviral vectors and electroporated plasmid DNA encoding the HCV non-structural region. Through stimulation of a cross-reactive T-cell response, chimpanzees were capable of resolving infection when challenged with virus differing from the vaccine by more than 13% at the amino acid level. Vaccination studies in chimpanzees are ongoing. There are many disadvantages to the use chimpanzees as models, including cost, ethics, and most recently, an NIH ban on the use of chimpanzees for biomedical research[10].

Tupaia infection with HCV

Tupaia belangeri is a tree shrew native to Southeast Asia. Tupaia has been shown to be susceptible to a variety of human viruses including herpes simplex virus, rotavirus,

and HBV. In 2002, Zhao *et al*[11] demonstrated effective hepatitis C replication and virion synthesis in primary tupaia hepatocytes. This group plated and infected primary tupaia hepatocytes with serum or plasma derived HCV from infected humans. Infection and effective replication was confirmed by reverse transcription polymerase chain reaction detection of negative strand RNA in hepatocytes as well as secretion of viral particles in culture medium. HCV RNA could be detected up to 14 d after plating. The enveloped virions produced were shown to be resistant to degradation by ribonuclease and could infect previously uninfected tupaia hepatocytes.

In 2010, Amako *et al*[12] reported a longitudinal study which followed HCV-infected tupaia over a three year period after inoculation with hepatitis C from a patient or viral particles from full length cDNA. The animals demonstrated mild inflammation and viremia during the acute infection followed later by development of liver steatosis, cirrhotic nodules and tumorigenesis. Moreover, serum from infected tupaia was harvested and inoculated into naïve tupaia resulting in acute infection, demonstrating effective replication and potential transmission of HCV.

HCV entry in tupaia has been shown to support current knowledge of human essential entry receptors. In 2011, Tong *et al*[13] cloned tupaia CD81, scavenger receptor class B member 1 (SCARB- I or SR-B I), claudin-1 (CLDN1) and occludin and demonstrated that entry of HCV pseudoparticles or cell culture-derived HCV was permitted by these molecules. Inhibition of CD81 or SR-B I blocked HCV entry. However, there may be structural variations between human and tupaia receptors that may allow for differences in efficiency of viral entry. For example, the subtle structural difference between human and tupaia CD81 was shown to alter the ability of the extracellular loop to bind to HCV glycoprotein E2 in a study by Tian *et al*[14]. This information may be helpful in elucidating potential drug targets to block viral entry in humans. Tupaia is, therefore, a promising and effective model for the ongoing study of HCV entry and replication. A disadvantage of the tupaia model is that unlike humans with HCV, these animals rarely maintain sustained virema[12,15].

Mouse models for HCV replication

Much research has been devoted to understanding why murine hepatic cells are naturally resistant to hepatitis C viral cell entry, and permit only inefficient replication in cell culture and in animals. Ultimately, overcoming this resistance could provide a valuable mouse model for HCV replication as well as development of potential immune strategies to block HCV entry and replication in humans.

Mouse hepatocytes have been shown to allow HCV entry in cell culture in cells genetically engineered to express human HCV-specific entry molecules including CD81 and occludin. However, in addition to the barrier to entry, murine cells also have a resistance towards rep-

lication, assembly and release of HCV. This was demonstrated by Long *et al*[16] who used a transcomplementation system of mouse hepatoma cell lines that contained a subgenomic HCV replicon for ectopic expression of HCV structural proteins, p7, NS2 and apolipoprotein E (ApoE). They were able to demonstrate that assembly and release occurred successfully in murine cells with expression of the aforementioned proteins including ApoE.

In order to overcome the barrier to murine infection by HCV, Washburn *et al*[17] developed a model consisting of a humanized mouse engineered by engraftment of human hepatocyte progenitors and human CD34+ human hematopoietic stem cells. This model was generated by the use of a fusion of the FK506 binding protein and caspase 8 under the control of the albumin promoter. This construct induced apoptotic elimination of host hepatocytes in Balb/C Rag2 (-/-) C-null mice. Ultimately these humanized mice were shown to harbor both human hepatocytes and T cells. Upon infection with HCV, the mice developed liver inflammation and fibrosis. A human T-cell immune response to HCV was observed. This study was limited by low levels of HCV infection demonstrated by absence of HCV RNA in the serum, and a lack of a B cell immune response due to absence of an engraftment of a complete immune system.

Similarly, Mercer *et al*[18] and Kneteman *et al*[19] developed a chimeric severe combined immune deficient (SCID)/urokinase-type plasminogen activator mouse model. This immune deficient mouse model has been shown to support proliferation of transplanted human hepatocytes, and more importantly, sustained HCV infection as demonstrated by detection of viral RNA within hepatocytes after intravenous inoculation. Production and release of viral particles were demonstrated by successful passage of infection through three generations of mice. The studies showed that when the human hepatocytes comprise the majority of liver cells (at least 80% of total hepatocytes) within chimeric SCID mice, infection with HCV can occur, and can result in liver failure. Further research revealed that human apolipoprotein (ApoB) and cholesterol ester transfer protein may play a role in allowing HCV infection in the chimeric SCID mice, and thus may offer a target to prevent viral entry within humans[20]. HCV-infected SCID mice have also been used to study direct antiviral agents including IFN alpha-2 anti-NS3 and anti-NS5B proteases. Responses were shown to parallel that of humans. More recently, Kamiya *et al*[21] tested anti-NS3-4A (telaprevir) in HCV-infected SCID mice to evaluate the pharmacokinetics and dynamics of the drug as it related to a dose-dependent reduction in HCV serum RNA. Studies on SCID mice thus offer the potential to serve as a bridge between *in vitro* and clinical trials for HCV antiviral agents. Limiting factors of the SCID mouse model include a lack of a complex immune system, and inability to achieve a fully humanized liver. Recent studies have shown promise with use of a herpes simplex virus type-1 thymidine kinase/ganciclovir system

for cell specific ablation as means of obtaining exclusive growth of human hepatocytes in the SCID mouse[22].

In order to create a mouse model permissive to HCV infection while maintaining complex immunity, Dorner *et al*[23] developed a humanized mouse model using genetic engineering to study viral entry and immunity. Mice were genetically engineered to express HCV-specific entry factors including CD81, occludin, SCARB-Ⅰ, and CLDN1. Using this model, it was demonstrated that human-specific CD81 and occludin were essential to all HCV entry into murine hepatocytes. Expression of SCARB-Ⅰ heightened HCV entry when expressed in combination with CD81 and occludin. Because this model used an immunocompetent mouse, viral replication and persistence of infection was limited. However, this model was also used to study passive immunization by administration of anti-CD81 antibodies and anti-E2 antibodies both of which decreased HCV infection. This model may offer future study of passive immunization or vaccination strategies to prevent acute infection of HCV before or after exposure.

Rat model for HCV infection

As discussed above, the search for an immunocompetent rodent host for HCV infection has been difficult. Although transplantation of human hepatocytes has been possible in mice, long-term HCV infection has only been shown in the setting of immunodeficiency, which limits study of immune responses. Ouyang *et al*[24] sought to overcome these barriers by creating an immunocompetent rat model that was tolerant to human hepatocytes. In order to achieve this, Huh7 human hepatoma cells were injected into the peritoneal cavities of fetal rats between gestation ages of 15-17 d. By injecting human hepatocytes during the development of the fetal immune system of the rat, specific tolerance to that specific cell type was achieved. Subsequent transplantation of human Huh7 cells into newborn tolerant rats resulted in survival and limited growth of the human cells without evidence of rejection as demonstrated by mixed lymphocyte assays. Colonies of cells bearing human liver cell markers increased in size, and were visualized within rat livers by immunohistochemical staining. Human albumin was detected in liver cells and in serum, and human hepatic mRNA was detected in hepatocytes, thus demonstrating active synthetic function of transplanted human Huh7 cells.

To study HCV infection, HCV isolated from human serum was inoculated into immunocompetent, tolerant, Huh7 cell-transplanted rats[25]. HCV RNA levels of 7.0×10^3 copies/mL were detectable in serum by week 4, and peaked at 20×10^3 copies/mL by week 12 after infection. Levels decreased thereafter. Moreover, biochemical evidence of hepatic inflammation was demonstrated by elevations of serum alanine aminotransferase beginning at 4 wk, and peaked at 3× the baseline level by the 13th week, after which levels declined. Light microscopy of liver sections showed mononuclear infiltrates in portal and central regions at times coinciding with detectable viremia. Controls without transplanted cells, tolerization, or HCV inoculation lacked any markers of HCV infection or hepatitis. The limitations of this rat model include low numbers of transplanted human hepatocytes as well as relatively low levels of viremia (22 500 copies/mL), in comparison to that in a typical human infection. However, this model offers a rodent model large enough to tolerate repeated blood and tissue sampling to study viral entry, replication, and immune-mediated hepatic injury as well as a screening tool to evaluate novel antiviral agents.

PROSPECTIVE

Hepatitis C viral infection is a growing health concern that leads to liver failure and hepatocellular carcinoma. The study of HCV has been limited due to a lack of appropriate and reliable animal models. However, much of our understanding of viral infection, replication and host immune responses has been gathered from animal data. Animal studies have historically utilized chimpanzees, but alternative models such as tupaia, mice and rats are now viable models for research.

Chimpanzees are advantageous models given their close genetic resemblance to humans, intact immune system which offers potential for study of innate and adaptive immune responses, and potential to study both treatments and prophylactic vaccinations. Additionally, important research with chimpanzees has resulted in the development of molecular clones of the virus for use in molecular and cellular research. Disadvantages include high cost, ethical constraints, and low susceptibility to chronic infection, thus limiting the study of HCV-related cirrhosis and hepatocellular carcinoma. Currently, chimpanzees are not available for biomedical research[10].

Advantages of the tupaia model include low cost, ease of propagation, immunocompetence, and capability to study cirrhosis and tumorigenesis. On the other hand, tupaia and humans are genetically very different, potentially limiting the applicability of tupaia data on viral infectivity and disease to humans. Reliability and reproducibility of infection with tupaia have been also been debated.

Xenograft mice constructed with human hepatocytes are useful models given the demonstrated persistent viremia, and development of cirrhosis. These models provide a unique opportunity to study viral activity within human hepatocytes in a living host. Limitations include difficulty with mouse reproduction for repeated sampling, and immunodeficiency that limits the study of the natural immunological response.

The immunocompetent mouse and rat are promising models given their relatively low cost, ease of propagation, and intact immune system. There is great potential for the study of immunologic mechanisms as well as treatment and vaccination agents using these models. Disadvantages include relatively low levels of viremia,

and possible limitation of persistent infection by a competent immune response.

Our knowledge of HCV and related disease has grown significantly from its discovery nearly three decades ago. Together with molecular and cellular approaches, continued animal research will undoubtedly play a critical role in development of new antiviral therapies, and our understanding of mechanisms involved in the pathogenesis of both acute and chronic HCV infections. Further research is needed to enhance the potential of the currently available models in order to optimize the cost of maintenance and propagation, viremia, and maximize the similarity of infection models as they relate to human infection.

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ORIGINAL ARTICLE

Proteome profiling of spinal cord and dorsal root ganglia in rats with trinitrobenzene sulfonic acid-induced colitis

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Abstract

AIM: To investigate proteomic changes in spinal cord and dorsal root ganglia (DRG) of rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis.

METHODS: The colonic myeloperoxidase (MPO) activity and tumor necrosis factor-α (TNF-α) level were determined. A two-dimensional electrophoresis (2-DE)-based proteomic technique was used to profile the global protein expression changes in the DRG and spinal cord of the rats with acute colitis induced by intracolonic injection of TNBS.

RESULTS: TNBS group showed significantly elevated colonic MPO activity and increased TNF-α level. The proteins derived from lumbosacral enlargement of the spinal cord and DRG were resolved by 2-DE; and 26 and 19 proteins that displayed significantly different expression levels in the DRG and spinal cord were identified respectively. Altered proteins were found to be involved in a number of biological functions, such as inflammation/immunity, cell signaling, redox regulation, sulfate transport and cellular metabolism. The over-expression of the protein similar to potassium channel tetramerisation domain containing protein 12 (Kctd 12) and low expression of proteasome subunit α type-1 (psma) were validated by Western blotting analysis.

CONCLUSION: TNBS-induced colitis has a profound impact on protein profiling in the nervous system. This result helps understand the neurological pathogenesis of inflammatory bowel disease.

INTRODUCTION

Inflammatory bowel disease (IBD) is defined as a group

of inflammatory conditions in the colon and small intestine, mainly including ulcerative colitis and Crohn's disease. The cause of IBD is suggested to be a nebulous combination of not only host genetic factors, but also immune dysfunction, dysbiosis, cellular oxidative stress and leakage of intestinal barrier[1]. Fundamental therapy for this condition has not yet been established because its etiology remains obscure. Unfortunately, the prevalence of IBD is continuing to increase in both Eastern and Western countries, causing enormous medical costs[2,3]. Beside intestinal disorders, many organs outside the gastrointestinal tract, such as the central nervous system, are involved in IBD[4]. Neuropathies, cerebrovascular events, white matter lesions, and visceral pain are common neurological manifestations[4]. These alterations may help explain some of the underlying comorbidities, such as hyperalgesia, seizure and anorexia[5,6]. Unfortunately, the exact mechanism for IBD needs further investigations.

This study focuses on the spinal cord and dorsal root ganglia (DRG) to reveal the neurological dimension in a trinitrobenzene sulfonic acid (TNBS)-induced active colitis model. Unlike previous studies that were based mainly on investigations of specifically selected gene/proteins, proteomic approach was applied in this study to reveal the global changes of proteins. The two-dimensional electrophoresis (2-DE) in combination with matrix-assisted laser desorption-time-of-flight/time-of-flight mass spectrometer (MALDI-TOF/TOF MS) have been widely used to probe into changes of protein profiles accompanied with diseases like cancer and hyperalgesia[7,8]. In the present study, this approach was applied to analyze the proteomic differences in lumbar enlargement of spinal cord and DRG in the rat model of TNBS-induced colitis. This study aimed to investigate whether changed protein profiles in the nervous system are in any way associated with neurological dimensions in IBD animal model.

MATERIALS AND METHODS

Animals and tissue processing
Male Sprague-Dawley rats (180-200 g in weight) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. Rats were kept at room temperature 23 ℃ ± 2 ℃ with an alternating 12 h light-dark cycle, and were allowed access to food and water *ad libitum*. All of the experimental protocols were carried out with the approval of the Committee on Use of Human and Animal Subjects in Teaching and Research of Hong Kong Baptist University and according to the Regulations of the Department of Health, Hong Kong, China.

Induction of colitis
Induction of colitis was adapted from the previously reported methods[9,10]. Briefly, under chloral hydrate (350 mg/kg, ip) anesthesia, colitis was induced in overnight-fasted rats ($n = 5$) by intra-colonic administration of 30 mg/kg of TNBS (Sigma, St. Louis, United States) dissolved in 50% ethanol solution at 8 cm from the anal

verge using a rubber catheter. The rats were kept upside-down for 1 min to ensure that the TNBS solution was not expelled immediately. The rats in control group ($n = 4$) received intra-colonic injection of saline.

Tissue preparation
On the 7th day after TNBS instillation, the rats were anesthetized with chloral hydrate (350 mg/kg, ip). Distal colon tissue was excised in two pieces. One piece was fixed in 4% paraformaldehyde, routinely embedded in paraffin, cut into 5 μm sections, mounted on glass slides and stained with hematoxylin and eosin to reveal structural features. The other piece of colon sample was frozen in liquid nitrogen and stored at -80 ℃ for measurement of myeloperoxidase (MPO) activity and tumor necrosis factor-α (TNF-α) level. The rat was then perfused with ice-cold normal saline. The spinal cord and DRG of the lumbosacral enlargement were dissected, immediately frozen and stored at -80 ℃ until use. Samples were firstly lysed in buffer (8 mol/L urea, 2 mol/L thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% NP-40, 2 mmol/L tribromophenol (TBP), 1 × protease inhibitor mix, 1 × nuclease mix, 1 mmol/L phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF), and 2% immobilized pH gradient (IPG) buffer, and then incubated on ice for 45 min. The lysed mixtures were centrifuged at 14 000 × *g* for 15 min at 4 ℃. The supernatant samples were determined by Bradford protein assay (BioRad, California, United States) and stored at -80 ℃.

Two-dimensional gel electrophoresis and image analysis
2-DE and image analysis were performed as previously described with some modifications[11]. Isoelectric focusing (IEF) was performed using IPGphor Ⅱ apparatus (Amersham, Sweden). Samples (150 μg protein/group, containing an equal amount of protein from each animal) were diluted in 250 μL rehydration solution (8 mol/L urea, 2% CHAPS, 0.4% dithiothreitol (DTT), 0.5% IPG buffer, 0.002% bromophenol blue) and loaded onto the IPG strips (13 cm, pH 3-10, NL) by 10 h rehydration at 30 V. Proteins were focused by using a step-wise voltage ramp: 500 V for 1 h, 1000 V for 1 h, and finally 8000 V for 6 h. The IPG strips were then incubated in the equilibration buffer (6 mol/L urea, 2% SDS, 30% glycerol, 0.002% bromophenol blue, 50 mmol/L Tris-HCl, pH 6.8) containing 1% DTT for 15 min with gentle agitation. The strips were then transferred to the equilibrating solution containing 2.5% iodoacetamide and agitated for 15 min, and subsequently were placed on top of a 12.5% uniform SDS-PAGE gel (150 mm × 158 mm × 1.5 mm). Separation in the second dimension was performed in Tris-glycine buffer (25 mmol/L Tris, 0.2 mol/L glycine, 0.1% SDS) at a constant current setting of 15 mA/gel initially for 30 min and 30 mA/gel thereafter. SDS-PAGE was terminated when the bromophenol blue dye front reached the lower ends of the gels. After 2-DE, gels were visualized using silver-staining[11]. All the raw images were digitalized using

a scanner (GS-800 calibrated densitometer, BioRad) and the Quantity One software (BioRad). Further analysis was completed using PDQuest (version 8.0, BioRad) mainly for spots' detection and quantification. The protein spots where the peak-volume ratio in the TNBS group changed more than 3-folds in comparison with the matched spots in the control group, were considered as differentially expressed, and were picked out for identification by tandem mass spectrometer (MS-MS).

In-gel digestion

Protein spots of interest were manually excised from the 2-D gels, and digested as previously described with small modification[12-14]. Briefly, the gel plugs were washed in 30 mmol/L potassium ferricyanide and 100 mmol/L sodium thiosulfate (1:1 v/v) for 5 min, and then washed in water twice. Subsequently, the gel plugs were equilibrated in 50 mmol/L ammonium bicarbonate for 20 min, then in 25 mmol/L ammonium bicarbonate and 50% acetonitrile (ACN), and finally soaked in 100% ACN until gel plugs became opaque. Thereafter, vacuum-dried gel plugs were rehydrated with 10 mg/mL of trypsin in 25 mmol/L ammonium bicarbonate (pH 8.0). Proteolysis of proteins was performed at 37 ℃ for 16-18 h. Supernatants were transferred into a new tube, and mixed with 1/2 volume of 1% trifluoroacetic acid to stop digestion. The samples were then vacuum dried at 45 ℃ for 1-2 h.

Protein identification by MS/MS

Protein identification was performed using a Autoflex Ⅲ MALDI-TOF/TOF mass spectrometer (Bruker, Germany) equipped with a 200 Hz N2 laser operating at 337 nm. Data were acquired in the positive ion reflector mode over a mass range of 800-4000 m/z using Bruker calibration mixture as an external standard. Bruker calibration mixture consists of the following peptides (monoisotopic mass of the singly protonated ion is given in parenthesis in Da); bradykinin (757.3992), angiotensin Ⅱ (1046.5420), angiotensin Ⅰ (1296.6853), substance P (1347.7361), bombesin (1619.8230), renin substrate (1758.9326), ACTH clip 1-17 (2093.0868), ACTH clip 18-39 (2465.1990) and somatostatin 28 (3147.4714). Keratin contamination peaks, matrix ion peaks and trypsin ion peaks were excluded from spectra. Typically 400 shots were accumulated per spectrum in MS mode and 2000 shots in MS/MS mode. The spectra were processed using the FlexAnalysis 3.0 and BioTools 3.1 software tools (Bruker, Germany). Protein identification was performed using Mascot (2.2.04, http://www.matrixscience.com) to search the international protein index (IPI) database. Peptide masses were matched with the theoretical peptides of all proteins in the IPI database using the Mascot search program. The following parameters were used for database searches: monoisotopic mass accuracy < 100 ppm, missed cleavages 1, carbamidomethylation of cysteine as fixed modification, oxidation of methionine as variable modifications. In MS/MS mode, the fragment ion mass accuracy was set at < 0.5 Da.

Determination of MPO activity and TNF-α level

The MPO activity was measured following the method as previously described[15,16]. Colonic TNF-α was determined using an enzyme-linked immunosorbent assay kit (Leinco Technologies, United States). The protein was quantified using a bicinchoninic acid protein assay kit (Thermo Scientific, United States).

Immunoblotting analysis

Two identified proteins: (1) similar to potassium channel tetramerisation domain containing protein 12 (Kctd12); and (2) proteasome subunit α type-1 (Psma1), were selected for the confirmation study. For Western-blot analysis, protein lysates were diluted in sample buffer and denatured at 100 ℃ for 5 min. Proteins (15 μg/lane) of interest were separated by 12% SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Nonspecific binding sites were blocked with 5% nonfat milk for 1 h at room temperature, then the blots were incubated at 4 ℃ overnight with rabbit antibody against mouse antibody against Psma1 (1:250 in TBST, Santa Cruz) or Kctd12 (1:500 in TBST, Santa Cruz). After washing, the membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000) in Tris-Buffered Saline and Tween 20 (TBST) with 5% nonfat milk against the primary antibody species for 1 h at room temperature. The immunoreaction was detected with the enhanced chemiluminescence (ECL) Western blotting kit (Invitrogen). Bands were visualized on Bio-max X-ray film (Kodak, Japan) and captured by a scanner. The optical densities of protein blots were analyzed using Image J software. The results were presented as the ratio of optical density of Kctd12 or Psma1 standardized to optical density of β-actin.

RESULTS

Establishment of IBD model

Rats developed hypomotility and diarrhea 1 d after TNBS treatment. Hematoxylin and eosin staining of distal colon revealed incrassation and edema in the mucosa and submucosa, hyperaemia and dilation of the blood vessel, and prominent neutrophilic infiltrates in the submucosal layer, indicating severe colonic inflammation (Figure 1A). TNBS group showed significantly elevated MPO activity (Figure 1B) and TNF-α level (Figure 1C), suggesting increased granulocyte recruitment and macrophage activation in the acute phase of inflammation.

Identified proteins by 2-DE-based proteomic technique

The representative 2-DE images of protein profiling changes in DRG and spinal cord is shown in Figure 2. Overall, a total of 26 spots differentially expressed in the DRG of these two groups were identified by the mass spectrometry (MS) analysis, 12 of which were up-regulated and 14 of which were down-regulated (Table 1). A total of 19 spots differentially expressed in the spinal cord of the two groups were identified by MS analysis, 9 of which

Figure 1 The establishment of colitis in trinitrobenzene sulfonic acid rats. Representative hematoxylin and eosin microscopic photos of the colon tissue (A) revealed inflammation in the sub-mucosa layer of trinitrobenzene sulfonic acid (TNBS) rats; measurement of myeloperoxidase (MPO) activity in wet colon tissue (B, 2.61 ± 2.47 *vs* 0.03 ± 0.01) and tumor necrosis factor-α (TNF-α) level in colonic total protein (C, 759.80 ± 81.07 *vs* 174.00 ± 31.92) revealed significantly elevated MPO activity and TNF-α level in TNBS treated group in comparison with saline control group. [a]$P < 0.05$ *vs* saline group.

Figure 2 Representative examples of the silver-stained two-dimensional electrophoresis gels show expression maps of proteins in dorsal root ganglia (A) and spinal cord (B) of trinitrobenzene sulfonic acid colitis group and saline control groups. TNBS: Trinitrobenzene sulfonic acid; DRG: Dorsal root ganglia; Gstp1: Glutathione S-transferase P; Sod2: Superoxide dismutase; Ndufv2: NADH dehydrogenase (ubiquinone) flavoprotein 2; Ndufb10: NADH dehydrogenase (ubiquinone) 1 β subcomplex 10; Fh1: Fumarate hydratase; Psat1: Phosphoserine aminotransferase; Kctd12: Potassium channel tetramerisation domain containing protein 12; Vdac2: Voltage-dependent anion-selective channel protein 2; Ywhae: The 14-3-3 protein epsilon; Tst: Thiosulfate sulfurtransferase; Hspa8: Heat shock cognate 71 kDa protein.

were up-regulated and 10 of which were down-regulated (Table 2). In particular, we found altered expression of (1) proteins involved in inflammatory/immune responses, such as Isoform B0a of heterogeneous nuclear ribonucleoproteins A2/B1 (Hnrnpa2b1) and proteasome subunit α type-1 (Psma1); (2) signal-related proteins, such as adenylyl cyclase-associated protein 1 (Cap1) and voltage-dependent anion-selective channel protein 2 (Vdac2); (3) proteins involved in sulfate transport, thiosulfate sulfurtransferase (Tst); (4) cellular enzymes involved in cell redox homeostasis, such as glutathione S-transferase P (Gstp1) and superoxide dismutase (Sod2); (5) metabolic enzymes, such

as fructose-bisphosphate aldolase C (Aldoc); (6) structure protein Lamin C2 (Lmna); and (7) chaperonins, such as heat shock cognate 71 kDa protein (Hspa8, or HSC70) and stress-induced phosphoprotein 1 (Stip1). Although previous studies have reported the contribution of a few proteins, such as down-regulated glutathione peroxiredoxin-1 (Prdx1) and malate dehydrogenase (Mdh2)[12], most of the proteins were first identified in TNBS-induced colitis (Table 3). Most importantly, the samples analyzed in previous studies were usually mucosa or submucosa of the colon, whereas this paper firstly investigated the global protein expression changes in the spinal cord and DRG of

Table 1 Significantly regulated proteins after trinitrobenzene sulfonic acid-induced colitis in dorsal root ganglia

Function	Cell component	Protein name	Abbreviation	Accession No.	Theoretical PI/Mr (kDa)	Sequence coverage (%)	MASSOT score	Change (TNBS)
Proteins involved in inflammatory/immune response								
Autoantigen in many autoimmune diseases	Cytoplasm, nucleus spliceosome	Isoform B0a of heterogeneous nuclear ribonucleoproteins A2/B1	Hnrnpa2b1	IPI00923129	8.74/32 572	16	220	↑
Hemopexin	Secreted	Hemopexin	Hpx	IPI00195516	7.58/52 060	22	597	↑
1. Accelerate the folding of protein 2. Immunosuppression	Cytosol, nucleus	Peptidyl-prolyl cis-trans isomerase A	Ppia	IPI00387771	8.34/18 091	32	363	↓
Proteins involved in cell signaling								
Growth protein	Membrane	Adenylyl cyclase-associated protein 1	Cap1	IPI00555187	7.16/51 899	5	145	↑
Cytoplasmic tetramerisation domain of voltage-gated K+ channel		Similar to potassium channel tetramerisation domain containing protein 12	Kctd12	IPI00359734	8.95/47 077	16	192	↑
1. Ion channel 2. Mitochondrial apoptosis	Mitochondrial outer membrane	Voltage-dependent anion-selective channel protein 2	Vdac2	IPI00198327	7.44/32 353	9	76	↓
Proteins involved in redox regulation								
Cell redox homeostasis	Mitochondria	Dihydrolipoyl dehydrogenase, mitochondrial	Dld	IPI00365545	7.96/54 574	5	141	↑
Antioxidant	Mitochondria	Superoxide dismutase [Mn], mitochondrial	Sod2	IPI00211593	8.96/24 887	22	130	↓
Xenobiotic metabolism and cellular defense	Nucleus	Glutathione S-transferase P	Gstp1	IPI00231229	6.89/23 652	12	255	↓
Eliminating peroxides	Mitochondria, cytosol	Peroxiredoxin-1	Prdx1	IPI00211779	8.27/22 323	9	108	↓
Proteins involved in chaperone function								
Chaperonins/heat shock proteins	Mitochondria	Heat shock cognate 71 kDa protein	Hspa8	IPI00208205	5.37/71 055	18	304	↓
Chaperonins/heat shock proteins	Nucleus, cytoplasm	Stress-induced-phosphoprotein 1	Stip1	IPI00213013	6.40/63 158	9	156	↑
Proteins involved in cellular structure								
Component of the nuclear lamina	Insoluble fraction, lamin filament, nuclear matrix	Lamin C2	Lmna	IPI00188879	6.22/52 661	16	136	↑
Proteins involved in cellular metabolism								
Oxidoreductase in valine and pyrimidine catabolic pathways	Mitochondria	Methylmalonate-semialdehyde dehydrogenase [acylating],mitochondrial	Aldh6a1	IPI00205018	8.44/58 396	4	68	↓
Glycolytic enzyme	Mitochondria	Fructose-bisphosphate aldolase A	Aldoc	IPI00231734	8.31/39 783	11	148	↓
Glycolytic enzyme	Axon, mitochondria	Aldoc 17 kDa protein	Aldoc	IPI00561972	6.84/16 666	14	88	↓
Glycolytic enzyme	Mitochondria	Dihydrolipoyllysine-residueacetyltransferase component of pyruvate dehydrogenase complex	Dlat	IPI00231714	8.76/67 637	7	79	↑
Glycolytic enzyme	Cytoplasm	Phosphoglycerate kinase 1	Pgk1	IPI00231426	8.02/44 909	16	184	↓
Glycolytic enzyme		Similar to pyruvate kinase 3		IPI00561880	7.15/58 264	17	231	↓
Glycolytic enzyme	Nucleus cytoplasm	Isoform M1 of pyruvate kinase isozymes M1/M2	Pkm2	IPI00231929	6.63/58 452	22	491	↑
1. Glycolytic enzyme 2. Transcription activation 3. Initiation of apoptosis	Cytoplasm	Similar to glyceraldehyde-3-phosphate dehydrogenase	RGD1565368	IPI00554039	8.44/36 045	14	347	↑
ATP energy transduction	Cytoplasm	Creatine kinase M-type	Ckm	IPI00211053	6.58/43 246	19	361	↑
ATP transducing	Mitochondria	Creatine kinase, mitochondrial 1, ubiquitous	Ckmt1	IPI00555166	8.58/47 331	21	231	↓

TCA cycle	Mitochondria	Isoform mitochondrial of fumarate hydratase, mitochondrial	Fh1	IPI00231611	9.06/54 714	5	115	↓
1. TCA cycle 2. Gluconeogenesis 3. Antioxidant	Mitochondria	Malate dehydrogenase, mitochondrial	Mdh2	IPI00197696	8.93/36 117	11	90	↓
Extrahepatic ketone body catabolism	Mitochondria	3-ketoacid-coenzyme A transferase 1, mitochondrial	Oxct1 Succinyl-CoA	IPI00766788	8.7/56 624	6	116	↑

↑: Elevated protein expression in TNBS group compared with saline group; ↓: Decreased protein expression in TNBS group in comparison with saline group. DRG: Dorsal root ganglia; TNBS: Trinitrobenzene sulfonic acid; Hnrnpa2b1: Heterogeneous nuclear ribonucleoproteins A2/B1; Cap1: Cyclase-associated protein 1; Kctd12: Potassium channel tetramerisation domain containing protein 12; Vdac2: Voltage-dependent anion-selective channel protein 2; Dld: Dihydrolipoyl dehydrogenase; Sod2: Superoxide dismutase; Gstp1: Glutathione S-transferase P; Prdx1: Peroxiredoxin-1; Stip1: Stress-induced phosphoprotein 1; Lmna: Lamin C2; Ckm: Creatine kinase M-type; Ckmt1: Creatine kinase, mitochondrial 1, ubiquitous; Fh1: Fumarate hydratase; Mdh2: Malate dehydrogenase; Oxct1 Succinyl-CoA: 3-ketoacid-coenzyme A transferase 1.

Figure 3 Immunoblotting analyses to validate the differential expression of proteasome subunit α type-1 (A, 0.53 ± 0.14 *vs* 1.81 ± 0.53) and potassium channel tetramerisation domain containing protein 12 (B, 1.21 ± 0.20 *vs* 0.56 ± 0.07) between trinitrobenzene sulfonic acid treated group and saline control group. The relative expression ratio standardized to β-actin. $^aP < 0.05$ *vs* saline group. TNBS: Trinitrobenzene sulfonic acid; Psma1: Proteasome subunit α type-1; Kctd12: Potassium channel tetramerisation domain containing protein 12.

rats with TNBS-induced acute colitis.

Validation by Western blotting analysis

In order to validate the proteomic data, two of the protein spots: Psma1, a protein involved in immunity, and Kctd12, a protein involved in voltage-gated potassium channel activity, were chosen for validation by Western blotting analysis. The comparison of samples derived from TNBS rats (lanes 1-4) and samples derived from saline control (lanes 5-8) revealed down-regulation of Psma1 (Figure 3A) in the spinal cord and up-regulation of Kctd12 (Figure 3B) in the DRG. These results confirmed the changed protein levels concluded from 2-DE.

DISCUSSION

Colitis persists for at least 28 d after TNBS colonic administration[17,18]. For the study of TNBS-induced colitis, rats were usually sacrificed 7 d after TNBS treatment[19], since TNBS-caused changes of gene expression profile were maximal at day 5 and day 7 after induction[20]. Consistent with previous studies, on day 7 after TNBS intraluminal treatment, we observed macroscopic and microscopic damage of the distal colon, demonstrating the presence of acute colitis. By using 2-DE in combination with MALDI-TOF/TOF MS based proteomic approach, we observed changed expression profiles not only in proteins participating in the immune/inflammatory response, but also in a broad range of proteins involved in cell signaling, sulfate transport, redox homeostasis, and cell metabolism. This result is consistent with previous studies revealing that in addition to inflammation/immunity response, TNBS-colitis affects the gene expression related to a numerous biological functions, such as signal transduction and cell metabolism[21,22]. The identified proteins from the spinal cord and DRG could be categorized into seven groups as follows.

Group 1: Proteins involved in inflammatory/immune responses

Consistent with previous results[23,24], the current study

Table 2 Proteins significantly regulated after trinitrobenzene sulfonic acid-induced colitis in spinal cord

Function	Cell component	Protein name	Abbreviation	Accession No.	Theoretical PI/Mr (kDa)	Sequence coverage (%)	MASSOT score	Change (TNBS)
Proteins involved in inflammatory/immune response								
1. Fatty acid catabolic process 2. Play a role in eicosanoid synthesis and inflammation	Cytoplasm	Isoform 1 of cytosolic acyl coenzyme A thioester hydrolase	Acot7	IPI00213571	7.16/37 936	14	167	↓
1. Glycolytic enzyme 2. Autoantigen	Cell membrane, cytoplasm	α-enolase	Eno1	IPI00464815	6.16/47 440	13	183	↓
Immunity	Cytoplasm, nucleus	Proteasome subunit α type-1	Psma1	IPI00191748	6.15/29 784	17	193	↓
Proteins involved in cell signaling								
1. Growth protein 2. Modulate calcium influx 3. Regulate the release of ICGRP	Cytoplasm	Dihydropyrimidinase-related protein 2	Dpysl2	IPI00870112	5.95/62 638	16	270	↑
Calcium ion signaling	Cytoplasm, cytoskeleton	Troponin C-like protein		gi223036	4.12/16 696	10	91	↓
1. Cell division 2. Apoptosis 3. Regulate insulin sensitivity	Melanosome, cytoplasm	14-3-3 protein epsilon	Ywhae	IPI00325135	4.63/29 326	15	165	↑
Proteins involved in sulfate transport								
Transferase detoxification	Mitochondrial matrix	Thiosulfate sulfurtransferase	Tst	IPI00366293	7.71/33 614	25	333	↓
Proteins involved in redox regulation								
Xenobiotic metabolism and cellular defense	Nucleus	Glutathione S-transferase P	Gstp1	IPI00231229	6.89/23 652	20	263	↓
Proteins involved in chaperone function								
Assist protein folding	Cytoplasm	T-complex protein 1 subunit γ	Cct3	IPI00372388	6.23/61 179	8	122	↑
Chaperonins/heat shock proteins	Mitochondria	Heat shock cognate 71 kDa protein	Hspa8	IPI00208205	5.37/71 055	11	172	↑
Chaperonins/heat shock proteins	Cytoplasm, nucleus	Stress-induced-phosphoprotein 1	Stip1	IPI00213013	6.4/63 158	7	143	↑
Proteins involved in cellular metabolism								
Glycolytic enzyme	Axon, mitochondria	Fructose-bisphosphate aldolase C	Aldoc	IPI00231736	6.67/39 658	10	189	↓
Glycolytic enzyme	Cytoplasm	Similar to phosphoglycerate kinase 1	RGD1560402	IPI00372910	6.15/43 604	10	151	↑
1. Glycolytic enzyme 2. Transcription activation 3. Initiation of apoptosis	Cytoplasm	Similar to glyceraldehyde-3-phosphate dehydrogenase	RGD1565368	IPI00554039	8.44/36 045	18	454	↑
ATP transducing	Mitochondria	Creatine kinase, mitochondrial 1, ubiquitous	Ckmt1	IPI00555166	8.58/47 331	12	155	↑
1. Electron transport in respiratory chain 2. Oxidoreductase	Mitochondria	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	Ndufv2	IPI00367152	6.23/27 703	24	253	↑
Electron transport in respiratory chain	Membrane, mitochondria	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 10	Ndufb10	IPI00202238	7.57/21 131	32	306	↓
1. Electron transport in respiratory chain 2. Mitochondrial dysfunction	Mitochondrial	Cytochrome b-c1 complex subunit 2, mitochondrial	Uqcrc2	IPI00188924	9.16/48 423	8	222	↓
Amino-acid (serine) biosynthesis		Phosphoserine aminotransferase	Psat1	IPI00331919	7.57/40 943	8	97	↓

↑: Elevated protein expression in TNBS group compared with saline group; ↓: Decreased protein expression in TNBS group in comparison with saline group; TNBS: Trinitrobenzene sulfonic acid; NADH: Nicotinamide adenine dinucleotide; Psma1: Proteasome subunit α type-1; Dpysl2: Dihydropyrimidinase-related protein 2; Ywhae: The 14-3-3 protein epsilon; Tst: Thiosulfate sulfurtransferase; Gstp1: Glutathione S-transferase P; Hspa8: Heat shock cognate 71 kDa protein; Stip1: Stress-induced phosphoprotein 1; Aldoc: Aldolase C; Ckmt1: Creatine kinase mitochondrial 1 ubiquitous; Ndufv2: NADH dehydrogenase (ubiquinone) flavoprotein 2; Ndufb10: NADH dehydrogenase (ubiquinone) 1 β subcomplex 10; Uqcrc2: Cytochrome b-c1 complex subunit 2; Psat1: Phosphoserine aminotransferase.

demonstrated immune regulation in the nervous system following peripheral inflammation. Hnrnpa2b1 hinders the double strand DNA break repair process by binding the DNA-dependent protein kinase complex[25], and is an autoantigen in autoimmune diseases such as rheumatoid arthritis and autoimmune hepatitis[26,27]. Thus, the up-

Table 3 Differentially expressed proteins identified in cellular and animal models of inflammatory bowel disease and in clinical samples of inflammatory bowel disease patients

Ref.	Animal model	Cell	Patient	Sample	Analytical technique (S)	No. of regulated proteins	Major findings	Protein name	Change
[68]		Human adenocarcinoma cells Dld-1 exposed to interferon-γ, interleukin-1 and interleukin-6			2D PAGE-MALDI-TOF-MS/MS	5	Protein biosynthesis	Tryptophanyl-tRNA synthetase	Up
							Redox regulation	Indoleamine-2, 3-Dioxygenase	Up
[14]		HT29 Cl.16E cell treated with interferon-γ			1D PAGE-LC ESI/QTOF-MS/MS	7	Structure protein	Histone H2A type-1B	Up
							Metabolic enzymes	Adenosylhomocysteinase	Up
							Redox regulation	Peroxiedonxin-1	Down
			CD patients	Intestinal epithelial cells		14	Structure protein	Histone H1.2, H2A.V, H2B Type 1-C/E/F/G/I, H3 Like, H4	Up
							Chaperone	Heat shock 70 kDa protein 5	Up
							Annexin	Annexin A1	Down
[69]			CD and UC patients	Endoscopic biopsies of colonic mucosa	Multi-epitope-ligand-cartographic immunofluorescence microscopy		Apoptosis	Caspase-3	Down
							Transcription regulation	NF-κ B	Up
[12]			UC patients	Colon biopsies	2D-MALDI-TOF-MS/MS	19	Negative regulation of cell proliferation and DNA replication	Prohibitin (PHB)	Down
							1. TCA Cycle 2. Gluconeogenesis 3. Antioxidant	Mitochondrial malate dehydrogenase (Mdh2)	Down
							Eliminating Peroxides	Thioredoxin peroxidase (Prdx1)	Down
							1. Ion Channel 2. Apoptosis	Voltage-dependent anion-selective channel protein 1 (Vdac1)	Down
							1. Intracellular signal transduction 2. Regulation of transcription, DND-dependent	Nuclear factor of activated T-cells cytoplasmic (NFAT C1)	Up
							Protein folding	Tumor rejection antigen 1 (TRA1)	Up
							Cell adhesion Host-virus interaction	Poliovirus receptor related protein 1 (PVRL1)	Up
[70]			CD and UC patients	Serum	SELDI-TOF-MS	4	Cytokine-mediated signaling	Platelet factor 4	Up
							Chronic inflammation	Myeloid related protein 8	Up
							Fibrin producing and inflammation	FIBA	Up
							Transferase	Haptoglobin alph2	Up
[71]			CD and UC patients	Intestinal epithelial cells	2D PAGE-MALDI-TOF-MS	17	Regulation of GTPase activity	Rho gdp dissociation inhibitor (GDI) α chain	Up
							Differentiation	Nicotinamide phosphor-ribosyltransferase	Up
							Calcium ion binding	Myosin regulatory light chain 2, nonsarcomeric	Up
[72]			CD patients	Serum	RP NANO-LC ESI/Q-TOF MS	8	Immunity	Complement C3	Up
							Blood coagulation	Fibrinogen α chain	Up
							Lipid transport	Apolipoprotein E	Down

[73]		UC patients	Mucosa/ submucosa	2D PAGE-LC-MS/MS	7	Cell adhesion	Protocadherin 17 precursor	Up
						Acute-phase response	α-1-Antitrypsin (precursor)	Up
						Muscle protein	Caldesmon	Up
						Structural molecule activity	Mutant desmin	Up
[74]	DSS treated Balb/C mice		Mucosa	2D-DIGE-MALTI-TOF	5	Isomerase	Protein disulfide isomerase family A, member 3	Down
[13]	TNBS treated SD rats		Lymphocytes	2D-MALDI-TOF-MS/MS	42	Redox regulation	Peroxiredoxin 6 (Prx6)	Down
						Apoptosis-related proteins	PYD and Card domain containing protein	Down
						DNA damage response	Proteasome activator complex subunit 2	Down
						Glycolysis	Phosphoglycerate mutase type B subunit	Down
						Xenobiotic metabolism and cellular defense	Glutathione S-transferase, Pi 2	Down
						Cytokine	Interleukin-12 p40 precursor	Up
						Proteins involved in cell growth, differentiation and signal trransduction	Nucleoside diphosphate kinases β isoform	Up
						Inflammatory factors	Myeloid-related protein	Up
						ATP transduction	ATP-citrate synthase	Up
						Redox regulation	Dismutase	Up
[49]	TNBS treated SD Rats		DRG	2D-MALDI-TOF-MS/MS	26	Xenobiotic metabolism and cellular defense	Glutathione S-transferase P (Gstp1)	Down
						Eliminating peroxides	Peroxiredoxin-1 (Prdx1)	Down
						1. Accelerate the folding of protein 2. Immunosuppression	Peptidyl-prolyl cis-trans isomerase A (Ppia)	Down
						1. Ion channel 2. Mitochondrial apoptosis	Voltage-dependent anion-selective channel protein 2 (Vdac2)	Down
						Cytoplasmic tetramerisation domain of voltage-gated K+ channel	similar to Potassium channel tetramerisation domain containing protein 12	Up
			Spinal cord		19	Xenobiotic metabolism and cellular defense	Glutathione S-transferase P (Gstp1)	Down
						1. Glycolytic enzyme 2. Autoantigen in many diseases	α-enolase (Eno1)	Down
						Immunity	Proteasome subunit α type-1 (Psma1)	Down

DRG: Dorsal root ganglia; TNBS: Trinitrobenzene sulfonic acid; CD: Crohn's disease; UC: Ulcerative colitis; DSS: Dextran sulfate sodium; NF: Nuclear factor.

regulated Hnrnpa2b1 in DRG of TNBS rats may suggest reduced DNA repair efficiency of neurons and activated autoimmunity in DRG. Ppia (also known as cyclophilin A) contributes to the pathogenesis of inflammation-mediated diseases by directly inducing leukocyte chemotaxis and expression of proinflammatory cytokine/chemokines through a NF-κB dependent pathway[28,29]. In addition, Ppia is a novel paracrine and autocrine modulator of endothelial cell functions in immune-mediated diseases[30]. The down-regulated expression of Ppia in DRG of TNBS rats might associate with impaired immune modulation following acute colitis. Eno1 is a multifunctional enzyme that plays a part in various processes such as glycolysis, growth control and allergic responses. It is an autoantigen in many diseases, however, its diagnostic value in IBD is still controversial[31]. The underexpressed Eno1

may suggest that glycolysis is blocked and gluconeogenesis is dominant, which may associate with diarrhea and emaciation caused by colitis. Psma1 and Acot7 may be associated with the anti-inflammatory effect of macrophages. Psma1 mediates lipopolysaccharide-induced signal transduction in the macrophage proteasome[32]. Acot7 hydrolyzes the CoA thioester of palmitoyl-CoA, an important precursor for proinflammatory and immunoactive eicosanoids. Acot7 gene is highly expressed in macrophages and up-regulated by lipopolysaccharide[33]. The down-regulated Psma1 and Acot7 expression in the spinal cord of TNBS rats may be associated with inhibited anti-inflammatory responses.

Group 2: Proteins involved in cell signaling

Group 2 consists of proteins involved in ion channel

function, cell growth and division. Potassium channels are important in shaping the action potential, excitability and plasticity of neurons[34]. Changes in the properties of potassium channels at the soma accompanied with hyperexcitability in nociceptive DRG neurons in animal with TNBS-induced ileitis[35]. We observed overexpressed kctd12 protein in DRG of the TNBS rats. This might be related to altered function of potassium channel in DRG and hyperalgesia in colitis rats. Voltage-dependent anion-selective channel protein 2 (Vdac2) inhibits mitochondrial apoptosis[36]. It is interesting that a significantly down-regulated Vdac2 protein expression was observed in the DRG of TNBS rats, indicating an up-regulated apoptosis signaling. Adenylyl cyclase-associated protein 1 (Cap1) is a growth protein involved in the cyclic AMP (cAMP) pathway. Inflammatory signals can activate cAMP-protein kinase A pathways, which correlates with electrophysiological hyperexcitability of DRG neuron[37]. And, cAMP plays a role in DRG axon regeneration[38]. The up-regulated Cap1 in DRG of TNBS rats is probably a sign of neuronal hyperexcitability and/or axon regeneration. The 14-3-3 protein epsilon (Ywhae) is a splice variant of the 14-3-3 protein, which modulates cell division and apoptosis[39]. Elevated Ywhae expression was observed in the spinal cord of injury rats[40]. Consistent with down-regulated Vdac2 expression in DRG, the elevated Ywhae expression in spinal cord of TNBS rats may also indicate enhanced apoptosis signaling. Dihydropyrimidinase-related protein 2 (Dpysl2) plays a role in axon guidance, neuronal growth cone collapse and cell migration. In rat brain after ischemic stroke, up-regulated Dpysl2 indicates an early neuronal defense mechanism involving active neuronal repair, regeneration and development[41]. The up-regulated Dpysl2 in the spinal cord of TNBS rats may be related to the neurite outgrowth/plasticity associated with immunoreaction.

Group 3: Proteins involved in sulfate transport

Thiosulfate sulfurtransferase (Tst) can detoxify H_2S. Dysregulation of Tst expression associates with inability to detoxify detrimental H_2S and could be a factor in cell loss and inflammation[42]. The down-regulated Tst expression in spinal cord of TNBS rats may indicate dysfunction of the Tst detoxification that is possibly related to cell damage and inflammation in acute colitis.

Group 4: Proteins involved in cell redox homeostasis

Glutathione S-transferase P (Gstp1) functions in xenobiotic metabolism and plays a central role in the cellular defense against harmful endogenous compounds and xenobiotics[43,44]. Gstp1 is distributed in neuronal perikarya and oligodendrocytes in the central nervous system (CNS), and in neuronal perikarya and satellite cells of the DRG[45]. Reduced level of Gstp1 indicates a declined antioxidative capacity which may contribute to the damage to motor neurons in the process of immune-mediated motor neuron injury[46]. The underexpression of Gstp1 in both spinal cord and DRG of TNBS rats might suggest

oxidative stress and damage in neuronal cells. Prdx1 may participate in the signaling cascades of growth factors and TNF-α by regulating the intracellular concentrations of H_2O_2. A recent study revealed that in dextran sulfate sodium (DSS) mice, the inflamed intestinal mucosa has a down-regulated Prdx6 expression in comparison with normal mucosa[13]. Consistently, down-regulated Prdx1 in DRG of rats with TNBS colitis suggesting oxidative stress occurred in DRG. Superoxide dismutase (Mn), mitochondrial (Sod2) is an important antioxidant defenser in nearly all cells exposed to oxygen[47]. Ulcerative colitis involves intestinal mucosal damage driven by reactive oxygen species (ROS), in particular, superoxide anion. At the stage of severe inflammation, suppression of superoxide dismutase activity and elevation of nitrous oxide systems activity occur concomitantly. The underexpression of Sod2 protein indicates oxidative stress existing in the DRG of TNBS rats. Taken together, the down-regulated Gstp1 expression in spinal cord and DRG of TNBS rats, along with decreased expression of Prdx1 and Sod2 in DRG of TNBS rats, suggest that TNBS rats may have a significantly declined antioxidative and cellular defense capacity in the nervous system. Interestingly, another protein involved in cell redox homeostasis, dihydrolipoyl dehydrogenase (Dld), has a higher expression in the DRG of TNBS rats. Dld is a source of ROS, capable of scavenging nitric oxide, and can serve as an antioxidant by protecting other proteins against oxidative inactivation[48].

Group 5: Chaperonins

In both the spinal cord and DRG of TNBS rats, we observed significant up-regulation of Stip1. It is a chaperone that mediates the association of the molecular chaperones, heat shock cognate 71 kDa protein (Hspa8) and heat shock protein 90. Hspa8 is a key component of stress responses induced by various noxious conditions[49]. It regulates protein biosynthesis and refolding of denatured proteins, and plays an essential role in protecting cells in intestinal mucosal inflammation, potentially by lessening the extent and severity of injury[50,51]. The up-regulated Stip1 expression was observed in both the DRG and spinal cord of TNBS rats, indicating stress responses in primary afferent and CNS. Hspa8 is up-regulated in the spinal cord and down-regulated in DRG of TNBS rats. As Hspa8 exhibits both protective and antigenic properties, and the Hspa8 expression may stem from neuron, satellite or immune cells[49,52], the conflicting Hspa8 expression in spinal cord and DRG merits further investigation.

Group 6: Structure protein

Lamins are components of the nuclear lamina, providing a framework for the nuclear envelope. The mechanical properties of the cytoskeleton and cytoskeleton-based processes (such as cell motility and cell polarization), depend critically on the integrity of the nuclear lamina[53]. The overexpressed Lmna protein was observed in DRG of TNBS rats, which may suggest altered cytoskeleton.

Figure 4 Schematic drawing summarizes the major findings that might associate with pathophysiological changes in rat nervous system caused by trinitrobenzene sulfonic acid-induced colitis. TNBS: Trinitrobenzene sulfonic acid; DRG: Dorsal root ganglia; Hnrnpa2b1: Heterogeneous nuclear ribonucleoproteins A2/B1; Gstp1: Glutathione S-transferase P; Prdx1: Peroxiredoxin-1; Sod2: Superoxide dismutase; Lmna: Lamin C2; Aldoa: Aldolase A; Aldoc: Aldolase C; Ckmt1: Creatine kinase, mitochondrial 1, ubiquitous; Ckm: Creatine kinase M-type; Ndufv2: NADH dehydrogenase (ubiquinone) flavoprotein 2; Ndufb10: NADH dehydrogenase (ubiquinone) 1 β subcomplex 10; Oxct1 succinyl-CoA: 3-ketoacid-coenzyme A transferase 1; Fh1: Fumarate hydratase; Mdh2: Malate dehydrogenase; Psat1: Phosphoserine aminotransferase; Cap1: Cyclase-associated protein 1; Kctd12: Potassium channel tetramerisation domain containing protein 12; Vdac2: Voltage-dependent anion-selective channel protein 2; Ywhae: The 14-3-3 protein epsilon; Tst: Thiosulfate sulfurtransferase; Stip1: Stress-induced phosphoprotein 1; Hspa8: heat shock cognate 71 kDa protein.

Group 7: Cell metabolic enzymes

Proteins involved in glycolysis: Significant down-regulation of glycolysis enzymes, for example, Aldoc 17 kDa protein, fructose-bisphosphate aldolase A (Aldoa), and a third enzyme similar to pyruvate kinase 3, was observed in the DRG of TNBS group. Consistent with this, decreased expression of Aldoc was observed in spinal cord of TNBS rats. Aldoc and Aldoa regulate the stability of the light-neurofilament mRNA[54], and Aldoc provides marked neuroprotection to Purkinje cells after trauma and AMPA-

mediated excitotoxicity[55]. The down-regulated Aldoc in spinal cord and DRG together with decreased Aldoa expression in DRG may suggest down-regulated neuroprotection in TNBS rats. In addition, inhibition or activation of other glycolysis enzymes may result in speeding up or slowing down certain steps in the glycolysis pathway, and reflecting adjustment to physiological/pathological changes compensating for the cellular energy or neuron apoptosis[49].

Proteins involved in adenosine triphosphate transduction: Creatine kinase serves as an energy reservoir for the rapid buffering and regeneration of adenosine triphosphate (ATP) to sites with high, fluctuating energy demands, such as the synapse[55]. In the present study, expression of creatine kinase, mitochondrial 1, ubiquitous (Ckmt1) in the spinal cord was significantly up-regulated in TNBS rats. In contrast, Ckmt1 expression was decreased but the expression of creatine kinase M-type (Ckm) was up-regulated in the DRG of TNBS rats. Given that selective localization of Ckm in neurons was postulated to reflect the specific energy requirements of the specialized cells, these alterations may indicate enhanced phosphocreatine energy shuttles in spinal cord and remodelled energy shuttles/circles in the DRG[56,57].

Proteins involved in mitochondrial respiratory chain: Cytochrome b-c1 complex subunit 2, mitochondrial (Uqcrc2) is implicated in mitochondrial ROS generation and inflammation. Uqcrc2 deficiency causes mitochondrial dysfunction and exacerbates allergic airway inflammation[58]. The down-regulated Uqcrc2 expression in the spinal cord of TNBS rats might associate with inflammatory responses. Nicotinamide adenine dinucleotide (NADH) dehydrogenase is a potent source of reactive oxygen species such as superoxide and hydrogen peroxide[59]. It is interesting that, in contrast to the up-regulated protein expression of NADH dehydrogenase (ubiquinone) flavoprotein 2 (Ndufv2) in the spinal cord of the TNBS group, the NADH dehydrogenase (ubiquinone) 1 β subcomplex 10 (Ndufb10) was down-regulated. As the specific cellular functions of these subcomplexes are still not well known, further investigation is warranted.

Proteins involved in ketone body catabolism: 3-keto-acid-coenzyme A transferase 1 (Oxct1 Succinyl-CoA) is a mitochondrial matrix enzyme that plays a central role in extrahepatic ketone body catabolism. In the DRG of TNBS rats, Oxct1 Succinyl-CoA showed a 4-fold higher expression than in control rats. This observation is consistent with accelerated hepatic gluconeogenesis as well as ketogenesis in patients with chronic IBD, which probably is a consequence of the altered hormonal milieu[60].

Proteins involved in tricarboxylic acid cycle: Malate dehydrogenase, mitochondrial (Mdh2) is a cellular antioxidant, an enzyme in the tricarboxylic acid (TCA) cycle and gluconeogenesis. Mdh2 was down-regulated in experi-

mental autoimmune uveitis oxidative stress, and in colon mucosa of ulcerative colitis patients[12,61]. Similarly, the down-regulated Mdh2 and isoform mitochondrial of fumarate hydratase (Fh1) expression in DRG of TNBS rats may indicate a TCA metabolic dysregulation under disease condition.

Proteins involved in serine biosynthesis: Phosphoserine aminotransferase (Psat1) is an active serine biosynthesis enzyme in the mammal brain. Patients with Psat1 deficiency present with intractable seizures and psychomotor retardation[62]. The significantly down-regulated Psat1 expression in spinal cord of the TNBS group may indicate dysregulation of serine biosynthesis and may be associated with seizure susceptibility in TNBS rats.

Our analysis provides an overview profiling the proteomic changes in the spinal cord and DRG of rats with TNBS-induced colitis. As summarized in Figure 4, intracolonal instillation of TNBS not only induces inflammatory/immune responses in the DRG and spinal cord, but also triggers broad alterations of protein involving cell signaling, cellular metabolism, redox regulation, stress response *etc*. The TNBS-induced colitis in rodents is an immunologically mediated colitis that accompanies with an increase in proinflammatory factors and systemic endotoxaemia[63]. Besides colonic and systematic changes, a series of alterations in the nervous system have been described, such as transient disruption of blood-brain-barrier to small molecules[64]; a marked inflammatory response within the CNS[22]; and neurosignaling activation in rodent primary afferent nerve as well as in DRG and spinal cord neurons[20,65,66]. These neurological alterations may relate to intrinsic neuronal excitability and help explain some of the underlying comorbidities, such as hyperalgesia, seizure and anorexia[5,6]. The neurologic manifestations of IBD are most likely primary immune-mediated disorders, possibly caused by proinflammatory cytokines that diminish neuron proliferation, increase apoptosis, increase neuronal excitability, exacerbate sickness and/or result in psychological symptoms[21,67]. Our results delineated a dramatic deviation of protein profiling from the carefully orchestrated physiological balance in the DRG and spinal cord of TNBS rats. These findings provide useful proteins for further investigation on the neurological manifestations of IBD.

COMMENTS

Background
Inflammatory bowel disease (IBD) is a systematic illness, whose etiology and pathophysiology is incompletely understood. Many organs outside the gastrointestinal tract are involved in IBD, including the nervous system (neuropathies, cerebrovascular events, white matter lesions). These pathological changes may associate with a variety of comorbidities, such as hyperalgesia, seizure, and anorexia, but the underlying mechanism remains poorly understand.

Research frontiers
Proteomics keeps a rapidly expanding field, with a wealth of reports regularly appearing on technology enhancements and scientific studies using these new instruments.

Innovations and breakthroughs

This study demonstrated that trinitrobenzene sulfonic acid (TNBS) colitis profoundly changed expression of not only proteins involved in inflammatory/immune responses, but also proteins involved in cell signaling, sulfate transport, redox homeostasis and cell metabolism.

Applications

This study provides an overview profiling the proteomic changes in the spinal cord and dorsal root ganglia (DRG) of rats with TNBS-induced experimental colitis. These findings provide useful proteins for further investigation on the neurological manifestations of IBD.

Peer review

In this study, the authors profiled the global protein expression changes in the DRG and spinal cord in rats with acute colitis induced by TNBS using a two-dimensional electrophoresis-based proteomic technique. They found that altered proteins were involved in a number of biological functions including inflammation/immunity, cell signaling, redox regulation, sulfate transport and cellular metabolism. Although the number of the samples examined in this study was small, this paper is well written and the results are interesting.

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ORIGINAL ARTICLE

Stem cell factor-mediated wild-type KIT receptor activation is critical for gastrointestinal stromal tumor cell growth

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Abstract

AIM: To clarify the biological role of stem cell factor (SCF)-mediated wild-type KIT receptor activation in gastrointestinal stromal tumor (GIST) growth.

METHODS: The co-expression of wild-type KIT receptor and SCF was evaluated in 51 GIST samples using mutation analysis and immunohistochemistry, and the results were correlated with clinicopathological parameters, including the mitotic count, proliferative index (Ki-67 immunohistochemical staining), mitotic index (phospho-histone H3 immunohistochemical staining) and apoptotic index (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling). Using primary cultured GIST cells, the effect of SCF-mediated wild-type KIT receptor activation was determined by western blotting, methyl thiazolyl tetrazolium (MTT), and apoptosis assays.

RESULTS: We found that wild-type KIT receptor and SCF protein were expressed in 100% and 76.5% of the 51 GIST samples, respectively, and the co-expression of wild-type KIT receptor and SCF was associated with known indicators of poor prognosis, including larger tumor size ($P = 0.0118$), higher mitotic count ($P = 0.0058$), higher proliferative index ($P = 0.0012$), higher mitotic index ($P = 0.0282$), lower apoptosis index ($P = 0.0484$), and increased National Institutes of Health risk level ($P = 0.0012$). We also found that the introduction of exogenous SCF potently increased KIT kinase activity, stimulated cell proliferation ($P < 0.01$) and inhibited apoptosis ($P < 0.01$) induced by serum starvation, while a KIT immunoblocking antibody suppressed proliferation ($P = 0.01$) and promoted apoptosis ($P < 0.01$) in cultured GIST cells.

CONCLUSION: SCF-mediated wild-type KIT receptor activation plays an important role in GIST cell growth. The inhibition of SCF-mediated wild-type KIT receptor activation may prove to be particularly important for GIST therapy.

Key words: Gastrointestinal stromal tumor; Stem cell factor; Wild-type KIT receptor; Cell growth; *In vitro*

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INTRODUCTION

The KIT receptor, encoded by the oncogene *c-kit*[1], is characterised structurally by five immunoglobulin-like extracellular domains and an intracytoplasmic domain that contains an adenosine triphosphate (ATP)-binding domain and a phosphotransferase domain, which are separated by an interkinase sequence[2-5]. Under physiological conditions, the stem cell factor (SCF) binds to KIT and induces KIT homodimerisation, resulting in the phosphorylation of its tyrosine residues. The tyrosine-phosphorylated KIT receptor subsequently becomes a new docking site for signal transduction molecules and induces substrate binding and phosphorylation[6]. Thus, the interaction between SCF and the KIT receptor is essential for normal hematopoiesis, melanogenesis, gametogenesis, and the growth and differentiation of mast cells and interstitial cells of Cajal (ICCs)[7,8]. Notably, mutations in the *c-kit* gene have been implicated in neoplasms arising from these cell lineages. Oncogenic mutations in *c-kit* cause a constitutive phosphorylation of the KIT receptor that is independent of SCF binding, leading to a cascade of intracellular signalling events that contribute to the abnormal proliferation and survival of these neoplastic cells[9,10].

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract, and they are believed to originate from ICC progenitor cells[11-13]. It has also been noted that approximately 90% of GIST cases have activating mutations in either the *c-kit* or platelet-derived growth factor receptor (PDGFR) A genes[14-16]. In addition, the emerging role of SCF in *c-kit*-mutant GISTs indicates that an autocrine-paracrine loop serves as a further mechanism of wild-type KIT receptor activation[17,18].

In this study, we demonstrated the co-existence of wild-type KIT receptor and SCF in primary GISTs by analysing the entire coding sequence of *c-kit* and the protein expression of KIT and SCF in these tumors, as suggested in a previous study[19]. Based on *ex vivo* assays, we further demonstrated that SCF-mediated wild-type KIT receptor activation affected GIST growth in a dual manner by stimulating proliferation and inhibiting the apoptosis of GIST primary cells. These data suggest that the inhibition of SCF-mediated wild-type KIT receptor activation may be particularly important for GIST therapy.

MATERIALS AND METHODS

Patients

Samples from 51 consecutive patients with GISTs who underwent surgery at Changhai Hospital (Shanghai, China) between January and October 2006 were subjected to histological analysis. In addition, GIST primary cells were isolated from three fresh GIST specimens from patients who underwent surgery at Changhai Hospital in 2009. The GIST diagnosis was confirmed as previously described[20-22], and all tumors were KIT protein (CD117)-positive. No patients had received imatinib prior to the surgical resection of the tumor. Demographic

Table 1 Correlations between the co-expression of wild-type KIT receptor and stem cell factor and clinicopathological factors in gastrointestinal stromal tumors

	Co-expression of wild-type KIT receptor and stem cell factor		
	Absent (*n* = 12)	Present (*n* = 39)	*P* value
Age (yr)	58.9 ± 13.2	52.8 ± 10.5	0.0524
Sex			
Male	6	18	0.8154
Female	6	21	
Tumor size (cm)	3.69 ± 1.68	6.21 ± 3.94	0.0118
Histological phenotype			
Spindle type	8	26	0.7262
Epithelioid/mixed type	4	13	
Cellularity			
Sparse	1	6	0.8000
Moderate/dense	11	33	
Tumor location			
Gastric	6	25	0.5931
Non-gastric	6	14	
Proliferative index	2.92 ± 2.23	7.21 ± 4.93	0.0012
pHH3			
≤ 5	12	24	0.0282
> 5	0	15	
Mitotic counts	3 ± 2.37	7.36 ± 6.36	0.0058
≤ 5	10	14	0.0004
> 5	2	25	
Apoptosis index	44.58 ± 19.00	32.59 ± 17.63	0.0484
c-kit mutation			
Presence	5	27	0.1659
Absence	7	12	
National Institutes of Health risk group			
Very low or low	10	10	0.0012
Intermediate or high	2	29	

data and clinical and histological features for all of the GISTs analysed in this study are summarised in Table 1.

The use of all human tissues was approved by the hospital's institutional committee for human research, and informed consent was obtained from all of the subjects.

Immunohistochemistry

Immunohistochemical staining was performed using the labelled streptavidin-biotin method (DAKO LSAB-2 Kit, Peroxidase, DAKO) according to the manufacturer's instructions. The following primary antibodies were used: CD117 (DAKO), Ki-67 (DAKO), SCF (Cell Signaling Technology, Inc.) and phospho-histone H3 (pHH3, Cell Signaling Technology). Parallel sections were used to examine the co-expression of KIT and SCF. For Ki-67 and pHH3, positive cells were counted in five randomised regions in the tumor component of each lesion, and the labelling index was calculated as follows: Labelling index (%) = (positive cell number/total cell number) × 100%.

In situ apoptosis

In situ apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL, Roche Diagnostics) staining, which was performed according to the manufacturer's instructions. The apoptotic index was calculated as follows: Apoptotic index (%) = (apoptotic cell number/total cell number) × 100%.

Polymerase chain reaction amplification and sequencing

Genomic DNA was extracted from cryopreserved (*n* = 51) or fresh (*n* = 3) specimens using a commercial kit (BBI, Canada). Next, *c-kit* exons 9, 11, 13, 14 and 17, as well as PDGFRA exons 12 and 18, were amplified using the following primer sequences and annealing temperatures (designed): *c-kit* exon 9 (5'TTTATTTTCCTAGAG-TAAGCCAGGG-3' and 5'-ATCATGACTGATA TGGTAGACAGAGC-3', at 56 ℃), *c-kit* exon 11 (5' -ATTATTAAAAGGTGAT CTATTTTT-3' and 5' -ACTGTTATGTGTACCCAAAAAG-3', at 60 ℃), *c-kit* exon 13 (5'-CACCATCACCACTTACTTGTTGTCT-3' and 5'-GACAGACAAT AAAAGGCAGCTTGGAC-3', at 67 ℃), *c-kit* exon 14 (5'-TCTCACCTTC TTTCTA-ACCTTTTC-3' and 5'-AACCCTTATGACCCCAT-GAA-3', at 54 ℃), *c-kit* exon 17 (5'GAACAT-CATTCAAGGCGTACTTTTG-3' and 5'-TTGAAA CTAAAAATCCTTTGCAGGAC-3', at 65 ℃), PDG-FRA exon 12 (5'-CTCTGGTGCACTGGGACTTT-3' and 5'-GCAAGGGAAAAGGGAGTCT T-3', at 60 ℃), and PDGFRA exon 18 (5'-ATGGCTTGATCCTGAGT-CATT-3' and 5'-GTGTGGGAAGTGTGGACG-3', at 60 ℃). Gene mutations were analysed through the direct sequencing of uncloned polymerase chain reaction (PCR) fragments. Samples that appeared to contain mutations were further examined for the presence of the wild-type *c-kit* gene by subcloning the purified PCR products using a TA cloning vector system (Stratagene, CA). Five independent subclones from each PCR were sequenced.

Cell isolation and short-term primary cell culture

GIST primary cells were isolated and cultured as described in the literature with minor modifications[23]. The nonnecrotic tissue was separated from fresh GIST samples and finely minced with curved scissors. The minced tissue was homogenised by being passed through a 15-gauge needle with a syringe five times and subsequently being passed through a stainless steel mesh (200 wires/inch). The cells were counted with a haemocytometer, and cell viability was determined by propidium iodide staining.

After centrifugation in phosphate buffered solution (PBS) at 4 ℃, cell pellets were resuspended in 1640 RPMI medium supplemented with 20% heat-inactivated foetal bovine serum (FBS), 1.0 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 0.5 mmol/L 3-isobutyl-1- methylxanthine to inhibit fibroblast growth. Medium changes were performed at 24 h after seeding and every two or three days before cell analysis.

Enzyme-linked immunosorbent assay for stem cell factor production

For the measurement of SCF production, the concentration of GIST primary cells was adjusted to 5 × 10^6 cells/mL, and the supernatants were collected 24 h later. Samples were stored at -80 ℃ for further analysis. SCF concentration was determined by enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with catching antibody (diluted to 5 μg/mL in PBS, 100 μL/well) at 4 ℃ overnight according to the manufac-

turer's instructions.

Western blotting

Protein extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with a specific antibody to demonstrate protein loading. Anti-phospho-KIT (pY703) antibody was purchased from Abcam (United Kingdom). Glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody (Santa Cruz Biotechnology) was used as an internal control. To examine the effects of exogenous SCF on KIT phosphorylation, GIST primary cells were incubated overnight with medium containing 0.5% FBS. Cells were subsequently stimulated with various concentrations of SCF for 15 min, and phospho-KIT was examined as described above.

In vitro proliferation assay

Cell proliferation was determined using a methyl thiazolyl tetrazolium (MTT) assay (Roche, United States) according to the manufacturer's instructions. In brief, GIST primary cells were seeded at a density of 8 × 10^3 cells/100 μL into 96-well plates and allowed to adhere. After 24 h, the original media were replaced with fresh media containing various concentrations of SCF (Pepro-Tech, United States) or KIT immunoblocking antibody (Sigma, United States) with 0.5% FBS. After 48 h of incubation, MTT was added to each well. The quantity of the formazan product measured at 572 nm was directly proportional to the number of live cells in the culture.

Flow cytometric analysis of apoptosis

GIST primary cells were subjected to serum withdrawal for 12 h in the presence or absence of exogenous SCF or KIT immunoblocking antibody to induce apoptosis. Subsequently, apoptosis was detected using an annexin V-fluorescein isothiocyanate staining kit (R and D Systems). The apoptotic index was reported as the percentage of annexin V-positive cells in the early and late stages of apoptosis.

Statistical analysis

Data are expressed as the mean ± SD or the median and 25th and 75th percentiles [median (Q1, Q3)] for continuous variables and as percentages for categorical variables. Continuous variables were compared using Student's *t* test or the Wilcoxon rank sum test for nonnormally distributed data. Correlations between categorical and continuous variables were assessed using the χ^2 or Fisher's exact test and *t* test, respectively. *P* values of less than 0.05 were considered to be significant. All analysis were performed with SPSS version 17.0 (SPSS, Chicago, IL).

RESULTS

Tumor genotypes

All 51 cryopreserved specimens were screened for mutations in the *c-kit* gene. Overall, 32 of 51 (62.7%) tumors

Figure 1 Heterozygous *c-kit* mutations in primary gastrointestinal stromal tumors. The mutant nucleotide sequence is indicated in the upper diagram, and the wild-type nucleotide sequence is indicated in the lower diagram. Nucleotide changes are shown in red capital letters. A: Cryopreserved specimens; B: Fresh specimens.

harboured *c-kit* mutations. Among the GISTs with *c-kit* mutations, 27 (52.9%) had mutations in exon 11 of *c-kit,* five had mutations in exon 9 (Figure 1), and none had mutations in exon 13, 14 or 17. The amino acid changes observed in the 27 tumors with exon 11 mutations were as follows: deletion in 16 (59.3%) tumors, substitution in 7 (25.9%), both deletion and substitution in 2 (7.4%), insertion in 1 (3.7%), and duplication in 1 (3.7%). Five cases harboured exon 9 *c-kit* mutations, including in-frame insertions (3 tumors; 60.0%) and missense mutations (2 tumors; 40%). GISTs without *c-kit* mutations were further examined for PDGFRA mutations; only four (7.8%) tumors harboured substitution mutations in PDGFRA exon 18. All *c-kit* mutations were heterozygous, as indicated by the presence of a wild-type *c-kit* allele in the nucleotide sequence (Figure 1A).

In the three fresh specimens analysed in this experiment, sequencing showed that GIST1 and GIST3 each harboured heterozygous *c-kit* exon 11 mutations, while GIST2 had no mutations in either *c-kit* or PDGFRA (Figure 1B).

Expression of stem cell factor in primary GISTS

The immunohistochemistry experiments shown in Figure 2 demonstrated that SCF expression was observed in both GIST cells and fibroblasts, while KIT and SCF were co-expressed in tumor cells. Given that GISTs contains only a small number of fibroblasts, a specimen was considered "positive" for SCF expression if only the tumor cells showed distinct cytoplasmic or membrane staining; otherwise, the specimen was considered "nega-

tive" for SCF expression. SCF protein was present in 39 (76.5%) of the 51 GIST samples. A morphometric study revealed more pHH3 and Ki-67 positive cells and lower apoptotic cells in SCF-positive GIST cases compared with SCF-negative GIST cases (Figure 2). These results suggest that SCF may participate in an autocrine-paracrine stimulatory loop within primary GISTs.

Correlations between SCF and clinicopathological variables

As shown in Table 1, the co-expression of wild-type KIT receptor and SCF was associated with known prognostic variables, including larger tumor size ($P = 0.0118$), higher mitotic count ($P = 0.0058$), higher proliferative index ($P = 0.0012$), higher mitotic index ($P = 0.0282$), lower apoptosis index ($P = 0.0484$), and an increased NIH risk level ($P = 0.0012$). These results suggest that the SCF-mediated activation of wild-type KIT may play an important role in tumor cell growth by directly promoting cell proliferation and inhibiting apoptosis. The co-expression of the wild-type KIT receptor and SCF was not associated with any other variable tested, including patient's age, sex, histological phenotype, cellularity, tumor location and *c-kit* gene mutation.

KIT activation in primary GISTS

In line with other published findings[24,25], KIT was phosphorylated at tyrosine 703 in 74.5% (38/51) of GISTs (Figure 3A), although the levels of phosphorylated KIT varied substantially from tumor to tumor, even between those with identical *c-kit* gene mutations.

Figure 2 The expression of stem cell factor in primary gastrointestinal stromal tumors. Sections of gastrointestinal stromal tumors (GISTs) (*n* = 51) were immunostained with stem cell factor (SCF), KIT (CD117), pHH3 and Ki-67 antibodies (magnification, ×200). Apoptosis was assessed *in situ* by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining (magnification, ×200). Representative images are shown. Bar = 50 μm.

Figure 3 Western blotting analysis of phosphorylated KIT. A: KIT phosphorylation (p-KIT Y703) was analysed in gastrointestinal stromal tumor (GIST) samples (*n* = 51). A representative western blotting of p-KIT Y703 (top) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (bottom) in patients with exon 11 or 9 mutations or wild-type c-kit-bearing tumors; B: GIST primary cells were incubated with 100 ng/mL stem cell factor (SCF) for the indicated times. KIT phosphorylation (p-KIT Y703) was analysed by western blotting.

Furthermore, KIT phosphorylation was not significantly associated with the presence of *c-kit* mutations (*P* = 0.6625). Thirteen tumors without detectable *c-kit* mutations showed strong KIT phosphorylation, which was consistent with a nonmutational mechanism of KIT activation.

To determine whether the KIT receptor was activated due to the presence of the wild-type KIT receptor in *c-kit*-mutant GISTs, we cultivated GIST primary cells in the presence of various concentrations of exogenous SCF. KIT was rapidly phosphorylated in response to

exogenous SCF in a time-dependent manner in GIST primary cells (Figure 3B). These findings suggest that the hyperphosphorylation of the wild-type KIT receptor was induced by exogenous SCF, confirming that SCF mediated the activation of the wild-type KIT receptor in *c-kit*-mutant GISTs.

Effects of wild-type KIT receptor activation on proliferation in GIST primary cells

To assess whether SCF-mediated wild-type KIT activation participated in the proliferation of GIST tumor cells, we analysed the proliferation rate of GIST primary cells exposed to exogenous SCF. Consistent with the finding that SCF induced KIT phosphorylation, exogenous SCF significantly increased cell proliferation in a dose-dependent manner after 72 h of treatment (Figure 4A).

The ELISA results showed that all three primary cell lines produced significant amounts of SCF (3.5 ± 2.33 pg/mL per 10^6 cells). Therefore, we examined the effect of SCF-mediated wild-type KIT activation on cell proliferation by inhibiting the interactions between the KIT receptor and endogenous SCF. As shown in Figure 4B, cell proliferation was significantly inhibited (by > 50%) after 72 h of treatment with a KIT immunoblocking antibody, suggesting that SCF-mediated wild-type KIT receptor activation plays a key role in controlling GIST cell proliferation through the SCF/KIT autocrine-paracrine loop.

Effects of wild-type KIT receptor activation on apoptosis in GIST primary cells

The capacity to regulate survival is an important feature of tumor cells. Therefore, we tested whether wild-type

A

B

Figure 4 Stem cell factor stimulates gastrointestinal stromal tumors cell proliferation *in vitro*. Gastrointestinal stromal tumor (GIST) primary cells were incubated for 72 h with exogenous stem cell factor (SCF) (0-100 ng/mL, A) or KIT immunoblocking antibody (100 ng/mL, B), cell proliferation was analysed by methyl thiazolyl tetrazolium assay. The data are presented as the mean ± SD (n = 4 for each). [a]P < 0.05, [b]P < 0.01 *vs* untreated cells.

KIT receptor activation could rescue GIST primary cells from serum deprivation-induced death. As shown in Figure 5, the addition of exogenous SCF (100 ng/mL, 12 h) significantly reduced the percentage of apoptotic cells, from 32.05% ± 2.65% to 18.55% ± 1.83% (P < 0.01) in GIST primary cells. In contrast, treatment with KIT immunoblocking antibody significantly increased the percentage of apoptotic cells from 32.05% ± 2.65% to 43.58% ± 2.94% (P < 0.01).

DISCUSSION

The present study showed that *c-kit* gene mutations occurred in 32 (62.7%) of the 51 GIST clinical samples, which is consistent with previous results[14-16]. All of these mutations were heterozygous, i.e., at least one wild-type *c-kit* allele was present in all tumors, which is consistent with a previous report that most GIST mutations were heterozygous[26]. The heterozygous nature of the receptor status in GISTs suggests that KIT receptor activation is not induced by *c-kit* gene mutations alone but rather by other mechanisms, such as the activation of ligand-dependent signal transduction pathways. Indeed, we noted that KIT activation, as manifested by receptor tyrosine phosphorylation, is a general phenomenon in GISTs, even those without *c-kit* mutations.

Constitutive receptor tyrosine kinase activation is believed to be important for tumor proliferation and progression, and in GISTs, KIT activation can serve as an initiating event in oncogenesis[27-29]. However, the results obtained in our study showed that SCF expression was positively correlated with mitotic activity. KIT was rapidly phosphorylated upon stimulation with exogenous SCF, suggesting that ligand-dependent hyperactivation is also a strong mitogen in GIST cells. This observation is consistent with the data reported by Hirano *et al*[18], who found that SCF-positive GIST cases had a significantly higher average MIB-1 labelling index and a larger average tumor size than did the SCF-negative cases. Similarly, Théou-Anton *et al*[17] detected SCF in up to 93% of the GISTs studied and speculated that KIT activation in GISTs may be caused partly by the presence of SCF within the tumors. However, no *in vitro* measurements were conducted in either of these two studies, and the possible role of SCF-mediated wild-type KIT receptor activation in GIST proliferation should be explored *in vitro* to assess its independent contribution to cell growth. Accordingly, we further examined the mitogenic activity of this molecule on GIST primary cells harbouring a heterozygous c-kit mutation. It was found that exogenous SCF markedly stimulated cell proliferation and KIT phosphorylation in all GIST primary cells, while the inhibition of the SCF/KIT interaction reduced cell proliferation, confirming that exogenous or endogenous SCF-mediated activation of wild-type KIT provided an important signal for GIST cell proliferation. Further, GIST882, which has a homozygous activating *c-kit* mutation[30], did not exhibit increased proliferation in response to supplemental SCF, and the exogenous SCF stimulation of GIST544 cells, which express a heterozy-

Figure 5 Stem cell factor inhibits gastrointestinal stromal tumor cell apoptosis *in vitro*. Representative flow cytometric contour of annexin V-fluorescein isothio-cyanate (FITC)/propidium iodide (PI) dual-colour flow cytometry after 12 h of treatment with exogenous stem cell factor (SCF) or KIT immunoblocking antibody. The lower right quadrant represents early apoptotic cells. The data are presented as the mean ± SD (*n* = 4 for each). [a]*P* < 0.01 *vs* untreated cells.

gous *c-kit* mutation, induces stronger KIT phosphorylation and cell growth[31]; these observations further validate our results.

Apoptosis is an active process that plays a key role in the development and maintenance of tissue homeostasis. The tumor growth rate partly depends on an excess of proliferation over apoptosis[32]. Indeed, the relationship between SCF-mediated wild-type KIT receptor activation and apoptosis has been explored extensively. For example, *c-kit* activation was found to suppress the apoptosis of normal murine melanocyte precursors[33], soft tissue sarcomas of neuroectodermal origin[34], neuroblastomas[35], and normal and malignant human haematopoietic cells[36]. Our results also support a role for SCF mediated wild-type KIT receptor activation in the survival of GIST primary cells because exogenous SCF rescues GIST primary cells from serum deprivation-induced death and may consequently prolong cell survival, while blocking the interactions between KIT receptor and endogenous SCF markedly reduces the viability of

these cells.

In summary, our results demonstrated that the SCF-dependent activation of the wild-type KIT receptor is specifically involved in promoting the cell growth of GISTs *via* autocrine-paracrine loop activation. Therefore, drugs targeted against GISTs should switch off the activation of both mutant and wild-type receptors to achieve an effective response.

COMMENTS

Background
Gastrointestinal stromal tumor (GIST) is the most common sarcoma of the intestinal tract. Imatinib has shown remarkable efficacy in the treatment of GISTs, which are notoriously refractory to conventional chemotherapy or radiation. However, a considerable proportion of GIST patients show primary or acquired resistance to Imatinib.

Innovations and breakthroughs
In this study, the authors showed that the co-expression of the wild-type KIT receptor and stem cell factor (SCF) was associated with known prognostic variables in a series of 51 patients. Authors further demonstrated that SCF-

mediated wild-type KIT receptor activation participated in GIST growth by stimulating the proliferation and inhibiting the apoptosis of GIST primary cells. These results suggest that SCF-mediated wild-type KIT receptor activation represents a new and potentially promising target for GIST therapy. Therefore, to achieve an effective response, drugs targeted against GISTs should switch off the activation of both mutant and wild-type receptors.

Applications
The study demonstrated that the SCF-dependent activation of the wild-type KIT receptor is specifically involved in promoting the cell growth of GISTs *via* autocrine-paracrine loop activation. Therefore, drugs that target wild-type receptor activation may be viable candidates for the treatment of GISTs and should be explored in future studies.

Peer review
This is a well written manuscript. The use of confocal microscopy would have been ideal for studying both KIT and SCF expression.

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ORIGINAL ARTICLE

Hepatocellular carcinoma and macrophage interaction induced tumor immunosuppression *via* Treg requires TLR4 signaling

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Abstract

AIM: To investigated the interaction between toll-like receptor 4 (TLR4)-activated hepatoma cells and macrophages in the induction of tumor-immune suppression mediated by CD4+CD25^{high} family of transcription factor P3 (FOXP3) regulatory T cells (Tregs).

METHODS: The proportion of FOXP3+ Tregs was identified in peripheral blood and tumor tissues of 60 hepatocellular carcinoma (HCC) patients. TLR4 expression was examined in tumor tissues and cell lines. The correlation was examined between FOXP3+ Tregs in peripheral blood and TLR4 expression of HCC tissues. Following activation of TLR4 in H22 murine hepatoma cells pre-incubated with lipopolysaccharide (LPS) and co-cultured with macrophage cell line RAW246.7, the synthesis of cytokines tumor necrosis factor-α, CCL22, and interleukin (IL)-10 by the two cell lines was detected and analyzed.

RESULTS: FOXP3+ Tregs were enriched in tumor sites, and circulating FOXP3+ Tregs were increased in HCC patients in correlation with multiple tumor foci and up-regulated TLR4 expression in HCC tissues. Semi-quantitative analysis indicated that TLR4 was over-expressed in HCC compared with the matched normal tissues. Cell cultivation experiments indicated that the mRNAs of IL-10 and CCL22 were significantly up-regulated in the RAW246.7 cell line when co-cultured with LPS pre-incubated H22 cells.

CONCLUSION: In hepatoma cell lines, TLR4 may indirectly facilitate the recruitment of Tregs to the tumor site and promote intrahepatic metastasis through its interaction with macrophages.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most com-

mon cancer worldwide, and is the third most common cause of cancer related deaths[1]. Previous studies have demonstrated that the majority of HCC patients develop tumor-specific immune responses; however, in most patients, tumors progress despite tumor-specific humoral and cellular immune responses. These findings imply that HCC escapes the anti-tumor immune response through various strategies.

Recently, many studies have suggested that the tumor microenvironment plays an important role in the establishment and progression of tumors. Lymphocytes contribute to the tumor microenvironment through immunity and inflammation. Through diverse strategies, tumor cells play an important role in the suppression of anti-tumor immunity in the surrounding microenvironment *via* interactions with infiltrated immune cells or macrophages, which in turn potentially facilitates growth of the tumor itself. Moreover, induction of the differentiation and/or recruitment of regulatory T cells (Tregs), a unique population of CD4+ T cells, potentially comprises one of the key mechanisms. Tregs are defined based on their expression of CD4, CD25 and forkhead, or winged helix family of transcription factor P3 (FOXP3), which is critical for the development and function of Tregs in mice and humans[2]. Tregs play a critical role in immunologic self-tolerance and suppression in the tumor immune response[3,4]. Early evidence indicates that Tregs (not FOXP3+ T cells but CD4+CD25+ T cells) are increased in patients with various types of cancer[5-7]. It is hypothesized that their systemic and/or local accumulation promotes tumor growth through suppression of the host anti-tumor response[8]. However, considerable uncertainty remains regarding the characteristics, functions, and regulation of Tregs. Therefore, we investigated the clinicopathologic significance of CD4+CD25^{high}FOXP3+ Tregs in HCC patients, and analyzed their ability to suppress the immune response. Elucidation of the mechanisms underlying Treg elevation is essential for the development of new approaches that aim to modulate the frequency and function of Tregs in order to enhance the efficacy of cancer immune-based therapies.

Toll-like receptors (TLRs) recognize specific structural regions of invading pathogens and initiate innate and adaptive immune responses; their expression has been detected in immune cells and also in many cancer cells[9]. Lipopolysaccharide (LPS), a ligand for TLR4, stimulates immune cells and triggers the production of inflammatory cytokines and other mediators *via* TLR4, which in turn regulates the host immune defense system and eliminates pathogens[10,11]. It has been reported that inflammatory cytokines induced by inflammatory stimuli counteract immune surveillance and facilitate tumor growth[12,13]. However, various other reports have suggested that diverse TLRs potentially exhibit different effects on Tregs due to differences in pathogens and immune environment, resulting in either increased suppression or abrogation of suppression[14-17]. The suppressive function of Tregs is tightly regulated to respond to the different requirements of immunity. The mechanism underlying the selective control of Treg function remains obscure. Precise modulation of the suppressive function of Tregs is crucial for the development of effective cancer immunotherapy. Thus, in this study we investigated whether TLR4 is expressed in HCC, and whether tumor TLR4 is functionally active in inducing cytokines, and we also examined the clinicopathological correlation between tumor TLR4 signaling and CD4+CD25^{high}FOXP3+ Tregs in tumor immune escape.

Currently, the roles of CD4+CD25^{high}FOXP3+ Tregs and TLR4 in HCC and their regulatory activity in the tumor microenvironment remain unclear. In the present study, we described the clinicopathological significance of Tregs in 60 HCC patients, and the expression of TLR4 in hepatic cancer cells. Our findings indicated that TLR4 ligation promotes the secretion of inhibitory cytokine interleukin (IL)-10 and chemokine CCL22 from co-cultured macrophages, but not from the tumor cells themselves. Furthermore, the prevalence of Tregs significantly correlated with the presence of multifocal tumor. Our results suggest a mechanistic path for the indirect modulation of CD4+CD25^{high}FOXP3+ Tregs *via* tumor TLR4 signaling, and demonstrate that interactions between hepatoma cells and macrophages induce anti-tumor immune suppression *via* Tregs.

MATERIALS AND METHODS

Ethics
This study was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The Ethics Committee of Tongji Medical College (Wuhan, China) approved the study protocol. All patients provided informed written consent before blood and tumor sampling.

Patient and samples
Blood samples were collected from 60 HCC patients who underwent hepatic resection in the Center of Hepatobiliary Surgery, Union Hospital, Wuhan, China from March 2008 to October 2008. Control blood samples were obtained from 20 healthy volunteers. HCC patients were pathologically diagnosed following surgical resection. Among the 60 HCC patients, there were 51 males and nine females aged 17-77 years (mean, 51.3 years). According to the International Union against Cancer tumor-node-metastasis classification[18], there were 19 (31.7%), 24 (40%) and 17 (28.3%) cases at stage Ⅰ, Ⅱ and Ⅲ, respectively. No patient was treated with local ablative therapy, chemotherapy, or immunotherapy prior to surgery. Clinical and laboratory characteristics of the HCC patients are shown in Table 1. Blood samples (3 mL) were collected 2-3 d before operation from each patient in the early morning. Samples were placed in ethylenediaminetetraacetic acid anticoagulant tubes for flow cytometric detection in our hospital. Next, 1 cm × 1 cm × 1 cm tumor and normal tissues were obtained from each patient intraoperatively, avoiding

Table 1 Clinical and laboratory characteristics of the 60 hepatocellular carcinoma patients

Items	Results
Age (yr) median (range)	53.5 (17-77)
Gender (male/female)	51/9
Virus (HBV/HCV)	48/4
TNM stage (I / II / III / IV)	19/24/17/0
AFP (μg/L), median (range)	350 (1.8-127 278)
Blood neutrophil (%)	62.29 ± 10.96
Blood monocyte (%)	7.21 ± 1.69
Hemoglobin concentration (g/L)	124.47 ± 17.04

TNM: Tumor-node-metastasis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: α-fetoprotein.

areas of necrosis, hemorrhage, and/or adipose tissues. One portion of each specimen was snap frozen in liquid nitrogen and the other part was fixed in 10% polyformaldehyde solution and embedded in paraffin.

Flow cytometric analysis

Peripheral blood mononuclear cells from each patient (100 μL) was separated into a heparinized container. Twenty microliters anti-human CD25-fluorescein isothiocyanate and anti-human CD4-PerCP was added to each tube, and incubated at room temperature in the dark for 15 min. After washing in 1 × phosphate-buffered saline, fixed broken membrane buffer 1 mL (1% paraformaldehyde and 70% ice-alcohol; pH 7.4) was added. The remaining lymphocytes were incubated in 20 μL FOXP3-PE at 4 ℃ for 30 min. Subsequently, cells were analyzed by flow cytometry (FACS Calibur, BD) with Cellquest software (Version 3.3, BD Biosciences-Pharmingen, United States). All conjugated antibodies described above and their isotype-matched monoclonal antibodies were purchased from BD, United States.

Lymphocytes were gated on forward and side scatter profiles followed by gating on CD4+ T cells, and these cells were then analyzed for CD25 expression. For FOXP3 expression analysis, cells inside the CD4+CD25^{high} gate were analyzed.

Western blotting

Nuclear protein extracts were prepared in sodium dodecyl sulfate (SDS) lysis buffer containing protease inhibitors, pre-stained molecular weight markers were denatured in laemmli buffer (10% glycerol, 2% SDS, 0.1 mol/L dithiothreitol, 50 mmol/L Tris, 0.01 mg/mL bromphenol blue; pH 6.8) at 90 ℃, and were separated by SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred onto polyvinylidene fluoride membrane in Trans-blot wet buffer (Bio-Rad Laboratories, United States). The membranes were blocked with 5% nonfat dry milk in 1 × tris-buffered saline (TBS), then incubated with 2 μg/mL mouse monoclonal anti-FoxP3 antibody (clone 22510; Abcam, United States) overnight at 4 ℃ followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) for 1 h at room temperature, and washed with 1

× TBS. Membranes were treated with enhanced chemiluminescence plus Western blotting detection kit (Transgen, Beijing, China), and bands were detected using STORM 840v2005 with ImageQuant software (GE, United States).

Cell lines and reagents

If not indicated otherwise, all substances were purchased from Gibco, United States. The H22 (murine) and HepG2 (human) hepatic cancer cell lines were a gift from Dr. Huang Bo (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China). The immortalized murine macrophage line RAW246.7 was preserved in our laboratory. The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. All experiments were performed under endotoxin-free conditions.

Co-culture assay

The various cellular components were grown in an artificial basement membrane in a modified Polyster-Transwell (Costar, United States) plate without direct cell-to-cell contact. 1×10^5 RAW246.7 cells/mL were seeded into the upper well of the Transwell plate (0.4 μm pore diameter), which consisted of a membrane permeable to liquids but not cells, whereas the lower well was filled to the top with RPMI + 10% fetal calf serum. H22 cells (1×10^6 cells/mL RPMI) were seeded into a 12-well plate. To activate TLR4 in H22 cells, LPS (Gibco, United States) was used at 1 μg/mL. The culture medium was removed after LPS-stimulating the H22 cells for 12 h. Next, the Transwells were inserted into the 12-well plate. Gene expression was compared between control and macrophages co-cultured with H22 cells after 24 h, and macrophages with or without conditioned tumor medium were analyzed using reverse transcriptase polymerase chain reaction (RT-PCR). All experiments were performed at least in triplicate.

RNA extraction and RT-PCR

Total RNA was extracted from patient samples, H22, HepG2, and RAW246.7 cells, using Trizol reagent (Invitrogen, United States). Using a first strand cDNA synthesis kit (ToYoBo, Shanghai, China), cDNA was generated with Oligo dT primer. Primers for TLR4, tumor necrosis factor-α (TNF-α), IL-10, CCL22 and β-actin were designed using Premier 5.0 software (Table 2). Primers were synthesized by Sangon Inc. Shanghai, China.

Each reaction mixture contained 2.5 μL 10 × buffer, 2.5 μL 2.5 mmol MgCl₂, 0.5 μL 10 mmol of dNTP, 0.2 μL 5 U/μL Taq DNA polymerase, 1 μL each of sense and antisense primer, and 1 μL cDNA in a final volume of 25 μL (Fermentas, United States). Reaction mixtures were incubated at 94 ℃ for 5 min to activate the Taq DNA polymerase, and then amplified using 40 cycles of 30 s at 94 ℃ (denaturation) and 40 s at annealing temperature for TLR4, TNF-α, IL-10, CCL22, and β-actin, respectively. PCR was performed using the Agarose Gel Electrophoresis Imaging Analysis System (Beijing, China).

Table 2 Primer sequences and conditions used for reverse transcription-polymerase chain reaction

Gene	Sequence	Product size (bp)	Annealing temperature (℃)
mTLR4	5′-GCTTTCACCTCTGCCTTCAC-3′ 3′-AGGCGATACAATTCCACCTG-5′	259	57
HuTLR4	5′-GAAATGGAGGCACCCCTTC-3′ 3′-GAATATTCCTTTGCATAGGT-5′	506	52
CCL22	5′-AAGACAGTATCTGCTGCCAGG-3′ 3′-GATCGGCACAGATATCTCGG-5′	141	57
IL-10	5′-GGTTGCCAAGCCTTATCGGA-3′ 3′-ACCTGCTCCACTGCCTTGCT-5′	190	60
TNF-α	5′-CATCTTCTCAAAATTCGAGTGACAA-3′ 3′-TGGGAGTAGACAAGGTACAACCC-5′	175	58
β-actin	5′-TCACCCACACTGTGCCCATCTACGA-3′ 3′-GATAACCGTTGCTCGCCAAGGCTAC-5′	300	50

TNF: Tumor necrosis factor; IL: Interleukin; TLR: Toll-like receptor.

Immunohistochemistry

To assess TLR4 protein expression in HCC and normal tissues, a polyclonal rabbit anti-human TLR4 (ab47093; Abcam, United States) was used. Paraffin-embedded sections (5-μm thick) were fixed in freshly prepared 10% paraformaldehyde for 5 min. After blocking the endogenous peroxidase activity with 0.3% hydrogen peroxide in TBS for 15 min, the sections were immersed in horse serum diluted 1:10 in TBS for 30 min to reduce nonspecific binding, and then incubated with the primary antibody overnight at 4 ℃ after washing in TBS. Next, the sections were incubated in biotinylated horse anti-mouse or goat anti-rabbit IgG for 30 min, and avidin-biotin-peroxidase complex for 30 min. After each step of the staining procedure, the sections were given three 5-min washes in TBS. Immunoreactivity (IR) was visualized using 1 mg/mL diaminobenzidine as chromogen and 0.01% hydrogen peroxide as substrate. The peroxidase reaction was stopped after 5 min with distilled water, and the sections were counter-stained with Toluidine blue, dehydrated, and then mounted with Entellan.

Slides were evaluated under a light microscope (× 400 magnification). For digital image analysis, the software Adobe Photoshop version 7.0 was used. Results were scored by two independent investigators as positive, heterogeneous, or negative. The two scores were averaged.

Statistical analysis

SPSS version 10.0 (SPSS Inc., United States) was used for statistical analysis. All data were expressed as mean ± SE. Statistical analyses were performed using the Student's *t* test. If there was evidence of non-normality, Kruskal-Wallis one-way analysis of variance was used to test the difference in median among the groups. To analyze the correlation between Tregs and TLR4, Spearman's rank correlation coefficients were performed. Difference was considered statistically significant at $P < 0.05$.

RESULTS

CD4+CD25^high^FOXP3+ Tregs accumulation in HCC tumor and peripheral blood

The prevalence of CD4+CD25^high^ and CD4+CD25^high^FOXP3+ Tregs was analyzed in the peripheral blood of 60 patients with HCC. The population of CD4+CD25^high^ and FOXP3+ Tregs as a percentage of total CD4+ T and CD4+CD25^high^ T cells, respectively, was identified by flow cytometry. Representative dot plots of HCC patients and controls are shown in Figure 1A. The frequency of CD4+CD25^high^ and FOXP3+ Tregs in HCC patients was significantly higher than in the healthy controls (Figure 1B). The expression of FOXP3 in HCC patients was detected using Western blotting analysis. As shown in Figure 1C and D, both tumor and normal tissues exhibited FOXP3 protein expression, although the expression was weaker in normal tissues.

Clinicopathologic characteristics of HCC patients and prevalence of circulating CD4+CD25^high^FOXP3+ Tregs

We analyzed the correlation between the proportion of CD4+CD25^high^FOXP3+ Tregs in the peripheral blood and the clinicopathological characteristics of the subjects (Table 3). The proportion of CD4+CD25^high^FOXP3+ Tregs was significantly higher in patients with high serum AFP levels and multiple tumor foci ($P = 0.009$). The proportion of CD4+CD25^high^FOXP3+ Tregs did not correlate with other clinicopathological characteristics of HCC patients ($P > 0.05$).

Considering the potential impact of multifocal tumor on tumor size, the correlation of the proportion of CD4+CD25^high^FOXP3+ Tregs with different tumor diameters was analyzed in 52 patients with a single lesion. Our findings indicated that the proportion of Tregs was low in patients with a small tumor (< 5 cm in diameter) (Figure 2).

Expression of TLR4 in patient samples and hepatoma cell lines

The expression of TLR4 in patient tumor samples and hepatoma cell lines was examined using RT-PCR. Tumor and normal tissues, as well as HepG2 and H22 cells, expressed TLR4 mRNA (Figure 3). We determined the relative expression of TLR4 mRNA (TLR4 mRNA level/β-actin mRNA level × 100%) and found that TLR4 mRNA expression level in HCC tissues was higher than in the normal tissues ($P = 0.01$) (Figure 3A). Additionally, immunohistochemical analysis confirmed the expression of TLR4 protein. TLR4 positive hepatocytes were present in paraffin-embedded sections. Moderate and strong IR for TLR4 was detected in 63.4% (38/60) of HCC specimens, and normal tissues displayed positive staining in 10% (6/60). TLR4 in cancer cells was stained more intensely than in the normal cells (Figure 3C). The distribution of TLR4 in a given HCC specimen was uneven, and the majority of positive hepatocytes exhibited expression on the membrane and in the cytoplasm. Scattered expression of TLR4 protein was also observed in a small number of liver sections derived from normal tissues.

Figure 1 CD4+CD25^high^FOXP3⁺ regulatory T cells accumulation in hepatocellular carcinoma and peripheral blood. A: Representative flow cytometry plots of CD4+CD25^high^ regulatory T cell (Treg) (region R2) and family of transcription factor P3 (FOXP3)+ Treg cells in peripheral blood from a healthy donor and hepatocellular carcinoma (HCC) patient; B: Percentage of CD4+CD25^high^ Treg and FOXP3+ Treg cells in peripheral blood from HCC patients and controls; C: The prevalence of CD4+CD25^high^ Treg (8.57% ± 6.31%, $P = 0.002$) and FOXP3+ Treg cells (67.51% ± 20.59%, $P < 0.05$) in peripheral blood from HCC patients was significantly higher than that of healthy donors (1.71% ± 1.59% and 43.35% ± 13.91%, respectively). Western blotting analysis of nuclear extracts prepared from HCC and normal tissues; D: The relative expression of FOXP3 protein (FOXP3 protein/β-actin protein × 100%). The FOXP3 protein expression in HCC tissues was stronger (0.72 ± 0.14) as compared with normal tissues (0.24 ± 0.05) ([a]$P < 0.05$). FITC: Fluorescein isothiocyanate.

Figure 2 Correlation of regulatory T cells with tumor size of a single tumor. Circulating levels of CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs) was low in patients with a small tumor (less than 5 cm in diameter). Comparisons of data of Tregs among 3 tumor size groups using a Kruskal-Wallis test. Statistically significant differences were found in all comparisons between the 3 groups (χ^2: 7.365, P = 0.0252). HCC: Hepatocellular carcinoma.

Upregulation of IL-10 and CCL22 expression in RAW246.7 cells elicited by co-cultivation with LPS preincubated hepatoma cells

Hepatoma cell line H22 cells were pre-incubated with LPS. The RAW246.7 cells and the H22 cells were co-cultured in a transwell system for 24 h. Semiquantitative RT-PCR was performed to detect the expression of TNF-α, IL-10, and CCL22 in different cell lines. RAW246.7 cells co-cultured with LPS pre-treated H22 cells exhibited significant up-regulation of mRNA for genes IL-10 and CCL22 ($P < 0.001$), whereas expression of TNF-α remained unchanged (Figure 4). Tumor cells without LPS pre-incubation induced negligible gene expression changes in co-cultured RAW246.7 cells. H22 cells alone did not produce IL-10 and CCL22 under conditions of TLR4 activation following LPS stimuli or not (data not shown). These findings, taken together, suggested that activated TLR4 on H22 cells facilitated the induction of cytokine secretion of IL-10 and CCL22 by RAW246.7 cells.

Correlation between proportion of CD4+CD25^{high}FOXP3+ Tregs in peripheral blood of HCC patients and TLR4 in HCC tissues

We investigated the associations between tumor Union for International Cancer Control (UICC) stage, circulating CD4+CD25^{high}FOXP3+ Tregs, and TLR4 in the HCC tissues. The expression level of TLR4 protein in 60 HCC specimens was positively correlated with the frequency of CD4+CD25^{high}FOXP3+ Tregs in peripheral blood (Figure 5A). There was an increased frequency of Tregs in the peripheral blood of HCC patients, which exhibited a high level of expression of TLR4 protein. However, there was no significant correlation between the number of CD4+CD25^{high}FOXP3+ Tregs and tumor UICC stage (Figure 5B).

DISCUSSION

Increased FOXP3+ Tregs in HCC tissues and peripheral blood

T cells play an essential role in the immunosurveillance

Table 3 Correlation between the proportion of CD4+CD25^{high}FOXP3+ regulatory T cells in peripheral blood and clinicopathological characteristics of hepatocellular carcinoma

Items	*n*	Treg (%)	*P* values
Sex			0.201
Male	51	68.95 ± 20.71	
Female	9	59.37 ± 18.97	
UICC/TNM stage			0.781
I	19	66.41 ± 20.28	
II - III	41	68.02 ± 20.97	
AFP (μg/L)			<0.001
≤ 20	23	53.13 ± 17.96	
> 20	37	76.45 ± 16.84	
HBsAg			0.611
Positive	48	68.20 ± 19.69	
Negative	12	64.78 ± 24.66	
Margin			0.981
Clear	29	67.45 ± 19.74	
Unclear	31	67.57 ± 21.69	
Capsule			0.058
Complete	28	62.14 ± 21.92	
Incomplete	32	72.22 ± 18.43	
Tumor diameter (cm)			0.245
≤ 5	23	59.84 ± 19.06	
6-9	18	75.94 ± 18.43	
≥ 10	19	68.81 ± 21.84	
Tumor number			0.009
Single	52	63.83 ± 20.55	
≥ 2	8	86.41 ± 6.18	
Cirrhosis			0.881
Presence	23	68.02 ± 20.42	
Absence	37	67.19 ± 20.98	
Tumor differentiation			0.152
WD	11	56.61 ± 24.98	
MD	7	69.60 ± 20.45	
PD	42	70.02 ± 18.59	

MD: Moderately differentiated; WD: Well differentiated; PD: Poorly differentiated; AFP: α-fetoprotein; Tregs: Regulatory T cells; TNM: Tumor-node-metastasis; UICC: Union for International Cancer Control.

and destruction of cancer cells. Among CD4+ T lymphocytes, CD4+CD25+ Tregs are thought to comprise a functionally unique subpopulation of T cells that act to maintain immune homeostasis. The lack of CD4+CD25+ Tregs results in various autoimmune syndromes. Alternatively, Tregs-mediated suppression potentially hinders an effective immune response, which is crucial for elimination of tumors and infection[19]. Humans with cancer exhibit increased numbers of peripherally circulating and tumor Tregs[5,20,21]. Ormandy *et al*[22] found that in HCC patients the number of Tregs in the peripheral blood was significantly increased. However, Yang *et al*[23] found that the proportion of CD4+CD25+ Tregs in the peripheral blood of HCC patients was significantly decreased. Too few samples and different patient inclusion criteria has potentially led to conflicting results in previous studies. This study presents evidence of an accumulation of FOXP3+ Tregs in HCC tumor tissues and peripheral blood. A few previous studies have also investigated the clinicopathologic significance of Tregs, but conclusions regarding correlations with various clinicopathologic features were contradictory. Our results demonstrated

Figure 3 Expression of toll-like receptor 4 mRNA in hepatocellular carcinoma patients and hepatoma cell lines. A: Reverse transcriptase polymerase chain reaction analysis of total RNA from hepatocellular carcinoma (HCC) and normal tissues. The toll-like receptor 4 (TLR4) mRNA expression in HCC tissues was stronger (2.08 ± 0.14) compared to normal tissues (0.48 ± 0.05) (P = 0.01); B: TLR4 mRNA expressed in human HepG2 and murine H22 hepatoma cell lines; C: Expression of TLR4 protein in HCC and normal tissues by immunohistochemistry. a, c: Paraffin-embedded sections of HCC exhibiting positive expression on the membrane and in the cytoplasm in several positive hepatocytes (arrows) [diaminobenzidine (DAB) ×400]; b, d: Paraffin-embedded sections of normal tissues showing weaker expression (DAB ×400); D: TLR4 was detected by immunohistochemistry in tissue sections of patients with HCC (frequency of positive hepatocytes for TLR4 in paraffin-embedded sections).

that the proportion of Tregs was not correlated with hepatitis and cirrhosis, which was in concordance with other studies[22,24]. Shen *et al*[25] indicated that the increased prevalence and expanded function of Tregs in the tumor microenvironment of HCC correlated with cancer stage. However, no significant differences between number of Tregs and tumor UICC stage were observed in our HCC patients; other research groups also confirmed our findings[26,27]. The discrepancy among different research groups potentially results from differences in patient profiles, e.g., Shen *et al*[25] selected 31 patients who had undergone hepatectomy for their study. Moreover, we demonstrated a positive correlation between the number of Tregs and serum AFP levels and multifocal tumor. In order to exclude the influence of multifocal tumor, we separately analyzed 52 HCC patients with a single lesion, and found that the proportion of Tregs was higher in patients with a larger tumor size (Figure 3, P = 0.0252). Our results suggested that the number of Tregs in HCC potentially contributes to the inhibition of effective anti-

tumor immune responses and promotes intrahepatic tumor metastasis.

Correlation between Tregs levels and expression of TLR4 in HCC tissues

Despite the important role of Tregs in controlling immune responses to self-antigen and non-self-antigens and their natural ligands, the molecular mechanisms underlying the regulation and recruitment of Tregs in the tumor microenvironment remain poorly understood. One family of receptors involved in immune regulation is TLRs, a class of receptors that recognizes pathogen-associated molecular patterns or endogenous inflammation-associated molecules[28]. Recently, TLRs were identified on cancer cells and T cells, including Tregs[9,11]. Thus, TLR-signaling directly or indirectly regulates the immunosuppressive function of Tregs in the immune response[29-32]. A few studies concerning indirect regulatory function have been conducted to clarify the complex cross-talk between TLRs and Tregs in tumors. Our data provide

Figure 4 Tumor necrosis factor-α, interleukin-10, and CCL22 mRNA expression in RAW246.7 and RAW246.7 cells co-cultured with H22 cells ± lipopolysaccharide. The bars indicate the relative amount of tumor necrosis factor (TNF)-α, interleukin (IL)-10 and CCL22 mRNA expression (reverse transcriptase polymerase chain reaction). LPS: Lipopolysaccharide. [b]$P < 0.001$ *vs* RAW co-culture.

Figure 5 Correlation between the expression of toll-like receptor 4 in hepatocellular carcinoma specimens, tumor Union for International Cancer Control stage, and CD4+CD25^{high} FOXP3+ regulatory T cell. A: Toll-like receptor 4 expression was positively correlated with the number of CD4+CD25^{high}FOXP3+ regulatory T cells (Tregs) in peripheral blood. Analysis of data was performed using Spearman's rank correlation coefficients ($n = 60$, $r = 0.411$, $P < 0.001$); B: According to Union for International Cancer Control/tumor-node-metastasis classification, there were 19 cases, 24 cases, and 17 cases at stage Ⅰ, Ⅱ and Ⅲ hepatocellular carcinoma, respectively. Statistically negative correlations were found in all comparisons between the 3 groups ($\chi^2 = 0.921$, $P = 0.631$) using the Kruskal-Wallis test.

some important clues regarding the interaction of TLR4 expression in tumor tissues and the number of Tregs in hepatocellular cancer. We demonstrated that TLR4 was expressed in diverse hepatoma cells and enriched in HCC tumor tissues, and that the expression of TLR4 in HCC positively correlated with the frequency of FOXP3+ Tregs in both circulation and tumor tissues ($P < 0.001$). These results suggest that the recruitment and proliferation of Tregs are indirectly regulated *via* TLR4 signaling.

Activation of TLR4 on tumor cells and its interaction with macrophages indirectly suppress anti-tumor immune response via recruitment of Tregs

Although some evidence implicates Tregs in the immunopathogenesis of cancer, suppressive mechanisms in the tumor microenvironment and the underlying mecha-

nisms of regulation remain poorly understood[33]. Early evidence suggests that CCL22 preferentially attracts activated antigen-specific T cells to dendritic cells[34]. Curiel recently demonstrated that tumor cells and microenvironmental macrophages in ovarian carcinoma produce the chemokine CCL22, which mediates Treg trafficking to the tumor[35]. Therefore, tumor cells potentially utilize this effect to attract Tregs to the microenvironment. Our data suggested that co-culture of macrophages with a hepatoma cell line leads to a significant increase in the expression of IL-10 and CCL22 ($P < 0.001$). The source of this CCL22 is co-cultured macrophages and hepatoma cells preincubated with LPS. IL-10 is a pleiotropic cytokine produced by myeloid cells and lymphocytes that displays immunoregulatory effects[36]. IL-10 inhibits the production of other cytokines such as IL-2, IL-12 and TNF-α

and plays an important role in suppression of T cell anti-tumor responses[37-39]. In this study, we demonstrated that co-cultured macrophages and LPS pre-treated hepatoma cells significantly up-regulate IL-10 expression. Our findings indicated that the activation of TLR4 on hepatoma cells indirectly modulates the suppressive function of Tregs and enhances Tregs recruitment by inducing cytokines, resulting in the immune escape of HCC.

The accumulation of FOXP3+ Tregs in HCC suggests a trend toward intrahepatic metastasis. The modulation of TLR4 activation on tumor cells links between Tregs suppressive function and the immunopathogenesis of human cancer. The association between TLR4-signaling and functional control of CD4+CD25^{high}FOXP3+ Tregs indicates intriguing potential opportunities to suppress anti-HCC immunity and improve therapeutic effectiveness for the patients. Our study provides the evidence which is useful for the development of an improved immunotherapeutic approach to HCC, and further studies are warranted.

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COMMENTS

Background

The survival of hepatocellular carcinoma (HCC) is limited in the majority of such cases. The active suppression of immune responses against tumor is a major barrier to the likely success of cancer immunotherapy. There is now compelling evidence implicating CD4+CD25^{high} family of transcription factor P3 (FOXP3)+ regulatory T cells (Tregs) as being key players driving immune suppression. However, precise function regulation of CD4+CD25^{high}FOXP3+ Tregs remains obscure.

Research frontiers

Toll-like receptors (TLRs) have recently emerged as a critical pathogen-associated molecular pattern of the innate immune system for detecting microbial infection and activation of dendritic cell maturation programs to induce adaptive immune responses. TLR4, an important member of TLRs family, plays a central role in phagocytosis, signal transduction and cell apoptosis. New evidence suggests that TLR signaling may directly or indirectly regulate the suppressive function of Treg cells. TLR4 signaling pathway activation induces production of massive cytokines, and finally intrigues the downstream nuclear factor kappa B to shift the balance between CD4+ T-helper and Treg cells, in ways that may facilitate tumor evasion of immune surveillance.

Innovations and breakthroughs

Currently, the roles of CD4+CD25^{high}FOXP3+ Tregs and TLR4 in HCC and their regulatory activity in the tumor microenvironment remain unclear. In the present study, authors described the clinicopathological significance of Tregs in 60 HCC patients, and the expression of TLR4 in hepatic cancer cells. The findings indicated that TLR4 ligation promotes the secretion of inhibitory cytokine interleukin-10 and chemokine CCL22 from co-cultured macrophages, not from the tumor cells themselves. Furthermore, the prevalence of Tregs significantly correlated with the presence of multifocal tumor. The results suggest a mechanistic path for the indirect modulation of CD4+CD25^{high}FOXP3+ Tregs *via* tumor TLR4 signaling, and demonstrate that interactions between hepatoma carcinoma cells and macrophages induce anti-tumor immune suppression *via* Tregs.

Applications

The authors report that activation of TLR4 on hepatoma cells followed by its interaction with macrophages may indirectly facilitate the recruitment of Tregs into tumor site and promote the intrahepatic metastasis, which suggests innate immunity mediated cellular interference maybe an effective therapeutic target in the treatment of HCC patients.

Terminology

CD4+CD25^{high}FOXP3+ Treg: Tregs are defined based on their expression of CD4, CD25 and forkhead, or winged helix FOXP3, which is critical for the development and function of Tregs in mice and humans. Tregs play a critical role in immunologic self-tolerance and suppression in the tumor immune response; TLR: To recognize specific structural regions of invading pathogens and initiate innate and adaptive immune responses; their expression has been detected in immune cells and also in many cancer cells. TLR4, an important member of TLRs family, plays a central role in phagocytosis, signal transduction and cell apoptosis.

Peer review

The article shows that TLR on hepatoma cell lines may indirectly facilitate the recruitment of Treg cells within tumor site *via* the potential activation of macrophages. This is a good exploratory study in which authors analyze the relationship of tumor TLR4 signaling pathway and CD4+CD25^{high}FOXP3+ Treg cells in tumor immune escape.

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ORIGINAL ARTICLE

Pyogenic liver abscesses associated with nonmetastatic colorectal cancers: An increasing problem in Eastern Asia

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Abstract

AIM: To elaborate the clinicopathologic features of colorectal cancer-related pyogenic liver abscess (PLA).

METHODS: Reported cases of colorectal cancer-related PLAs were collected from the literature published up to October 2011 and evaluated for their clinicopathologic features. Data of collected cases included demographics, clinical presentation, microbial findings and treatment. Categorical variables were compared by χ^2 analysis and continuous variables were evaluated using Student's t test.

RESULTS: A total 96 cases of colorectal cancer-related PLA were collected from the previous literature. Most patients (60%) were male and 40% cases occurred in the age group of 61-70 years. Apart from some special types of PLA, there were significant differences in the microbiological spectrum between Eastern Asia and non-Eastern Asian countries, which implied different risk factors and courses of the disease. Gram negative bacteria especially *Klebsiella pneumoniae* (*K. pneumoniae*) PLA was predominant in Eastern Asia (80.0%) in contrast to non-Eastern Asian countries ($P < 0.01$). Meanwhile, most of the Eastern Asian patients exhibited smaller size of liver abscess and atypical presentation. Sigmoid colon and rectum (72.73%) were the main sites of tumor in Eastern Asian patients, whereas tumor sites were uneven among most of the non-Easter Asian PLA patients.

CONCLUSION: *K. pneumoniae* PLA was strongly associated with colorectal cancer, especially those occurring in sigmoid colon and rectum, in elderly Eastern Asian male patients.

INTRODUCTION

Liver is the most common organ to develop abscesses.

Pyogenic liver abscess (PLA), once predominantly a disease of young adults as a consequence of post-appendicitis pylephlebitis, nowadays occurs more frequently in elderly patients, with hepatobiliary tract diseases or intra-abdominal infections including cholecystitis, suppurative cholangitis, suppurative pylephlebitis, diverticulitis and peritonitis[1]. Meanwhile, as we cannot find significant underlying causes of PLA, the term "cryptogenic abscesses" is used. For all types of PLA, mucosal defect present within digestive tract lesions or a compromised mucosal barrier allowing a route for bacteria invasion into the portal system with subsequent hematogenous spread to the liver is regarded as the key process[2].

Previous studies revealed that a few cryptogenic PLA patients were related to neoplasms[3]. This type of PLA was regarded as a warning indicator of silent cancers. Recently, many cases of PLA associated with nonmetastatic colorectal carcinoma have been reported worldwide. Interestingly, this PLA in Eastern Asia seems to have greater morbidity and exhibits differences in clinical characteristics. However, there were few studies which analyzed this phenomenon, neither was there any recommendation or consensus for treatment. Therefore, we reviewed published case reports from worldwide literature and retrospectively investigated the etiology, clinical characteristics and treatment of PLA complicated with nonmetastatic colorectal carcinoma.

MATERIALS AND METHODS

Source of data
Data from the available medical literature were systematically reviewed and pooled to analyze. MEDLINE (United States National Library of Medicine, Bethesda, MD), EMBASE (Elsevier Science, New York, NY) and CNKI National Knowledge Infrastructure) bibliographic databases were searched and relevant studies in form of case-control studies, case series or case report published in English language were retrieved using the keywords: "hepatic/liver abscess", "malignant cancer", "colorectal cancer" or "bacterium". The relevant article references in English and other languages were also collected.

Data extraction and quality control
A medical information scientist performed the literature retrieval and the initial screening of relevant studies, and a medical doctor reviewed and coded all studies. Cases were scrutinized to exclude any duplicate reports of the same patients. Many studies reported only aggregate results. Whenever possible, data were extracted both at an aggregate level within each study and at a patient level. The individual cases without individual identification were also excluded. We excluded studies or individuals with missing data from specific analyses. As a result, the number of patients in each analysis varied.

Database
The collected cases were evaluated individually, and de-

tails were extracted and computerized for further analysis. Coded potential prognostic determinants included patient demographics, microbial, clinical and laboratory findings, and the authors' affiliations. The data were pooled at both the aggregate and patient levels to determine the distribution of the underlying disease, site of infection, and other pertinent variables.

Statistical analysis
All collected data were transcribed into a Microsoft Excel spreadsheet and analyzed using SPSS software, version 14.0 (SPSS). Continuous variables were compared using analysis of variance, and categorical variables were compared using the χ^2 test. $P < 0.05$ was regarded as significantly different.

RESULTS

Removing irrelevant articles, and articles published in internal journal and reviews, we collected a total of 32 articles with 96 cases from 623 publications in the international literature up to September 2011. Two case-control studies[4,5] and 30 case reports[3,6-34] were included. Most of articles were single case report (26/32). Sixteen cases included in aggregate series[2,15,27-31,33] and 32 cases with individual information summarized in one article published in Japanese were also extracted[7]. Among the 32 collected articles, 25 articles were in English, 2 were in Japanese, 1 was in Chinese, 1 was in Spanish, 1 was in French and 1 was in Hebrew.

Global distribution of reported cases
Although cases of colorectal cancer-related PLA were originally reported in Western countries[2,27-31,33], most of reported cases (80.21%) were published in the Eastern Asian countries/regions, especially in Japan (40 cases), China (26 cases) and Korea (8 cases) (Table 1). The reported number of colorectal cancer-related PLA has increased significantly over the past two decades. Approximately, 90% patients have been reported since 1990 after colonoscopy and percutaneous transhepatic abscess drainage (PTAD) became common in clinical practice. In Eastern Asian countries/regions, the number of cases was increasing rapidly, with a growth rate of approximately 4-5 times every decade from 1981 to 2011 (Figure 1).

Demographical evaluation
The average age of the patients in the present series was 64.4 ± 10.1 years. A male-to-female ratio of 1.5:1 was calculated for the overall series and for patients of different areas. There was no significant difference in the average age between gender groups (male 65.0 years *vs* female 63.4 years, $P > 0.05$) and geographical groups (Eastern Asia 64.6 years *vs* non-Eastern Asia 63.3 years, $P > 0.05$). In the overall series from both Eastern Asia and non-Eastern Asian groups, approximately 40% patients fell into the age groups of 61-70 years (Figure 2).

Table 1 Documented cases collected from the international literature

Country/region	No. of cases	No. of articles
Eastern Asia		
Japan	40	36[6-10]1
China	26	6[4,5,11-14]
Korea	8	1[15]
Singapore	3	1[16]
Middle East and Europe		
Israel	3	3[17-19]
Italy	2	2[20,21]
Spain	2	2[22,23]
Portugal	1	1[24]
France	1	1[25]
United Kingdom	1	1[26]
North and Central America		
United States	7	7[2,27-32]
Canada	1	1[33]
Netherlands Antilles	1	1[34]
Total	96	63

[1]Other 33 cases reported in a Japanese article reference were included[8].

Table 2 Constituent ratio of pus bacterial cultures *n* (%)

Pathogens	Eastern Asia (*n* = 49)	Non-Eastern Asia (*n* = 9)	Total (*n* = 58)
Bacteria			
Gram negative bacteria			
Klebsiella pneumoniae	28 (57.14)	1 (11.1)	29 (50.0)
Fusobacterium species	4 (8.16)	0	4 (6.90)
Bacteroides species	2 (4.08)	1 (11.1)	3 (5.17)
Escherichia coli	0	1 (11.1)	1 (1.72)
Pseudomonas aeruginosa	1 (2.04)	0	1 (1.72)
Gram positive bacteria			
Streptococcus species	1 (2.04)	3 (33.3)	4 (6.90)
Enterococcus faecium	2 (4.08)	0	2 (3.44)
Polymicrobial	0	2 (22.2)1	2 (3.44)
Amoebae	2 (4.08)	0	2 (3.44)
Negative	9 (18.37)	1 (11.1)	10 (17.24)

[1]Pus cultures showed mixed infection in two patients: *E. corrodens*, *Candida albicans* and *Candida glabrata*; *Peptostreptococcus anaerobius*, *Bacteroides melaninogenicus* and *Peptostreptococcus spp*.

Figure 1 Growth trend of reported cases in different countries/regions from 1960 to 2011.

Figure 2 Age distribution of reported cases in different countries/regions.

Microbiology

According to bacteria culture results from 58 patients, *Klebsiella pneumoniae* (*K. pneumoniae*) was the most common pathogen (50.0%), followed by *Fusobacterium species* (6.90%), *Streptococcus species* (6.90%), *Bacteroides species* (5.17%), *Enterococcus faecium* (3.44%), *Escherichia coli* (3.44%) and *Pseudomonas aeruginosa* (3.44%). There were two cases of amoebic liver abscess and two cases with mixed infection. In addition, 10 patients had negative results of pus cultures (Table 2). There was a significant difference between Eastern Asian and non-Eastern Asian groups in gram stains of pathogens. Among the gram-negative pathogens, *K. pneumoniae* was more dominant in Eastern Asian than in non-Eastern Asian groups (Figure 3).

Morphologic characteristics of liver abscess

All liver abscess cases were finally diagnosed with B-mode ultrasonic and/or computed tomography scanning. Ac-

cording to the image results of the 66 patients, 66.7% abscesses formed in the right liver lobe, 18.2% in the left lobe and 12.1% in two lobes (Figure 4). There was no significant difference in the location of liver abscess between Eastern Asian and non-Eastern Asian groups (Figure 5). Thirty-one reported cases had individual data of liver abscess size, which was 5.31 ± 2.11 cm on average. The average abscess size of Eastern Asian patients was smaller than that of non- Eastern Asian patients (*P* < 0.05). Moreover, there was no significant difference in the average size between gender groups, age groups and pathogen groups (Figure 6).

Clinical manifestations and diagnosis

The clinical manifestation of 25 patients was pyogenic liver abscess, including fever and chill (92.0%), abdominal pain (68.0%), abdominal tenderness (64.0%) and nausea/vomiting (45.0%). No more than 40% patients had the chief complaint of atypical symptoms of tumor, which included anemia (40.0%) and weight loss (32.0%). Only approximately 10% of the patients had bowl cancer symptoms, including bloody stool (12.0%) and alterations

Figure 3 Categorization of pathogens in Eastern Asian and non-Eastern Asian patients. A: Distribution of Gram-negative and Gram-positive pathogens; B: Distribution of *Klebsiella pneumoniae* (*K.P*) and non-*K.P*. There were significant differences in categories of pathogens between Eastern Asian and non-Eastern Asian patients ($P < 0.01$).

Figure 4 Distribution of colorectal cancer and liver abscesses.

Figure 5 Probability of abscess in different liver lobes in Eastern Asian and non-Eastern Asian patients. There was no significant difference between the two groups ($P > 0.05$).

colon (40.9%), followed by rectum (27.3%), ascending (18.2%), transverse (7.6%) and descending colon (6.0%) (Figure 4). Among the 23 patients with pathological reports, colorectal adenocarcinoma (81.8%) and medium differentiation degree (66.7%) were the most common pathological type and differentiation degree, respectively. There was no difference between Eastern Asian and non-Eastern Asian groups in the sites of colon tumors. However, tumors occurred more often in sigmoid colon and rectum in Eastern Asia group (Figure 8).

Treatment for colorectal cancer-related PLA

All of the patients were treated with broad-spectrum antibiotics. The most commonly used antibiotics were cephalosporins with or without metronidazole, followed by fluoroquinolones with or without metronidazole, ampicillin and gentamicin, carbapenems, and gentamicin (Table 3). PTAD was extremely successful as initial treatment for liver abscess. The type of treatment (antibiotics combined with/without PTAD) chosen may have been influenced by several factors (e.g., clinician/radiologist's decision and others). There was no significant difference in the distribution of demographic characteristics (age, gender, geographic distribution and pathogens) between the study and the comparison groups (Table 4). Until the date of the submission of the report, 17 patients had been followed up for an average of 15.4 ± 15.44 mo, all

in bowel habits (4.0%) (Figure 7). None of the cases had abdominal mass, and the digital rectal examinations were negative. Infection indices of the 25 patients with details of laboratory examination revealed a significantly increased level of white blood cell count ($17.9\text{-}5.22 \times 10^9/\text{L}$) and C-reactive protein (17.8 ± 7.49 mg/dL); and a moderately elevated level of alanine aminotransferase and total bilirubin. However, colorectal cancer-related biomarkers (including CA19-9 and carcinoembryonic antigen) did not elevate in most of the patients.

Monitoring of occult colorectal cancers

Most of the diagnoses of colorectal cancer were made by coloscopy (77.3%) and barium enema (29.2%). The most common site of tumor formation was sigmoid

Figure 6 Univariate analysis of abscess size in colorectal cancer related pyogenic liver abscess patients. A: Abscess sizes in different geographical groups; B: Abscess sizes in different gender groups; C: Abscess sizes in different age groups; D: Abscess sizes in different pathogenic groups. There was significant difference in liver abscess sizes between Eastern Asian and non-Eastern Asian patients ($P < 0.05$). *K.P*: *Klebsiella pneumoniae*.

Figure 7 Clinical presentations of colorectal cancer related pyogenic liver abscess patients.

Figure 8 Probability of cancer in different colorectal regions in Eastern Asian and non-Eastern Asian patients. A: Probability of cancer appeared in the right and left hemi-colon; B: Probability of cancer in sigmoid and rectum and other regions. Sigmoid and rectum were the more common cancer sites, compared with other regions ($P < 0.05$).

were kept stable with no tumor recurrence.

DISCUSSION

Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women all over the world. Previous studies have reported rapid increases in colorectal cancer incidence rates, especially in economically transitioning countries[35,36]. Many Eastern Asian countries, such as China, Japan, South Korea, and Singapore, have experienced a 2-4 folds increase in the incidence of colorectal cancer during the past few decades[37]. Common manifestations of colon cancer are

Table 3 Treatment for liver abscess complicated with colorectal carcinoma

Sex	Age, yr	Count of abscess	Anti-infection treatment	Surgical treatment	Ref.
Female	46	Mutiple[1]	Cephalosporin	Sigmoidectomy	[4]
Female	79	Single	Carbapenem	PTAD + low anterior rectal resection	[5]
Male	66	Single	Broad spectrum antibiotic[6]	PTAD + low anterior rectal resection	[6]
Male	73	Single	Broad spectrum antibiotic[6]	PTAD + sigmoidectomy	[7]
Male	65	Single	Broad spectrum antibiotic[6]	PTAD	[7]
Male	67	Single	Broad spectrum antibiotic[6]	PTAD	[7]
Male	81	Mutiple	Broad spectrum antibiotic[6]	Laparoscopic-assisted sigmoidectomy	[8]
Male	67	Mutiple[2]	Cephalosporin	PTAD + polypectomy under colonoscopy	[11]
Male	67	Single	Broad spectrum antibiotic[6]	PTAD + polypectomy under colonoscopy	[12]
Female	84	Single	Broad spectrum antibiotic[6]	None[7]	[12]
Female	82	Mutiple[3]	Cephalosporin	PTAD + sigmoidectomy	[13]
Female	57	Single	Cephalosporin + carbapenem + moxifloxacin + gentamicin	PTAD + sigmoidectomy + chemotherapy	[14]
Female	68	Single	Cephalosporin	PTAD + low anterior rectal resection	[16]
Male	67	Single	Cephalosporin	Polypectomy under colonoscopy	[16]
Male	77	Single[4]	Cephalosporin + ciprofloxacin + gentamicin	PTAD[7]	[16]
Female	66	Single	Broad spectrum antibiotic[6]	PTAD + sigmoidectomy	[18]
Male	64	Single	Broad spectrum antibiotic[6]	PTAD + right hemi-colectomy + partial hepatectomy	[19]
Male	60	Mutiple	Piperacillin + aminoglycoside	Right hemi-colectomy	[20]
Female	50	Single	Broad spectrum antibiotic[6]	Right hemi-colectomy + partial hepatectomy + chemotherapy	[21]
Male	68	Three	Amoxicillin + clavulanic acid	PTAD + sigmoidectomy	[22]
Male	72	Single	Cephalosporin + metronidazole + gentamicin	PTAD + right hemi-colectomy	[23]
Male	64	Two	Cephalosporin + metronidazole	Sigmoidectomy and radiotherapy	[24]
Female	82	Single	Cephalosporin +metronidazole	PTAD	[26]
Male	52	Mutiple[5]	Broad spectrum antibiotic[6]	Sigmoidectomy + chemotherapy + gemcitabine	[32]
Male	55	Single	Ampicillin + sulbactam + gentamicin	PTAD + sigmoidectomy	[34]

[1]Patient had endophthalmitis with brain, lung abscesses complicated with liver abscesses; [2]Patient developed *S. bovis* bacteremia with complications of endocarditis, osteomyelitis and silent splenic abscess after four episodes of *Klebsiella pneumoniae* liver abscess within 3 years; [3]Patient experienced three episodes of liver abscess within 1 year; [4]Complicated with pulmonary infections; [5]Patient experienced two episodes of liver abscess within 3 mo; [6]Details were not recorded; [7]Patients refused to have further treatments. PTAD: Percutaneous transhepatic abscess drainage.

Table 4 Different therapy for colorectal cancer related liver abscess patients *n* (%)

	No. of patients		*P* value
	Antibiotics alone (*n* = 16)	Antibiotics + drainage (*n* = 42)	
Gender			0.057
Male	13 (81.2)	23 (54.8)	
Female	3 (18.8)	19 (45.2)	
Age (yr)			0.631
< 60	2 (12.5)	5 (11.9)	
> 60	14 (87.5)	37 (88.1)	
Geographic distribution			0.564
Eastern Asia	13 (81.2)	35 (83.3)	
Non-Eastern Asia	3 (18.8)	7 (16.7)	
Pathogen			0.498
K. pneumoniae	6 (37.5)	14 (33.3)	
Non-*K. pneumoniae*	16 (62.5)	28 (66.7)	

K. pneumoniae: *Klebsiella pneumoniae*.

alteration in bowel habit, rectal bleeding and abdominal pain. Besides, liver abscess during the course of an undiagnosed colon cancer may also occur as the initial manifestation of the disease, even without associated metastasis. This PLA had been reported worldwide and was regarded as the herald of colorectal cancers.

Recently, the number of new cases of colorectal cancer-related PLA is soaring in Eastern Asia, and this trend is worthy of concern. We found that 80% of the reported cases in the whole world occurred in the Eastern Asian countries, especially in Japan, China and Korea. The demographic features of the Eastern Asian and non-Eastern Asian patients in this study were non-specific. The mean age was 64.4 years, and male to female ratio was about 1.5:1. However, microbiological feature was different between Eastern Asian and non-Eastern Asian patients. The bacteriologic analysis in our series revealed that *K. pneumoniae* was the most common pathogen in Eastern Asian patients. Our observation was also consistent with the entire incidence of *K. pneumoniae* PLA in Eastern Asian population[38-40].

Clinical features of the colorectal cancer-related PLA patients in this study were non-specific. The most common clinical presentations were fever, chills, abdominal pain, and nausea or vomiting. In contrast, only 10% patients had bowl cancer symptoms and the diagnosis of colorectal cancer was extremely difficult. Colonoscopy was considered as an effective screening method for diagnosis of colorectal cancer. Most (77.3%) of colorectal cancer patients in our study were confirmed by colonoscopy. The colon site of tumor mostly involved was the sigmoid colon (40.9%), followed by the rectum (27.3%), ascending colon (18.2%), transverse colon (7.6%) and descending colon (6.0%). There was significant difference of tumor site between Eastern Asian and non-Eastern Asian patients. Thus, Eastern Asian physicians should be vigilant in monitoring colorectal cancers, especially in the sigmoid colon and rectum.

In our case series, before colorectal cancers were found,

several patients had experienced recurrence of PLA after PTAD treatment[13,15,32]. Destruction of the mucosal barrier of colon and the repeated bacterial translocation was the pathogenesis of liver abscesses in such patients. Thus, although broad spectrum antibiotics combined with PTAD as first-line treatment for the management of PLA had been accepted by most physicians[41], operative intervention for colorectal lesions remains crucial. Meanwhile, in the elderly with recurrent cryptogenic PLA, colonoscopy is suggested and required to avoid misdiagnosis of colorectal cancer.

To our knowledge, this is the first attempt to systemically review the cases of colorectal cancer-related PLA worldwide. However, there still remain limitations to our retrospective study. Incomplete data collection was found during our review of literatures. Some clinical features appeared to have been overlooked; in particular, the relatively non-specific clinical symptoms were missing. In addition, although more reported cases were observed in Eastern Asian than non Eastern Asian countries, the real incidence of colorectal cancer-related PLA is still unknown. Epidemiological investigation with a larger sample size is needed for further analysis.

In conclusion, colorectal cancer-related PLA is an increasing life-threatening disease in Eastern Asia in the recent two decades. In the absence of significant manifestation, the search for the underlying cause of the pyogenic liver abscess should be an integral part of the management of liver abscesses. The association with a colorectal cancer is rare but should be taken into consideration. Early and appropriate surgical treatment can achieve good prognosis.

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COMMENTS

Background
Colorectal cancers related-pyogenic liver abscess (PLA) is a special hepatic infection and regarded as the herald of colon cancer. Recent publications from Eastern Asia revealed a considerable morbidity in this region. Knowledge of etiology and clinical features, when possible, play an important role in the successful treatment of colorectal cancers-related PLA patients.

Research frontiers
PLA, once occurs more frequently in elderly patients with hepatobiliary tract disease or intra-abdominal infections. Recently, many cases of PLA associated with nonmetastatic colorectal carcinomas have been reported in the worldwide. Interestingly, this PLA in Eastern Asia seems to have a greater morbidity and exhibits differences in clinical characteristics. However, there have been few studies to analyze this phenomenon.

Innovations and breakthroughs
This is the first attempt to systemically review the cases of colorectal cancer-related PLA worldwide. A total of 96 cases of colorectal cancers-related PLAs

were collected from the international literature and evaluated in clinicopathologic features. This study results revealed that *Klebsiella pneumoniae* (*K. pneumoniae*) PLA was tightly related with colorectal cancer (especially those located in sigmoid colon and rectum) in elderly Eastern Asian males.

Applications
By understanding the different clinicopathological features between patients from Eastern Asia and non-Eastern Asia countries, this study may represent a future strategy for therapeutic intervention in the treatment of patients with colorectal cancer-related PLA.

Terminology
PLA, occurs more frequently in elderly patients with hepatobiliary tract diseases or intra-abdominal infections. For all types of PLA, mucosal defects present within digestive tract lesions are regarded to be the key process. Recent studies revealed that a few PLA patients were related to neoplasms. This type of PLA exhibited different features and was regarded as a warning indicator of silent cancers.

Peer review
The authors reviewed the literature to evaluate features of colorectal cancer-related pyogenic liver abscess. It revealed that there was clear differences in the microbiological spectrum between Asian and non-Asian cases. Gram-negative bacteria especially *K. pneumoniae* PLA was predominant in Eastern Asia. Meanwhile, most of the Eastern Asian patients exhibited smaller size of liver abscess and atypical presentation. Sigmoid colon and rectum were the main sites of tumor in these patients. The results are interesting and may represent a clear understanding of colorectal cancer-related PLA in Eastern Asian patients.

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ORIGINAL ARTICLE

Targeting X-linked inhibitor of apoptosis protein inhibits pancreatic cancer cell growth through p-Akt depletion

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Abstract

AIM: To determine whether lentivirus-mediated shRNA targeting the X-linked inhibitor of apoptosis protein (XIAP) gene could be exploited in the treatment of pancreatic cancer.

METHODS: Human pancreatic cancer cells Panc-1, Mia-paca2, Bxpc-3 and SW1990, infected with lentivirus, were analyzed by real-time polymerase chain reaction (PCR). Western blotting was used to examine XIAP protein levels, survivin and p-Akt to confirm the result of real-time PCR and determine the possible mechanism. The 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure IC_{50} to determine chemosensitivity to the chemotherapeutic drugs 5-fluorouracil (5-FU) and gemcitabine. A colony assay, MTT assay and a tumorigenicity experiment were used to study cell proliferation *in vitro* and *in vivo*. Caspase-3/7 activity, 4',6-diamidino-2-phenylindole-staining and flow cytometric measurements were used to study apoptosis in SW1990 cells.

RESULTS: XIAP proteins were found to be differentially expressed among pancreatic cancer cell lines Panc-1, Mia-paca2, Bxpc-3 and SW1990. Data of real-time PCR and Western blotting showed that XIAP was reduced persistently and markedly by lentivirus-mediated shRNA. Downregulation of XIAP by transfection with XIAP shRNA resulted in decreased p-Akt expression. XIAP shRNA also inhibited the growth of pancreatic cancer cells *in vitro* and *in vivo*, enhanced drug-induced apoptosis and increased chemosensitivity to 5-FU and gemcitabine. Results also suggest that inhibition of XIAP and subsequent p-Akt depletion may have an anti-tumor effect through attenuating the ability of cancer cells to survive.

CONCLUSION: Lentivirus-mediated gene therapy is an attractive strategy in the treatment of pancreatic cancer and justifies the use of lentivirus in pancreatic cancer gene therapy studies.

Jiang C, Yi XP, Shen H, Li YX. Targeting X-linked inhibitor of apoptosis protein inhibits pancreatic cancer cell growth through

INTRODUCTION

Pancreatic cancer is one of the most aggressive human malignancies, with an extremely poor prognosis and a 5-year survival rate of only approximately 5%[1], partially due to the very little possibility of surgical resection and resistance to chemo-radiotherapy. Disordered apoptosis and abnormal proliferation have been linked with development of malignancy and treatment resistance[2,3]. Apoptosis, also termed programmed cell death, occurs *via* extrinsic or intrinsic signal transduction pathways[4,5]. Therefore, further understanding of the molecular mechanisms, the relationship between pancreatic cancer chemoresistance and disordered apoptosis and abnormal proliferation, can be important in trying to circumvent resistance to cancer therapy[6].

To date, 8 human inhibitor of apoptosis protein (IAP) family members [X-linked IAP (XIAP), cIAP1, cIAP2, IAP-like protein 2, melanoma IAP, neuronal apoptosis inhibitory protein, survivin and baculovirus IAP repeats repeat-containing ubiquitin conjugating enzyme] have been identified. XIAP, a member of the IAP family, plays an important role in regulating both apoptosis and cell proliferation. XIAP is one of the most important members of the IAP family. It is highly expressed in malignant tumor cells and promotes tumor cell invasion, metastasis, growth, survival and chemoresistance. It is reported that XIAP antagonists such as second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI increase caspase activity, and not only directly induce apoptosis of many types of tumor cell lines *in vitro*, but also suppress growth of established tumors in xenograft models in mice *in vivo*, while displaying little toxicity to normal tissues. These findings validate XIAP as a target for cancer gene therapy. XIAP is a key factor in malignancy development and treatment resistance, which is associated with disordered cell apoptosis and abnormal proliferation. It is clear that XIAP is one of the most efficient caspase inhibitors of the 8 proteins, and inhibition of apoptosis by XIAP is mainly coordinated through binding to initiator caspase-9 and effector caspase-3 and caspase-7[7-9]. In addition to caspase inhibition, XIAP induces nuclear factor-κB and mitogen-activated protein kinase activation during transforming growth factor-β and bone morphogenetic protein receptor signaling and overexpression. Akt (protein kinase B) represents a subfamily of serine/threonine kinases that promotes cell survival[10]. XIAP and Akt are functionally linked in maintaining homeostasis between cell death and cell proliferation[11]. XIAP may be a core link between the cell apoptosis signaling pathway and the cell survival signaling pathway. Targeting XIAP might simultaneously influence cell apoptosis and proliferation.

RNA interference (RNAi) is a sequence specific post-transcriptional gene silencing factor, which has been extensively used in the study of gene function and gene therapy for cancer[12]. The use of chemically synthesized small interfering RNA (siRNA) or siRNA-encoding plasmids to produce RNAi in mammalian cells by transfection is still limited in clinical application due to some disadvantages including transient expression, and low transfection efficiency especially in non-dividing cells and when it is necessary to generate long-time gene silencing *in vivo*. A lentivirus-based vector is considered to be a promising gene delivery tool because of its ability for specific, highly stable and functional knockout of gene expression in both dividing and non-dividing cells compared with retroviral vectors. In addition, there is minimal immunogenicity associated with lentivirus vectors compared with adenoviral vectors[13-15].

In this study, we constructed lentivirus vectors encoding shRNA targeting the human XIAP gene to study the possible mechanisms of the XIAP gene in regulating apoptosis and proliferation in pancreatic cancer. There was an inhibitory effect of XIAP gene shRNA on the growth of SW1990 cells *in vitro* and *in vivo*, which would be useful for the development of gene therapy approaches for pancreatic cancer treatment in clinical application.

MATERIALS AND METHODS

Construction and production of lentivirus vectors

Three self-complementary hairpin DNA oligos targeting XIAP mRNA and a negative control were synthesized and cloned into a lentivirus vector. A self-inactivating lentivirus vector pGCSIL-PUR (Genechem, Shanghai, China) containing a cytomegalovirus-driven puromycin and a U6 promoter upstream of the cloning restriction sites (*Age*I and *Eco*RI) was used. Three coding regions corresponding to targeting human XIAP (GenBank Accession No: NM_001167) were selected as shRNA target sequences under the guide of the shRNA design protocol. We constructed 3 shRNA-XIAP and negative control lentivirus vectors, namely Lv-X1, Lv-X2, Lv-X3 and Lv-Xnc, respectively (Table 1). Oligonucleotides were annealed and inserted between the *Age*I and *Eco*RI restriction sites of the lentivirus vector. They were confirmed by restriction mapping and DNA sequencing. Lentivirus vector DNA and packaging vectors (pHelper1.0, pHelper2.0) were then transfected into 293T cells. Forty-eight hours later, the supernatant containing the lentivirus particles was collected, filtered through the 0.45 μm cellulose acetate filters, and the titer of lentiviruses was determined by hole-by-dilution titer assay. The virus titers produced was approximately 10^9 TU/mL.

Cell culture and infection

SW1990, Panc-1, Mia-paca2 and Bxpc-3 human pancre-

Table 1 Sequences selected as target sequences of RNA interference against human X-linked inhibitor of apoptosis protein (NM_001167)

Name	Target sequences selected	Sequence cloned into the vector
Lv-X1	GGTGAAGGTGATAAAGTAA	f: 5'-CCGGTAGGTGAAGGTGATAAAGTAATTCAAGAGATTACTTTATCACCTTCACCTA TTTTTG-3'
		r: 5'-AATTCAAAAATAGGTGAAGGTGATAAAGTAATCTCTTGAATTACTTTATCACCTTCACCTA-3'
Lv-X2	CTTGAGGAGTGTCTGGTAA	f: 5'-CCGGCACTTGAGGAGTGTCTGGTAATTCAAGAGATTACCAGACACTCCTCAAGTGTTTTTG-3'
		r: 5'-AATTCAAAAACACTTGAGGAGTGTCTGGTAATCTCTTGAATTACCAGACACTCCTCAAGTG-3'
Lv-X3	GTGGTAGTCCTGTTTCAGC	f: 5'-CCGGAAGTGGTAGTCCTGTTTCAGCTTCAAGAGAGCTGAAACAGGACTACCACTTTTTTTG-3'
		r: 5'-AATTCAAAAAAAGTGGTAGTCCTGTTTCAGCTCTCTTGAAGCTGAAACAGGACTACCACTT-3'
Lv-Xnc	TTCTCCAACGTGTCACGT	f: 5'-CCGGTTCTCCAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTG-3'
		r: 5'-AATTCAAAAATTCTCCAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAA-3'

atic cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin) at 37 ℃ in a humidified incubator containing 5% CO_2. SW1990 cells were maintained in DMEM and was plated into 6-well plates at 1×10^5 cells per well. Overnight when the cells reached 30%-50% confluence, they were infected with viral particles in the presence of polybrene (5 μg/mL final concentration) and ENi.S (Genechem) for 8 h at a multiplicity of infection (MOI) of 50, and then added to fresh medium. The transfected SW1990 cells were subcultured at an appropriate density in fresh DMEM and 90% of the cells were transfected at 5 d post-transfection as indicated by the expression of green fluorescent protein (GFP) (pGCSIL-GFP empty vector was used to observe the transfection efficiency of lentivirus particles). Pooled stable transfectants were established using puromycin (Sigma-Aldrich, St Louis, MI, United States) selection. Puromycin was added into the medium to select stably transfected cells at a concentration of 1 μg/mL. Puromycin-resistant colonies were picked up 14 d after transfection and stable transfectant cells were maintained in medium containing 0.5 μg/mL puromycin.

Quantification by real-time polymerase chain reaction
Total RNA was isolated using Trizol (Invitrogen). M-MLV reverse transcriptase (Fermentas) was used to create cDNA according to the manufacturer's instructions: 1 μg RNA, Oligo dt18 as primer, 42 ℃ for 60 min, 70 ℃ for 5 min. Quantitative real-time polymerase chain reaction (RT-PCR) assays were carried out using SYBR TAQ real-time kits (TaKaRa Biotechnology, Otsu, Japan) and RT-PCR amplification equipment ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, United States). The PCR primers used to detect XIAP and β-actin were as follows: XIAP, upstream primer 5'-GACAG-TATGCAAGATGAGTCAAGTCA-3', downstream primer 5'-GCAAAGCTTCTCCTCTTGCAG-3', with a product length of 93 bp; β-actin, upstream primer 5' -ACTCTTCCAGCCTTCCTTCC-3', downstream primer 5'-GTACTTGCGCTCAGGAGGAG-3', with a product length of 232 bp. The quantitative RT-PCR parameters and analysis of results were performed as normal.

Western blotting analysis
The cell extracts were prepared with lysis buffer radio immunoprecipitation assay containing 50 mmol/L Tris-HCl, pH 7.3, 150 mmol/L NaCl, 2% NP-40, 0.5% deoxycholate, 2 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L NaF, and 1% Protease Inhibitor Cocktail (Pierce, Rockford, IL, United States). Total protein concentration was measured using the BCA assay kit (Sigma, Inc.) with bovine serum albumin as a standard according to the manufacturer's instructions. Western blotting was performed with primer and secondary antibodies: (1) Goat antihuman XIAP polyclonal antibody (R and D Systems Inc., Minneapolis, MN, United States) 1:2000, secondary antibody, 1:10 000; (2) Rabbit antihuman survivin antibody (Novus Biologicals, Inc.) 1:1000, secondary antibody, 1:10 000; (3) Rabbit antihuman p-Akt antibody (Abcam, Inc.) 1:300, secondary antibody, 1:10 000; and (4) Mouse antihuman β-actin monoclonal antibody (Sigma, Inc.) 1:10 000, secondary antibody, 1:10 000. Densitometry was performed by Quantity One image analysis software.

3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay
Cells were plated in 96-well plates at 5×10^3 (to test drug sensitivity) or 1×10^3 (for the growth curves) per well. Cell growth was examined by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after lentivirus transfection once a day for 8 d. The cells were cultured in DMEM overnight to ensure that they adhered to the wall of the plates. Various concentrations of 5-fluorouracil (5-FU) or gemcitabine were added to the medium. After incubation for 72 h, 20 μL of 5 mg/mL MTT (Sigma, Inc.) was added to the medium and cultured for another 4 h, then 150 μL of dimethyl sulfoxide (Sigma, Inc.) was added into each well and shaken for 10 min. Absorbance of each well was read using a Bio-Rad model 550-microplate reader (Bio-Rad Co., CA, United States) at a wavelength of 490 nm. Semilogarithmic curves were drawn for cell survival and the logarithm of the drug concentration by SPSS16.0. The 50% inhibitory concentration (IC_{50}) was determined according to the curves.

Colony assay
Approximately 1×10^3 non-transfected control cells and SW1990 cells stably transfected with Lv-Xnc, Lv-X1, Lv-X2, Lv-X3 were plated in 10-cm culture dishes. After 28 d, cells were fixed with methanol and stained with 0.1% crystal violet. Colonies were counted by visual inspection.

Caspase-3/7 activity

Caspase-3/7 activity was evaluated using the Caspase-Glo 3/7 Assay (Promega, Madison, United States) according to the manufacturer's instructions. Cells (1×10^5 cells per well) were seeded in a 6-well plate incubated at 37 ℃ overnight, then specific concentrations of 5-FU or gemcitabine were added into the medium. The ultimate concentrations for both 5-FU and gemcitabine were 1, 10 and 0.1, 1 μg/mL. Plates were further incubated at 37 ℃ for 72 h and luminescence was measured at 3 s delay-time, 10 s duration using SIRIUS Luminometer V3.2 (Berthold, Inc., Germany). Caspase-3/7 activity was normalized to the number of viable cells (as determined by trypan blue staining). Caspase-3/7-fold induction was determined as the ratio between caspase-3/7 activities in treated and control cells.

4',6-diamidino-2-phenylindole staining

Cells were seeded into 24-well plates on sterile round glass coverslips at a density of 2×10^4 cells per well. The cells were incubated in the medium with various concentration of 5-FU or gemcitabine for 72 h. Cells were then washed once with phosphate-buffered saline (PBS) and fixed in PBS containing 4% paraformaldehyde and 10% sucrose at room temperature for 15 min in the dark. Cells were labeled with 4',6-diamidino-2-phenylindole (DAPI) in PBS (1 μg DAPI/mL) at room temperature for 2 min in the dark. Thereafter, cells were washed twice with PBS and once with distilled water, and mounted in glycergel (60%, 4 μL). Staining was visualized *via* fluorescence microscope. The apoptosis index (AI) of cultured SW1990 cells with different lentivirus transfection was calculated using the following formula. AI (%) = apoptotic cells/total cells × 100%.

Flow cytometric measurements

Apoptosis was measured with an annexin V-fluorescein isothiocyanate Apoptosis Detection Kit (Beyotime institute of biotechnology, China). Cells were seeded in 6-well culture plates and divided into the following groups: non-transfected control, SW1990 cells stably transfected with Lv-Xnc, Lv-X1; SW1990 + 5-FU, Lv-Xnc + 5-FU, Lv-X1 + 5-FU; SW1990 + gemcitabine, Lv-Xnc + gemcitabine, Lv-X1 + gemcitabine. Each group contained three culture flasks. When the cells were 70%-80% confluent, cells were added with 1 μg/mL 5-FU or 0.1 μg/mL gemcitabine. After 72 h, the cells were harvested and washed in cold PBS. Annexin V and PI staining were carried out using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol. Apoptotic cells were immediately analyzed by fluorescence-activated cell sorting analysis.

Tumorigenicity experiments

To determine whether the Lv-X1 silence XIAP gene could inhibit tumor development *in vivo*, non-transfected control cells, Lv-Xnc control, Lv-X1 transfected SW1990 cells (1.5×10^7 cells in 200 μL DMEM) were injected subcutaneously into the left axilla of BALB/c nude mice (6 mice per group). Tumor growth was monitored every 4 d in 2 dimensions with a vernier caliper, and tumor size was calculated according to the formula $V = a^2b/2$, where a and b are the shortest and longest diameters, respectively.

Statistical analysis

All data are expressed as mean ± SD. Analysis was performed using analysis of variance or the Student t test. The relationship between XIAP protein level and IC_{50} was analyzed by Pearson linear correlation analysis. The criterion for significance was $P < 0.05$. All the statistical analysis was performed by SPSS16.0.

RESULTS

XIAP overexpression is associated with greater chemotherapeutic drug chemoresistance

Levels of XIAP expression were highest in Panc-1 and SW1990 cell lines with a higher degree of 5-FU and gemcitabine chemoresistance than Mia-paca2 and Bxpc-3, which expressed XIAP at relatively lower levels (Figure 1A and B).

Selection of the most effective suppression XIAP specific shRNA vector

In order to exclude an off-target silencing effect mediated by specific shRNA, we designed 3 different sequences targeting XIAP and selected the most effective Lv-shRNA in this study. Real-time RT-PCR was performed after transfection and selection with puromycin. The XIAP mRNA expression in Lv-X1, Lv-X2 and Lv-X3 transfected SW1990 cells were reduced by 62.48% ± 7.67%, 49.62% ± 4.7% and 54.47% ± 2.7%, respectively, compared with the Lv-Xnc transfected control ($P < 0.05$). In addition, no difference was observed between the Lv-Xnc control and the SW1990 control ($P > 0.05$) (Figure 1C). Western blotting revealed that the inhibition efficiencies on XIAP protein expression by Lv-X1, Lv-X2, and Lv-X3 lentivirus were consistent with that on the targeted genes' mRNA expression. XIAP protein was knocked down in Lv-X1, Lv-X2 and Lv-X3 transfected SW1990 cells, its expression demonstrated a significant reduction in Lv-X1 (5.98% ± 0.7%), Lv-X2 (12.32% ± 0.9%) and Lv-X3 (13.52% ± 2.2%) transfected SW1990 cells compared with the Lv-Xnc transfected control ($P < 0.05$). In addition, no difference was observed between the Lv-Xnc control and the SW1990 control ($P > 0.05$) (Figure 1D). According to the results of RT-PCR and Western blotting, Lv-X1 was the most effective lentivirus vector and thus we used it in the following research. To validate the specificity of RNAi targeting XIAP, we also determined the level of anther IAP family protein, survivin. The results showed that survivin was not affected by any constructed lentivirus (Figure 1D).

Figure 1 X-linked inhibitor of apoptosis protein expression analysis and selection of the RNAi target for X-linked inhibitor of apoptosis protein. A, B: The X-linked inhibitor of apoptosis protein (XIAP) protein level and IC_{50} for Panc-1, SW1990, Mia-paca2 and Bxpc-3 cell lines. R^2 = 0.87 (Spearman correlation) between IC_{50} and relative XIAP expression, $P < 0.05$; C: Relative expression of XIAP mRNA after transfection and selection with puromycin; D: Downregulatory effect of lentivirus-mediated RNAi on XIAP in SW1990 cells. Lv-X1, Lv-X2, Lv-X3 efficiently suppressed XIAP expression. The results also showed that survivin was not affected by any lentivirus. Lv-Xnc, which was transfected with the non-sense lentivirus vector, was set as the calibrator with the relative expression value of "1". β-actin was used as the internal loading control in three independent experiments. 5-FU: 5-fluorouracil.

Suppression of XIAP expression enhances drug-induced cytotoxicity and inhibits cell proliferation *in vitro* and *in vivo*

To determine the IC_{50}, cells were exposed to 1000, 100, 10, 1, 0.1, 0.01 and 0.001 μg/mL 5-FU or gemcitabine for 72 h. The IC_{50} was calculated from MTT cytotoxicity assay data. Lv-X1 (0.2 ± 0.01 μg/mL for 5-FU, 0.14 ± 0.03 μg/mL for gemcitabine), Lv-X2 (0.72 ± 0.08 μg/mL for 5-FU, 0.42 ± 0.02 μg/mL for gemcitabine), Lv-X3 (0.5 ± 0.05 μg/mL for 5-FU, 0.28 ± 0.01 μg/mL for gemcitabine) inhibited the IC_{50} significantly ($P < 0.05$ *vs* Lv-Xnc), and Lv-X1 was the most effective. Lv-Xnc control had no effect on the IC_{50} ($P > 0.05$ *vs* SW1990 control) (Figure 2A). When compared with SW1990 and Lv-Xnc control cells, Lv-X1, Lv-X2 and Lv-X3 infected SW1990 transfected cells showed much slower growth, especially Lv-X1 infected SW1990 transfected cells. XIAP shRNA lentivirus transfection knockdown of XIAP significantly inhibited the proliferation of cultured SW1990 cells *in vitro* at the time points from day 4 to day 8 ($P < 0.05$) (Figure 2B). The result of the colony formation assay indicated that the number of colonies of Lv-X1 cells (55.26% ± 3.74%) were much less than that of Lv-Xnc (100.6% ± 5.07%) and non-transfected cells (100% ± 6.04%) ($P < 0.05$) (Figure 2C). To determine whether Lv-X1 knockout of the XIAP gene could inhibit tumor development *in vivo*, SW1990 control cells, Lv-Xnc control, and Lv-X1 infected SW1990 cells were injected into BALB/c nude mice and tumor growth was monitored every 4 d. At the time points of day 16 to day 28 after SW1990 implantation, the tumorigenicity experiments revealed that suppression of XIAP in the Lv-X1 group significantly inhibited the growth of the transplanted tumor in nude mice, which was consistent with the results achieved from the experiments *in vitro* ($P < 0.05$). For example, the tumor volume in nude mice at day 28 after the inoculation of SW1990 cells in groups of non-transfected, Lv-Xnc and Lv-X1 were 1223.28 ± 176.14, 1173.45 ± 149.61 and 532.83 ± 84.59 mm^3, respectively. When compared with SW1990 and Lv-Xnc control cells, Lv-X1 infected SW1990 transfected cells developed much smaller tumors in the nude mice ($P < 0.05$) (Figure 2D).

XIAP specific silencing enhances drug-induced activation of caspase-3/7 and enhances drug-induced apoptosis

5-FU or gemcitabine induced apoptosis in cancer cells, *via* caspase activation and inhibition of apoptosis by XIAP, was mainly coordinated through binding to effector caspase-3 and caspase-7. Thus, we sought to determine the effect of XIAP silencing on caspase activities after exposure to different concentrations of 5-FU or gemcitabine for 72 h. 5-FU or gemcitabine-induced caspase-3/7 activity was markedly increased after transfection of Lv-X1 (4.41 ± 0.51-fold, 23.31 ± 8.14-fold at 1,

Figure 2 Lentivirus-mediated shRNA targeting the X-linked inhibitor of apoptosis protein gene inhibits the growth of human pancreatic cancer SW1990 cells *in vitro* and *in vivo* and promotes chemosensitivity to drugs. A: Lv-X1, Lv-X2, Lv-X3 promoted 5-fluorouracil (5-FU) or gemcitabine-induced cytotoxicity. The IC$_{50}$ was determined by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay after exposure to 5-FU or gemcitabine for 72 h; B: The growth curves of different stably transfected cell lines; C: Results of colony formation assay. Lv-X1 had much fewer colonies than Lv-Xnc and untransfected cells. These experiments were performed 3 times; D: SW1990, Lv-Xnc and Lv-X1 cells growth in BALB/c nude mice ($P < 0.05$, from day 12 to day 28).

10 μg/mL 5-FU, respectively; 20.26 ± 0.96-fold, 36.62 ± 3.77-fold at 0.1, 1 μg/mL gemcitabine, respectively), but was unaffected after transfection of Lv-Xnc control. In addition, blank Lv-X1 (1.07 ± 0.03-fold) and Lv-Xnc (1.03 ± 0.01-fold) caspase-3/7 activity, although slightly increased, were not significantly different compared with SW1990 control (Figure 3A), which was also confirmed by DAPI staining and flow cytometry (FCM) (Figure 3B and C). We used DAPI staining to observe the morphological changes of apoptosis and the apoptosis index of DAPI staining analysis was consistent with caspase-3/7 activity (Figure 3B). To validate the results of DAPI staining, SW1990 and stably transfected SW1990 cells (Lv-Xnc, Lv-X1) were stained with annexin V and PI and analyzed by FCM. Cell apoptosis analysis indicated that downregulation of XIAP was not associated with a significantly increased spontaneous apoptosis rate (there were no obvious differences in apoptosis rates among SW1990 (3.03% ± 0.49%), Lv-X1 (5.06% ± 0.54%) and Lv-Xnc (4.21% ± 0.36%) ($P > 0.05$)), while the apoptosis of Lv-X1 + 5-FU (12.7% ± 0.50%) or Lv-X1 + Gem (13.68% ± 0.56%) was significantly increased compared with SW1990 and Lv-Xnc control cells ($P < 0.05$) (Figure 3C). All these results showed that the lentivirus-mediated inhibition of XIAP expression did not lead to acceleration of the apoptosis of SW1990 cells.

Downregulation of XIAP with Lv-X1 decreased p-Akt levels

We observed that decreased expression of XIAP resulted in inhibition of cell proliferation according to the results of the growth curves and colony formation assay of different stably transfected cell lines (Figure 2B-D). Caspase-3/7 activity, DAPI staining and FCM analysis of Lv-X1 groups although slightly increased, showed no significant differences compared with controls (Figure 3A-C). It is reported that apoptotic pathways in cancer functionally crossover with survival pathways PI3K/Akt, and compared with the Lv-Xnc group, XIAP protein expression levels in cells transfected with Lv-X1 were reduced by 92.55% ± 0.78% ($P < 0.05$), and p-Akt protein expression in Lv-X1 transfection groups was reduced by 94.63% ± 0.32% ($P < 0.05$) (Figure 4). We found that downregulation of XIAP with Lv-X1 decreased p-Akt levels, which might explain the phenomenon.

DISCUSSION

It has been reported that XIAP is overexpressed in many human malignancies such as ovarian carcinoma, laryngeal cancer, esophageal cancer, breast cancer, hepatoma cells, colon cancer, and pancreatic cancer. Upregulated levels of XIAP expression have been correlated with

Figure 3 Apoptosis analysis. A: Relative enzymatic activity of caspase-3/7; B: Apoptosis examined by 4',6-diamidino-2-phenylindole (DAPI) staining (200×), apoptosis index (%) = Apoptotic cells/Total cells × 100% (Seen in a same microscopic field and 3 randomized microscopic fields chosen for counting) using DAPI staining. Either gemcitabine or 5-fluorouracil (5-FU) kills cancer cells by inducing apoptosis, and X-linked inhibitor of apoptosis protein knocked out by Lv-X1 can enhance the capacity of the 2 drugs; C: Flow cytometry: The apoptotic rate of Lv-X1 and Lv-Xnc slightly increased but there was no significant difference among SW1990, Lv-X1 and Lv-Xnc. While the apoptotic rate of Lv-X1 + 5-FU and Lv-X1 + gemcitabine increased (12.7% and 13.68%, respectively, *P* < 0.05). FITC: Fluorescein isothiocyanate.

tumor resistance to chemotherapy or radiotherapy, and some researchers have reported that inhibition of XIAP by siRNA, plasmid or adenovirus mediated-shRNA can reduce tumor cell growth, induce apoptosis and enhance the sensitivity of tumor cells to chemotherapeutic or radiotherapeutic agents[16-21]. Though chemically synthesized siRNA can be introduced into cells *via* traditional delivery strategies including liposomes, polyethyleneimine, or electroporation, these methods have short gene silencing effects and the effects cannot be passed to cell progeny. Other gene delivery systems such as adenovi-

rus showed transient expression of the transgene and immunogenicity. Lentivirus vectors were developed to overcome these disadvantages. Recently, the lentivirus vector-mediated gene therapy including RNAi or overexpression has shown great promise in pancreatic cancer and other tumors because of its long-term gene expression and high efficiency in transducing dividing and non-dividing cells[22-25].

As we know, Panc-1 and Mia-paca2 are poorly differentiated; SW1990 and Bxpc-3 are moderately differentiated. In our study, human pancreatic cancer cell lines

Figure 4 Immunoblot analysis for p-Akt in SW1990 cells after stable transfection. Downregulation of X-linked inhibitor of apoptosis protein with Lv-X1 largely decreased p-Akt protein levels and survivin was not affected (6 mo in culture).

with higher levels of XIAP protein displayed greater 5-FU or gemcitabine chemoresistance (Figure 1A and B).

In this study, we found that lentivirus-based vectors were extremely efficient in transducing SW1990 cells. *In vitro*, 79% of the cells expressed GFP at a MOI of 50. In addition, GFP expression was stable up to 6 mo in culture (data was not shown). To determine whether lentivirus-mediated shRNA could stably inhibit the expression of XIAP, pooled clones were expanded, and stably transfected cells were lysed for Western blotting analysis 6 mo later. The results showed that the expression of XIAP in Lv-X1 cell was still markedly reduced (Figure 4). It indicated that lentivirus-based shRNA resulted in a persistent gene knockdown instead of a transient inhibitory effect.

Furthermore, the strong inhibition of XIAP by lentivirus could inhibit proliferation of SW1990 cells (Figure 2B-D), enhance drug-induced apoptosis and promote chemosensitivity to chemotherapeutic drugs, which also were reported in other studies (Figure 3A-C)[26,27], and some drugs induce apoptosis through downregulation of cell survival proteins and upregulation of death receptors *via* the reactive oxygen species-mediated upregulation of the CHOP (CCAAT/enhancer-binding protein-homologous protein) pathway[28]. Some studies showed that silencing the XIAP gene resulted in a significant induction of apoptosis in pancreatic cancer cells Mia-paca2 and AsPC-1[29]. In this study, we did not find that downregulation of XIAP was associated with a significantly increased spontaneous apoptosis rate (Figure 3A-C), which was consistent with some other previous studies[30]. To validate the specificity of RNAi targeting XIAP and to avoid off-target phenomenon, we transfected the cells with lentivirus carrying 3 different shRNA sequences against XIAP and determined the level of another IAP family protein survivin (Figures 1-4). The results showed that survivin was not affected by any constructed lentivirus, which was not consistent with previous studies[31].

One of the greatest challenges for researchers in new treatment strategies for pancreatic cancer is the obvious need to test them in preclinical *in vivo* animal models that have a good probability of being predictive of similar activity in humans. The most used models are xenografts

of human tumors grown subcutaneously in immunodeficient mice such as BALB/c nude mice[32]. In this study, we determined whether SW1990 cells stably transfected with lentivirus have reduced tumorigenicity. In the xenograft model, BALB/c nude mice that received injection of Lv-X1 cells developed tumors of a smaller size compared with control, and also showed higher levels of XIAP protein and greater tumorigenicity (Figure 2D). These findings were consistent with some previous studies in xenograft models, in which tumors from the parental pancreatic carcinoma cells with stable XIAP knockdown clones showed growth retardation[33].

Akt, which has been shown to have both prosurvival and antiapoptotic functions, is a serine/threonine kinase that is known to have at least 3 isoforms (Akt1, Akt2 and Akt3), all of which are activated by phosphoinositide 3-kinase (PI3K), and p-Akt is an activity form of Akt. It is reported that total Akt (phosphorylated + non-phosphorylated) was not altered by XIAP, nor was the expression of the p85 subunit of PI3K, suggesting a direct influence of XIAP upon Akt activation rather than an upregulation of Akt expression[34]. One of the Akt substrates identified to have antiapoptotic effects is Bad, which is the proapoptotic Bcl-2 family member that initiates apoptosis *via* binding antiapoptotic Bcl-2 family members and results in the release of cytochrome c from mitochondria. Akt has also been shown to directly phosphorylate and inactivate caspase-9[10,35], which is coordinated through binding to XIAP. Recently, XIAP was added to the list of Akt substrates. Akt has been shown to prevent the ubiquitination and degradation of XIAP *via* phosphorylation both *in vitro* and *in vivo*, and XIAP is a downstream target of Akt and a potentially important mediator of the effect of Akt on cell survival[36]. XIAP is also thought to promote Akt activity, XIAP acting as an E3 ubiquitin ligase for PTEN and promotes Akt activity by regulating PTEN content and compartmentalization, while XIAP silencing reduces constitutive mono- and poly-ubiquitination of PTEN, increases PTEN protein levels, and prevents nuclear accumulation of PTEN[37]. Furthermore, it has been reported that suppression of XIAP by either siRNA or antisense adenovirus of XIAP induced apoptosis and inhibited Akt-stimulated cell survival in ovarian cancer cells and uterine cancer cells[11,34]. These results are significant because they suggest a feedback regulation of XIAP and Akt. In this study, we found stable downregulation of XIAP with Lv-X1 in SW1990 pancreatic cancer cells markedly decreased the p-Akt protein level (Figure 4). Perhaps XIAP is a potentially important mediator of the effect of Akt on cell survival in pancreatic cancer cells as well as in ovarian and uterine cancer cells. While XIAP upregulates Akt phosphorylation and requires Akt for its function, so stable downregulation of XIAP markedly decreases the p-Akt protein level in SW1990 pancreatic cancer cells. Thus, it appears that there is an intricate, coordinated regulatory system at play between XIAP and the PI3K/Akt signaling pathway. Additional studies are necessary

to determine the precise molecular mechanism by which XIAP regulates the Akt survival pathway.

In summary, our findings demonstrate for the first time that suppression of XIAP expression *via* lentivirus-mediated shRNA represents a novel strategy for chemosensitizing pancreatic cancer cells to chemotherapeutic drugs. Our study indicates that lentivirus-mediated inhibition of XIAP is an attractive therapeutic strategy in the treatment of pancreatic cancer and justifies the use of lentivirus in cancer gene therapy studies. However, emergence of replication competent lentivirus *in vivo*, transcriptional targeting affected by the chromosomal integration site and risk of oncogene activation by the lentivirus are current problems, and an effective and safe protocol should be developed. Thus, there remains a long road before lentivirus-mediated shRNA targeting XIAP can be introduced into clinical use.

COMMENTS

Background

Pancreatic cancer is one of the most aggressive human malignancies with an extremely poor prognosis and a 5-year survival rate of only approximately 5%, partially because of the low possibility of surgical resection and resistance to chemo-radiotherapy.

Research frontiers

It has been reported that X-linked inhibitor of apoptosis protein (XIAP) is overexpressed in many human malignancies. The upregulated levels of XIAP expression have been correlated with tumor resistance to chemotherapy or radiotherapy. Lentivirus vector-mediated gene therapy has great promise in pancreatic cancer because of its long-term gene expression and high efficiency. Thus it is necessary to determine whether lentivirus-mediated shRNA targeting XIAP gene could be exploited in the treatment of pancreatic cancer.

Innovations and breakthroughs

XIAP proteins were found to be differentially expressed among pancreatic cancer cell lines. Downregulation of XIAP by transfection with XIAP shRNA resulted in decreased p-Akt expression. Moreover, it could inhibit the growth of pancreatic cancer cells *in vitro* and *in vivo* and enhance drug-induced apoptosis and promote chemosensitivity to chemotherapeutic drugs 5-fluorouracil and gemcitabine. Results also suggest that inhibition of XIAP and subsequent p-Akt depletion may have an anti-tumor effect through attenuating the ability of cancer cells to survive. Perhaps XIAP is a potentially important mediator of the effect of Akt on cell survival in pancreatic cancer cells. It appears that there is an intricate, coordinated regulatory system at play between XIAP and the phosphatidylinositol 3-kinases/Akt signaling pathway.

Applications

The results suggest that suppression of XIAP expression *via* lentivirus-mediated shRNA represents a novel strategy for chemosensitizing pancreatic cancer to chemotherapeutic drugs. Lentivirus-mediated inhibition of XIAP is an attractive therapeutic strategy in the treatment of pancreatic cancer and justifies the use of lentivirus in cancer gene therapy studies.

Terminology

XIAP: A member of the IAP family, plays an important role in regulating both apoptosis and cell proliferation; Apoptosis or programmed cell death: Disordered apoptosis and abnormal proliferation have been linked to malignancy development and treatment resistance; Akt: Also termed protein kinase B, represents a subfamily of serine/threonine kinases that promotes cellular survival. RNAi: RNA interference, a sequence specific posttranscriptional gene silencing process, which has been extensively used in the study of gene function and gene therapy for cancer.

Peer review

This is an interesting manuscript. The data is solid, but it is still a long way from lentivirus-mediated shRNA targeting XIAP to clinical use.

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Impact of ribavirin dose on retreatment of chronic hepatitis C patients

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Abstract

AIM: To study the efficacy and factors associated with a sustained virological response (SVR) in chronic hepatitis C (CHC) relapsing patients.

METHODS: Out of 1228 CHC patients treated with pegylated interferon (PEG-IFN) and ribavirin (RBV), 165 (13%) had a relapse. Among these, 62 patients were retreated with PEG-IFN-α2a or -α2b and RBV. Clinical, biological, virological and histological data were collected. Initial doses and treatment modifications were recorded. The efficacy of retreatment and predictive factors for SVR were analyzed.

RESULTS: An SVR was achieved in 42% of patients. SVR was higher in young (< 50 years) (61%) than old patients (27%) (*P* = 0.007), and in genotype 2 or 3 (57%) than in genotype 1 or 4 (28%) patients (*P* = 0.023). Prolonging therapy for at least 24 wk more than the previous course was associated with higher SVR rates (53% *vs* 28%, *P* = 0.04). Also, a better SVR rate was observed with RBV dose/body weight > 15.2 mg/kg per day (70% *vs* 35%, *P* = 0.04). In logistic regression, predictors of a response were age (*P* = 0.018), genotype (*P* = 0.048) and initial RBV dose/body weight (*P* = 0.022). None of the patients without a complete early virological response achieved an SVR (negative predictive value = 100%).

CONCLUSION: Retreatment with PEG-IFN/RBV is effective in genotype 2 or 3 relapsers, especially in young patients. A high dose of RBV seems to be important for the retreatment response.

INTRODUCTION

Major advances have been made in the treatment of chronic hepatitis C (CHC) over the last decade. However, only 50% of patients will achieve a sustained virological response (SVR) with the combination of pegylated interferon (PEG-IFN)-α and ribavirin (RBV), the reference standard of care[1,2]. Hence, non-response and relapse are major issues. Approximately 30% of CHC patients with undetectable hepatitis C virus (HCV) RNA at the end of therapy (EOT) will experience relapse[3].

Although its mode of action is not completely understood, RBV is clearly needed to improve SVR rates when combination therapy with PEG-IFN is prescribed[4]. The optimal dose of RBV to maintain the highest SVR rates differs according to genotype. The recommended RBV dose is 1000/1200 mg/d and 800 mg/d in HCV genotype-1 and genotype-2 or -3 infected patients, respectively[2,5]. Controversial studies showed that RBV dose, as well as RBV reduction and/or discontinuation during the first 12-24 wk of treatment could have an impact on the treatment response[6-12].

In addition to RBV dose and cumulative exposure, viral and host factors associated with a virological response were identified in naïve patients. The likelihood of a response is higher when patients have an early and long period of undetectable HCV RNA[13]. Patients who attain a rapid virological response and early virological response (EVR) have lower rates of relapse[14]. Also, older age, advanced liver fibrosis, high baseline viral load, infection with HCV genotype 1 and co-infection with human immunodeficiency virus (HIV) are known factors associated with treatment failure[2,13,15,16]. Recent data suggests that the type of PEG-IFN also has an impact on the outcome of HCV treatment. In the IDEAL trial, PEG-IFN-α2a and PEG-IFN-α2b in combination with RBV were compared. Although the EOT response was lower with PEG-IFN-α2b, higher relapse rates were observed with PEG-IFN-α2a. Therefore, the rates of SVR did not differ between the two types of PEG-IFN[13].

In contrast to the well-defined management of new HCV patients, relapsers are a challenge nowadays. There are no proven guidelines for retreatment of relapsers. Retreatment studies have been made based on heterogeneous groups. The majority of reports included both non-responders and relapsers and different previous therapies: IFN monotherapy, IFN plus RBV or PEG-IFN monotherapy. Overall SVR rates of 13%-50% were obtained in retreatment of patients who failed previous IFN-based therapy, with a higher SVR in former relapsers than in non-responders[17-21].

Thus, retreatment of relapsers with PEG-IFN plus RBV has not been well studied. The aim of this study was to evaluate, outside of trials, the efficacy of retreatment, and predictors of response, in a population of CHC relapsers after a previous course of PEG-IFN and RBV.

MATERIALS AND METHODS

Patient selection

Patients with CHC who relapsed after a previous course of PEG-IFN-α2a or PEG-IFN-α2b in combination with RBV were eligible. Patients previously treated for at least 12 wk, with undetectable HCV RNA at the end of treatment and recurrence of viremia during 24-wk post-treatment follow-up were included in this retrospective cohort study. Exclusion criteria were co-infection with human immunodeficiency virus or HBV, the presence of any other cause of liver disease, decompensated liver disease and a history of hepatocellular carcinoma.

Study design

Patients were treated with PEG-IFN-α2a at a dose of 180 μg per week, plus weight-based oral RBV as previously described or PEG-IFN-α2b at the standard dose of 1.5 μg/kg body weight per week, in combination with oral RBV at a dose of 800-1200 mg per day, according to genotype and body weight[2,22]. The duration of therapy was determined according to genotype, duration of previous therapy, initial virological response and tolerability. Treatment prolongation and high RBV dose administration were decided case by case according to the physicians' discretion. All patients had a post-treatment follow-up of at least 24 wk.

Assessments

Serum HCV RNA level was measured at treatment initiation, treatment week 4, every 12 wk during the treatment period; and during post-treatment follow-up at weeks 4, 12 and 24. HCV RNA was detected qualitatively with the use of transcription-mediated assay (VERSANT HCV RNA Qualitative Assay; Siemens Medical Solution Diagnosis), which has a sensitivity of 9.6 IU/mL. A rapid virological response (RVR) was defined as undetectable HCV RNA at week 4 of treatment. An EVR was defined according to HCV viral load at week 12 and categorized as: no EVR (reduction of less than 2 log in HCV viral load compared with the baseline level); partial EVR (pEVR): reduction greater than 2 log; and complete EVR (cEVR): undetectable HCV RNA. Response to treatment was based on HCV RNA measurement at the end of therapy and at week 24 of follow-up. Non-responders were defined as detectable HCV RNA at EOT. Relapsers were defined as HCV RNA undetectable at EOT but detectable within the 24-wk follow-up period. An SVR was defined as negative HCV RNA 24 wk after cessation of therapy. Pretreatment liver biopsies were analyzed by a single pathologist using the META-VIR scoring system.

Patients were evaluated for tolerability and safety by physical examination and laboratory evaluation, including hematological and biochemical analyses. Dose reductions or discontinuation of PEG-IFN or RBV (or both)

Table 1 Baseline characteristics *n* (%)	
	All patients (*n* = 62)
Male gender	45 (72.6)
Mean age, yr ± SD	52 ± 9
Mean weight, kg ± SD	76 ± 14
Mean BMI, kg/m^2 ± SD	26 ± 4
Abnormal ALT	54 (90)
Abnormal GGT	36 (67)
Mean hemoglobin, g/dL ± SD	14.8 ± 1.5
HCV RNA	
> 600 000 IU/mL	11 (28)
METAVIR fibrosis score	
F2	20 (34)
F3	11 (19)
F4	23 (39)
Steatosis	
< 5%	13 (21)
5%-30%	23 (37)
> 30%	26 (42)

BMI: Body mass index; ALT: Alanine aminotransferase; GGT: γ-glutamyl transferase; HCV: Hepatitis C virus.

were performed when appropriate, in accordance with guideline recommendations.

Statistical analysis

Univariate analysis was performed to evaluate treatment response and baseline characteristics. Categorical variables were compared using χ^2 or F tests. Continuous variables were analyzed with the Student t test or Mann-Whitney U test as appropriate. Predictors of response were identified and entered in a stepwise logistic regression in order to assess their association with SVR. Statistical significance was defined as $P < 0.05$ and all comparisons were two-tailed. Statistical analysis was performed using SPSS, version 12.0 (SPSS Inc., Chigago, IL).

RESULTS

Patient population

Of 1228 CHC patients treated with a combination of PEG-IFN-α plus RBV in the Hepatology Department of Hôpital Beaujon, 165 (13%) patients were identified as relapsers and were eligible for this study. Retreatment was proposed for 75 patients. Among these, 62 consecutive patients were retreated between April 2003 and June 2008 and finished their follow-up period. Retreatment was prescribed with the same type of PEG-IFN-α used in the prior PEG-IFN combination treatment in 53% of patients. Median duration of therapy was 48 wk (16-72 wk). Retreatment was at least 24 wk longer than previous therapy in 51% of patients. Initial dose of RBV was >13.3 mg/kg per day in 54%. A high dose of RBV (daily doses > 15.2 mg/kg[22]) was prescribed in 16% of patients.

Baseline demographic, clinical, biochemical, virological and histological characteristics are summarized in Table 1. The mean age of the patients was 52 years, and approximately 73% were male; 57% had a body mass index (BMI) > 25 kg/m^2. Serum alanine aminotransferase

Table 2 Treatment characteristics		
	No. of patients, *n* (%)	SVR (%)
Overall population	62 (100)	42
Type of PEG-IFN (retreatment)		
PEG-IFN-α2a	43 (69)	40
PEG-IFN-α2b	19 (31)	47
RBV ⩾ 13.3 mg/kg per day	34 (54)	35
RBV ⩾ 15.2 mg/kg per day	10 (16)	70
Treatment duration 24 wk longer than previous course	31 (51)	53
Patients with RBV ⩾ 15.2 mg/kg per day and 24 wk longer duration	6 (10)	67

Sustained virological response (SVR) according to different types of pegylated interferon (PEG-IFN), dose of ribavirin (RBV) and duration of therapy.

and γ-glutamyl transferase (GGT) levels were abnormal in 90% and 67% of patients, respectively. Forty-eight patients were infected with HCV genotype 1. High viral load (> 600 000 IU/mL) was observed in 28%. Necro-inflammatory activity was mild (A1) in 51% of patients, 34% had F2 fibrosis, 19% had advanced fibrosis (F3) and 39% had cirrhosis (F4). Steatosis was absent (< 5%) in 21%, mild (5%-30%) in 37%, and moderate or severe (> 30%) in 42% of patients.

Response to treatment

After retreatment with PEG-IFN and RBV, the overall SVR rate was 42%. An EOT response was achieved by 77% of patients (48/62); among them, 46% (22/48) again experienced a relapse. Patients < 50 years achieved a higher SVR rate (61%) when compared to older patients (27%) ($P = 0.007$). Female and male patients had SVR rates of 53% and 38%, respectively, but with no significant difference ($P = 0.28$). There was a trend for higher SVR rates in patients with normal baseline GGT (61% *vs* 36%, $P = 0.081$) and lower BMI (mean BMI 24.6 in SVR *vs* 26.5 in non responder, $P = 0.071$). In addition, patients infected with genotype 2 or 3 had higher SVR than those with genotype 1 or 4 (57% *vs* 28%, $P = 0.023$) (Figure 1A). SVR rates were similar regarding low and high viral load (41% *vs* 36%, $P = 0.77$). Necro-inflammatory activity, fibrosis and steatosis did not influence SVR rates.

Treatment responses according to dose and duration are summarized in Table 2. There was no difference between retreatment response with PEG-IFN-α2a or PEG-IFN-α2b regarding EOT (74% *vs* 84%, $P = 0.52$) and SVR rate (40% *vs* 47%, $P = 0.56$). Relapse rates were similar between groups (35% *vs* 37%, $P = 0.68$). In patients retreated with a different type of PEG-IFN-α from prior therapy, SVR was achieved in 36%, similar to that in patients retreated with the same PEG-IFN, who attained an SVR rate of 46% ($P = 0.39$). Retreatment for at least 24 wk longer than the previous therapy was associated with a higher SVR rate (53% *vs* 28%, $P = 0.044$). A high initial dose of RBV was associated with a higher likelihood of SVR. Although EOT response rates

Figure 1 Sustained virological response rates according to age, genotype and initial ribavirin dose. A: Sustained virological response (SVR) rates according to age, genotype; B: SVR rates according to initial ribavirin dose.

Figure 2 Predictive value of complete early virological response. Among 46 patients with complete early virological response (cEVR), 25 (54%) patients achieved an sustained virological response (SVR) and 21 (46%) were non-responders (17 patients had a relapse and 4 patients had a non-response). Positive predictive value of cEVR for SVR = 54% (*P* = 0.003). Negative predictive value of cEVR for SVR = 100% (*P* = 0.003). HCV: Hepatitis C virus; NR: Non response.

were not statistically different between groups (90% *vs* 75%, *P* = 0.43), patients who received > 15.2 mg/kg per day had a superior SVR rate when compared to patients receiving lower doses (70% *vs* 35%, *P* = 0.041) (Figure 1B). These results were related to a lower rate of relapse among patients with a high dose of RBV (20% *vs* 39%). Regarding RBV dose reduction, no impact on SVR rates was observed (43% among those patients without a reduction *vs* 33% with a dose reduction, *P* = 0.75).

Tolerability and dose reduction
Retreatment with combination therapy was well tolerated. Seventy-nine percent of patients did not reduce their initial dose of PEG-IFN and/or RBV. Only 4 patients (6.6%) had a reduction in PEG-IFN dose, 3 of whom had clinical intolerance with asthenia, and one had marked neutropenia. The reduction in RBV dose was necessary in 12 patients (19.7%), with anemia being the major reason (58%). Among all patients, only 2 had cumulative RBV doses lower than 80% of the predicted dose. High initial doses of RBV did not seem to influence RBV reduction. In patients with an initial dose of RBV > 13.3 mg/kg per day and in those with > 15.2 mg/kg per day, 26% and 30% of patients needed RBV dose reduction. The treatment was stopped earlier than the proposed therapy duration in 11 patients (18%). Among these, 10% did not achieve a virological response at week 24, and treatment was discontinued.

Predictors of response
The factors identified in bivariate analysis as possibly associated with SVR and entered in a logistic regression model were: age, genotype, and high dose RBV. In the stepwise logistic regression analysis, the predictors of SVR were age [< 50 years *vs* ≥ 50 years; odds ratio (OR), 4.26; 95% CI: 1.28-14.19], genotype (G2/3 *vs* G1/4; OR, 3.55; 95% CI: 1.01-12.46), and high dose RBV (> 15.2 mg/kg per day *vs* <15.2 mg/kg per day; OR, 6.99; 95% CI: 1.32-36.97). Younger patients with genotype 2 or 3 can attain an SVR of 59%, while older patients, infected with genotype 1 or 4 only had an SVR of 10%. None of the patients of older age, genotype 1 or 4, and RBV initial dose < 15.2 mg/kg per day achieved an SVR.

Predictive value of rapid virological response and early virological response
RVR was assessed in 39 patients; 18% (7/39) achieved an RVR, and 5 of these achieved an SVR [positive predictive value (PPV) = 71%]. In addition, RVR had a negative predictive value (NPV) for SVR of 88% (*P* = 0.007).

At week 12, 55 patients were classified according to the 3 categories of EVR described earlier. pEVR and cEVR were observed in 11% and 84% of patients. Patients with no EVR (3/55) or pEVR (6/55) did not attain an SVR (NPV = 100%). Among the 46 patients with cEVR, 4 (9%) were non-responders, 17 (37%) were relapsers and 25 (54%) had an SVR. cEVR had a PPV and a NPV for SVR of 54% and 100%, respectively (*P* = 0.003) (Figure 2).

DISCUSSION
Retreatment of CHC patients who have failed prior antiviral therapy is an important clinical issue. Our study evaluated the efficacy of retreatment of CHC patients who relapsed after combination therapy with PEG-IFN

plus RBV. The overall SVR rate achieved was 42%. An important point of our study is the inclusion of a homogeneous population of prior relapsers to the PEG-IFN-α plus RBV combination therapy. Most previous studies analyzed the efficacy of retreatment with PEG-IFN plus RBV based on groups composed mainly of patients who failed conventional IFN-based therapy without distinguishing between non-responders and relapsers, or between monotherapy and combination therapy. Jacobson *et al*[21] demonstrated that SVR rates decreased according to previous conventional IFN-based therapy status: 42% in conventional IFN (cIFN) plus RBV relapsers, 21% in cIFN monotherapy non-responders, and 8% in cIFN plus RBV non-responders. These data were also confirmed by several other studies: retreatment of previous relapsers to cIFN plus RBV could achieve SVR rates of 41%-58%, while for patients who were non-responders, only 4%-26% achieved an SVR[17-21]. The same relationship was observed in previous failures to PEG-IFN and RBV: 33% in prior relapsers and 14% in prior non-responders, with an overall SVR of 22%[23].

The SVR rate of 42% observed in our study was slightly higher than that described in the EPIC3 clinical trial, where prior PEG-IFN plus RBV relapsers attained an SVR of 33%[23]. In our study, HCV genotype was an important predictor for SVR. Patients infected with genotype 2 or 3 attained the highest rates of SVR (60% in genotype 2 and 56% in genotype 3). Thus, a higher proportion of genotype non-1 infected patients in the current study (52% *vs* 20% in EPIC3 trial) could account for this difference. In addition, the EPIC trial used less sensitive qualitative assays that could result in misclassification of EOT responders, increasing the number of relapsers that were in fact non-responders, with a lower probability of SVR.

Young age and genotype 2 or 3 were factors associated with treatment response as previously reported[2,13,15]. We did not find a relationship between low baseline viral load or low fibrosis stage and better response to therapy. These factors have been described in controversial studies with the treatment of naïve and IFN-experienced patients, and their impact on the response in relapsers could have less strength[7,13,19,23,24].

Retreatment with only PEG-IFN-α in patients who failed to respond to the other PEG-IFN-α has been described as an alternative strategy. However, in our study no gain was observed in patients who received a different type of PEG-IFN-α. This finding is consistent with the REPEAT trial, where prior non-responders to PEG-IFN-α2b were retreated with PEG-IFN-α2a. Only 9% of SVR was observed in the regimen of 48 wk retreatment[25]. Besides, this trial demonstrated higher SVR rates in the group retreated for 72 wk (14%)[26]. In the current study, the SVR rate was also improved with longer duration of therapy. Thus, retreatment for at least 24 wk longer than the previous course is important to increase the probability of SVR in relapsers and non-responders.

Some controversial studies have suggested that exposure to RBV is critical for attaining an SVR. At first, adherence to therapy was considered extremely important. McHutchison *et al*[8] demonstrated that at least 80% adherence to therapy enhanced SVR. They found a continuous, increasing relationship between adherence and SVR in genotype 1. These findings were also observed in another study with genotype 1-naïve patients, where a linear relationship between exposure and the SVR rate was observed at the first 12 wk of treatment[7]. Also, a study with RBV discontinuation in a subset of HCV RNA-negative patients at week 24 showed an increase in the rate of virological breakthrough and relapse[9]. In contrast, in our study no relation was found between dose reduction of RBV and SVR. However, the rate of RBV reduction was 20% and only 2 patients did not have at least 80% of the predicted RBV doses.

Recent studies suggested that high-dose RBV schedules reduced relapse rates and increased SVR in difficult-to-treat selected patients[10-12]. In a pilot study with 10 genotype 1 patients, higher RBV doses were associated with more frequent and serious adverse events, but the SVR rate was 90%[11]. Also, Fried *et al*[10] reported a study with 188 treatment-naïve, genotype 1 and high viral load patients. Patients who received an RBV dose of 1600 mg/d had superior SVR rates when compared with standard doses (1200 mg/d). Our data demonstrated a clear relation between high initial dose of RBV[22] and SVR rates. Patients with RBV dose >15.2 mg/kg per day achieved an SVR rate of 70%, while only 26% of patients with lower doses attained an SVR.

Our study demonstrates that an RVR in a relapser retreatment population is attained by 18%, of whom 71% achieved an SVR. Prediction of non response on treatment was more marked with EVR analyses. If the patient did not achieve a cEVR, no SVR was observed (NPV = 100%). Hence, the presence of detectable HCV RNA at week 12 is a good indication to stop treatment in relapsers and it is as relevant as for naïve or non-responding patients[3,19,25].

Specifically targeted antiviral therapies for hepatitis C are currently under evaluation in clinical trials. These new drugs are mostly effective and have been studied in genotype 1 patients[27-29]. Telaprevir, an antiprotease NS3-NS4A, increases SVR rates in genotype 1 naïve and non-responding patients, but it has limited activity against genotype 2 and 3[30]. Besides, even when these medications will be available outside trials, they will not be accessible worldwide. For these reasons, PEG-IFN and RBV still have a role on hepatitis C retreatment, in particular in young patients infected with non genotype 1.

In conclusion, our study shows that retreatment of prior relapsers after treatment with a combination of PEG-IFN plus RBV may be effective. As observed with naïve patients, genotype is crucial for a treatment response. Better results of retreatment are obtained in patients with genotype 2 or 3 and of younger age. In addition, in this subset of patients, higher SVR rates

are achieved with increased doses of RBV, without a marked increase in adverse events or dose reductions. Thus, a high dose schedule of RBV is recommended if retreatment is proposed. Also, prolonging therapy for at least 24 wk more than the previous course enhances SVR rates. Finally, the absence of a cEVR as defined by detectable HCV RNA at week 12 should be considered a stopping rule in the retreatment of relapsers.

COMMENTS

Background
Only 50% of chronic hepatitis C (CHC) patients treated with the combination of pegylated interferon (PEG-IFN)-α and ribavirin (RBV), the standard treatment, will achieve a sustained virological response (SVR). Therefore, patients with no response or relapse after PEG-IFN and RBV treatment are a major issue. Approximately 30% of CHC patients with undetectable hepatitis C virus (HCV) RNA at end of therapy (EOT) will experience relapse.

Research frontiers
Retreatment of CHC patients with relapse to antiviral therapy is a current clinical issue. There are no specific recommendations about type, dose and duration of retreatment in this particular situation. In this research area, different dose schedules and duration of PEG-IFN and RBV therapy have been evaluated in order to increase the SVR in patients with a previous relapse to this antiviral therapy.

Innovations and breakthroughs
This study shows in a real life cohort that retreatment of relapsers after prior treatment with a combination of PEG-IFN plus RBV may be effective. Better results of retreatment are obtained in patients with genotype 2 or 3 and of younger age as is observed in naïve patients. Moreover, in this subset of patients, higher SVR rates are achieved with increased doses of RBV (> 15.2 mg/kg per day), without a marked increase in adverse events or dose reductions. Also, lengthening therapy for at least 24 wk more than the previous course enhances SVR rates.

Applications
The study suggests that retreatment of patients with a relapse after treatment with PEG-IFN and RBV may be effective, especially in patients with genotype 2 or 3 who are of younger age. In order to increase SVR in this particular situation, high dose RBV and longer duration of therapy should be proposed.

Terminology
In CHC patients, treatment responses to the combination of PEG-IFN and RBV are defined by a virological parameter (HCV RNA analysis) rather than a clinical endpoint. The most important definitions are: SVR if HCV RNA remains undetectable 24 wk after EOT, non response if HCV RNA is positive at EOT, and relapse if HCV RNA is undetectable at EOT but detectable within 24-wk follow-up period.

Peer review
The authors revealed that SVR was achieved in 42% of the retreated patients, and that initial dose/weight of RBV was an important predictor of SVR.

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BRIEF ARTICLE

Investigation of compensatory postures with videofluoromanometry in dysphagia patients

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Abstract

AIM: To investigate the effectiveness of head compensatory postures to ensure safe oropharyngeal transit.

METHODS: A total of 321 dysphagia patients were enrolled and assessed with videofluoromanometry (VFM). The dysphagia patients were classified as follows: safe transit; penetration without aspiration; aspiration before, during or after swallowing; multiple aspirations and no transit. The patients with aspiration or no transit were tested with VFM to determine whether compensatory postures could correct their swallowing disorder.

RESULTS: VFM revealed penetration without aspiration in 71 patients (22.1%); aspiration before swallowing in 17 patients (5.3%); aspiration during swallowing in 32 patients (10%); aspiration after swallowing in 21 patients (6.5%); multiple aspirations in six patients (1.9%); no transit in five patients (1.6%); and safe transit in 169 patients (52.6%). Compensatory postures guaranteed a safe transit in 66/75 (88%) patients with aspiration or no transit. A chin-down posture achieved a safe swallow in 42/75 (56%) patients, a head-turned posture in 19/75 (25.3%) and a hyperextended head posture in 5/75 (6.7%). The compensatory postures were not effective in 9/75 (12%) cases.

CONCLUSION: VFM allows the speech-language therapist to choose the most effective compensatory posture without a trial-and-error process and check the effectiveness of the posture.

INTRODUCTION

Swallowing is a coordinated activity that enables solids and liquids to pass uninterrupted from the mouth to the

stomach. Dysphagia occurs when this process is altered by organic or functional alterations at the level of swallow initiation or esophageal emptying[1].

Oropharyngeal dysphagia can manifest as one or more symptoms that are specific for oropharyngeal dysfunction, which help the clinician distinguish it from esophageal dysphagia. Typical symptoms include the inability to chew, a delayed or absent swallow initiation, a bolus delay located in the neck, nasal regurgitation, a need to swallow repeatedly to clear food or fluid from the pharynx, coughing after aspiration, and dysphonia[2,3].

Oropharyngeal dysphagia is usually a manifestation of a systemic disease rather than a disease specific to the oropharynx. This manifestation occurs in one-third of all stroke patients and has a 20%-50% prevalence in conditions such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis[3-5].

Videofluoroscopy (VFS) is the gold standard in the study of oropharyngeal dysphagia because it provides information on the presence and severity of the major categories of dysfunction, including the presence, timing and severity of aspiration[6,7].

Videofluoromanometry (VFM) correlates fluoroscopic events with manometric data. For example, the narrow upper esophageal sphincter (UES) opening can be distinguished from uncoordinated UES relaxation, and the weak propulsive pharyngeal forces can be distinguished from an increased outflow resistance. Moreover, VFM is especially useful for subsequent treatment planning[2].

Characterization of a swallowing disorder allows the patient to adopt compensatory swallowing postures, change their diet to permit safe oral feeding, and delay the use of percutaneous endoscopic gastrostomy in degenerative diseases[8]. The aim of our study was to evaluate the effectiveness of compensatory postures to ensure safe oropharyngeal transit with videofluoromanometric guidance.

MATERIALS AND METHODS

From January 2008 to December 2010, 321 dysphagia patients (171 male, 150 female, aged 18-87 years, mean age: 57 years) were enrolled in this study. All patients gave their written consent. The past medical history, present symptoms, and alimentary status were obtained from all patients. All patients underwent a morphofunctional logopedic evaluation according to recent guidelines[9,10].

Through the VFM assessment, the dysphagia patients were classified as follows: safe transit; penetration without aspiration; aspiration before, during, or after swallowing; multiple aspirations and no transit. The patients with aspiration or no transit were investigated to determine whether compensatory postures could correct the swallowing disorder. Based on manometric data, a chin-down posture, head-turned posture or hyperextended head posture was tested to obtain safe swallowing. Patients with multiple aspirations were excluded from the search for compensatory postures. These patients, who are often

Table 1 Normal values of videofluoromanometry

	Normal values measured with VFM
Tongue base pressure	130 ± 70 mmHg
UES	
Resting pressure	90 ± 30 mmHg
Contraction pressure	240 ± 80 mmHg
Residual pressure	< 10 mmHg
Relaxation duration	1.5 ± 0.4 s
Bolus progression	> 4 cm/s
Pharyngeal pressure	60 ± 20 mmHg

VFM: Videofluoromanometry; UES: Upper esophageal sphincter.

in an advanced disease stage, do not follow the operator commands during the VFM assessment and do not cooperatively assume the compensatory postures. Additionally, these subjects may only somewhat benefit from these compensatory postures[11]. The VFM study consisted of a parallel execution of VFS and manometry. A simultaneous manometric evaluation analyzed the tongue base pressure (the contact pressure between the posterior tongue thrust and the pharyngeal wall), UES tone (resting pressure, contraction pressure and residual pressure) and the bolus transit coordination, which are useful for selecting the compensatory postures[12,13] (Table 1).

A Dyno Compact computerized system (MENFIS Biomedica s.r.l., Bologna, Italy) was used. This system was equipped with the following: (1) A graphics card for managing radiographic images; and (2) AVIUS-dedicated software, which enables digital-quality recording (PAL/NTSC, composite video or S-video) of the VFS study in AVI format with a 320 × 240 resolution and 25 Hz acquisition frequency. The delay introduced by the image digitization process was approximately 200 ms; therefore, for analytical purposes, the images could be considered synchronized with the manometric recordings. The concurrent pressure measurements were performed with a manometry catheter with endoluminal five-channel, solid-state microtransducers 2 cm apart at an angle of 120°-90°. The catheter was inserted through the nasal cavity into the stomach. The value recorded was used for the calibration of 0. Afterwards, a pull through was performed and the catheter was withdrawn to allow for the positioning of the transducers. Transducer 1 was placed at Passavant's ridge to evaluate the correct closure of the rhinopharynx during swallowing and phonation. Transducers 2, 3 and 4 were placed in the pharynx. Transducer 5 was placed at the UES, and the correct placement was determined by the appearance of the characteristic M wave. During image acquisition, the video images and manometric trace were displayed in real time as a full screen image on the personal computer monitor. A cursor indicated the exact correspondence between the video images and the traces. Following the acquisition, the video and manometric trace could be analyzed during real-time reproduction or at reduced or increased speed, or it could be paused for a frame-by-frame analysis. The examinations were acquired with the patient standing or

Table 2 Results of videofluoromanometry

	ALS	Stroke	MS	PD	Post-surgery	AD	Others	Total
Safe transit	41	21	22	28	3	14	40	169
Penetration without aspiration	22	14	8	10	-	7	10	71
Aspiration before swallowing	4	5	1	3	1	3	-	17
Aspiration during swallowing	12	5	3	5	4	3	-	32
Aspiration after swallowing	10	2	2	3	2	2	-	21
Multiple aspirations	4	-	-	-	-	2	-	6
No transit	1	-	-	2	-	2	-	5
Total	94	47	36	51	10	33	50	321

ALS: Amyotrophic lateral sclerosis; MS: Multiple sclerosis; PD: Parkinson's disease; AD: Alzheimer's disease. Others: Gastroesophageal reflux disease, achalasia, chest pain, diverticula.

seated if the patient was unable to remain standing.

VFM began with a baseline evaluation (without contrast) to study the motility of the vocal chords and soft palate. The VFM proceeded with barium contrast medium (Prontobario HD suspension, Bracco SpA, Milan; 250% w/v) at a dose of 5-15 mL that was optimized for the patient to evaluate swallowing, with particular attention paid to aspiration. This was made possible by previous evaluation by a speech therapist. The patients were asked to hold the bolus in their mouth for several seconds and to swallow when asked by the operator. All phases of the process were video-recorded first in the anteroposterior and then the laterolateral view[9,14]. If an impaired bolus transit or aspiration in the airways was detected, an evaluation of the ideal bolus size and monitoring the effectiveness of the compensatory postures were used to investigate correcting the dysfunction.

The compensatory posture was selected for each patient based on the specific swallowing dysfunction that was considered to have caused the aspiration. For an effective oropharyngeal bolus transit while swallowing, the following compensatory postures were tested. The chin-down posture involved tucking the chin to the neck. The change in head position inverted the epiglottis into a more protective position over the airway entry, which reduced the airway entrance space and increased the size of the vallecular spaces[12,15,16]. The head-turned posture narrowed the ipsilateral piriform sinus and sent the bolus to the contralateral sinus. In addition, it determined the decrease in UES pressure, delayed its closure during swallowing, and optimized the bolus propulsion[17,18]. The hyperextended head posture facilitated the bolus transit using the force of gravity. This posture is indicated for reduced pharyngeal peristalsis[4].

RESULTS

VFM in 169 (52.6%) of 321 enrolled patients showed safe transit of the contrast medium. In 71/321 cases (22.1%), penetration of the contrast medium into the laryngeal lumen without aspiration below the glottic level was observed, and we did not suggest compensatory postures for these patients. In 5/321 (1.5%) cases, there was no transit.

In 76/321 patients (23.7%), aspiration was observed. In 70/76 (92.1%) patients, VFM revealed a single aspiration, and in 17/70 (24.3%) cases, aspiration occurred before swallowing, in 32/70 (45.7%) during swallowing, and in 21/70 (30%) after swallowing. In 6/76 cases (7.9%), there were multiple aspirations, and compensatory postures were not sought in these patients.

Compensatory postures (Figures 1 and 2) through VFM guidance were tested in the 70 patients with a single aspiration and the five with no transit (Table 2). Among the aspiration cases, 24 (34%) had no spontaneous reflex cough. The 17 patients with aspiration before swallowing suffered from disorders of the oral phase, such as a deficit of lip closure (17.6%), reduced lifting of the soft palate (41.2%), and reduced (29.4%) or disorganized tongue movement (11.8%). The tongue-palate contact was incomplete in 4/17 (23.5%), with leakage and aspiration before voluntary swallowing due to a fraction of the bolus moving into the pharynx and being aspired without manometric alterations. In 8/17 cases (47%), the manometric evaluation showed that the tongue base pressure was < 60 mmHg during swallowing. In all 17 patients, a total resolution of the disorder was obtained by adopting the chin-down posture.

Aspiration during swallowing occurred in 32 (45.7%) cases. Eighteen of these (56.3%) were related to a reduced laryngeal closure (10/32) or elevation (8/32), and 14 were related to a UES disorder, with a residual pressure during relaxation of > 10 mmHg (9/14, 28.1%) and an early closure of the UES compared to the end of the pharyngeal contraction (5/14, 15.6%).

In 14/18 (78%) patients with reduced laryngeal closure or elevation, the chin-down posture (Figure 2) resolved the aspiration, whereas in 10/14 (71%) patients with UES disorders, the aspiration was corrected with the head-turned posture.

The compensatory postures were not effective in 8/32 (25%) patients. In the 21 cases of aspiration after swallowing, the bolus was inhaled due to stagnant contrast medium in the pharynx. The contrast medium was noted in 5/21 (23.8%) cases at the piriform sinuses, in 4/21 (19.1%) at the glossoepiglottic valleys, and in 12 (57.1%) at both locations. Aspiration after swallowing in 4/21 (19.1%) cases resulted from incomplete release

Table 3 Results of compensatory postures in selected patients

	ALS	Stroke	MS	PD	Post-surgery	AD	Others	Total
Swallowing disorders								
Aspiration before swallowing	4	5	1	3	1	3	-	17
Aspiration during swallowing	12	5	3	5	4	3	-	32
Aspiration after swallowing	10	2	2	3	2	2	-	21
No transit	1	-	-	2	-	2	-	5
Compensation postures								
Chin down	13	6	5	6	5	7	0	42
Head turned	10	4	1	3	0	1	0	19
Head hyperextended	1	0	0	2	0	2	0	5
No compensation	3	2	0	2	2	0	0	9

ALS: Amyotrophic lateral sclerosis; MS: Multiple sclerosis; PD: Parkinson's disease; AD: Alzheimer's disease.

Figure 1 Videofluoromanometry study. A: Aspiration after swallowing (black arrow) with stagnation in the glossoepiglottic valleys and piriform sinuses (white arrows). Incomplete upper esophageal sphincter (UES) release (arrow); B: The same occurred with the head-rotated posture; stasis persisted at the pharyngo-esophageal junction (white arrow), but there was no aspiration of the contrast medium. The UES was fully open (dashed box).

Figure 2 Videofluoromanometry study. A: Aspiration during swallowing (arrow) for the reduced laryngeal closure with an incomplete upper esophageal sphincter (UES) release; B: The same occurred in the course of the chin-down posture; this posture resolved the aspiration because it placed the epiglottis in a more protective position of the airways and restricted the airway entrance.

of the UES, with a residual pressure of > 10 mmHg. In 4/21 (19.1%) cases, the resting pressure of the UES was > 150 mmHg. In 1/21 (4.8%) cases, the pharyngeal dysfunction and stagnation of contrast medium was unilateral. The chin-down posture solved the aspiration in 11/21 cases (52.4%), and the head-turned posture was useful in 9/21 patients (42.9%). In 1/21 (4.7%) cases, there was no discomfort reduction.

In the five cases of dysphagia with no transit, the disorder was characterized by alterations in the initiation of the swallowing reflex without a peristaltic wave from the tongue base; all of these cases benefited from the

hyperextended head posture (Table 3).

DISCUSSION

Swallowing therapy has the primary goal of maintaining oral feeding and achieving safe and efficient swallowing, which is important to ensure a good quality of life. Different swallowing techniques have been adopted rapidly during the past decade and are used almost worldwide. However, few studies have investigated how each technique affects swallowing[13,15,19].

When treating patients with oropharyngeal dysfunction, an ideal approach to the assessment and treatment

is working in a team with a radiologist, gastroenterologist and a speech-language pathologist. In the present study, a multi-specialist assessment team evaluated each enrolled patient to assess the swallowing dysfunction and test the adequacy of therapy to ensure safe transit. VFM provides a parallel acquisition of videofluoroscopic and manometric data and plays a crucial role in therapeutic planning.

We did not employ dietary modifications, including increases in the volume or density of food, for radioprotection reasons, but a single bolus of contrast medium was optimized for the patient.

Three different compensatory postures, including chin down, head turned and head hyperextended, were adopted. Each patient was tested before the posture was deemed appropriate. The compensatory postures restored a safe transit in 66/75 patients (88%). The chin-down posture was effective in 42/66 patients (63.6%). This posture was useful in all patients with aspiration before swallowing because it promoted bolus control in the oral cavity until the swallowing reflex was elicited. In patients with aspiration during swallowing and reduced laryngeal closure or elevation, the chin-down posture protected the airway from aspiration. In patients with aspiration after swallowing, dropping the tongue base and the contrast medium stasis in the pharynx fostered the bolus flow into the esophagus.

The head-turned posture was useful in 19/66 (28.8%) patients with both aspiration during swallowing and aspiration after swallowing with a residual pressure or uncoordinated UES relaxation due to the decrease in UES pressure and increase in UES opening time. This posture was useful also in one patient with stroke and unilateral pharyngeal failure because it excluded the affected side from the bolus transit.

The hyperextended head posture was effective in all five patients without transit. This posture uses gravity to aid swallowing; therefore, this posture is recommended for all cases with impaired lingual propulsion but should be suggested only after verifying that the transit occurs safely[4].

The compensatory postures were not effective in 9/75 patients (12%). This finding was related to a massive aspiration in 3/9 cases, poor compliance due to an advanced stage of disease in 4/9 cases, and surgery in 2/9 cases.

Silent aspiration, defined as aspiration in the absence of reflex cough, should also be mentioned. This event occurred in 24/70 (34.3%) patients with a single aspiration; specifically, three with aspiration before swallowing, 10 during swallowing, and 11 after swallowing. In other studies, this phenomenon occurred in approximately half of those patients who aspirated and was significantly more frequent in those with a history of laryngeal pathology[20].

Silent aspiration has major importance[21] because it promotes the early onset of complications, such as aspiration pneumonia. Aspiration pneumonia is three times more frequent in dysphagia patients than those without[22],

and is the most common cause of death in patients with neurological disorders associated with dysphagia[23].

The present study had some limitations, including the use of an intraluminal manometric catheter, which is considered a non-altering device for swallowing, and the poor availability and high cost of this imaging method.

Compared to other studies[13,15], this study included the largest number of patients. The effectiveness of the compensatory postures in the oropharyngeal dysphagia patients was evaluated with combined videofluorography and manometry. In our experience, manometry has extensively evaluated deglutition disorders. Consequently, this method has chosen the better posture and defined the more effective therapeutic strategy.

In conclusion, there is not a single compensatory posture for each type of aspiration. Only VFM accurately defines the pathogenic mechanism of the swallowing deficit; helps the speech-language therapist choose the most effective compensation posture without a trial-and-error process; and checks the effectiveness at the same time. The chin-down, head-turned and head hyperextension postures are efficacious in several swallowing disorders. These postures can be evaluated during the examination itself, are easy to learn, and ensure good patient compliance. This study, complemented by a professional treatment team, guarantees a more accurate diagnosis and greater therapeutic efficacy than the individual modalities for evaluating dysphagia patients.

COMMENTS

Background
Choking, aspiration pneumonia, malnutrition and dehydration can complicate dysphagia. An exact characterization of the pathogenetic mechanisms of dysphagia helps define the therapeutic approach to reduce the risk of complications.

Research frontiers
Videofluoromanometry (VFM) is the gold standard for swallowing studies and analyzing the mechanisms underlying dysphagia.

Innovations and breakthroughs
There is not a single compensatory posture for each type of aspiration. Only VFM accurately defines the pathogenic mechanism of the swallowing deficit; helps the speech-language therapist choose the most effective compensation posture without a trial-and-error process; and checks the effectiveness at the same time.

Applications
VFM explores the effectiveness of the compensatory postures. This method may help manage dysphagia.

Peer review
This is a novel approach for the assessment of swallowing and the anatomy and physiology of the laryngopharyngeal segment. The authors seem to know the radiological aspects of the subject.

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BRIEF ARTICLE

Diagnostic value for extrahepatic metastases of hepatocellular carcinoma in positron emission tomography/computed tomography scan

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Abstract

AIM: To evaluated the value of ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/computed tomography (CT) scan in diagnosis of hepatocellular carcinoma (HCC) and extrahepatic metastases.

METHODS: A total of 138 patients with HCC who had both conventional imaging modalities and ^{18}F-FDG PET/CT scan done between November 2006 and March 2011 were enrolled. Diagnostic value of each imaging modality for detection of extrahepatic metastases was evaluated. Clinical factors and tumor characteristics including PET imaging were analyzed as indicative factors for metastases by univariate and multivariate methods.

RESULTS: The accuracy of chest CT was significantly superior compared with the accuracy of PET imaging for detecting lung metastases. The detection rate of metastatic pulmonary nodule ≥ 1 cm was 12/13 (92.3%), when < 1 cm was 2/10 (20%) in PET imaging. The accuracy of PET imaging was significantly superior compared with the accuracy of bone scan for detecting bone metastases. In multivariate analysis, increased tumor size (≥ 5 cm) ($P = 0.042$) and increased average standardized uptake value (SUV) uptake ($P = 0.028$) were predictive factors for extrahepatic metastases. Isometabolic HCC in PET imaging was inversely correlated in multivariate analysis ($P = 0.035$). According to the receiver operating characteristic curve, the optimal cutoff of average SUV to predict extrahepatic metastases was 3.4.

CONCLUSION: ^{18}F-FDG PET/CT scan is invaluable for detection of lung metastases larger than 1 cm and bone metastases. Primary HCC having larger than 5 cm and increased average SUV uptake more than 3.4 should be considered for extrahepatic metastases.

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INTRODUCTION

Most patients with hepatocellular carcinoma (HCC) present with underlying liver disease, usually cirrhosis, hepatitis B and hepatitis C virus infection[1,2]. Screening and surveillance programmes based on periodic ultrasonography and α-fetoprotein (AFP) among high-risk patients could establish of early diagnosis and provide more effective treatments of HCC[3]. With advances in variable treatment modalities, the prognosis of HCC has been much improved[4-7]. With prolonged survival of HCC patients, the incidence of extrahepatic metastases has been reported up to 42%[8]. Precise evaluation of extrahepatic metastases of HCC is important because treatment modality could be determined belong to the results.

Positron emission tomography (PET)/computed tomography (CT) scan using ^{18}F-fluorodeoxyglucose (FDG) is now well established as a noninvasive diagnostic tool for diagnosis, staging and monitoring of a variety of malignant tumors[9,10]. However, in detection of primary HCC, the sensitivity of ^{18}F-FDG PET/CT scan has been reported not sufficiently high (50%-55%) because of its variable ^{18}F-FDG uptake pattern[11-14].

Several studies were performed for investigation of usefulness of ^{18}F-FDG PET/CT scan in detection of extrahepatic metastases of HCC. A previous study reported that the sensitivity for the detection of extrahepatic metastasis was 79%[15,16]. However, there are few detailed reports to compare ^{18}F-FDG PET/CT scan with conventional imaging modalities.

In this study, we evaluated the value of ^{18}F-FDG PET/CT scan in diagnosis of primary HCC and extrahepatic metastases. Furthermore, we suggest several clinical factors and tumor characteristics including ^{18}F-FDG PET/CT scan findings that indicate extrahepatic metastases in diagnosis of HCC.

MATERIALS AND METHODS

Patients

We conducted a retrospective chart review of patients with HCC at Soonchunhyang University Hospital who had both conventional imaging modalities and ^{18}F-FDG PET/CT scan done within at least a month between November 2006 and March 2011. During this period, all patients diagnosed with HCC who were newly diagnosed

or reevaluated after treatment underwent ^{18}F-FDG PET/CT scan. A total of 138 patients were enrolled for this study. Eighty-eight patients were treatment-naïve and the other 50 patients were previously treated for HCC (tumor resection, transcatheter arterial chemoembolization, radiofrequency ablation, systemic chemotherapy). The diagnosis of primary HCC was based on contrast enhanced abdomen CT or magnetic resonance imaging (MRI), where hyperattenuation in the arterial phase and early washout in the delayed phase were considered definitely diagnostic. Elevations in tumor markers such as AFP, protein induced by vitamin K antagonist Ⅱ (PIVKA Ⅱ) levels were considered suggestive of HCC. Ultrasound-guided needle biopsy was performed when considered necessary. This study was approved by the local institutional review board and was conducted in accordance with the principles set forth in the Declaration of Helsinki.

Conventional imaging modalities

Chest X-ray and contrast enhanced chest CT for evaluation of lung metastases were performed. If there are suspicious lesions, repeated contrast enhanced chest CT was examined within 3 mo. Whole body bone scan for evaluation of bone metastases was performed. If there are suspicious lesions, bone MRI was conducted for definite diagnosis or repeated bone scan was followed within 3 mo. Regional and distant lymph node metastases were determined according to contrast enhanced CT. If there are suspicious lesions, repeated contrast enhanced CT was examined within 3 mo to observe interval size difference. Some metastatic lesions were diagnosed with pathologic confirmation, but most metastatic lesions were clinically diagnosed because of difficult access to deep lesions and too small size to do a biopsy.

Intrahepatic tumor size was measured by the greatest diameter in treatment-naïve patients, and the greatest diameter including viable portion from the first diagnosis in previously treated patients.

^{18}F-FDG PET/CT scan

^{18}F-FDG PET/CT scan was performed with a Biograph 2 (Siemens Medical Solution, Knoxville, TN, United States). All patients fasted for at least 6 h before ^{18}F-FDG injection. Serum glucose levels measured at the time of ^{18}F-FDG injection were less than 150 mg/dL in all patients. Approximately 370-500 MBq of ^{18}F-FDG was injected intravenously and an emission scan (2.5 min/bed position) was performed from the knees to the head 40 min after of ^{18}F-FDG injection in the two dimensional imaging mode. A transmission scan (3 min/bed position) was then obtained with a rotating ^{68}Ge source.

^{18}F-FDG PET images were interpreted by one over 30 years experienced nuclear medicine physician. If no significant ^{18}F-FDG uptake was detectable in the tumor compared to normal liver tissue by ^{18}F-FDG PET/CT scan, this was considered isometabolic HCC, hypermetabolic HCC for increased ^{18}F-FDG uptake, and

Table 1 Patients baseline characteristics		
	n = 138	%
Age	59.6 ± 11.1 (range: 33-84)	
Sex		
M/F	114/24	82.6/17.4
Etiology of liver disease		
HBV/HCV/alcohol/unknown	89/15/10/24	64.5/10.9/7.2/17.4
AFP (ng/mL)	9512.9 ± 23 026.4	
PIVKA Ⅱ (mAU/mL)	854.2 ± 871.7	
Tumor morphology		
Nodular/infiltrating	78/60	56.5/43.6
Tumor size (mm)	69.8 ± 45.2	
Tumor number		
1/⩾ 2	52/86	37.7/62.3
PVTT		
Yes	54	39.1
Child-Pugh classification		
A/B/C	77/56/5	55.8/40.6/3.6
Tumor stage[1]		
Ⅰ / Ⅱ / Ⅲ / Ⅳa / Ⅳb	7/34/26/22/49	5.1/24.6/18.8/15.9/35.5
SUV		
Iso-/hypometabolism	42/3	30.4/2.2
Hypermetabolism	93	67.4
Maximum	5.32 ± 2.38	
Average	4.03 ± 1.26	
TNR (SUV ratio)	1.60 ± 0.49	
Extrahepatic metastases	50	36.2
Lung	23	46.0
Lymph nodes	22	44.0
Bone	11	22.0
Others[2]	5	10.0

[1]Tumor stage based on the Modified Union for International Cancer Control Tumor Node Metastasis staging system; [2]Others: Adrenal gland, peritoneal carcinomatosis, Morrison's pouch. HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: α-fetoprotein; PIVKA Ⅱ: Protein induced by vitamin K antagonist Ⅱ; PVTT: Portal vein tumor thrombosis; SUV: Standardized uptake value; TNR: Tumor-to-nontumor ratio.

hypometabolic HCC for decreased ^{18}F-FDG uptake. Isometabolic or hypometabolic HCC was excluded from quantitative evaluation. For measurement of ^{18}F-FDG uptake, region of interest (ROI) was placed over tumor lesion including the area of maximum activity. The highest value of ^{18}F-FDG uptake in ROI is defined as maximum standardized uptake value (SUV) and the average value of ^{18}F-FDG uptake in ROI is defined as average SUV. Then, ROI was placed over nontumor area sized 20 mm × 20 mm for estimation of average ^{18}F-FDG uptake of nontumor area. SUV was calculated by as follows; mean tissue activity (kBq/mL) × calibration factor × body weight (kg)/injected dose (MBq). The tumor-to-nontumor ratio (TNR, SUV ratio) was calculated by average tumor SUV/average nontumor SUV.

Histologic examination
Histologic examination was performed to assess the histologic grade of HCC (*n* = 50). Twenty-nine patients were indicated for tumor resection at diagnosis, the other 21 patients were performed ultrasound-guided needle biopsy. According to histologic grade, the tumors were divided into low-grade (well-differentiated and moderately-differentiated type) and high-grade (poorly-differentiated

and undifferentiated type). As 4 patients were revealed as combined HCC-CC (cholangiocarcinoma), a total of 46 patients were analyzed.

Statistical analysis
Data are expressed as the mean ± SD, range, or *n* (%) as appropriate. When comparing the baseline characteristics of patients with 2 different groups, chi-square test and Fisher's exact test were used for categorical data, and the Student *t* test and *U* test were used for continuous variables. We performed receiver operating characteristic (ROC) curve analysis to compare the diagnostic performance of conventional and PET imaging for detection of extrahepatic metastasis. To estimate risk factors for extrahepatic metastases of HCC, univariate and subsequent multivariate logistic regression analysis were used. The overall cumulative survival rate was analyzed using the Kaplan-Meier method, and differences in survival between the groups were compared using a log-rank test. Data analysis was performed using SPSS 17.0 and MedCalc.

RESULTS

Patient characteristics
Patient characteristics are summarized in Table 1. Eighty-six patients (62.3%) had multiple lesions and 54 patients (39.1%) had portal vein thrombosis. Child-Pugh class A was 77 patients (55.8%), 56 patients (40.6%), and stage Ⅳb was 49 patients (35.5%) based on the modified Union for International Cancer Control Tumor Node Metastasis staging system.

Correlation between ^{18}F-FDG uptake and tumor differentiation
Forty-five of 138 patients (32.6%) with HCC did not have ^{18}F-FDG uptake. Therefore, SUV (maximum and average) was calculated in 93 patients (67.4%). The maximum SUV was 5.32 ± 2.38, average SUV was 4.03 ± 1.26, and tumor-to-nontumor ratio (TNR) was 1.60 ± 0.49 (Table 1). We analyzed the correlation of histologic grade in HCC with clinical factors and tumor characteristics including ^{18}F-FDG PET/CT scan findings (Table 2). Forty-six patients were performed tumor resection or ultrasound-guided needle biopsy and assessed the histologic grade.

In HCC with isometabolism, low-grade HCC was found in 14 patients and high-grade HCC in 2 patients; Isometabolic HCC tended to be histologically low-grade rather than high-grade (*P* = 0.061). In hypermetabolic HCC, maximum SUV value was higher in high-grade HCC than low-grade HCC (5.75 ± 2.15 *vs* 3.75 ± 0.74, *P* = 0.027) although average SUV and TNR (SUV ratio) was not different between two groups (Table 2).

Diagnostic value of imaging modalities for detection of extrahepatic metastases
The results of the detection rate of conventional imaging modalities and ^{18}F-FDG PET/CT scan for extrahepatic metastases in HCC are summarized in Table 3.

Table 2 Correlation of histologic grade with clinical factors and tumor characteristics *n* (%)

	Low-grade (*n* = 34)	High-grade (*n* = 12)	*P* value[1]
Age	61.7 ± 8.4	56.5 ± 6.7	0.055
Sex			0.260
M/F	32 (94.1)/2	10 (83.3)/2	
Etiology of liver disease			0.431
HBV/HCV/alcohol/unknown	21/2/2/9	9/0/1/2	
AFP (ng/mL)	2892.0 ± 9255.6	2960.5 ± 9554.0	0.930
< 200/≥ 200	26 (76.5)/8	10 (83.3)/2	
PIVKA Ⅱ (mAU/mL) (*n* = 20/*n* = 7)	758.4 ± 823.1	798.6 ± 879.6	1.000
< 40/≥ 40	3 (15)/17	1 (16.7)/6	
Tumor morphology			0.441
Nodular/infiltrating	24 (70.6)/10	8 (66.7)/4	
Tumor size			0.643
< 5/≥ 5	16 (47.1)/18	7 (58.3)/5	
Tumor number			0.297
1/≥ 2	20 (58.8)/14	9 (75)/3	
PVTT			0.259
Yes	8 (23.5)	1 (8.3)	
Child-Pugh classification			0.563
A/B/C	30 (88.2)/4/0	11(91.7)/1/0	
T stage			0.537
1/2/3/4	3/15/9/7	1/7/2/2	
SUV			
Isometabolism	14 (41.2)	2 (16.7)	0.061
Hypometabolism	2	0	
Hypermetabolism (*n* = 18/*n* = 10)			
Maximum	3.75 ± 0.74	5.75 ± 2.15	0.027
Average	3.30 ± 0.42	4.15 ± 1.17	0.226
TNR (SUV ratio)	1.33 ± 0.22	1.64 ± 0.52	0.286

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: α-fetoprotein; PIVKA Ⅱ: Protein induced by vitamin K antagonist Ⅱ; PVTT: Portal vein tumor thrombosis; SUV: Standardized uptake value; TNR: Tumor-to-nontumor ratio. [1]Statistical significance test was done by *U* test.

Twenty-three patients were diagnosed of clinical lung metastases showing interval size increase on follow up chest CT. Fifteen patients were test positive on ^{18}F-FDG PET/CT scan, 14 patients were true positive and 1 patient turned out to be false positive revealing non-tuberculosis mycobacterium infection on percutaneous transthoracic needle aspiration (Figure 1). The detection rate of metastatic pulmonary nodule ≥ 1 cm was 12/13 (92.3%), when < 1 cm was 2/10 (20%) (*P* = 0.0003). The sensitivity, specificity, and accuracy for detection of lung metastases in HCC by ^{18}F-FDG PET/CT scan were 60.9%, 99.1% and 92.6%, respectively (Table 3). Contrast enhanced chest CT all detected for lung metastases in HCC and 2 lesions turned out to be false positive which did not show size increase during follow up chest CT. Therefore, the sensitivity, specificity and accuracy were 100%, 98.2% and 98.5%, respectively. The accuracy of chest CT was significantly superior compared with the accuracy of PET imaging for detecting lung metastases by comparison of ROC curves (*P* = 0.000, CI 0.0888-0.294) (Table 3).

Twenty-two patients were diagnosed of regional or distant lymph node metastases showing arterial phase enhancement and interval size increase on follow up contrast enhanced CT. The sensitivity of ^{18}F-FDG PET/CT

Table 3 Diagnostic value of ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography scan and conventional imaging modalities for detection of extrahepatic metastases

	Lung metastases (*n* = 23)		Lymph node metastases (*n* = 22)		Bone metastases (*n* = 11)	
	TP	TN	TP	TN	TP	TN
PET (+)	14	1	20	4	11	0
Conventional (+)	23	2	22	4	7	4
	PET	Conventional	PET	Conventional	PET	Conventional
Sensitivity, %	60.9	100	90.9	100	100	63.6
Specificity, %	99.1	98.2	96.5	96.5	100	96.8
Accuracy, %	92.6	98.5	95.6	97.1	100	94.1
PPV, %	93.3	92	83.3	84.6	100	63.6
NPV, %	92.5	100	98.2	100	100	96.8
Comparison of ROC curves	*P* = 0.000 (CI: 0.0888-0.294)		*P* = 0.269		*P* = 0.010 (CI: 0.0481-0.348)	

TP: True positive; TN: True negative; PPV: Positive predictive value; NPV: Negative predictive value; ROC: Receiver operating characteristic; PET: Positron emission tomography.

scan for lymph node metastases was 90.9% showing lower than 100% in conventional imaging modalities. Both ^{18}F-FDG PET/CT scan and contrast enhanced CT detected 4 lesions as a positive test which turned out to be false positive. The accuracy of both images was not different by comparison of ROC curves (*P* = 0.269) (Table 3).

Eleven patients were diagnosed of bone metastases, ^{18}F-FDG PET/CT scan detected all of these lesions. However, bone scan failed to identify 4 patients and 4 suspicious of metastases turned out to be false positive. Therefore, the sensitivity, specificity and accuracy of ^{18}F-FDG PET/CT scan in diagnoses of bone metastases were 100%, 100% and 100%, respectively. The sensitivity, specificity and accuracy of bone scan were 63.6%, 96.8% and 94.1%, respectively. The accuracy of PET imaging was significantly superior compared with the accuracy of bone scan for detecting bone metastases by comparison of ROC curves (*P* = 0.010, CI: 0.0481-0.348) (Table 3).

Three patients with adrenal metastases were all detected by abdomen CT, but ^{18}F-FDG PET/CT scan failed to detect metastasis in one patient. There were 2 patients who were suspicious of cervical lymph node metastasis on both ^{18}F-FDG PET/CT scan and neck CT which turned out to be Warthin's tumor on needle biopsy.

Indicative factors for extrahepatic metastases in ^{18}F-FDG PET/CT scan

Elevated AFP (≥ 200 ng/mL), elevated PIVKA Ⅱ (≥ 40 mAU/mL), infiltrative tumor morphology, larger tumor size (≥ 5 cm), multiple tumors in liver, portal vein tumor thrombosis, advanced T stage, increased SUV uptake and high-grade HCC were associated with the presence of extrahepatic metastases of HCC (Table 4).

In multivariate analysis, increased tumor size (≥ 5 cm) (*P* = 0.042) and increased average SUV uptake (*P* = 0.028) were indicative factors for extrahepatic metastases in HCC (Table 4). Isometabolic HCC in ^{18}F-FDG PET/CT scan was inversely correlated with extrahepatic me-

Figure 1 A 51 year-old-female with hepatocellular carcinoma was suspicious of lung metastasis (arrows) in both chest computed tomography and ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography scan which turned out to be nontuberculosis mycobacterium infection on percutaneous transthoracic needle aspiration. A: High-resolution computed tomography (CT); B: Contrast-enhanced CT; C: ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET); D: PET/CT scan.

Figure 2 The area under the receiver operating characteristic curve to estimate the optimal cutoff of average standardized uptake value to predict extrahepatic metastasis. AUROC: Area under receiver operating characteristic.

tastases ($P = 0.035$). According to the ROC curve, the optimal cutoff of average SUV to predict extrahepatic metastases was > 3.4 (Figure 2). Therefore, when the average SUV in ROI is higher than 3.4, we should consider the possibility of extrahepatic metastases with poor prognosis.

Cumulative survival rate was studied by intrahepatic tumor size, average SUV, isometabolic HCC, and extra-

hepatic metastasis after dividing average SUV group into two groups by the cutoff 3.4. The survival rate was significantly higher in group with tumor size < 5 cm (1-year survival rate; 69.1% *vs* 25.9%, $P = 0.000$) (Figure 3A), average SUV < 3.4 (1-year survival rate; 57.1% *vs* 19.2%, $P = 0.000$) (Figure 3B), and isometabolic HCC (1-year survival rate; 78.0% *vs* 28.3%, $P = 0.000$) (Figure 3C). There were 2 clinical factors that affected survival rate of HCC by Cox proportional hazard analysis. Child-pugh class [class B: odds ratio (OR) 4.784, CI: 2.575-8.891, $P = 0.000$; class C: OR 10.787, CI: 3.579-32.511, $P = 0.000$] and metastases (OR 2.069, CI: 1.152-3.715, $P = 0.015$) were significantly associated with survival rate (Table 5).

DISCUSSION

Several investigators quantitatively evaluated glucose utilization in HCC with ^{18}F-FDG PET/CT scan and showed its usefulness for assessing characterization of tumor[17]. Increased tumor ^{18}F-FDG uptake is highly reflected the enzymatic activity of glucose metabolism and the histologic grading of HCC[17,18]. Well-differentiated HCC cells exhibit an ^{18}F-FDG metabolism similar to that of normal liver tissue, whereas undifferentiated HCC cells do not do so[17,19]. ^{18}F-FDG PET/CT scan was not sensitive than ultrasound and serum AFP levels for diagnosing HCC in HBV carriers[14]. Because of its limitations for intrahepatic lesions, ^{18}F-FDG PET/CT scan is not suitable as

Table 4 Clinical factors and tumor characteristics of extrahepatic metastases in hepatocellular carcinoma

	Metastasis (*n* = 50)	No metastasis (*n* = 88)	P value Uni	Multi
Age	58.7 ± 11.1	60.1 ± 11.1	0.480	
Sex			0.285	
M/F	39/11	75/13		
Etiology of liver disease			0.215	
HBV/HCV/alcohol/unknown	31/3/4/12	58/12/6/12		
AFP (ng/mL)	15 568.8 ± 28 119.6	6072.1 ± 18 882.3	0.019	0.254
< 200/≥ 200 (%)	21/29 (58.0)	59/29 (33.0)		
PIVKA Ⅱ (mAU/mL)	1320.5 ± 784.0	630 ± 827.9	0.001	
< 40/≥ 40 (*n* = 25/*n* = 52) (%)	2/23 (92.0)	18/34 (65.4)		
Tumor morphology			0.000	0.126
Nodular/infiltrating (%)	17/33 (66.0)	61/27 (30.7)		
Tumor size	88.9 ± 40.2	58.9 ± 44.5	0.000	0.042[1]
< 5/≥ 5 (%)	7/43 (86.0)	48/40 (45.5)		
Tumor number			0.000	0.382
1/≥ 2 (%)	7/43 (86.0)	45/43 (48.9)		
PVTT			0.000	0.330
Yes (%)	30 (60.0)	24 (27.2)		
Child-Pugh classification			0.474	
A/B/C	35/11/4	68/19/1		
T stage			0.000	0.197
1/2/3/4 (%)	1/4/12/33 (66.0)	7/34/26/21 (23.9)		
SUV				
Isometabolism (%)	3 (6.0)	39 (44.3)	0.000	0.035
Hypometabolism	0	3		
Hypermetabolism (*n* = 47)				
Maximum	5.89 ± 2.55	4.74 ± 2.05	0.019	0.517
Average	4.38 ± 1.35	3.68 ± 1.05	0.006	0.028[2]
TNR (SUV ratio)	1.70 ± 0.51	1.50 ± 0.45	0.048	0.352
Pathology			0.042	
Low-/high-grade (*n* = 12/*n* = 34) (%)	7 (58.3)/5	27 (79.4)/7		

[1]1.06-31.8; [2]1.3-127.9. HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: α-fetoprotein; PIVKA Ⅱ: Protein induced by vitamin K antagonist Ⅱ; PVTT: Portal vein tumor thrombosis; SUV: Standardized uptake value; TNR: Tumor-to-nontumor ratio.

Table 5 Clinical factors that affected survival by multivariate analysis

	Odds ratio	CI	P value
Child class B	4.784	2.575-8.891	0.000
Child class C	10.787	3.579-32.511	0.000
AFP (> 200 ng/mL)	1.825	0.998-3.338	0.051
Tumor size (> 5 cm)	1.004	1.000-1.009	0.060
Metastases	2.069	1.152-3.715	0.015

a screening tool for detection of intrahepatic recurrence after tumor resection or liver transplantation[16].

In our study, 45 of 138 patients (32.6%) with HCC did not have ^{18}F-FDG uptake. Isometabolic HCC tended to be histologically low-grade (*P* = 0.061) and showed superior survival rate (*P* = 0.000). In this aspect, ^{18}F-FDG PET/CT scan might be useful for the prediction of outcome in patients with hepatocellular carcinoma. Yang *et al*[20] reported PET imaging could be a good preoperative tool for estimating the post-LT risk of tumor recurrence. It reported the overall survival rate was significantly lower in high SUV and high TNR group. Especially, TNR was independent predictor of survival in HCC patients in multivariate analysis[18,21]. The blood glucose level is often high in patients with cirrhosis[22],

affecting the SUV in the tumor region[23]. Therefore, TNR, tumor-to-nontumor SUV ratio more strongly correlated with characteristics of HCC than SUV[21]. In our study, the cumulative survival rate in the group with average SUV less than 3.4 was significantly higher than in the group with average SUV more than 3.4.

Extrahepatic metastases of HCC was occurred in 36.2% (29.5% in treatment-naïve patients) in our study which have been reported to occur in 13.5%-42%[24-27]. Major metastatic sites from HCC are the lung, lymph nodes, bone, and adrenal gland consistent with other reports[24-26,28].

It is known that lungs are both the most common site of metastases and the most common site of the first detectable metastases[16,24,25,28]. Chest X-ray is inexpensive, may serve as a baseline investigation to evaluate abnormalities, however, the detection rate of pulmonary metastases is low. Gielen *et al*[29] reported 10/19 patients with pulmonary metastases were not identified with chest X-ray in patients with colorectal cancer. To compare chest CT with ^{18}F-FDG PET/CT scan, the accuracy of chest CT was higher than ^{18}F-FDG PET/CT scan in our study. The detection rate of ^{18}F-FDG PET/CT scan was only 20% when metastatic pulmonary nodule < 1 cm. Therefore, to detect early lung metastases from

Figure 3 Cumulative survival rate in patients with hepatocellular carcinoma by intrahepatic tumor size (A), average standardized uptake value (B), isometabolic hepatocellular carcinoma (C) and extrahepatic metastasis (D). SUV: Standardized uptake value.

HCC, chest CT should be performed at regular intervals.

The diagnosis of regional or distant lymph node metastases is determined by interval size increase and arterial phase enhancement in abdomen CT/MRI, chest CT and neck CT[25]. It is well documented that patients who have cirrhosis also have benign enlarged lymph nodes[30]. In our study, 4 patients were detected for lymph node metastases, which turned out to be false positive in both ^{18}F-FDG PET/CT scan and contrast enhanced CT. Therefore, follow up CT is critical for determination of metastases even when increased ^{18}F-FDG uptake in ^{18}F-FDG PET/CT scan is observed. Lymph node metastasis is difficult to confirm due to poor accessibility for biopsy. If suspicious lesions were identified at conventional imaging or ^{18}F-FDG PET/CT scan, it should be clinically confirmed during follow-up imaging.

In our study, all bone metastases of HCC were detected by ^{18}F-FDG PET/CT scan whereas bone scan could not detect 4 lesions and 4 abnormal uptakes were false positive based on bone MRI and follow up imaging. Other studies have also reported PET imaging is more sensitive than bone scan[15,16,27]. Whole body bone scan is a routine modality in detecting bone metastases; however, lesions may remain invisible in the absence of an osteo-

blastic response. Furthermore, bone scan is not likely to differentiate healing fractures and degenerative disease from bone metastases[31]. Based on these results, ^{18}F-FDG PET/CT scan is more sensitive and specific diagnostic tool than bone scan for evaluation of bone metastases.

Although extrahepatic metastases of HCC are common, undergoing ^{18}F-FDG PET/CT scan in all HCC patients may not be cost-effective. Selected patients who are suspected of extrahepatic metastases of HCC should be performed of ^{18}F-FDG PET/CT scan. A previous study reported majority of patients (87%) with extrahepatic HCC had intrahepatic stage III (10%) and stage IVa (76%) tumors[25]. Natsuizaka *et al*[24] and Uka *et al*[26] also reported patients with more advanced intrahepatic tumor stage at the first diagnosis of HCC developed extraheaptic metastases more frequently. Especially, tumor diameter is a well-known predictor of extrahepatic metastases[27,28]. Our results demonstrated tumor size ($\geq$ 5 cm) ($P = 0.042$) was predictive factors for extrahepatic metastases in HCC which was strongly correlated with cumulative survival rate.

As previously mentioned, the most common site of the first detectable metastasis is lung. Our data showed that the sensitivity of ^{18}F-FDG PET/CT scan to detect

lung metastases was only 60.9%. Therefore, we suggest that patients with diagnosed of HCC should undergo chest CT at initial diagnosis of HCC. Sixteen of 22 patients (72.7%) with lymph node metastases and 6 of 11 patients (54.5%) with bone metastases were not accompanied by lung metastases, so the patients at high risk of extrahepatic metastases or who was diagnosed of lung metastases by chest CT should be considered performing ^{18}F-FDG PET/CT scan to identify other extrahepatic metastases.

Our data showed that average SUV in ^{18}F-FDG PET/CT scan is indicative factor for extrahepatic metastases, staging evaluation for metastases should be done carefully at regular interval in patients with high average SUV uptake. The SUV is well correlated with histologic differentiation and cumulative survival rate, therefore we can apply this information in clinical settings to make a decision for the treatment and predict the prognosis. PET imaging is highly sensitive for the diagnosis of bone metastases, it should be considered to be done when patients are suspicious of bone metastases, but negative results in bone scan.

There were limitations to our study. (1) It was a retrospective study; (2) We did not confirm the extrahepatic metastases by biopsy; and (3) In histologic grading of intrahepatic HCC, needle biopsy is prone to sampling error as only limited area of the tumor is analyzed microscopically.

In conclusion, ^{18}F-FDG PET/CT scan has a limitation for detection of intrahepatic tumor, but meaningful for prediction of prognosis and planning for staging evaluation. In aspect of a screening tool of extrahepatic metastasis of HCC, ^{18}F-FDG PET/CT scan is invaluable for detection of lung metastases larger than 1 cm and bone metastases. In evaluation of lymph node metastases, follow-up imaging is crucial for clinical diagnosis. We suggest that primary HCC having larger than 5 cm and increased average SUV uptake more than 3.4 should be considered for extrahepatic metastases.

COMMENTS

Background

With advances in variable treatment modalities, the prognosis of hepatocellular carcinoma (HCC) has been much improved. With prolonged survival of HCC patients, the incidence of extrahepatic metastases has been increased.

Research frontiers

Positron emission tomography (PET)/computed tomography (CT) scan using fluorodeoxyglucose (FDG) is now well established as a noninvasive diagnostic tool for diagnosis, staging and monitoring of a variety of malignant tumors. However, the role in diagnosis of primary HCC and extrahepatic metastases has not been reported sufficiently.

Innovations and breakthroughs

^{18}F-FDG PET/CT scan has a limitation for detection of intrahepatic tumor, but meaningful for prediction of prognosis and planning for staging evaluation. The detection rate of metastatic pulmonary nodule $\geq$ 1 cm was 12/13 (92.3%), when < 1 cm was 2/10 (20%) in PET imaging. The accuracy of PET imaging was significantly superior compared with the accuracy of bone scan for detecting bone metastases. In multivariate analysis, increased tumor size ($\geq$ 5 cm) (P = 0.042) and increased average standardized uptake value (SUV) uptake (P = 0.028) were predictive factors for extrahepatic metastases.

Applications

The study results suggest that ^{18}F-FDG PET/CT scan is invaluable for detection of lung metastases larger than 1 cm and bone metastases. Authors suggest that primary HCC having larger than 5 cm and increased average SUV uptake more than 3.4 should be considered for extrahepatic metastases.

Terminology

PET/CT scan: PET/CT scan depicts the spatial distribution of metabolic or biochemical activity in the body. PET/CT scan has revolutionized many fields of medical diagnosis, by adding precision of anatomic localization to functional imaging.

Peer review

This is a well-organized study in which authors analyze the substantial role in the diagnosis of extrahepatic metastases in HCC. Furthermore, the results are interesting that average SUV could suggest the prognosis of HCC. In patients with higher average SUV more than 3.4 should be carefully follow-up for the possibility of extrahepatic metastases.

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BRIEF ARTICLE

Noninvasive assessment of hepatic fibrosis in Egyptian patients with chronic hepatitis C virus infection

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Abstract

AIM: To evaluate the accuracy of specific biochemical markers for the assessment of hepatic fibrosis in patients with chronic hepatitis C virus (HCV) infection.

METHODS: One hundred and fifty-four patients with chronic HCV infection were included in this study; 124 patients were non-cirrhotic, and 30 were cirrhotic. The following measurements were obtained in all patients: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin, prothrombin time and concentration, complete blood count, hepatitis B surface antigen (HBsAg), HCVAb, HCV-RNA by quantitative polymerase chain reaction, abdominal ultrasound and ultrasonic-guided liver biopsy. The following ratios, scores and indices were calculated and compared with the results of the histopathological examination: AST/ALT ratio (AAR), age platelet index (API), AST to platelet ratio index (APRI), cirrhosis discriminating score (CDS), Pohl score, Göteborg University Cirrhosis Index (GUCI).

RESULTS: AAR, APRI, API and GUCI demonstrated good diagnostic accuracy of liver cirrhosis (80.5%, 79.2%, 76.6% and 80.5%, respectively); *P* values were: < 0.01, < 0.05, < 0.001 and < 0.001, respectively. Among the studied parameters, AAR and GUCI gave the highest diagnostic accuracy (80.5%) with cutoff values of 1.2 and 1.5, respectively. APRI, API and GUCI were significantly correlated with the stage of fibrosis (*P* < 0.001) and the grade of activity (*P* < 0.001, < 0.001 and < 0.005, respectively), while CDS only correlated significantly with the stage of fibrosis (*P* < 0.001) and not with the degree of activity (*P* > 0.05). In addition, we found significant correlations for the AAR, APRI, API, GUCI and Pohl score between the non-cirrhotic (F0, F1, F2, F3) and cirrhotic (F4) groups (*P* values: < 0.001, < 0.05, < 0.001, < 0.001 and < 0.005, respectively; CDS did not demonstrate significant correlation (*P* > 0.05).

CONCLUSION: The use of AAR, APRI, API, GUCI and Pohl score measurements may decrease the need for liver biopsies in diagnosing cirrhosis, especially in Egypt, where resources are limited.

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INTRODUCTION

Egypt has the highest prevalence of adult hepatitis C virus (HCV) infection in the world, affecting an average of 15%-25% of the population in rural communities[1,2]. Worldwide, HCV is one of the major causes of chronic liver diseases, which include inflammation, fibrosis and cirrhosis. Furthermore, HCV has been associated with increased morbidity and mortality in hepatocellular carcinoma[3-5].

Although liver biopsy is an invasive procedure and includes a risk of complications, such as pain, pneumothorax, puncture of other viscera and hemorrhage, it is still the gold standard for grading the severity of necroinflammation and staging the extent of liver fibrosis in patients with chronic HCV infection[6-8].

In addition to the added cost, liver biopsy cannot be performed universally in all patients with impaired hemostasis of any origin[9]. The procedure is known to underestimate liver fibrosis when small tissue samples are collected, and it is prone to intra- and inter-observer variation[10-13]. Moreover, several studies have suggested that liver biopsy is far from being a perfect diagnostic tool because its accuracy in detecting pathology is dependent on the size of the biopsy[14-17]. Previous reports have proposed that a liver biopsy sample should contain a minimum of 5 portal tracts and be at least 15 mm in length to be considered adequate[18-20]. Other authors have recommended even larger samples[21]. In 2003, a French survey reported that liver biopsy may be refused by up to 59% of patients[22]. In 2005, an Italian survey reported major discrepancies among hepatologists regarding when and how to take a liver biopsy from the same subgroup of chronic hepatitis C patients[23].

Considering these limitations, many studies have recently focused on the development of non-invasive markers as surrogates of liver biopsy[24-34]. An accurate assessment of hepatic fibrosis can be achieved with various markers and indices. In this study, we aimed to assess the validity of six markers of hepatic fibrosis, including the ratio of aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (AAR), AST to platelet ratio index (APRI), age platelet index (API), cirrhosis discriminating score (CDS), Göteborg University Cirrhosis Index (GUCI) and Pohl score, in grading fibrosis and diagnosing early cirrhosis as an accurate alternative to liver biopsy in patients with chronic HCV infection in a country known to have a high prevalence of the disease[1,2].

MATERIALS AND METHODS

Patients
This study included 154 patients with chronic HCV infection. They were selected from the gastroenterology and hepatology clinics of the Faculty of Medicine, Cairo University, Egypt, over the period from March 2009 to November 2010. All selected patients were potential candidates for interferon therapy.

Exclusion criteria
Patients with chronic hepatitis B infection, autoimmune hepatitis, decompensated liver disease, hepatocellular carcinoma, history of previous antiviral therapy and presence of absolute contraindication for liver biopsy were excluded from this study.

Methods
All patients were subjected to full history intake, thorough physical examination and the following laboratory test measurements: serum ALT, AST, albumin, total bilirubin, prothrombin time and concentration, complete blood count, HCV antibody (anti-HCV), hepatitis B surface antigen (HBsAg), HCV-RNA by quantitative polymerase chain reaction (PCR), circulating autoantibodies (ANA, ASMA), abdominal ultrasonography and ultrasonographic guided liver biopsy.

Liver biopsies were performed using 18-20 gauge Trucut needles (GMSS.N, GHATWARY MEDICAL). To assess necroinflammation, the grade of activity was evaluated using a modified hepatic activity index: mild (0-6), moderate (7-12) and severe (13-18). Fibrosis was staged according to the METAVIR scoring system from F0 to F4. Based on the results obtained from histopathological assessment of their liver biopsies, patients were divided into two groups:the non-cirrhotic group (F0, F1, F2 and F3) and the cirrhotic group (F4).

Definition of the noninvasive indices
The following ratios, scores and indices[24-34] were calculated and compared with the results of histopathological examination: (1) AAR; (2) APRI, calculated using the following equation: (AST/upper limit of normal)/platelet count $(\times 10^9/L) \times 10$; (3) API, calculated by summing the scores awarded for the following patient laboratory results (a possible value of 0-10): age (in years) < 30 = 0; 30-39 = 1; 40-49 = 2; 50-59 = 3; 60-69 = 4; $\geq$ 70 = 5; platelet count $(\times 10^9/L)$: $\geq$ 225 = 0; 200-224 = 1; 175-199 = 2; 150-174 = 3; 125-149 = 4; < 125 = 5; (4) CDS, calculated by summing the scores awarded for the following patient laboratory results (a possible value of 0-11): platelet count $(\times 10^9/L)$: > 340 = 0; 280-339 = 1; 220-279 = 2; 160-219 = 3; 100-159 = 4; 40-99 = 5; < 40 = 6. ALT/AST ratio: > 1.7 = 0; 1.2-1.7 = 1; 0.6-1.19 = 2; < 0.6 = 3. International normalized ratio (INR): < 1.1 = 0; 1.1-1.4 = 1; > 1.4 = 2; (5) GUCI, calculated using the following equation: normalized AST $\times$ INR $\times$ 100/platelet count $(\times 10^9/L)$; and (6) Pohl score,which was considered positive if the AAR was $\geq$ 1 and the platelet

Table 1 The demographic and laboratory data of all patients (mean ± SD)

Item	Non-cirrhotic group (F0, F1, F2 and F3) n (124)	Cirrhotic group (F4) n (30)	P value
Age, yr	37.19 ± 9.58	47.87 ± 7.76	0.0002
Gender, n (%)			
Male	86 (69.35)	18 (60)	
Female	38 (30.65)	12 (40)	
AST (IU/mL)	48.84 ± 42.7	61 ± 20.4	0.01
ALT (IU/mL)	60.235 ± 42.3	57.7 ± 24.69	0.68
Alkaline phosphatase (U/L)	81.654 ± 38.4	111 ± 42.5	0.004
Total bilirubin (mg/dL)	0.787 ± 0.30	1.003 ± 0.38	0.07
Albumin (g/dL)	5.803 ± 0.789	4.1 ± 0.42	0.004
INR	1.127 ± 0.092	1.254 ± 0.12	0.0001
Platelet count (/mm^3)	213.75 ± 66.1	151.87 ± 73.79	0.001
HCV viraemia, IU/mL	893 015.72 ± 1 571 254.86	347 974.86 ± 536 542.77	0.23

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio; HCV: Hepatitis C virus.

Table 2 Mean values (± SD) of aspartate aminotransferase-to-alanine aminotransferase ratio, aspartate aminotransferase-to-platelet ratio index, age platelet index, cirrhosis discriminating score and Göteborg University Cirrhosis Index in non-cirrhotic and cirrhotic groups of chronic hepatitis C virus infected patients

Variable	Non-cirrhotic group (F0, F1, F2 and F3), n (124)	Cirrhotic group (F4), n (30)	P value
AAR	0.84 ± 0.31	1.23 ± 0.47	0.001
APRI	0.078 ± 0.09	0.118 ± 0.07	0.02
API	2.98 ± 2.21	5.87 ± 1.99	0.0001
CDS	5.48 ± 1.36	6 ± 1.31	0.17
GUCI	0.913 ± 1.27	1.6573 ± 0.89	0.001
Pohl score	+ve 12 (9.67%)	+ve 12 (40%)	0.004
	-ve 112 (90.3%)	-ve 18 (60%)	

AAR: Aspartate aminotransferase-to-alanine aminotransferase ratio; APRI: Aspartate aminotransferase-to-platelet ratio index; API: Age platelet index; CDS: Cirrhosis discriminating score; GUCI: Göteborg University Cirrhosis Index.

count was $< 150 \times 10^9$/L. The Ethics committee at our institution approved the study, and all patients provided informed consent before participating in this study.

Statistical analysis

Descriptive statistics included range, mean ± SD, median, frequencies (number of cases) and percentages when appropriate. Comparisons of numerical variables between the study groups were made using the Mann Whitney U test for independent samples. To compare categorical data, the Chi squared (χ^2) test was used. When the expected frequency was less than 5, the Exact test was used instead. Accuracy was represented using the terms sensitivity and specificity. Receiver operator characteristic analysis was used to determine the optimum

Table 3 The correlation between age and variable laboratory data, and the stage of fibrosis and grade of necroinflammatory activity

Variable	Stage of fibrosis		Grade of activity	
	Correlation coefficient	P value	Correlation coefficient	P value
Age	0.4	0.0003	0.3	0.005
AST	0.3	0.003	0.3	0.006
ALT	0.2	0.07	0.9	0.1
Alkaline phosphatase	0.3	0.006	0.2	0.08
Total bilirubin	0.2	0.07	0.3	0.003
Albumin	-0.3	0.002	-0.2	0.08
INR	0.4	0.001	0.2	0.13
Platelet count	-0.5	0.000001	-0.4	0.0002
HCV RNA load	-0.07	0.5	0.07	0.5

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio; HCV: Hepatitis C virus.

cutoff value for the studied diagnostic markers. Various variables were tested for correlation using the Spearman rank correlation equation for non-normal variables. P values less than 0.05 were considered statistically significant. Normality of data was checked by the Kolmogorov Smirnov test. Most of our markers violated the normal assumption; therefore, the data were analyzed using non-parametric tests. Two-tailed tests were used where appropriate. Multivariate logistic regression determined only API to be significantly associated with diagnosis of cirrhosis in our cases. No other variable was found to be a significant predictor of cirrhosis. All statistical calculations were performed using the computer programs Microsoft Excel 2007 (Microsoft Corporation, NY, United States) and SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, United States) version 15 for Microsoft Windows.

RESULTS

Demographic and baseline laboratory data of non-cirrhotic and cirrhotic patients are shown in Table 1.

Our findings demonstrated a statistically significant correlation for AAR, APRI, API, GUCI and Pohl score between the cirrhotic and non-cirrhotic patients; CDS was not found to be significant. Pohl score was positive (indicating cirrhosis) in 40% of cirrhotic patients, whereas it was positive in only 9.67% of non-cirrhotic patients, with a P value of 0.004 (Figure 1 and Table 2).

Patient age, AST and platelet count correlated significantly with both the grade of activity and the stage of fibrosis. However, neither ALT nor HCV RNA load demonstrated statistically significant correlations with the grade of activity or the stage of fibrosis. With regard to other laboratory parameters, INR, albumin and alkaline phosphatase levels were significantly correlated with stage of fibrosis but not with grade of activity, whereas serum bilirubin was significantly correlated with grade of activity but not with stage of fibrosis (Table 3).

The results of our study revealed a significant correla-

Table 4 The correlation between the aspartate aminotransferase-to-alanine aminotransferase ratio, aspartate aminotransferase- to-platelet ratio index, age platelet index, cirrhosis discriminating score and Göteborg University Cirrhosis Index and the grade of necroinflammatory activity and the stage of fibrosis

Variable	Stage of fibrosis		Grade of activity	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
AAR	0.2	0.054	0.1	0.23
APRI	0.4	0.00006	0.4	0.001
API	0.6	0.000001	0.5	0.00002
CDS	0.4	0.0002	0.2	0.056
GUCI	0.5	0.0001	0.3	0.003

AAR: Aspartate aminotransferase-to-alanine aminotransferase ratio; APRI: Aspartate aminotransferase-to-platelet ratio index; API: Age platelet index; CDS: Cirrhosis discriminating score; GUCI: Göteborg University Cirrhosis Index.

tion between APRI, API and GUCI, and both the grade of activity and the stage of fibrosis. CDS correlated significantly with the stage of liver fibrosis but not with the grade of necroinflammatory activity. In contrast, the AST/ALT ratio had no significant correlation with either the stage of fibrosis or the grade of activity (Table 4).

For non-invasive diagnosis of liver cirrhosis (F4), using AAR, APRI, API and GUCI, Table 5 and Figure 2 show the cutoff values, sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), and area under the receiver operating characteristics curve of these parameters.

DISCUSSION

Although it is costly, requires hospitalization for at least 6-18 h, is invasive and carries a risk of complications with an associated morbidity rate between 0.3% and 0.6% and mortality rate of 0.05%, liver biopsy remains the gold standard for assessing liver histology[34-36]. However, limitations of liver biopsy include the underestimation of fibrosis stage, given that only 1/50 000 of the organ is removed[37], and the reported inter- and intra-observer discrepancies rates of 10%-20%[38,39].

In this study, we found that the optimal cutoff AAR-value for diagnosing cirrhosis was ≥ 1.2, with a sensitivity of 46%, specificity of 88.7% and PPV and NPV of 50% and 87.3%, respectively. These results support previous findings by Giannini *et al*[40], who recommended an AAR value of ≥ 1 as a cutoff value for diagnosing cirrhosis. However, Ehsan *et al*[41] reported a higher cutoff value (≥ 1.5) for diagnosing cirrhosis, with a sensitivity of 44% and a specificity of 91%.

Elevation of the AST/ALT ratio in cirrhotic patients may be explained by the reduction in AST clearance, which leads to an increase in serum AST levels. In addition, advanced liver disease may be associated with mitochondrial injury, resulting in increased release of AST present in the mitochondria and cytoplasm[42].

Thrombocytopenia in patients with advanced fibrosis

Figure 1 Positive and negative Pohl score in non-cirrhotic and cirrhotic patients with chronic hepatitis C virus infection.

Figure 2 Sensitivity and specificity of age platelet index, Göteborg University Cirrhosis Index, aspartate aminotransferase to platelet ratio index and aspartate aminotransferase/alanine aminotransferase ratio in diagnosing cirrhosis in patients with chronic hepatitis C virus infection. API: Age platelet index; GUCI: Göteborg University Cirrhosis Index; APRI: Aspartate aminotransferase to platelet ratio index; AAR: Aspartate aminotransferase/alanine aminotransferase ratio.

may be due to reduced hepatic production of thrombopoietin, increased splenic sequestration of platelets secondary to portal hypertension or the myelosuppressive action of HCV[43,44].

Results from the current study revealed a significant correlation between APRI and both the stage of liver fibrosis and the grade of activity. The optimal cutoff APRI value for the diagnosis of cirrhosis was ≥ 1.36, which was consistent with findings by Ichino *et al*[45] and Ehsan *et al*[41], who reported cutoff values of 1.3 and 1.5, respectively.

In the present study, we found a significant correlation between API and both the stage of fibrosis and the grade of activity (*P* < 0.001 for both). Our results revealed that the optimal AP index cutoff value for the diagnosis of cirrhosis was ≥ 5.5, with 60% and 80.6% sensitivity and specificity, respectively, and 42.86% and 89.29% PPV and NPV, respectively. The results of the current study are in agreement with the results of previous studies by Lackner *et al*[46] and Poynard *et al*[47].

Results from this study showed that there was a significant correlation between GUCI and both the stage of liver fibrosis and the grade of activity. We recommend a GUCI value of ≥ 1.56 as an optimal cutoff value for

Table 5 The accuracy of different ratios and indices in the diagnosis of early liver cirrhosis

Item	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUROC	*P* value
AAR	1.2	46	88.7	50	87.3	80.5	0.761	0.002
APRI	1.36	46.7	87.1	46.7	87.1	79.2	0.697	0.018
API	5.5	60	80.6	42.9	89.3	76.6	0.826	0.000
GUCI	1.56	60	85.5	50	89.8	80.5	0.783	0.001

AAR: Aspartate aminotransferase-to-alanine aminotransferase ratio; APRI: Aspartate aminotransferase-to-platelet ratio index; API: Age platelet index; GUCI: Göteborg University Cirrhosis Index; PPV: Positive predictive value; NPV: Negative predictive value; AUROC: Area under the receiver operating characteristics.

the diagnosis of cirrhosis, with 60% sensitivity, 88.7% specificity, and a PPV and NPV of 89.83% and 80.52%, respectively. These results supported those reported by Islam *et al*[48], who found a significant correlation between GUCI and both stage of fibrosis and grade of activity. Similar results were reported by Ehsan *et al*[41], who recommended a GUCI cutoff value of $\geq$ 1.5 for the diagnosis of cirrhosis, with 89% specificity and 74% sensitivity.

In the present study, we found a statistically significant correlation ($P = 0.004$) between positive Pohl score (AAR $\geq$ 1, and platelet count < 150×10^9/L) and the presence of cirrhosis (F4). These findings supported the results of Pohl *et al*[27] and Lackner *et al*[46], who confirmed the diagnostic accuracy of the Pohl score in significant fibrosis and cirrhosis.

In our study, there was a significant correlation between CDS and stage of liver fibrosis ($P < 0.001$), but the relationship was not significant with regard to the grade of activity ($P = 0.056$). The CDS values were not significant between the cirrhotic and non-cirrhotic patients ($P = 0.17$), which disagreed with results reported by Ichino *et al*[45], who recommended a CDS value of $\geq$ 8 as a cutoff value for the diagnosis of cirrhosis.

Some studies showed no correlation between the histological outcome and HCV-RNA levels, while other reports suggested that the viral titer may influence the severity of liver damage and that high titer viremia correlates with the most severe liver damage[49]. The current study revealed no significant correlation between HCV RNA load as measured by quantitative PCR and both the grade of activity and fibrosis stage.

Our results agreed with the studies conducted by Lee *et al*[50] and Saleem *et al*[51]. In contrast, Kato *et al*[52] found significantly higher HCV RNA loads in patients with chronic active hepatitis and cirrhosis compared to those with chronic persistent hepatitis. These discrepancies could be attributed to the fact that serum HCV RNA load is not a stable parameter because it fluctuates[53]. In addition, a high amount of circulating HCV does not always imply a more active state of viral replication in the liver nor does it indicate a more severe degree of liver disease. HCV is known to replicate both within the liver as well as in extra-hepatic sites[54,55].

In conclusion, the API index, APRI, AST/ALT ratio and GUCI showed good accuracy, moderate sensitivity, and high specificity for the diagnosis of early cirrhosis. These measures also demonstrated significant correlation with both the stage of liver fibrosis and the grade of activity. The combination of these non-invasive biochemical markers may replace the requirement for liver biopsy, particularly for cases with cirrhosis or early cirrhotic changes in which the procedure has known limitations and complications.

COMMENTS

Background
Hepatitis C virus (HCV) is one of the major causes of chronic liver diseases worldwide. It has been associated with increased morbidity and mortality in hepatocellular carcinoma. In patients with chronic HCV infection, liver biopsy is essential to the assessment of hepatic fibrosis. Evaluating the degree of fibrosis is an important step in determining the need and priority for treatment with antiviral drugs. However, liver biopsy is a costly and invasive procedure with a risk of complications and a tendency to underestimate liver fibrosis. Hence, alternative non-invasive diagnostic tools are needed.

Research frontiers
In the area of liver cirrhosis assessment, the focus of research is on how to use biochemical markers and indices [aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (AAR), AST to platelet ratio index (APRI), age platelet index (API), cirrhosis discriminating score (CDS), Göteborg University Cirrhosis Index (GUCI) and Pohl score] calculated from simple routine laboratory tests, such as serum levels of bilirubin, ALT, AST, albumin and platelet count, to determine the severity of liver fibrosis and to evaluate their accuracy in comparison to liver biopsy.

Innovations and breakthroughs
The results showed that APRI, API, GUCI and CDS were significantly correlated with the degree of liver fibrosis. AAR, APRI, API, GUCI and Pohl score can accurately diagnose early liver cirrhosis. AAR and GUCI gave the highest accuracy for the diagnosis of liver cirrhosis (80.5%). These simple biochemical markers, especially when used in combination, may decrease the use of liver biopsy in the assessment of fibrosis and diagnosis of cirrhosis in patients with chronic HCV infection.

Applications
The study results suggest that these biochemical markers can identify significant fibrosis and cirrhosis in patients with chronic HCV; their combined application may decrease the need for liver biopsy, thereby reducing its associated costs and complications. Important fields for further study include the use and evaluation of these markers for repeated assessment in monitoring the progression of liver fibrosis and its regression following interferon treatment in patients with chronic hepatitis C.

Terminology
CDS, GUCI and Pohl score are indices calculated to develop noninvasive diagnostic markers of liver fibrosis depending on simple biochemical tests such as platelet count, AST and ALT.

Peer review
In this paper, the authors focused on the noninvasive assessment of liver fibrosis in Egyptian patients with chronic HCV infection using different indexes. It is potentially interesting and well-written and provides useful information in a selected population with a high prevalence of chronic HCV infection.

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BRIEF ARTICLE

Overexpression of metastasis-associated in colon cancer 1 predicts a poor outcome of hepatitis B virus-related hepatocellular carcinoma

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Abstract

AIM: To investigate the intratumoral expression of metastasis-associated in colon cancer 1 (MACC1) and c-Met and determine their clinical values associated with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC).

METHODS: A retrospective study admitted three hundred fifty-four patients with HBV-related HCC. The expression and distribution of MACC1 and c-Met were assessed by quantitative real-time polymerase chain reaction and immunohistochemistry staining. Prognostic factors influencing survival, metastasis and recurrence were assessed.

RESULTS: Intratumoral MACC1 level was found to be associated with HCC disease progression. Both median tumor-free survival (TFS) and overall survival (OS) were significantly shorter in the postoperative HCC patients with high intratumoral MACC1 expression, as compared to those with low intratumoral MACC1 levels (TFS: 34 mo *vs* 48.0 mo, $P < 0.001$; OS: 40 mo *vs* 48 mo, $P < 0.01$). Multivariable analysis indicated that high MACC1 expression or co-expression with c-Met were independent predictors for HCC clinic outcome ($P < 0.001$).

CONCLUSION: High intratumoral MACC1 expression can be associated with enhanced tumor progression and poor outcome of HBV-related HCC. MACC1 may serve as a prognostic biomarker for postoperative HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third cause of death from cancers worldwide[1]. The incidence of HCC in China is high, and most cases are associated with chronic hepatitis B virus (HBV) infection[2]. Hepatocarcinogenesis is a complex process associated with the accumulation of multiple genetic and epigenetic changes during the initiation, progression and maturation of this fatal disease[3,4]. As such, intensive research efforts have been carried out to determine the physiological, cellular and molecular mechanisms of HCC, in the hope of developing effective preventative measures and improved treatment strategies.

The metastasis-associated in colon cancer 1 (MACC1) gene was identified by a genome-wide screen of human colon cancer samples, and its expression was closely related to the metastasis of colon cancers[5]. Subsequent clinical studies have suggested that MACC1 might be an important predictor for metastasis and recurrence of colon cancers. Further studies have revealed that MACC1-induced tumorigenesis is correlated with enhanced hepatocyte growth factor (HGF)/c-Met signaling[6,7]. MACC1 functions as a transcription factor, and one of its target promoters is that of the receptor tyrosine kinase c-Met gene. Binding of MACC1 to the promoter has been demonstrated to stimulate c-Met transcription, ultimately inducing activation of the HGF/c-Met signaling pathway and enhancing cell proliferation, motility, and metastasis[8,9]. MACC1 is normally expressed in healthy liver tissues, but marked overexpression is frequently observed in HCC clinical samples[10]. To date, however, the clinical significance of MACC1 overexpression in HCC and of the correlation between MACC1 and the c-Met signaling in the disease state remain unknown. It is intriguing to speculate that MACC1 may contribute to HCC onset and progression, and therefore may represent a readily-detectable biomarker for tumor recurrence and/or metastasis in postoperative HCC patients.

In this study, we sought to determine the expression levels of MACC1 in HBV-related HCC at different disease stages and analyze its correlation with clinical outcome. In addition, we evaluated the related levels of its transcriptional target, c-Met. Our data indicated that MACC1 expression levels represent an effective prognostic factor for HBV-related HCC patients who undergo hepatectomy.

MATERIALS AND METHODS

Patients, clinical characteristics, and tissue sampling

Tumor samples were obtained from 412 consecutive patients admitted to The 302nd Hospital (Beijing, China) with HBV-related HCC from December 2004 to June 2006. The diagnosis of HCC was based on the criteria of the European Association for the Study of the Liver[11]. By using the Barcelona Clinic Liver Cancer (BCLC) staging classification system[12], 148 patients were classified as stage A, 144 as stage B, and 120 as stage C. All patients,

Table 1 Basic clinical characteristics of patients with hepatitis B virus-related hepatocellular carcinoma (*n* = 354)

Clinical features	Stage A	Stage B	Stage C
Cases (*n*)	138	96	120
Median age (yr, range)	55 (24-68)	53 (29-70)	52 (21-72)
Male/female	118/20	82/14	106/14
Median tumor diameter (cm, range)	2.5 (1.5-3)	4.0 (3-5)	4.5 (2-6)
AFP (μg/L, > 400/≤ 400)	45/93	40/56	85/35
HBV DNA (+/-)	78/60	57/39	76/44
HBeAg (+/-)	50/88	32/64	54/66
ECOG PS (0/1/2)	92/30/16	60/25/11	25/55/40
Child-Pugh (A/B)	92/46	67/29	58/62
Tumor number (single/multinodular)	123/15	56/40	67/53
Invasion of portal vein (+/-)	0/138	0/96	120/0
Tumor differentiation (high/intermediate/low)	45/66/27	27/38/31	20/50/50

HBV: Hepatitis B virus; ECOG PS: Eastern Cooperative Oncology Group Performance Status Scale; AFP: α- fetoprotein; HBeAg: HBV e antigen.

except for those at stage C, had undergone surgical resection. Among all patients, 36 had an incomplete resection, 12 died from other causes without recurrence, and 10 were lost to follow-up for non-medical reasons. Thus, the total study population was composed of 354 patients (Table 1). Matched non-tumor tissue samples were obtained from all surgical resected participants and were generally taken from a distance of more than 2 cm from the tumor tissue. Tumor samples from the stage C individuals were obtained by using the Single Action Biopsy Device (Promex Technologies, United States) and target tissues were identified by the following criteria: solitary lesions, or up to three nodules ≤ 6 cm in size; partial portal vein thrombosis or vena cana invasion; absence of extrahepatic metastasis; and preserved liver function (Child-Pugh A or ≤ B8 with serum bilirubin levels under 51.3 μmol/L). In addition, ten normal liver tissues were obtained from four cases of hepatic hemangioma and six patients with hepatic cyst, none of which had a history of viral hepatitis or liver cirrhosis.

Each of the samples were divided and either prepared for histochemical staining or snap-frozen in liquid nitrogen for RNA extraction for use in subsequent reverse transcription (RT)-polymerase chain reaction (PCR). For hematoxylin and eosin (HE) and immunohistochemical staining, the tissues were fixed in 10% formalin and paraffin-embedded.

The study protocol was approved by The 302nd Hospital Research Ethics Committee, and written informed consent was obtained from all participants or their legal guardian. None of the patients had received prior treatment for HCC, including radiation or chemotherapy. Patients were followed up every 2 mo within the first postoperative year and at approximately 3-4 mo intervals thereafter. Routine evaluation included physical examination, chest roentgenography, blood chemistry analysis, HBV-DNA test, and measurement of tumor markers (carcinoembryonic antigen and α-fetoprotein). Chest

and abdominal computed tomography, brain magnetic resonance imaging and a bone scintiscan were performed every 6 mo for three years after surgery. Additional examinations were performed if any symptoms or signs of recurrence were detected.

Determination of the mRNA levels of MACC1 and c-Met

The levels of the mRNA transcripts of MACC1 and c-Met were determined by quantitative real-time PCR, as described previously[13]. β-actin mRNA expression was used as an internal control and the relative gene expression values were calculated by the $2^{-\Delta Ct}$ method using Sequence Detection System 2.1 software. Total RNA was isolated from the tissues by using an RNA isolation kit (Qiagene, Germany) and following the manufacturer's instructions. The concentration of RNA was determined by spectrophotometric measurement at A_{260}, and the purity was verified by the $A260/A280$ ratio (> 1.8 was sufficiently pure). A total of 2 μg RNA was used for the preparation of cDNA by reverse transcriptase-PCR (SYBR PrimeScript RT-PCR Kit with SYBR Premix Ex Taq; Takara, Japan). The following PCR primers were used: MACC1 cDNA (136 bp), 5'-TTCTTTTGATTCCTCCGGTGA-3' (F) and 5'-ACTCTGATGGGCATGTGCTG-3' (R); c-Met cDNA (173 bp), 5'-GCAGGTTGTGGTTTCTCG-3' (F) and 5'-TGCAGCCCAAGCCATTCA-3' (R); and β-actin cDNA (125 bp), 5'-CGGGAAATCGTGCGTGAC-3' (F) and 5'-AGGCAGCTCGTAGCTCTTCT-3' (R). The cDNA equivalent of 50 ng of the original RNA was used in the PCR. The 50 μL reactions for MACC1 or c-Met were run for 40 cycles as follows: predenaturation at 95 ℃ for 30 s, denaturation at 95 ℃ for 5 s, annealing and extension at 60 ℃ for 30 s. The target mRNA was normalized to the corresponding β-actin signal. Measurements were performed in triplicate.

Assessment of MACC1 and c-Met in HCC by immunohistochemistry

Paraffin-embedded tissues from resected tumor and non-tumor tissues or biopsied tumor samples were cut for serial microtome sections with 4 μm thickness. After hematoxylin and eosin staining, the samples were assessed by two independent pathologists using Edmondson criteria[14]. Samples were classified as: well differentiated, corresponding to Edmondson's Grade Ⅰ or Ⅰ-Ⅱ; moderately differentiated, corresponding to Edmondson's Grade Ⅱ or Ⅱ-Ⅲ; or poorly differentiated, corresponding to Edmondson's Grade Ⅲ or Ⅲ-Ⅳ.

Two additional serial sections from each individual were prepared for MACC1 and c-Met immunohistochemical staining. Monoclonal rabbit anti-human antibody against MACC1 (1:50; Sigma, United States) and rabbit anti-human antibody against c-Met (1:250; Abcam, Hong Kong) were used. Detection of MACC1 and c-Met was carried out with 3-amino-9-ethylcarbozole (AEC; Zhongshan Bio, China) and diaminobenzidine (DAB; R and D Systems, United States), respectively. Positive stain-

ing was indicated by a prominent brownish or red pigmentation. In each case, a negative control was prepared using phosphate buffered saline as the first antibody to ensure the specificity of immunostaining.

The extent of positive staining for MACC1 was scored as follows[15]: 0, ≤ 10%; 1, > 10%-25%; 2, > 25%-50%; 3, > 50%-75%; and 4, > 75%. The intensity of the special staining was scored as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong. The final score was obtained by multiplying the extent scores and intensity scores, which produced values in a range from 0 to 12. Scores from 9-12 were defined as a strong staining pattern (++), scores from 0-4 were defined as negative expression (-), and scores from 6-8 were defined as an intermediate staining pattern (+). All the staining was evaluated and characterized by two independent pathologists.

Serological assays

Hepatitis B surface antigen (HBsAg), anti-HBs, HBeAg, anti-HBe and anti-HBc were detected using a commercially-available kit (Roche Diagnostics, United States) and electrochemiluminescence immunoassay analyzers (E170; Modular Analytics, Roche Diagnostics). HBV DNA was extracted from 200 μL of plasma sample from each study participant using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics Applied Science, Germany) and following the manufacturer's instructions. The viral titer and genotype of HBV were determined by a real-time PCR-based method that used fluorescent hybridization probes and a LightCycler PCR machine (Roche Diagnostics). This method consisted of two steps that were carried out in a single tube: the first step used real-time PCR to quantify the viral DNA and the second step used melting curve analysis of the final PCR product to genotype the virus. Details of the design and experimental conditions of this assay are available from the manufacturer. This assay showed a broad linear distribution for HBV titers that ranged from 10^2 to 10^{11} copies/mL, with a lower detection limit of $1-5 \times 10^2$ copies/mL.

Statistical analysis

The primary endpoint of the study was tumor-free survival (TFS) and the secondary endpoint included overall survival (OS) and follow-up for over 48 mo. TFS was calculated from the date of resection to the date when tumor recurrence was diagnosed. OS was calculated from the date of commencement of resection to the date of death or last follow-up[16]. All statistical analyses were performed with SPSS version 16.0 software. Continuous data were expressed as median and range. A comparison between the groups was performed using the χ^2 test. Survival rates were estimated by the Kaplan-Meier method and compared by the log rank test. The Cox proportional hazards model was used to determine the independent factors on survival and recurrence, based on the variables selected in univariate analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Increased intratumoral MACC1 mRNA is related to HCC progression

We analyzed the MACC1 mRNA levels in surgically-resected samples from 234 patients at BCLC stage A or stage B and in biopsied tumor tissues from 120 patients at BCLC stage C. MACC1 mRNA in tumor tissues was found to be increased gradually with the stage of HCC progression (Figure 1A and B). The intratumoral MACC1 mRNA levels detected in samples from HCC stage A (0.002281 ± 0.001972), B (0.003031 ± 0.003451) and C (0.009015 ± 0.004972) were about 3-, 4- and 14-fold higher than that in normal liver tissues (0.000592 ± 0.0000451), respectively. We next performed a paired comparison of gene expression for the 234 patients at stage A and stage B, for which we had matched tumor tissues and adjacent non-tumor liver tissues. The ratio of MACC1 mRNA in cancerous tissue relative to that of the matched paratumors (the T:N ratio) was about 5.4-fold higher in the stage B group than in the stage A group (1.25 ± 0.3 *vs* 0.23 ± 0.05, $P = 0.009$; Figure 1C). Thus, these data indicated that the MACC1 mRNA level in HCC tumors was associated with tumor progression.

We next determined the protein levels of MACC1 in tumor and paratumor tissues by analyzing immunohisto-chemistry scores. MACC1 protein levels were found to be significantly higher in malignant tissues than in paratumor tissues or normal liver tissues (both, $P < 0.001$). Compared with the corresponding peritumor tissue or normal liver tissues, tumors from 30 of 138 (22%) patients at stage A, 40 of 96 (41.6%) at stage B, and 80 of 120 (67%) at stage C displayed increased MACC1 expression (Figure 1D). Tumor cells demonstrated mild to strong positive MACC1 cytoplasmic staining (++) and apparent nuclear signals in some cases (Figure 1E).

Intratumoral MACC1 mRNA level correlates with clinical parameters in HCC patients

Patients with HCC ($n = 354$) were divided into two groups according to the median intratumoral MACC1 mRNA levels. The first group was composed of low intratumoral MACC1 mRNA (< 0.006732; range: 0.000050-0.036147) and the second of high intratumoral MACC1 mRNA (≥ 0.006732; range: 0.000050-0.036147). Following comparative analysis of these two groups, intratumoral MACC1 mRNA level was found to be associated with HCC clinical staging, age, portal vein invasion and tumor differentiation. However, no significant correlation was found between the intratumoral MACC1 mRNA level and gender, lesion number, α-fetoprotein level or Child-Pugh class (Table 2).

Intratumoral MACC1 expression is correlated with c-MET mRNA levels in HCC patients

c-Met is a well-known proto-oncogene and transcriptional target of MACC1. We next investigated whether overexpression of MACC1 corresponded to increased transcription of c-Met in HCC tissues. We found that intratumoral c-Met mRNA levels were consistent with the extent of MACC1 expression and were up-regulated in conjunction with tumor progression of HCC ($P < 0.01$; Figure 1A and B). In the 234 HCC patients with stage A and stage B, the expression of c-Met was increased in the 105 patients with high MACC1 expression, but decreased in the 129 patients with low MACC1 expression (0.058561 ± 0.017539 *vs* 0.024734 ± 0.018754, $P = 0.041$; Figure 2A). Among these 234 patients, 67 (28.6%) displayed elevated expressions of both MACC1 and c-Met, 93 (39.7%) had low expressions of both MACC1 and c-Met, and 38 (16.2%) had a high expression of MACC1 but a low expression of c-Met, while 36 (15.4%) had a high expression of c-Met but low expression of MACC1. MACC1 mRNA level was closely correlated with the corresponding intratumoral c-Met mRNA expression ($r = 0.360$, $P < 0.001$) (Figure 2B).

Further analysis of the 234 patients found that HCC patients with both MACC1 and c-Met high intratumoral co-expression had a median OS of 32 mo [95% confidence interval (CI): 25-41]. In contrast, those with both MACC1 and c-Met low intratumoral expression had a median OS of 48 mo (log-rank, $P < 0.001$; Figure 2D). Likewise, the patients with both MACC1 and c-Met high intratumoral expression had significantly shorter median TFS (28 mo, 95% CI: 22-33 mo) than those with both MACC1 and c-Met low expression (48 mo, log-rank $P < 0.001$; Figure 2C).

Increased intratumoral MACC1 expression is predictive of high risk of recurrence and poor survival in postoperative HCC patients

We followed up the 234 patients (stage A and stage B) after resection for a median of 30 mo (range: 6-48 mo). Seventy-nine of the 105 HCC patients (75.2%) with high intratumoral MACC1 mRNA levels experienced recurrent tumors, and 34 cases had extrahepatic metastasis. The 1-, 2- and 3-year recurrence-free survival rates were 81%, 67% and 39%, respectively. In contrast, only 41 of the 129 (31.8%) patients with low intratumoral MACC1 expression experienced recurrence, and 12 of those had extrahepatic metastasis. The 1-, 2- and 3-year TFS was 92%, 88% and 82%, respectively. Compared to those with low intratumoral MACC1 expression, patients with high intratumoral MACC1 had significantly high rate of recurrence and extrahepatic metastasis (both, $P < 0.001$). Generally, the HCC patients with high intratumoral MACC1 mRNA levels had a significantly shorter median TFS (34 mo, 95% CI: 30-37 mo) than those with low intratumoral MACC1 mRNA levels (48.0 mo, log-rank $P < 0.001$; Figure 3A). In addition, post-resected patients with low intratumoral MACC1 expression had a median OS of 48 mo, while those with high intratumoral MACC1 expression had 40 mo (95% CI: 34-45 and log-rank $P < 0.01$; Figure 3B). A total of 152 patients with a ratio of T:N MACC1 expression < 1 had a median TFS of 48 mo, compared to 36 mo (95% CI: 25-47) for the 82 patients who had a ratio ≥ 1 (log-rank, $P < 0.001$; Figure 3C).

Figure 1 Analysis of metastasis-associated in colon cancer 1 and c-Met expression in liver tissues. A: Representative metastasis-associated in colon cancer 1 (MACC1) and c-Met mRNA in intratumoral [T: T1 as hepatocellular carcinoma (HCC) stage A, T2, T3 as HCC stage B] and matching paratumor tissues (N: N1, N2 and N3) and normal liver tissues (C) by reverse-transcription polymerase chain reaction (RT-PCR); B: Comparison of MACC1 and c-Met expression levels in 10 normal liver tissue, and HCC with stage A (n = 138), stage B (n = 96), stage C (n = 120) by real-time quantitative-PCR; C: The ratio for MACC1 mRNA levels in HCC stage A and stage B tumor tissues relative to matching paratumor tissues; D: Comparison of the intratumoral and peritumoral MACC1 expression in HCC stage A and stage B, as determined by immunohistochemistry; E: Immunohistochemical staining of MACC1 expression in HCC. a-c: MACC1 and c-Met expression in tumor and matching paratumor tissues in one same HCC patient (× 200). a: HE showed the tumor and paratumor cells; b: MACC1 expression was higher in tumor cytoplasm than in paratumor cells; c: c-Met was expressed on the tumor cell membrane, but no staining on nontumor liver cell membrane; d-f: Representative expression of MACC1 in HCC tumor tissues (× 400). d: MACC1 positive staining occurred mainly in the cytoplasm; e: Nuclear staining of MACC1 in cancer cells; f: Relatively weak staining of MACC1 in an early stage HCC cancer cells, as compared to paratumor cells.

Patients with a ratio of T:N MACC1 expression < 1 had a median OS of 48.0 mo, compared to 41 mo (95% CI: 36-45) for those with a ratio of T:N MACC1 expression ≥ 1 (log-rank, P < 0.001; Figure 3D).

Univariate statistical analysis showed that the TFS was associated with intratumoral MACC1 expression, and recurrence-free survival was related to tumor number, tumor differentiation, MACC1 expression, and co-

Table 2 Metastasis-associated in colon cancer 1 mRNA expression and clinical characteristics of hepatocellular carcinoma patients *n* (%)

Variable	Cases (*n* = 354)	MACC1 mRNA high expression group	MACC1 mRNA low expression group	*P*
Gender				0.214
Male	306	157 (88.7)	149 (84.2)	
Female	48	20 (11.3)	28 (15.8)	
Age (yr)				< 0.001
≥ 55	185	70 (39.5)	115 (65.0)	
< 55	169	107 (60.5)	62 (35.0)	
Tumor size (cm)				0.087
≥ 3	196	90 (50.8)	106 (59.9)	
< 3	158	87 (49.2)	71 (40.1)	
Tumor thrombus				0.007
Yes	120	72 (40.7)	48 (27.1)	
No	234	105 (59.3)	129 (72.9)	
Tumor number				0.356
Single	246	119 (67.2)	127 (71.8)	
Multinodular	108	58 (32.8)	50 (28.2)	
Stage				0.007
Early-middle	234	105 (59.3)	129 (72.9)	
Advanced	120	72 (40.7)	48 (27.1)	
Differentiation				< 0.001
High	92	21 (11.9)	71 (40.1)	
Moderate	154	92 (52.0)	62 (35.0)	
Low	108	64 (36.1)	44 (24.9)	
AFP				0.056
≤ 400	184	83 (46.9)	101 (57.1)	
> 400	170	94 (53.1)	76 (42.9)	
HBeAg				0.190
Positive	136	62 (35.0)	74 (41.8)	
Negative	218	115 (65.0)	103 (58.2)	
HBV DNA				0.159
Positive	211	99 (55.9)	112 (63.3)	
Negative	143	78 (44.1)	65 (36.7)	
Child-Pugh				0.326
A	217	104 (58.8)	113 (63.8)	
B	137	73 (41.2)	64 (36.2)	

HBV: Hepatitis B virus; MACC1: Metastasis-associated in colon cancer 1; AFP: α-fetoprotein; HBeAg: HBV e antigen.

expression of MACC1 and c-Met. The median OS was associated with Child-Pugh class, score from Eastern Cooperative Oncology Group performance status scale (ECOG PS), tumor differentiation, MACC1 mRNA levels, co-expression of MACC1 and c-Met, tumor number, and tumor size. Further multivariate analysis using the Cox hazards model revealed that a high MACC1 expression or co-expression with c-Met was an independent poor prognostic factor for TFS and OS. The combined expression of both MACC1 and c-Met increased these prognostic values, as compared to MACC1 overexpression alone (Table 3).

DISCUSSION

Consistent with the multifactorial aetiology of HCC and the long latent period of tumor formation, a large variety of cancer genes are involved in the multistep process of human hepatocarcinogenesis[3,4]. In order to identify suit-

able prognostic markers and therapeutic targets, it is essential to analyze gene expression and proteomic changes by evaluating a large series of HCC patients and samples at different disease stages. Moreover, since metastasis or recurrence is the major cause of death of postoperative HCC patients, early identification of subjects at high-risk for either of these processes is necessary to improve OS rates. The clinical factors related to tumor invasiveness, such as tumor size, number, histological type and vessel invasion, are considered the most related to risk for recurrence and the most useful for prediction of HCC patient outcome[17-19]. Molecular biology studies have identified many biological factors that may act as potential tumor prognostic markers. The fact that HCC metastasis is a multistep process involving many factors[20-22] has led to attempts to develop a panel of multiple biomarkers that will facilitate tumor diagnosis and prediction of tumor metastasis and recurrence.

MACC1 was recently identified as being involved in metastasis of colon cancers, presumably by up-regulating c-Met transcription. Thus, to elucidate the MACC1-related mechanism of HCC and identify potential targets for molecular-based therapies we investigated the expression of MACC1 and c-Met in HBV-induced HCC.

We determined that intratumoral MACC1 expression was significantly up-regulated in most of the late stage HCC tissues examined. We further found that MACC1 overexpression was associated with higher c-Met expression in HCC and the intratumoral MACC1 mRNA level alone or in combination with that of c-Met can serve as an independent predictive factor for recurrence and survival of postoperative HCC patients.

We statistically analyzed the correlation of intratumoral MACC1 mRNA levels and clinical parameters of HCC patients. High intratumoral MACC1 mRNA levels were significantly associated with clinical stage, age, vessel invasion, and tumor differentiation. MACC1 mRNA levels were found to gradually increase with the progression of HCC, especially at the advanced HCC stage, and this process was accompanied by invasion of the portal vein. Shirahata *et al*[23] also showed that MACC1 expression was significantly correlated with vascular invasion, as it was in our study. Furthermore, intratumoral MACC1 protein was localized mainly in the cell cytoplasm, where the levels increased from low to robust in conjunction with tumor progression, indicating that MACC1 may represent an effective biomarker of tumor progression. Previous studies in colon cancers had also found that intratumoral MACC1 was up-regulated, as compared to levels detected in matched peritumoral or normal colon mucosa, regardless of tumor stage classification[5]. In our study of hepatic cancer, we found that the paratumor livers in some HCC patients had a relatively strong expression of MACC1; this was especially the case for those patients with TFS shorter than 6 mo after resection, implying that the pathogenesis of HCC and colon cancers is likely distinct. However, considering that all of the patients examined in our study had a background of chronic HBV

Table 3 Univariate and multivariate analyses of variables associated with tumor-free survival and overall survival

Variables	TFS				OS			
	Univariate P	Multivariate			Univariate P	Multivariate		
		Hazard ratios	95% CI	P		Hazard ratios	95% CI	P
Child-Pugh (A/B)	0.184	NA	NA	NA	0.034	1.342	1.016-1.747	0.041
Tumor differentiation (high/intermediate/low)	0.037	1.213	0.743-1.980	0.441	0.038	1.133	0.683-1.679	0.624
Tumor number (≥ 2/1)	0.021	1.012	0.675-1.517	0.354	0.040	1.107	0.732-1.575	0.630
ECOG PS (0/1/2)	0.078	NA	NA	NA	0.045	1.079	0.893-1.530	0.679
MACC1 (low/high)	< 0.001	1.489	1.071-1.801	0.013	< 0.001	1.508	1.079-1.835	0.012
MACC1 and c-Met expression (both/one)	< 0.001	1.929	1.207-3.083	0.006	< 0.001	1.539	1.172-2.208	0.010

TFS: Tumor-free survival; OS: Overall survival; ECOG PS: Eastern Cooperative Oncology Group Performance Status Scale; MACC1: Metastasis-associated in colon cancer 1; NA: Not applicable.

Figure 2 c-Met expression and correlation with metastasis-associated in colon cancer 1 in 234 hepatocellular carcinoma patients received resection. A: Comparison of c-Met expression level in hepatocellular carcinoma (HCC) patients with high or low intratumoral metastasis-associated in colon cancer 1 (MACC1) mRNA expression; B: c-Met level from 234 HCC patients receiving resection specimens were plottered against MACC1 levels from the same patients. Linear regression analysis showed a significant positive correlation between c-Met and MACC1 ($r = 0.360$, $P < 0.001$); C: Kaplan-Meier analysis of MACC1 and c-Met co-expression effects on tumor-free survival; D: Kaplan-Meier analysis of MACC1 and c-Met co-expression effects on overall survival.

infection and liver cirrhosis, it is possible that the above-mentioned difference is due to the chronic hepatitis B infection.

Based on our immunohistochemistry data, the over-expression rates of MACC1 in HCC ranged from 22% to 67% in patients at different stages. MACC1 staining occurred mainly in the cytoplasm of non-cancerous or cancerous cells, but some cancerous cells showed significantly strong MACC1 staining in the nucleus. Studies in colon cancer have also identified MACC1 in the nuclear compartment of cancerous cells. It has been theorized that nuclear localization of MACC1 in conjunction with high c-Met levels contribute to the later development of distant metastases[5]. The actual clinical significance of the nuclear translocation of MACC1 in HCC requires further investigation.

It has been well documented that MACC1 binds to the c-Met promoter, and this transcription regulation event is crucial for tumor metastasis as induced by HGF/c-Met signaling[9]. A regulatory feedback mechanism ex-

Figure 3 Metastasis-associated in colon cancer 1 expression level associated with outcomes of hepatocellular carcinoma patients receiving resection. A, B: Kaplan-Meier estimates of metastasis-associated in colon cancer 1 (MACC1) expression effects on tumor-free survival (TFS) (A) and overall survival (OS) (B); C, D: Kaplan-Meier estimates of ratio of T:N MACC1 expression effects on TFS (C) and OS (D).

ists in that HGF is able to promote the translocation of MACC1 to the nucleus, where MACC1 controls the promoter activity and hence expression of c-Met, thereby regulating c-Met-mediated signaling[6]. Our data showed that c-Met mRNA in intratumoral tissues was significantly higher than that in peritumoral tissues. Co-expression analysis showed a high consistency of MACC1 and c-Met mRNA in HCC tumors. Further investigation is required, however, to confirm whether MACC1 directly drives c-Met expression in HCC.

Previous studies on primary colon cancers indicated that the negative and positive predictions of MACC1 mRNA levels for distant metastases were 80% and 74%, respectively[5,24]. MACC1 can induce migration, invasion and proliferation of cultured cells[5], and can promote metastasis of tumor cells into liver and lung in various xenograft models. A current study in lung adenocarcinoma demonstrated that MACC1 overexpression was associated with postoperative recurrence[25]. In the present study, follow-up of 234 stage A and stage B HCC patients who received curative therapy revealed that a high intratumoral MACC1 expression level is correlated with a high rate of recurrence and extrahepatic metastasis. The median TFS and OS were much shorter in patients with high expression of both MACC1 and c-Met. High expression of intratumoral MACC1 was predictive of a poor outcome of HBV-related HCC, but when combined with high c-Met expression the predictive value for recurrence and metastasis increased.

Collectively, our data showed that intratumoral MACC1 expression is closely associated with tumor progression in HBV-induced HCC. Furthermore, elevated expression of MACC1 was statistically associated with poor outcome of these patients, suggesting that MACC1 is a novel predictor for recurrence and metastasis of postoperative HCC patients.

ACKNOWLEDGMENTS

COMMENTS

Background

Due to metastasis or recurrence is the major cause of death of postoperative hepatocellular carcinoma (HCC) patients, early identification of subjects at high-risk for either of these processes is necessary to improve overall survival rates. It has been known that hepatocarcinogenesis is a complex process associated with the accumulation of multiple genetic and epigenetic changes during the initiation, progression and maturation, and molecular biology studies have identified many biological factors that may act as potential tumor prognostic mark-

ers, in the hope of developing effective preventative measures and improved treatment strategies.

Research frontiers

The metastasis-associated in colon cancer 1 (MACC1) gene was identified by a genome-wide screen of human colon cancer samples, and its expression was closely related to the metastasis of colon cancers. Further studies have revealed that MACC1-induced tumorigenesis is correlated with enhanced hepatocyte growth factor/c-Met signaling.

Innovations and breakthroughs

The clinical significance of MACC1 overexpression in HCC and of the correlation between MACC1 and the c-Met signaling remain unknown. In the present study, authors sought to determine the expression levels of MACC1 in HBV-related HCC at different disease stages and analyze its correlation with clinical outcome. In addition, the authors evaluated the related levels of its transcriptional target, c-Met. The research indicated that MACC1 expression levels represent an effective prognostic factor for HBV-related HCC patients who undergo hepatectomy. The data showed that intratumoral MACC1 expression is closely associated with tumor progression in HBV-induced HCC. Furthermore, elevated expression of MACC1 was statistically associated with poor outcome of these patients.

Applications

The study results implied that the MACC1 is a novel predictor for recurrence and metastasis of postoperative HCC patients.

Terminology

The MACC1 was recently identified as being involved in metastasis of colon cancers, presumably by up-regulating c-Met transcription.

Peer review

The research is very important and the result is exciting and there is some clinical value, that is, intratumoral MACC1 expression may serve as a biomarker to predict recurrence or metastasis of postoperative HCC. The manuscript has a certain readability.

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BRIEF ARTICLE

Investigation of the effect of military stress on the prevalence of functional bowel disorders

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Abstract

AIM: To investigate the morbidity of functional bowel disorders (FBD) under military stress conditions in order to lay foundations for the prevention and treatment of this disease.

METHODS: Four hundred and fifty-seven soldiers who were assigned to specified services and 471 soldiers who were assigned to routine services were enrolled using cluster sampling, with the latter as a control group. They were surveyed using the Rome Ⅲ FBD standard questionnaire. The FBD symptom questionnaire included FBD-related symptoms, severity, duration or attack time, and accompanying symptoms.

RESULTS: The morbidity of the military stress group (14.6%) was significantly higher than in the control group (9.98%) (χ^2 = 4.585, P < 0.05). The incidence of smoking, abdominal pain and acid regurgitation (χ^2 = 4.761, P < 0.05) as well as the ZUNG anxiety/depression scores (χ^2 = 7.982, P < 0.01) were also significantly higher in the military stress group compared with the control group. ZUNG anxiety (χ^2 = 11.523, P < 0.01) and depression (χ^2 = 5.149, P < 0.05) scores were higher in the FBD group compared with the non-FBD group. The differences in the ZUNG self-rated anxiety and depression scales between the 2 groups were statistically significant (χ^2 = 14.482, P < 0.01 and χ^2 = 6.176, P < 0.05).

CONCLUSION: The morbidity of FBD was higher under military stress conditions.

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Key words: Military stress; Functional bowel disorders; Soldier; Self-rating anxiety; Depression scale

INTRODUCTION

Functional bowel disorders (FBD) is the generic term for disorders of bowel motor and secretary function without organic changes, which are diagnosed according to symptoms after the exclusion of lesions such as inflammation, infection, tumor and other structural disorders[1-3]. FBD includes 5 diseases, irritable bowel syndrome, functional abdominal bloating, functional constipation, functional diarrhea and unspecified functional bowel disorder. FBD are common clinical diseases which significantly affect the quality of patients' lives and incur considerable medical costs. A large number of studies have proved that

stress is the primary induction factor of FBD. Military stress is the emotional reaction of soldiers under military conditions, and mainly manifests as a state of tension[4,5]. There are few studies regarding the effect of military stress on FBD[6], and thus this study investigated the effects of stress by comparing FBD morbidity in soldiers conducting specialized operations with those carrying out regular tasks.

MATERIALS AND METHODS

Objects
Five hundred armed soldiers (mean age 20.7 ± 1.9 years) who were transferred from one province to another in China between April 2009 and May 2010 to handle emergencies were classified as the military stress group; Five hundred armed soldiers (mean age 20.14 ± 1.65 years) from the same province who conducted routine tasks were classified as the control group. All of the soldiers were male and garrisoned in the local area at least 1 year. Both groups were comparable in age, weight, height, the length of military service, education background, duty time, training time and garrison time.

Methods
Questionnaire: The FBD symptom questionnaire including FBD-related symptoms, severity, duration or attack time, and accompanying symptoms, was made with reference to Rome Ⅲ FGIDs functional gastrointestinal disorder standard questionnaire[7], and in combination with the practical conditions of the soldiers in the Chinese People's Armed Police. Psychological factors were investigated using the ZUNG Anxiety Scale and ZUNG Depression Scale.

Quality control of the questionnaire: The questionnaires were distributed according to lists of soldiers by responsible persons in every unit, and were filled in immediately after professional staff gave instructions and answered questions. All questionnaires were checked by a specially designated person after their return. The response rate and acceptance rate were 95.20% (476/500) and (452/476), respectively, in the military stress group, and 96.20% (481/500) and (471/481), respectively, in the control group.

Statistical analysis
The results were input into Epi Info 2003 software to establish data library and analyzed by SPSS18.0 statistical software; the χ^2 test was performed on categorical data. It was statistically significant at $P < 0.05$.

RESULTS

Morbidity of FBD
The rates of FBD in the military and control groups were 14.60% (66/452) and 9.98% (47/471), respectively. The difference between the two groups was statistically significant ($P < 0.05$, Table 1).

Prevalence of primary symptoms
There were 14 primary symptoms of FBD in the questionnaire. Individuals in the sampled populations could have one or more gastrointestinal symptoms. The prevalence of the primary symptoms is presented in Table 1.

Comparison of food habits and intake in soldiers with or without FBD
The food habits of soldiers with FBD were significantly different from those without FBD ($P = 0.000$-0.001). The occurrence of bad habits such as engorgement, being particular about food, omophagia, taking cold drinks, eating hot or spicy food, drinking tea and coffee was more frequent in the FBD group than in the non-FBD group (Table 2); the proportion of soldiers who had few or no bad food habits was smaller in the FBD group compared with the non-FBD group ($P = 0.000$-0.001); the proportion of soldiers who ate a lot of vegetables and fruit was smaller in the FBD group compared with the non-FBD group, while the proportion of soldiers who ate few vegetables and fruit was higher in the FBD group compared with the non-FBD group ($P = 0.000$); the proportion of soldiers who ingested many dairy products was higher in the non-FBD group compared with the FBD group, while the proportion of soldiers who ingested few dairy products was smaller in the FBD group compared with the non-FBD group ($P = 0.000$); the proportion of soldiers who drank coffee was higher in the FBD group compared with the non-FBD group, while the proportion of soldiers who drank tea was smaller in the FBD group compared with the non-FBD group ($P = 0.000$-0.001).

Comparison of the ZUNG self-rating anxiety and depression scales
The proportion of soldiers who had a score > 40 in the ZUNG self-rating anxiety scale was higher in the military stress group (11.97%) than in the control group (5.52%), and was statistically significant ($P < 0.01$). The proportion of soldiers who had a score > 40 in the ZUNG self-rating depression scale was also higher in the military stress group (68.29%) than in the control group (58.60%), and was statistically significant ($P < 0.05$).

DISCUSSION

Military stress[8,9] is a type of emotional reaction appearing in soldiers under military conditions, and mainly manifests as tension. Military stress can be considered as a kind of stimulated or emotional state[10,11]. Military stress cannot be simplistically considered as a negative reaction. It can be understood as a psychological problem only when stress induces changes in the cognition, emotions and behavior of soldiers to severely reduce their efficiency in military missions, and is mainly manifested by an inability to take part in daily military training, to adapt to the military environment or to join in fighting[12,13].

In recent years, more studies have focused on the effect of stress on gastrointestinal function[14], but few have paid attention to the effects of military stress on

Table 1 Comparison of morbidity and prevalence of primary symptoms of functional bowel disorders in the military stress and control groups n (%)

	Military stress group	Control group	χ^2	P
Disease name				
Irritable bowel syndrome	28/452 (6.19)	16/471 (3.40)	3.972	< 0.05
Functional abdominal bloating	0/452 (0.00)	0/471 (0.00)		> 0.05
Functional constipation	23/452 (5.09)	20/471 (4.25)	0.443	> 0.05
Functional diarrhea	9/452 (1.99)	8/471 (1.70)	0.108	> 0.05
Non-specific functional bowel disorder	6/452 (1.33)	3/471 (0.64)	1.135	> 0.05
Total	66/452 (14.6)	47/471 (9.98)	4.585	< 0.05
Primary symptom (No. of person with symptoms)				
Nausea	133/452 (29.42)	74/471 (15.71)	24.931	< 0.01
Vomiting	74/452 (15.71)	53/471 (11.25)	5.849	< 0.05
Abdominal distension	145/452 (32.08)	103/471 (21.87)	12.230	< 0.01
Acid regurgitation	113/452 (25.00)	64/471 (13.59)	19.329	< 0.01
Heartburn	61/452 (13.50)	43/471 (9.13)	4.397	< 0.05
Foreign body sensation in throat	85/452 (18.81)	78/471 (16.56)	0.800	> 0.05
Substernal pain	70/452 (15.49)	47/471 (9.98)	6.312	< 0.05
Hiccough	135/452 (29.87)	82/471 (17.41)	19.899	< 0.01
Food regurgitation	101/452 (22.34)	61/471 (12.95)	14.068	< 0.01
Abdominal pain	31/452 (6.86)	13/471 (2.76)	8.483	< 0.01
Constipation	142/452 (31.42)	98/471 (20.81)	13.492	< 0.01
Diarrhea	121/452 (26.77)	86/471 (18.26)	9.602	< 0.01
Encopresis	15/452 (3.32)	9/471 (1.91)	1.808	> 0.05

Table 2 Food intake of soldiers with and without functional bowel disorders n (%)

Food habit	Much	Moderate	Less	Little or not	Total
With functional bowel disorder					
Engorgement	10 (15.9)	21 (32.5)	20 (31.1)	13 (20.3)	64 (100)
Omophagia	6 (8.8)	10 (15.7)	23 (34.9)	26 (40.1)	65 (100)
Particular about food	12 (18.9)	19 (29.4)	15 (22.8)	20 (30.3)	66 (100)
Cold drinks	14 (20.6)	22 (33.3)	20 (30.6)	10 (15.2)	66 (100)
Spicy food	22 (33.4)	21 (32.1)	17 (26.3)	5 (7.7)	65 (100)
Dairy products	20 (30.9)	24 (37.2)	15 (24.1)	6 (9.3)	64 (100)
Vegetables	17 (27.6)	33 (51.7)	12 (19.2)	1 (1.6)	63 (100)
Fruit	14 (22.6)	26 (39.5)	18 (28.4)	7 (10.8)	65 (100)
Without functional bowel disorder					
Engorgement	3 (5.1)	10 (16.3)	18 (29.2)	29 (48.3)	60 (100)
Omophagia	3 (5.8)	6 (10.4)	15 (24.9)	35 (59.3)	59 (100)
Particular about food	5 (8.0)	10 (17.8)	11 (19.8)	32 (55.1)	58 (100)
Cold drinks	7 (11.6)	16 (26.3)	17 (28.6)	21 (34.4)	61 (100)
Spicy food	10 (16.4)	16 (27.1)	15 (25.8)	19 (31.7)	60 (100)
Dairy products	16 (25.9)	23 (37.2)	12 (19.1)	11 (17.7)	62 (100)
Vegetables	24 (37.4)	28 (43.7)	6 (9.2)	5 (7.9)	63 (100)
Fruit	22 (34.6)	24 (37.5)	12 (18.7)	6 (9.4)	64 (100)

gastrointestinal function[15,16]. The results in this study suggested that FBD was significantly higher in the military group (14.60%) compared with the control group (9.98%). Meanwhile, the rates of smoking, abdominal pain, and acid regurgitation, and the ZUNG anxiety and depression scores were also significantly higher in the military group compared with the control group. The increased incidence of FBD under military stress might be due to the dual regulatory effects of the autonomic nervous system and the endocrine system on the movement and secretion of the alimentary tract, which are directly or indirectly affected by the central nervous system[17,18]. The anatomical structures of the nervous and endocrine system overlap with that of the emotional center[19,20], thus after tension and emotional changes induced by military stress conditions arrive at the emotional center, the gastrointestinal regulatory center will also be excited, and therefore, gastrointestinal discomfort will likely occur or be aggravated[21,22].

It has been reported[23] that there are significant differences between individuals in the length of time psychological stress is sustained. Overall, although a psychological stress reaction may be alleviated within 10 d in about 85% soldiers, it persists in about 15% soldiers after 10 d. The following measures should be adopted to deal with the increased morbidity of FBD induced by military stress: a focus on daily training activity[24,25], with simulation of various duty environments, and enhanced quality of psychological and mental preparation for emergencies; the soldiers should actively take part in the handling of an emergency situation, have a specific daily schedule

with adequate rest periods, and be given medical treatment if necessary. Non-combat casualties resulting from illness will be decreased and should guarantee that military duties will be better accomplished[26].

Overall, FBD is an old problem, but there are still areas in the pathogenesis of the disease to explore, and which may involve a wide range of research, including cell biology, neurophysiology, immunology, endocrinology, behavior and other fields of medicine and psychology. Linking the clinical problem with stress may directly lead to a clinical benefit for all patients.

COMMENTS

Background
Functional bowel disorders (FBD) is a generic name for disorders in bowel motor and secretary function without organic changes, and is diagnosed according to symptoms after the exclusion of lesions such as inflammation, infection, tumor and other structural disorders. It is a common clinical disease which significantly affects the quality of patients' lives and incurs medical costs. A large number of studies have shown that stress is the primary induction factor of FBD.

Research frontiers
There are few studies of the effect of military stress on FBD, and thus this research tried to investigate these effects through comparing the morbidity in soldiers conducting specialized tasks with those undertaking regular tasks.

Innovations and breakthroughs
Four hundred and fifty-seven soldiers who were assigned to specified services and 471 soldiers who were assigned to common services were enrolled using cluster sampling, with the latter as the control group, and then they were surveyed according to the Rome Ⅲ FBD standard questionnaire.

Applications
To provide foundations for the prevention and treatment of this disease, authors investigated the morbidity of FBD under military stress conditions.

Terminology
FBD: Disorders of bowel motor and secretary function without organic changes, diagnosed according to symptoms after the exclusion of organic lesions.

Peer review
Overall, this is an interesting study which shows clearly that the morbidity of FBD was higher under military stress conditions.

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BRIEF ARTICLE

Ultrasound-guided microwave ablation for abdominal wall metastatic tumors: A preliminary study

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Abstract

AIM: To evaluate the feasibility, safety and efficacy of ultrasound-guided microwave (MW) ablation for abdominal wall metastatic tumors.

METHODS: From August 2007 to December 2010, a total of 11 patients with 23 abdominal wall nodules (diameter 2.59 cm ± 1.11 cm, range 1.3 cm to 5.0 cm) were treated with MW ablation. One antenna was inserted into the center of tumors less than 1.7 cm, and multiple antennae were inserted simultaneously into tumors 1.7 cm or larger. A 21 gauge thermocouple was inserted near important organs which required protection (such as bowel or gallbladder) for real-time temperature monitoring during MW ablation. Treatment outcome was observed by contrast-enhanced ultrasound and magnetic resonance imaging (MRI) [or computed tomography (CT)] during follow-up.

RESULTS: MW ablation was well tolerated by all patients. Six patients with 11 nodules had 1 thermocouple inserted near important organs for real-time temperature monitoring and the maximum temperature was 56 ℃. Major complications included mild pain (54.5%), post-ablation fever (100%) and abdominal wall edema (25%). All 23 tumors (100%) in this group were completely ablated, and no residual tumor or local recurrence was observed at a median follow-up of 13 mo (range 1 to 32 mo). The ablation zone was well defined on contrast-enhanced imaging (contrast-enhanced CT, MRI and/or contrast-enhanced ultrasound) and gradually shrank with time.

CONCLUSION: Ultrasound-guided MW ablation may be a feasible, safe and effective treatment for abdominal wall metastatic tumors in selected patients.

INTRODUCTION

Clinically, the incidence of primary abdominal wall malignant tumors is low. Usually metastasis or local infiltration is the major cause of abdominal wall metastatic tumors. A number of abdominal wall tumors occur during or after therapy, and are difficult to cure. Currently, most studies report that the main treatment for abdominal

wall tumors is resection[1], however some patients are unable to undergo resection due to tumor stage. Small subcutaneous lesions can be easily resected, whereas it is technically difficult for radical excision of large lesions, especially those which invade muscles. Moreover, surgical reconstruction is also troublesome, and a significant number of patients require abdominoplasty for larger abdominal wall tumors[2-4]. Other treatments have been used for abdominal wall tumors, such as radiotherapy, chemotherapy and thermal ablation. Radiotherapy requires patients to have optimal health status, while chemotherapy often plays an additional role and is not used as a radical cure. Thermal ablation is a minimally invasive technique, and has been widely used for the treatment of primary and metastatic liver cancer in past decades and is well established[5-8]. High intensity focused ultrasound (HIFU) and radiofrequency ablation have been used in abdominal wall metastatic tumors with curative effect[9]. Microwave (MW) ablation for the treatment of liver tumorsis relatively low-risk and has favorable therapeutic efficacy[10,11]. Compared with radiofrequency ablation, MW energy does not appear to be limited by charring and tissue desiccation, thus, thermal efficiency may be considerably higher with MW systems than with radiofrequency systems[12-15]. To our knowledge, there are no reports assessing the efficacy and safety of MW ablation for abdominal wall tumors under ultrasound guidance.

Thus, the purpose of this study was to assess the effectiveness of MW ablation for abdominal wall tumors under ultrasound guidance in the short and medium term, and to identify the possible complications that determine the rate of therapeutic success.

MATERIALS AND METHODS

Patients
From August 2007 to December 2010, eleven patients with 23 abdominal wall tumors were enrolled in this retrospective study (Table 1). The patients were 6 men and 5 women aged 35-68 years (mean age, 54.18 ± 9.14 years), and tumor size ranged from 1.3 cm to 5.0 cm in maximum diameter (mean diameter 2.59 ± 1.11 cm, range 1.3 cm to 5.0 cm). Before MW ablation, 2 patients were treated with chemotherapy, 1 patient was treated with chemotherapy and radiotherapy, and 1 patient with immunotherapy. Informed consent was obtained from all patients at enrollment. The inclusion criteria for this study were as follows: (1) The entire tumor could be clearly seen on ultrasound; (2) The tumor size was no more than 5 cm in diameter; (3) The tumor was located more than 5 mm from the skin surface; (4) The tumor had not adhered to the peritoneum or bowels; and (5) The tumor had not invaded the bones. The exclusion criteria were as follows: (1) Severe cardiopulmonary disease; (2) Severe coagulation abnormalities (prothrombin time more than 25 s, prothrombin activity higher than 40%, and platelet count higher than 40 cells $\times$ 10^9/L); and (3) Infection. All selected patients chose MW abla-

tion on the basis of tumor stage which made them inoperable, or had comorbidities, advanced age, or refused to undergo surgery. All abdominal wall nodules were metastatic lesions. In five cases abdominal wall tumors had metastasized from hepatocellular carcinoma (HCC); two cases metastasized from adrenal glands, whose pathological types were adrenal cortical carcinoma and pheochromocytoma. The remaining four cases metastasized from lung, ovary, bladder and kidney; and the corresponding pathological types were lung adenocarcinoma, ovarian peritoneal serous papillary carcinoma, bladder transitional cell carcinoma and renal clear cell carcinoma. The primary liver lesions in five patients were treated with MW ablation, and the primary lesions in the remaining patients were resected. Abdominal wall metastatic tumors in three patients with HCC were caused by needle tract seeding, which appeared 9, 11 and 22 mo after liver puncture.

Equipment
A KY2000 MW ablation system (Kangyou Medical Instruments, Nanjing, China) consisting of two independent MW generators, two flexible coaxial cables and two water-pumping machines, which could drive two 15-gauge cooled-shaft antennae simultaneously was used. The generators are capable of producing 1-100 W of power at 2450 MHz. The cooled shaft antenna was coated with polytetrafluoroethylene to prevent adhesion, which can also be clearly seen on ultrasound. The antenna is designed to minimize power feedback and provide tissue with optimal energy deposition. Three types of antennae were applied according to the size and location of the tumor, the antennae tips were 0.5, 0.7 and 1.1 cm, respectively. For tumors smaller than 2 cm, an antenna tip of 0.5 cm was chosen, while for tumors larger than 3 cm, a tip of 1.1 cm was selected. The MW machine is also equipped with a thermal monitoring system with 21-gauge thermocouple needles, which can be placed percutaneously at a designated location to monitor the temperature during real-time ablation.

Ablation procedures
Before treatment, all patients were scanned using contrast-enhanced computed tomography (CT)/magnetic resonance imaging (MRI) and ultrasound, and an appropriate puncture route was chosen by ultrasound. After local anesthesia with 1% lidocaine, the antenna was inserted percutaneously into the tumor and placed at designated sites under ultrasound guidance. Histologic diagnoses were confirmed by guided sonography with an 18-gaugecutting-edge needle through an automated biopsy gun device before inserting the antenna, and specimens were taken from different parts of the tumor (one to three pieces). One antenna was inserted into the center of tumors less than 1.7 cm, and multiple antennae were inserted into tumors 1.7 cm or larger. General anesthesia (Propofol, 6-12 mg/kg per hour; Ketamine, 1-2 mg/kg) was applied after correct placement of

Table 1 Patient and tumor characteristics

No.	Age (yr)	Sex	Tumor type	Tumor number	Tumor size (cm)	Antenna	Antenna number	Ablation power (W)	Ablation time (min)	Session	Follow up (mo)
1	59	M	HCC	1	1.8	T11	1	50	6	1	31
2	35	M	HCC	1	2.1	T11	1	50	8	1	26
3	68	M	HCC	2	4.3	T11	2	40	5	2	19
					4.8	T11	2	50	16.5	1	19
4	57	F	Ovary serous papillary adenocarcinoma	1	2.2	T7/T11	2	45	2	2	18
5	55	F	Lung adenocarcinomas	2	1.3	T11	1	50	3.5	1	18
					1.7	T11	1	50	3.5	1	18
6	56	F	Adrenocortical carcinoma	4	4	T11	2	60	12	1	9
					3.1	T11	2	30	12	1	9
					3.3	T7/T11	2	30	16	1	9
					2.5	T5	2	50	5	1	9
7	58	M	HCC	1	1.3	T11	2	50	5	1	3
8	48	M	Bladder adenocarcinoma	2	5	T11	2	40	15	2	13
					2.1	T11	2	50	11.8	1	13
9	59	F	Renal clear cell carcinoma	5	2.2	T11	2	60	5	1	15
					2.1	T11	2	50	5.5	1	15
					2.1	T11	2	50	6	1	15
					2	T7/T11	2	45	4	1	15
					1.6	T11	1	45	3.8	1	15
10	58	F	HCC	1	2.6	T5	1	50	13	1	1
11	42	M	Adrenal pheochromocytoma	3	3.1	T7	1	50	8	1	4
					2.8	T5	2	50	6.5	1	4
					1.4	T5	1	50	1.2	1	4

M: Male; F: Female; HCC: Hepatocellular carcinoma.

antennae, and MWs were then emitted[16,17]. Two antennae were used simultaneously during MW ablation to achieve a larger ablation zone. If the tumor was adjacent to bowel, gallbladder or other important tissues, a 21 gauge thermocouple was inserted close to these tissues for real-time temperature monitoring during MW ablation. The treatment session ended if the transient hyperechoic zone between the antennae merged and covered the target region on gray-scale ultrasound. Simultaneously, according to our previous clinical experience, the temperature of the thermal needles should not exceed the target temperature to avoid heat injury in these organs[18]. For tumors with subcutaneous invasion, an ice bag was placed on the skin to avoid scalding during MW ablation.

Postprocedural observation and follow-up

After MW ablation, patients were closely monitored for possible complications such as fever, skin burns and pain which were also documented. All patients underwent contrast-enhanced ultrasound 3 d after MW ablation to assess treatment efficacy. If residual tumor (hyper-enhanced area on contrast-enhanced ultrasound) was found, a further session was planned or patients entered the follow-up protocol, which consisted of contrast-enhanced CT, MRI and/or contrast-enhanced ultrasound 1, 3 and 6 mo after MW ablation, and every 6 mo thereafter. Enhanced areas on the abdominal wall were assumed to represent viable tumors. If residual or recurrent tumor on the abdominal or chest wall was detected, a further MW ablation session was planned if the lesion still met the inclusion criteria.

RESULTS

MW ablation was well tolerated by all patients. The output power ranged from 30 W to 50 W, an output setting of 50 W was routinely used during ablation sessions, relatively lower than that used in liver lesions. Four of 23 tumors were adjacent to the intestinal tract, one nodule was adjacent to the gallbladder, and 1 nodule was adjacent to the gallbladder and intestinal tract. A thermocouple was inserted adjacent to these high-risk locations for real-time temperature monitoring. In this study, we used 1 thermocouple with a maximum temperature of 56 ℃ in 6 patients with 11 nodules. The treatment time was no more than 16.5 min (mean time 7.6 ± 4.3 min, range 2 min to 16.5 min). Twenty-one of 23 tumors were successfully eradicated following one MW session. The other two tumors underwent two MW sessions; one tumor was near the adrenal gland, and the other was a tumor 5 cm in maximum diameter. All 23 tumors (100%) in this group were completely ablated which was confirmed by follow-up imaging during a period of 1-32 mo. No major complications were encountered after MW ablation. Six patients experienced grade 1 according to standardization of terms and reporting criteria for image-guided tumor ablation[19]. Post-ablation fever was encountered in all patients, but each patient's temperature was lower than 38 ℃ and no drugs were needed. No skin burns were observed in the treated area, however, the treated area was slightly swollen in 3 patients. The patients were followed up until January 20, 2011. The median survival period of the 11 patients after MW ablation was 15.0 mo. During a mean follow-up of 13 mo (range 1-32 mo), three patients died of primary

tumor progression, however, the treated tumors were un-enhanced on follow-up contrast-enhanced images. In the other 8 patients, the ablation zones were well defined on contrast-enhanced images, and gradually shrank with time (Figure 1). Two patients developed distant metastases, one patient was treated with repeated sessions of MW ablation, and the other underwent HIFU.

DISCUSSION

In the past few decades, the treatment of abdominal wall tumors, especially metastasis, has evolved[9,20]. In addition to traditional surgical resection, there are many other treatments, such as transarterial embolization[20] radiofrequency ablation and HIFU[21]. Surgical resection is the first choice for abdominal tumors, although it carries a risk of hemorrhage and possible post-operative incisional hernia, and patients usually require reconstruction, such as abdominoplasty[22]. Some patients may not be surgical candidates due to poor medical conditions[23]. It is difficult to create a safety margin to eradicate possible microscopic tumor foci using transarterial embolization of the feeding vessel of the abdominal tumor, and may cause ischemic changes. Thus, it is rarely used in abdominal tumors. Compared with surgical resection, HIFU is a less invasive alternative to surgical resection. However, due to the bio-effects of focused ultrasound during the procedure, heat diffusion out of the focal region is inevitable and can damage the surrounding tissues.

We have also made some progress with the antennae used in MW ablation. Three types of antennae were used in this study according to tumor size and location, and the antennae tips were 0.5, 0.7 and 1.1 cm, respectively. According to a preliminary study, an antenna tip of 1.1 cm can ablate 2-2.6 cm *ex vivo* porcine livers with the output power of 60 W and the setting time of 10 min[24]. Based on these preliminary experiments, an antenna tip of 0.7 cm and 0.5 cm can ablate 2.4-2.6 cm and 2.2-2.4 cm *ex vivo* porcine livers with the output power of 60 W and the setting time of 10 min, respectively. The new type tips are safer, because the ablation zone is relatively small which avoids burning the skin in the superficial tumor during the procedure. Therefore, percutaneous MW ablation may be clinically feasible for superficial tumors. Based on our previous experience in MW ablation for HCC, we performed MW ablation on abdominal tumors. Treatment efficacy was encouraging. For tumors smaller than 4 cm, radical cure was achieved in all nodules within no more than 12 min and no tumor recurrence was noted during follow-up. In order to achieve the same effect, tumors larger than 4 cm needed several sessions. MW ablation, is a relatively new technique and can be used in different types of tumors[25-30], the primary effectiveness rate is equal to HIFU and radiofrequency ablation[16,31]. The favorable effectiveness of MW ablation may be attributed to its potential advantages[32], such as larger volume of ablation zone, reduced treatment time, less influence on the perfusion-mediated heat sink effect, higher thermal efficiency and the possibility of placing multiple

antennae simultaneously[33-35], especially compared with radiofrequency ablation. Results suggest that, like other techniques, MW ablation may be safe and effective for abdominal wall tumors, it may also represent a competitive alternative to surgical resection and other therapies. The high effectiveness rate of MW ablation may due to the following 4 reasons: (1) The new-type antenna used in this study was capable of ablating superficial tumors more securely; (2) There were relatively strict inclusion and exclusion criteria; (3) All operations were performed by experienced doctors (Yu XL and Liang P); and (4) Real-time temperature monitoring served as an indicator for predicting reliable safety margins. No severe complications were observed in this study. Unlike the treatment of liver tumors, abdominal tumor ablation has its own complication of abdominal wall edema. We studied three patients who had abdominal wall edema and found that the ablated tumors were all located in muscles and with subcutaneous invasion. Compared with parenchymal organs, muscle tissue lacks relative capsules and can not accumulate heat. During the ablation procedure, heat overflow in the muscle bundle can readily lead to abdominal wall edema. In order to ablate completely, the actual ablation zone should be larger than the size of the tumor, which will ablate normal tissues (such as fatty tissue or muscle tissue) and cause edema in a short time. Fortunately, the abdominal wall edema seen in three patients was very mild and all patients recovered within a short time (1-3 mo) without special treatment. For the ablation of specific tumors, such as pheochromocytoma, we used low power at the beginning of MW ablation, and changed the power according to the blood pressure. The use of antihypertensive drugs during the procedure should be taken into account, as pheochromocytomas can release catecholamine which could result in blood pressure fluctuation. There are key points during MW ablation which can reduce the incidence of complications: (1) The MW antenna was inserted in the deepest area of the tumor; (2) Increasing the angle of puncture between the antenna and transducer, that is insert the antenna along the long axis of the tumor for conformable ablation; and (3) For tumors with subcutaneous invasion, the transient hyperechoic zone did not exceed the dermal layer in the gray-scan ultrasound, and an ice bag was placed on the skin to avoid scalding. Although this was not a randomized, controlled study of traditional techniques, the low complication rate, minimal side effects, rapid recovery and lower costs (compared with radiofrequency ablation in China) strongly favor MW ablation as an optional curative approach for abdominal wall tumors. This study has some limitations: (1) Only 11 patients were included in this study. More patients should be recruited in order to assess the efficacy of this treatment; (2) Follow-up was relatively short and we are uncertain of the long-term results; and (3) The study did not include a comparison with other treatments.

In conclusion, our preliminary results showed that ultrasound-guided MW ablation appeared to be effective in the treatment of abdominal wall tumors. Further stud-

Figure 1 Ultrasound findings in a 56-year-old woman with abdominal wall tumor metastasized from liver and adrenal gland cancer. A: Contrast-enhanced magnetic resonance imaging (MRI) scan shows a lesion with hyperenhancement in T2-weighted images in the abdominal wall (arrow); B: Arterial phase in contrast-enhanced ultrasound (CEUS) shows hyperenhancement within the lesions (arrow); C: CEUS scan obtained 3 d after microwave (MW) ablation shows a hypoechoic area with no enhancement, suggestive of complete necrosis (arrow); D: Contrast-enhanced MRI scan shows a lesion with hypoenhancement in T2-weighted phase MR image obtained 1 mo after MW ablation revealing complete ablation. Contrast-enhanced MRI scan shows a lesion with hypoenhancement in T2-weighted images in the abdominal wall (arrow); E: Sonogram obtained before MW ablation shows hypoechoic nodule of 2.48 cm in maximum diameter in the abdominal wall (arrow); F: Arterial phase in CEUS shows hyperenhancement within the lesions (arrow); G: Sonogram obtained during MW ablation shows one antenna being inserted into the nodule; H: Abdominal wall edema occurred at the right lumbar in the arterial phase of contrast-enhanced CT (arrow).

ies are warranted to observe its long-term efficacy and   the results should be compared with other treatments.

COMMENTS

Background

A number of abdominal wall tumors occur during or after therapy, and are difficult to cure. Currently, the main treatment for abdominal wall tumors is resection, while the excision rate is low. Microwave (MW) ablation for the treatment of liver tumors has relatively low-risk and favorable therapeutic efficacy. However, there are no reports assessing the efficacy and safety of MW ablation for abdominal wall tumors under ultrasound guidance.

Research frontiers

MW ablation, as a relatively new technique, and can be applied to different types of tumors. This research was concerned with applying MW ablation in patients with abdominal wall metastatic tumors, and to improve the effectiveness rate of this technique.

Innovations and breakthroughs

Unlike the commonly used antenna, the authors made some progress with the antennae used in this study. Three types of antennae were used according to tumor size and location, and the antennae tips used were 0.5, 0.7 and 1.1 cm, respectively. These new type tips are safer, because the ablation zone is relatively small which avoids burning the skin in the superficial tumor during the procedure. Therefore, percutaneous MW ablation may be clinically feasible for superficial tumors.

Applications

The study results suggest that ultrasound-guided MW ablation may be a feasible, safe and effective treatment of abdominal wall metastatic tumors in selected patients.

Terminology

Image-guided tumor ablation: The term tumor ablation is defined as the direct application of chemical or thermal therapies to a specific focal tumor (or tumors) in an attempt to achieve eradication or substantial tumor destruction.

Peer review

The authors present short and medium-term outcomes for ultrasound-guided MW ablation for abdominal wall metastatic tumors. The procedure was well tolerated by all patients with the most significant complication being abdominal wall edema which resolved without treatment in all cases.

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BRIEF ARTICLE

Analysis of risk factors for polypoid lesions of gallbladder among health examinees

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Abstract

AIM: To investigate the prevalence and risk factors of polypoid lesions of gallbladder (PLG) among the health examinees in the Shanghai region, China.

METHODS: A total of 11 816 subjects who underwent health examinations in our hospital between August 2010 and February 2011 were analyzed retrospectively. Among them, there were 7174 men and 4642 women. PLG was diagnosed by the real-time ultrasonography. Those with the body mass index (BMI) $\geqslant$ 28 were considered to be obese. Blood biochemical indices were detected with the fully automatic biochemical analyzer and hepatitis B surface antigen (HBsAg) was tested by the automated enzyme immunoassay. The correlations between the prevalence of PLG and age, sex, BMI, serum cholesterol (T-Cho), triglycerides (TG), blood sugar, HBsAg, high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), gallstone and fatty liver were investigated. After univariate analysis of 11 variables, stepwise logistic regression analysis was performed to explore the risk factors of PLG.

RESULTS: There was a significant difference in sex, T-Cho, HBsAg, HDL-C, LDL-C and fatty liver between the PLG-positive group and the PLG-negative group (332/163 *vs* 6842/4479, P = 0.003; 22/473 *vs* 295/11 026, P =0.013; 92/403 *vs* 993/10 328, P = 0.001; 47/448 *vs* 332/10 989, P = 0.001; 32/463 *vs* 381/10 940, P = 0.001; 83/412 *vs* 3260/8061, P = 0.001). No significant difference was found in the age, BMI, TG, blood sugar and gallstone between the two groups (47.3 ± 26 *vs* 45.1 ± 33, P = 0.173; 59/436 *vs* 1097/10 224, P = 0.102; 52/443 *vs* 982/10 339, P = 0.158; 17/478 *vs* 295/11 026, P = 0.26; 24/471 *vs* 395/10 926, P = 0.109). Logistic regression analysis showed that the sex, HBsAg and HDL-C were independent risk factors for the development of PLG in a descending order of HDL-C > HBsAg > sex.

CONCLUSION: In healthy people, the male gender, positive HBsAg, and low HDL-C confer higher risks of PLG development.

INTRODUCTION

The prevalence of polypoid lesions of gallbladder (PLG), a common clinical gallbladder disease, is about 3%, with an increasing trend[1,2]. PLG is the general term of the limited abnormal accumulations of mucous membrane tissue of the gallbladder or the limited lesion projecting into the lumen of the gallbladder. Clinically, the types of polypoid growth of the gallbladder mainly includes cholesterol polypoid/cholesterosis, inflammatory polyp, cholesterosis with fibrous dysplasia of gallbladder, adenomyomatosis, hyperplastic cholecystosis and adenocarcinoma. Ultrasonography (US) is a convenient and non-traumatic modality used to profile the gallbladder and the position of the lesion. The application of US has improved significantly the detection rate of PLG. It is of great clinical significance to analyze the risk factors of PLG in an attempt to improve its prevention and diagnosis. This study retrospectively analyzed the prevalence and risk factors of PLG among 11 816 health examinees in our clinical center. Through the univariate and multivariate analyses, this study aimed to provide the first-hand evidences for the primary prevention of PLG.

MATERIALS AND METHODS

Ethics
This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study protocol was approved ethically by Putuo Hospital. All patients provided informed written consent.

Subjects
A total of 11 816 subjects, including 7174 men and 4642 women with an average age of 48.6 ± 31 years (range, 15-86 years) who underwent health examinations in our health center between August 2010 and February 2011, were included in this study.

Diagnosis of polypoid lesions of gallbladder
The subjects were examined with ultrasonography using a real-time scanner with a 3.5 MHz array transducer (Philips En Visor and Philips-iU22) in the early morning after fasting for about 8-12 h. They were required to stay supine or change the position when it is necessary. The gallbladder was observed through multiple cross sections to detect the size, shape, number, location, internal echo, basal part, local cyst wall and the movement of lesions with the position change.

The diagnosis of gallbladder polyps was established according to the following criteria: (1) Spherical, mulberry-like or papillary projections, derived from either pedunculated or narrow bases, and no change after position change; (2) Multiple echogenic spots which could be found in any part of the gallbladder, e.g., the gallbladder neck, body or bottom, especially in the body and bottom of the gallbladder; (3) Small echogenic spots, usually less than 10 mm; and (4) Hyperechoic (more visible) or medium echoic structures without acoustic shadow.

Measurement of body weight
We measured the body mass index (BMI) of the subjects following "The Prevention and Control Guideline for Overweight and Obesity among Chinese Adults"[3], and BMI was calculated by dividing the mean weight by the mean height squared (kg/m^2). BMI $\geq$ 28 was defined as obesity.

Determination of blood biochemical indices and hepatitis B surface antigen
Blood serum samples of 5 mL were routinely collected intravenously in the morning before breakfast. Cholesterol (T-Cho), triglyceride (TG), high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C) and blood glucose levels were detected and analyzed using a Hitachi 7020 Automatic Biochemical Analyzer. Hepatitis B surface antigen (HBsAg) was detected with an Italy RB 138 Automated Enzyme Immunoassay Analyzer. The reagents used were offered by the Shanghai Kehua Bioengineering Co., Ltd, Shanghai, China. The tests were undertaken strictly according to the instructions of the manufacturers.

Statistical analysis
Results were expressed as mean ± SD. We analyzed 11 variables with univariate analysis to compare the differences between the two groups. Variables (age, sex, BMI, T-Cho, TG, HDL-C, LDL-C, glucose, HBsAg, gallstones and fatty liver) were defined as independent variables and the PLG was defined as a dependent variable. They were examined in a multivariate model using forward stepwise maximum likelihood logistic regression to identify the risk factors of PLG (α = 0.05). The variable assignment is shown in Table 1. Data were analyzed using the SPSS version 13.0 statistical software and significance was set at P < 0.05.

RESULTS

General data of patients with PLG
The overall prevalence of PLG found among the health examinees was 4.2% (495/11 816). The incidence of PLG was 4.6% (332/7174) in men and 3.5% (163/4642) in women. Overall, males had a significantly higher prevalence of PLG than females (4.6% *vs* 3.5%, *P* = 0.003). In this group, the incidence of obesity was 9.8% (1156/11 816); the rates of increased T-Cho, TG and LDL-C were 2.7% (317/11 816), 8.8% (1034/11 816) and 3.5% (413/11 816), respectively; the rate of high blood sugar was 2.6% (312/11 816), and the incidence of low

Table 1 Instructions of assignment of variables for polypoid lesions of gallbladder

	Female = 0	Male = 1
BMI	(< 28) = 0	(≥ 28) = 1
T-Cho	Normal or decreased = 0	Increased = 1
TG	Normal or decreased = 0	Increased = 1
HDL-C	Normal or increased = 0	Decreased = 1
LDL-C	Normal or decreased = 0	Increased = 1
Blood glucose	Normal or decreased = 0	Increased = 1
HBsAg	(-) = 0	(+) = 1
Gallstones	(-) = 0	(+) = 1
Fatty liver	(-) = 0	(+) = 1

BMI: Body mass index; T-Cho: Cholesterol; TG: Triglyceride; HDL-C: High density lipoprotein; LDL-C; Low density lipoprotein; HBsAg: Hepatitis B surface antigen.

Table 2 Results of univariate analysis of the relevant factors of polypoid lesions of gallbladder

	PLG positive	PLG negative
Age (yr)	47.3 ± 26	45.1 ± 33
Sex, male/female	332/163[a]	6842/4479
BMI, ≥ 28/< 28	59/436	1097/10 224
T-Cho, increased/normal or decreased	22/473[a]	295/11 026
TG, increased/normal or decreased	52/443	982/10 339
Glucose, increased/normal or decreased	17/478	295/11 026
HBsAg, +/-	92/403[a]	993/10 328
HDL-C, decreased/normal or increase	47/448[a]	332/10 989
LDL-C, increased/normal or decreased	32/463[a]	381/10 940
Gallstones, +/-	24/471	395/10 926
Fatty liver, +/-	83/412[a]	3260/8061

[a]*P* < 0.05 *vs* negative PLG. PLG: Polypoid lesions of gallbladder; BMI: Body mass index; T-Cho: Cholesterol; TG: Triglyceride; HBsAg: Hepatitis B surface antigen; HDL-C: High density lipoprotein; LDL-C; Low density lipoprotein.

HDL-C was 3.2% (379/11 816); and the incidence of gallstone, the fatty liver and HBsAg (+) was 3.5% (419/11 816), 28.3% (3343/11 816) and 9.2% (1085/11 816), respectively. There were significant differences in sex, T-Cho, HBsAg, HDL-C, LDL-C and fatty liver between the PLG-positive group and the PLG-negative group (332/163 *vs* 6842/4479, *P* = 0.003; 22/473 *vs* 295/11 026, *P* = 0.013; 92/403 *vs* 993/10 328, *P* = 0.001; 47/448 *vs* 332/10 989, *P* = 0.001; 32/463 *vs* 381/10 940, *P* = 0.001; 83/412 *vs* 3260/8061, *P* = 0.001). No significant difference was found in the age, BMI, TG, blood sugar and gallstone, between the two groups (47.3 ± 26 *vs* 45.1 ± 33, *P* = 0.173; 59/436 *vs* 1097/10 224, *P* = 0.102; 52/443 *vs* 982/10 339, *P* = 0.158; 17/478 *vs* 295/11 026, *P* = 0.26; 24/471 *vs* 395/10 926, *P* = 0.109) (Table 2).

Logistic regression analysis of relevant risk factors for PLG

Logistic regression analysis showed that sex, HBsAg and HDL-C were independent risk factors for PLG, in a descending order of HDL-C > HBsAg > sex. The subjects with lower HDL-C had a 3.346 times higher risk of PLG than those who had normal or higher HDL-C. The

Table 3 Logistic regression analysis of multiple relevant factors of polypoid lesions of gallbladder

Variables	OR	95% CI	*P* value
Sex	1.843	1.245-2.789	0.0035
HBsAg	2.563	1.875-3.418	< 0.001
HDL-C	3.346	2.932-4.133	< 0.001

OR: Odds ratio; HBsAg: Hepatitis B surface antigen; HDL-C: High density lipoprotein.

risk of PLG was 2.563 times higher in HBsAg-positive subjects than in HBsAg-negative ones. Men had a 1.843 times higher risk of PLG than women (Table 3).

DISCUSSION

PLG, which is often neglected due to lack of significant clinical signs or symptoms, is a common disease found in the ultrasound examinations. In recent years, with changes of diet, acceleration of the pace of life, increasing health awareness and the popularity of ultrasonography, the detection rate of PLG tends to increase, and nearly 85% of PLG are detected in a routine physical examination. It is reported that the prevalence of PLG in the Western society is 1.0%-6.9%[4-6], which is significantly lower than in the Asians. Park *et al*[7] reported that PLG prevalence in the South Korea was about 6.1%. Lin *et al*[8] reported a prevalence of 9.5% in Taiwan. After the logistic regression analysis of 34 669 cases, Lin *et al*[8] showed that the male gender was an independent risk factor of PLG. The domestic studies showed a prevalence of 3% in healthy adults in our country[1,2]. The Logistic regression analysis in this study showed that the gender was an independent risk factor for PLG and males bear a significantly higher risk of PLG than females. The risk of PLG in men was 1.843 times higher than in women.

In China, hepatitis B virus carriers account for 7.2% of the population. HBsAg infection may lead to acute or chronic hepatitis. In acute hepatitis, gallbladder wall thickening, volume change and abnormal bile composition can occur and the normal systolic and diastolic functions may be disrupted[9]. Cholesterol polyp is the most common type among PLGs. Compared with non-hepatitis B patients, chronic hepatitis B patients are prone to PLG and the possible causes include: (1) Liver cell cholesterol metabolic disorders may lead to the alteration of the bile composition and quantity, and increased cholesterol in the bile of the gallbladder may easily crystallize and precipitate on the gallbladder wall, resulting in abnormal deposition; (2) Hepatitis B virus activates the immune system to produce autoimmune inflammation, leading to an increased activity of the gallbladder macrophages for cholesterol phagocytosis; (3) Gastrointestinal hormone secretion and metabolic disorder may cause tension adjustment disorder of the sphincter of Oddi, causing increased bile viscosity and poor drainage; and (4) Damages of the liver Kupffer cells may compromise the

detoxification of microbial toxins and phagocytic functions. Together with the small bile ducts and capillary damage, microorganisms and toxins can invade the gallbladder[10]. Our data showed that compared with PLG-negative group, PLG-positive group had a significantly higher incidence of hepatitis B virus infection. The logistic regression analysis showed that positive HBsAg was a risk factor for PLG. Lin *et al*[8] also reported that positive HBsAg was an independent risk factor for PLG. But there are some contrary reports[11]. The inconsistent findings may be related to the number of cases, sex ratio, ethnic differences and other factors. The role of hepatitis B virus in PLG still deserves further studies.

PLG formation mechanisms are very complicated, involving many interacting factors. The mechanisms for cholesterol polyps, the main type of PLG, have been most frequently reported. It has been reported that cholesterol polyps are related to the metabolism of cholesterol in bile. Khairy *et al*[12] found that in 74 patients with cholesterol polyps, 63 (85.1%) patients had elevated plasma cholesterol levels. However, other studies showed that higher plasma T-Cho and TG levels and the incidence of PLG were not necessarily correlated with PLG[13,14]. Ivanchenkova *et al*[15] and Zák *et al*[16] found that plasma HDL-C levels in patients with cholesterol polyps were significantly lower than in the control group, while LDL-C levels were significantly increased. Our data showed that compared with PLG-negative group, T-Cho and LDL-C levels were significantly higher in PLG-positive group while HDL-C levels were significantly lower. The TG level showed no significant difference between the two groups. Consistent with the report by Cantürk *et al*[17], our study showed that low HDL-C level was a risk factor for PLG. Currently, whether the cholesterol deposited in the gallbladder is from the plasma and the relevance between plasma TG level and PLG still remain unclear. Most studies have focused on the mechanisms of absorption and excretion of cholesterol by the mucosa of the gallbladder[18].

Although PLG is an independent disease, it is closely related to the occurrence of the gallbladder stone, which is commonly seen in PLG patients. Studies showed that PLG was often accompanied with stones, and Ito *et al*[19] reported that the rate of PLG with stones was 12%. Colecchia *et al*[20] believed that metabolic disorders of cholesterol existed in both PLG and gallbladder stones, which shared the common pathogenesis. However, these two diseases were not necessarily correlated. Our study showed that the incidence of the gallbladder stone was not significantly different between PLG-positive and PLG-negative groups.

In recent years, the prevalence of diabetes, fatty liver and obesity has been increasing year by year, affecting more and more people at younger ages. These three morbidities all belong to metabolic disorders and their roles in PLG are not consistent among previous reports[11,21,22]. Our study demonstrated that the incidences of fatty liver were not statistically different between PLG-positive and

PLG-negative groups, nor the plasma glucose and obesity. The logistic regression analysis showed that diabetes, fatty liver and obesity were not risk factors of PLG.

The exact mechanisms underlying PLG pathogenesis are still not clear. This retrospective analysis has demonstrated that low HDL-C level, male gender and positive HBsAg are the risk factors for PLG, and these findings will provide the related evidence and guidance for health education, and prevention and treatment of PLG.

COMMENTS

Background
Polypoid lesions of gallbladder (PLG) are tumor or tumor-like projections, referring to any mucosal projection into the lumen of the gallbladder which is usually non-neoplastic (> 95%), but may infrequently be neoplastic (< 5%) in nature. The diagnosis of gallbladder polyps is relatively easy by ultrasonography. Although numerous studies have focused on gallbladder polyps, little has been known about factors associated with the occurrence of PLG. The authors aimed to investigate the prevalence and possible risk factors of PLG in a health screening population of Shanghai region.

Research frontiers
The incidence of PLG has an increasing tendency in recent years. The reports and analysis on the risk factors of PLG were not consistent. The literatures mostly focus on gender, lipid metabolism disorders, gallstones, hepatitis B, glucose metabolism disorders, gallbladder local inflammation and so on. Inconsistencies may be related to race, lifestyle, culture, geographic characteristics worldwide, as well as experimental design and other factors.

Innovations and breakthroughs
In China, especially in the Shanghai region, there have been few larger sample analyses on the risk factors of PLG. In this study, the authors reported that the male gender, positive hepatitis B surface antigen, and low high-density lipoprotein are high-risk factors for developing PLG in healthy people.

Applications
The study is conducive to the prevention and treatment of PLG. The relationship between gender, hepatitis B and high-density lipoprotein and PLG is worthy of further studies.

Peer review
It is an interesting topic. The authors analyzed the risk factors for polypoid lesions of gallbladder in a population of health examinees in the Shanghai region.

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BRIEF ARTICLE

WWOX induces apoptosis and inhibits proliferation of human hepatoma cell line SMMC-7721

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Abstract

AIM: To investigate the effects of the *WWOX* gene on the human hepatic carcinoma cell line SMMC-7721.

METHODS: Full-length *WWOX* cDNA was amplified from normal human liver tissues. Full-length cDNA was subcloned into pEGFP-N1, a eukaryotic expression vector. After introduction of the *WWOX* gene into cancer cells using liposomes, the WWOX protein level in the cells was detected through Western blotting. Cell growth rates were assessed by methyl thiazolyl tetrazolium (MTT) and colony formation assays. Cell cycle progression and cell apoptosis were measured by flow cytometry. The phosphorylated protein kinase B (AKT) and activated fragments of caspase-9 and caspase-3 were examined by Western blotting analysis.

RESULTS: WWOX significantly inhibited cell proliferation, as evaluated by the MTT and colony formation as-says. Cells transfected with WWOX showed significantly higher apoptosis ratios when compared with cells transfected with a mock plasmid, and overexpression of WWOX delayed cell cycle progression from G1 to S phase, as measured by flow cytometry. An increase in apoptosis was also indicated by a remarkable activation of caspase-9 and caspase-3 and a dephosphorylation of AKT (Thr308 and Ser473) measured with Western blotting analysis.

CONCLUSION: Overexpression of *WWOX* induces apoptosis and inhibits proliferation of the human hepatic carcinoma cell line SMMC-7721.

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INTRODUCTION

The tumor suppressor gene *WWOX* is localized in a common fragile site FRA16D (locus 16q23.3-24.1). Protein encoded by *WWOX* is an oxidoreductase containing two WW protein interaction domains. The biological role of the protein is not yet defined, although there are hypotheses that it may play a part in steroid hormones me-

tabolism and ErbB4 receptor signaling pathway[1,2]. Low expression level of the *WWOX* gene has been observed in many types of cancers[3-15], possibly due to the loss of heterozygosity or epigenetic changes, such as methylation of CpG islands in promoter region. Several researches have revealed loss of heterozygosity of *WWOX* locus in gastric[7], pancreatic[6], esophageal[3] and lung[4] cancers. The role of WWOX in hepatic carcinoma is not well understood, and few studies have reported the effects of WWOX on hepatic carcinoma. In this study, we investigated the apoptotic effects of the *WWOX* gene on the human hepatic carcinoma cell line SMMC-7721.

MATERIALS AND METHODS

Materials

The eukaryotic expression vector pEGFP-N1 and *Escherichia coli* DH5α competent cells are routinely maintained by the central laboratory at our hospital. The hepatoma cell line SMMC-7721 was obtained from the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modification of Eagle's medium Dulbecco (DMEM) culture medium was purchased from Gibco BRL (Gaithersburg, United States). Fetal bovine serum was obtained from Sijiqing Biological Engineering Material (Hangzhou, China). The following materials were used: RNeasy Protect Mini-kit (QIAGen Co., Germany), SMARTTM PCR cDNA synthesis kit (Clontech Co., United States), DNA gel extraction kit (Dalian TaKaRa Co., China), plasmid mini-preparation kit (Shanghai Huasun Biotechnology Co., China), KOD-Plus DNA polymerase (TOYOBO Co., United States), T4 DNA ligase and the HindⅢ and Kpn I restriction enzymes (New England Biolabs, United States), Lipofectamine 2000 (Invitrogen, United States), anti-WWOX, anti-phospho-AKT(pThr308 and Ser473), cleaved caspasse-9 and caspase-3 monoclonal antibodies (Santa Cruz Co., United States), and horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Zhongshan Co., China). Nucleic acid sequencing was performed by Shanghai Yingjun Bioengineering Co., China. The *WWOX* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were synthesized by Shanghai Yingjun Bioengineering Co., China.

Cell lines and culture conditions

SMMC-7721 cells were cultured in DMEM medium (Hy-Clone Inc, United States) supplemented with 10% new calf bovine serum in a 37 ℃ and 5% CO₂ incubator.

Construction of pEGFP-N1-WWOX vector and establishment of cell line SMMC-7721 that stably expresses WWOX

The *WWOX* open reading frame was amplified from a cDNA clone using the forward primer 5'GGAAGCTTTT-GGAGCGGGGAGTGAG-3' and the reverse primer 5' GGATCCCAGCAGTTGTTGAAGTACA-3', which introduced *Kpn* I and *Hin*DⅢ restriction endonuclease sites. *WWOX* cDNA digested with *Kpn* I and *Hin*dⅢ was

cloned into a pEGFP-N1 eukaryotic expression vector. The resulting vector was transfected into SMMC-7721 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). An empty vector of pEGFP-N1 was used as a negative control. After 24-48 h, the transient transfection efficiency was determined under an Olympus fluorescence microscope. The cells were then passaged at appropriate ratios to six-well plates. The next day, the cells were cultured in the presence of 1000-2000 g/mL G418 (Life Technology, Paisley, Scotland), which was increased in concentration in a stepwise manner over 14 d. Cells highly expressing green fluorescent protein (GFP) were selected.

Western blotting

Protein was extracted from cultured cells using lysis buffer. After a 30-min incubation on ice, the lysates were heated at 100 ℃ for 15 min and centrifuged at 12 000 × *g* for 15 min at 4 ℃. Lysates containing an equal amount of protein (25 μg) were dissolved in sodium dodecyl sulphate (SDS) sample buffer, separated on 12% SDS slab gels, and transferred electrophoretically onto polyvinylidene difluoride membranes. Equal protein loading and transfer were confirmed by Ponceau S staining. After being blocked with 5% non-fat dry milk in Tris-Buffered Saline and Tween 20 (10 mmol Ttris-HCl, pH 8.0, 100 mmol/L NaCl and 0.05% Tween), the membrane was incubated at 4 ℃ overnight with the appropriate primary antibodies. Following washing, horseradish peroxidase conjugated secondary antibody was applied to the membrane. Proteins bound by the secondary antibody were visualized by electrochemiluminescence (Amersham Bioscience) according to the manufacturer's instructions. The expression of GAPDH was measured as a control, and each experiment was performed in triplicate.

Cell growth assays

Cell growth was determined by the methyl thiazolyl tetrazolium (MTT) assay (Sigma, United States). Briefly, 1×10^4 cells were seeded onto 96-well plates with four replicates for each condition. Approximately 72 h later, MTT reagent was added to each well at 5 mg/mL in a 20 μL volume, and the reaction was incubated for another 4 h. The formazan crystals formed by viable cells were subsequently solubilized in dimethyl sulfoxide, and the absorbance (*A*) at 490 nm was measured.

Plate colony formation assay

Approximately 100 cells were added to each well of a six-well culture plate. After incubation at 37 ℃ for 15 d, cells were washed twice with phosphate-buffered saline (PBS) and stained with Giemsa solution. The number of colonies containing ≥ 50 cells was counted under microscope [plate clone formation efficiency = (number of colonies/number of cells inoculated) × 100%]. Each experiment was performed in triplicate.

Cell cycle analysis

Forty-eight hours after treatment, logarithmically grow-

Figure 1 WWOX protein levels in SMMC-7721 cells. 1: Control group; 2: Empty vector transfection group; 3: pEGFP-*WWOX* transfection group.

Figure 2 Growth of SMMC-7721 cells examined by plate colony formation. Overexpression of WWOX (pEGFP-N1-WWOX/SMMC-7721) resulted in a decrease in the number of formed colonies compared with the SMMC-7721 and control vector (pEGFP-N1/SMMC-7721) cells (*P* < 0.05).

ing cells were collected and washed with PBS three times and fixed with 75% ethanol at -20 ℃ for at least 1 h. After extensive washing with PBS, the cells were suspended in Hank's balanced salt solution containing 50 mg/mL RNase A (Boehringer Mannheim) and 50 mg/mL propidium iodide (PI) (Sigma-Aldrich), incubated for 1 h at room temperature, and were analyzed by FACScan (Becton Dickinson).

Apoptosis assays
Apoptosis was analyzed 48 h after treatment using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions.

Statistical analysis
Data were presented as the mean ± SD. Comparisons of experimental values between cisplatin-treated cells and untreated controls were conducted using analysis of variance or the Kruskal-Wallis rank test. Statistical significance was defined as *P* < 0.05.

RESULTS

Overexpression of WWOX in the cell line SMMC-7721
To study the biological functions of WWOX, we introduced WWOX into SMMC-7721 cells using a pEGFP-N1

Table 1 Methyl thiazolyl tetrazolium assay

Cell line	Optical density value
SMMC-7721	1.77 ± 0.20
pEGFP-NI/SMMC-7721	1.78 ± 0.13
pEGFP-Nl-WWOX/SMMC-7721	1.12 ± 0.23

The cell growth of parental SMMC-7721, control vector and WWOX over-expressing cells was examined by methyl thiazolyl tetrazolium assay over a 3-d period. The cell growth of the WWOX expressing cells (pEGFP-N1-WWOX/SMMC-7721) was reduced compared with the wild-type (SMMC-7721) and control vector (pEGFP-N1/SMMC-7721) cells (*P* < 0.05).

Figure 3 Overexpression of WWOX inhibits the proliferation of SMMC-7721 cells, as demonstrated by the methyl thiazolyl tetrazolium assay. There were no significant differences between the parental SMMC-7721 cell line and the control vector cell line based on the *P* values.

eukaryotic expression vector containing the *WWOX* gene. Seven stably transfected cell clones were obtained. Western blotting analysis with anti-GFP antibodies showed that WWOX-pGFP fusion protein in the SMMC-7721 cell clones was highly expressed compared with control cells and control-vector cells (Figure 1).

WWOX inhibits cell growth in vitro
To analyze the function of WWOX, we studied the rate of cell growth in the WWOX-expressing SMMC-7721 cells. The results from the colony formation assay indicated that SMMC-7721 cells overexpressing WWOX formed significantly fewer colonies than did the control clone cells and the control-vector cells (*P* < 0.05) (Figure 2). Cells transfected with *WWOX* also showed significantly decreased cell proliferation compared with control cells and control-vector cells when examined by the MTT assay (Table 1, Figure 3).

Overexpression of WWOX arrests the cell cycle in G1 and induces apoptosis of SMMC-7721 cells
To detect the effect of WWOX overexpression on the cell cycle, we measured the cell cycle distribution in WWOX-expressing SMMC-7721 cells. In these lines, there was a marked decrease in the S-phase population, while the G1 population was significantly increased compared with the control vector and wild type SMMC-7721 cells (*P* < 0.05). Neither cell lines showed significant changes in the G2 population (Figure 4, Table 2). Cells transfected with pEGFP-N1-*WWOX* demonstrated more apoptosis than did cells transfected with the mock

Figure 4 Cell cycle distribution of parental SMMC-7721, control vector (pEGFP-N1), and *WWOX* overexpressing (pEGFP-N1-WWOX) cells determined by fluorescently activated cell sorting caliber cytometry. The G1/S transition was inhibited in *WWOX* overexpressing cells compared with parental and control vector transformed cells (*P* < 0.05).

Figure 5 Cell apoptosis measured by flow cytometry using Annexin V/propidium iodide double staining. Cells transfected with pEGFP-N1-WWOX showed more apoptosis than parental cells or cells transfected with the mock plasmid (*P* < 0.05). PI: Propidium iodide; FITC: Fluorescein isothiocyanate.

Table 2 Overexpression of WWOX retards cell cycle progression from G1 to S phase

Group	Cell cycle		
	G1	S	G2
SMMC-7721	41.23 ± 2.12	34.52 ± 4.13	22.54 ± 3.12
pEGFP-Nl/SMMC-7721	40.45 ± 1.32	33.3 ± 3.11	21.24 ± 1.31
WWOX/SMMC-7721	64.23 ± 4.34	16.13 ± 2.65	17.12 ± 3.24

plasmid or the parent cells (*P* < 0.05) (Figure 5).

Caspase-9 and caspase-3 activation by WWOX

Expression of cleaved caspase-9 and caspase-3 was up-regulated, as measured by Western blotting in cells that were transfected with pEGFP-N1, compared with either

the cells transfected with a control vector or parental wild-type cells (Figure 6).

Phosphorylation of Akt decreased by WWOX

To evaluate the effect of WWOX on Akt/PKB activity, the phosphorylation level at Akt Thr308 and Ser473 was examined with specific phospho-Akt antibodies. Western blot analysis showed that WWOX significantly reduced the level of Akt/PKB phosphorylation (Figure 7).

DISCUSSION

Hepatic carcinoma is a highly invasive and clinically challenging tumor, and its molecular basis remains poorly understood. We used a gain-of-function approach by

Figure 6 Overexpression of WWOX activates the expression of caspase-9 and caspase 3 protein. A: Expression of cleaved caspase-9 was upregulated in pEGFP-WWOX cells compared with parental and control vector cells; B: Protein expression of cleaved caspase 3 was upregulated in pEGFP-WWOX cells compared with control-vector cells and parental SMMC-7721. Data are presented as mean ± SD ($P < 0.05$). 1: Control group; 2: Empty vector transfection group; 3: pEGFP-WWOX transfection group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Figure 7 Overexpression of WWOX suppresses the phosphorylation of p-AKT(Thr308) and p-AKT(Ser473). A: p-AKT(Thr308) levels were decreased in pEGFP-WWOX cells compared with parental and control vector transformed cells by Western blotting analysis; B: p-AKT(Ser473) levels were decreased in pEGFP-WWOX cells compared with parental and control vector transformed cells by Western blotting analysis. Data are presented as mean ± SD ($P < 0.05$). 1: Control group; 2: Empty vector transfection group; 3: pEGFP-WWOX transfection group. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

introducing *WWOX* into wild-type cells to investigate the effect of the *WWOX* gene on SMMC-7721, the human hepatic carcinoma cell line. Our data suggest that WWOX can significantly inhibit cell proliferation and induce cell apoptosis of the hepatic carcinoma cell line SMMC-7721. Overexpression of *WWOX* delayed the cell cycle progression from G1 into S phase, as demonstrated by flow cytometry.

Apoptosis plays a central role in tumor development, and a lack or failure of apoptosis leads to the develop-ment of many tumors, including hepatocarcinoma[16,17]. This suggests that induction of apoptosis in tumor cells might be an effective approach for delaying tumor progression. In this study, we found that overexpression of *WWOX* induces apoptosis in the hepatic carcinoma cell line SMMC-7721.

There are, at least, two broad extrinsic and intrinsic pathways that lead to apoptosis[18-20]. The extrinsic pathway begins with the binding of Fas ligand (FasL or CD95L) to the Fas receptor (CD95) and results in the

recruitment of Fas-associated protein with death domain and pro-caspase-8 to the Fas complex. This increase in the local concentration of pro-caspase-8 leads to its autocatalysis and activation. Activated caspase-8 cleaves pro-caspase-3, which then undergoes autocatalysis to form active caspase-3, a principle effector caspase of apoptosis. The intrinsic apoptosis pathway always begins with mitochondrial damage, which results in the release of cytochrome C from the damaged mitochondria. In the cytosol or on the surface of the mitochondria, cytochrome C is bound to the protein Apaf-1 (apoptotic protease activating factor), which activates the initiating caspase, caspase-9, which then activates caspase-3[21,22]. The caspase families play an important role in the apoptosis-signaling pathway. The caspases are present in the cytoplasm under normal conditions as inactive proenzymes, and most of them are activated by proteolytic cleavage when the cell undergoes apoptosis[23,24]. Both caspase-8 and caspase-9 can activate the effector caspase, caspase-3, by proteolytic cleavage, and the subsequent processes result in nuclear DNA fragmentation and the formation of apoptotic bodies. This indicates that activation of caspase-3 is a central event for the process of apoptosis. Based on these results, we speculate that WWOX allows the release of cytochrome C from mitochondria, resulting in the activation of caspase-9 and caspase-3 in sequence and finally induces apoptosis of HCC cells. Consistent with this hypothesis, the results of Western blotting showed that WWOX overexpression induced the activation of caspase-9 and caspase-3.

Our work also shows that WWOX downregulates the phosphorylation of Akt/PKB at Thr308. Akt/PKB[25,26], the major downstream effector of the PI3-kinase, is a Ser/Thr protein kinase that plays a crucial role in the regulation of several cellular signaling pathways. Akt/PKB is a regulator of cell survival and apoptosis, and its activation in a variety of cells can protect against apoptosis. Akt/PKB is phosphorylated at two regulatory sites, Thr308 and Ser473, which are essential for its activation. Activated Akt/PKB can phosphorylate BAD, IκB kinase, glycogen synthase kinase-3b, and the forkhead transcription factors[27,28], leading to their inactivation and cell survival. It has been reported that the phosphorylation of caspase-9 can regulate its activity[29]. Akt phosphorylates pro-caspase-9 at Ser196, which inhibits the proteolytic processing of pro-caspase-9.

WWOX blocks the activation of Akt, thereby attenuating the activity of a major anti-apoptotic pathway and inducing cell apoptosis. It remains unclear how WWOX affects Akt phosphorylation, as it does not affect PI3-kinase activity directly[30]. Other potential consequences of WWOX inhibition, such as the modulation of the RAS-signaling pathway, the expression of p53 and other members of the B-cell lymphoma 2 family, such as myeloid cell leukemia-1[31,32], the activation of the sphingomyelin-ceramide pathway, and interference with nuclear factor-κB[33] merit further investigation in the future.

In conclusion, WWOX may play a key role in tumor cell proliferation and carcinogenesis. Overexpression of WWOX can suppress the growth of HCC cells by inhibiting cell growth and inducing cell apoptosis. Apoptosis is induced by WWOX through the activation of the caspase cascade, which is correlated with the phosphorylation of Akt/PKB. These results suggest a potential role for WWOX as an effective chemotherapeutic and chemopreventive strategy against human liver cancer.

COMMENTS

Background
Several researches have revealed loss of heterozygosity of *WWOX* locus in gastric, pancreatic, esophageal and lung cancers. The role of *WWOX* in hepatic carcinoma is not well understood, and few studies have reported the effects of *WWOX* on hepatic carcinoma. In this study, the authors investigated the apoptotic effects of the *WWOX* gene on the human hepatic carcinoma cell line SMMC-7721.

Research frontiers
This is the first report about *WWOX* gene relevant to human hepatoma cell line SMMC-7721.

Innovations and breakthroughs
By cloning the *WWOX* gene and transferring it into hepatocellular carcinoma cell line SMMC-7721, the authors investigated the growth-inhibiting and apoptosis-inducing effects of *WWOX* gene on human hepatoma cell line SMMC-7721 and concluded that the over-expression of *WWOX* gene could induce apoptosis and inhibit the growth of hepatic carcinoma cell line SMMC-7721.

Applications
WWOX may have a potential role in development of chemotherapeutic and chemopreventive strategies against liver cancer.

Terminology
The tumor suppressor gene *WWOX* is localized in a common fragile site FRA16D (locus 16q23.3-24.1). Protein encoded by *WWOX* is an oxidoreductase containing two WW protein interaction domains.

Peer review
The manuscript describes the results of studies on the effect of tumor suppressor gene, *WWOX*, expression in SMMC-7721. This is an interesting and well presented study, with thorough introduction and the succinct discussion of the topic.

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CASE REPORT

Surgical resection of a solitary para-aortic lymph node metastasis from hepatocellular carcinoma

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Abstract

Lymph node (LN) metastases from hepatocellular carcinoma (HCC) are considered uncommon. We describe the surgical resection of a solitary para-aortic LN metastasis from HCC. A 65-year-old Japanese man with B-type liver cirrhosis was admitted for the evaluation of a liver tumor. He had already undergone radiofrequency ablation, transcatheter arterial chemoembolization, and percutaneous ethanol injection therapy for HCC. Despite treatment, viable regions remained in segments 4 and 8. We performed a right paramedian sectionectomy with partial resection of the left paramedian section of the liver. Six months later, serum concentrations of alpha-fetoprotein (189 ng/mL) and PIVKA-2 (507 mAU/mL) increased. Enhanced computed tomography of the abdomen revealed a tumor (20 mm in diameter) on the right side of the abdominal aorta. Fluorine-18 fluorodeoxyglucose positron emission tomography revealed an increased standard uptake value. There was no evidence of recurrence in other regions. Esophagogastroduodenoscopy and colonoscopy revealed no malignant tumor in the gastrointestinal tract. Para-aortic LN metastasis from HCC was thus diagnosed. We performed lymphadenectomy. Histopathological examination revealed that the tumor was largely necrotic, with poorly differentiated HCC on its surface, which confirmed the suspected diagnosis. After 6 mo tumor marker levels were normal, with no evidence of recurrence. Our experience suggests that a solitary para-aortic LN metastasis from HCC can be treated surgically.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor in the world and accounts for about 500 000 deaths per year[1]. Long-term outcomes after hepatic resection remain unsatisfactory because of the high incidence of postoperative recurrence[2]. Although intrahepatic metastases are the most common type of

recurrence, extrahepatic metastases from HCC have an important impact on long-term survival[3]. The most frequent site of hematogenous metastases is the lung, followed by the adrenal gland and skeleton[4,5]. However, lymph node (LN) metastases from HCC are uncommon, with an reported prevalence of 2.2% in a series of Japanese patients who underwent hepatic resection[4]. LN metastases are usually associated with systematic metastases, and there is currently no standard treatment[6]. The 5-year survival rate of patients with LN metastases from HCC is about 20%[7], but successful resection of a solitary LN metastasis is expected to result in better outcomes[8]. We describe the surgical resection of a solitary para-aortic LN metastasis from HCC.

CASE REPORT

A 65-year-old Japanese man with B-type liver cirrhosis was admitted for the evaluation of a liver tumor. He had already undergone radiofrequency ablation, transcatheter arterial chemoembolization, and percutaneous ethanol injection therapy for HCC. Despite treatment, HCC remained viable. We therefore considered surgical resection. The patient had undergone the repair of an abdominal aortic aneurysm with a vascular prosthesis 1 year previously. Laboratory examinations revealed the serum hemoglobin concentration to be 14.7 g/dL (normal, 14 to 17 g/dL); the platelet count was $9.7 \times 10^4/\mu L$ (normal, 12 to $38 \times 10^4/\mu L$); the total bilirubin level was 2.4 mg/dL; the direct bilirubin level was 1.9 mg/dL; the albumin level was 4.1 g/dL; the serum creatinine level was 1.24 mg/dL (normal, < 1.2 mg/dL); and the prothrombin time was 90.8%, which indicated Child-Pugh class A disease. The indocyanine green clearance rate of 15 min was 3.5% (normal, < 10%). The serum concentration of PIVKA-2 was 1226 mAU/mL (normal, < 37 mAU/mL), whereas the serum concentration of alpha-fetoprotein (AFP) was 563.7 ng/mL (normal, < 10 ng/mL). Enhanced computed tomography (CT) of the abdomen revealed a tumor, 20 mm in diameter, in segment 4 of the left lobe on early-phase images, with washout on late-phase images. Abdominal CT angiography revealed a diffuse high-density area, including lesions previously treated by radiofrequency ablation, in segment 8 of the right hepatic lobe. The high-density area was visible on arterial-phase images and washed out on late-phase images (Figure 1). The tumors in both segments were diagnosed as viable HCCs. We performed hepatic right paramedian sectionectomy with partial resection of the left paramedian section of the liver. During the operation, a tumor thrombus was found in a peripheral portal vein in segment 8. Histopathologically, the tumor contained broad areas of necrosis and fibrosis, and the HCC had a moderate-grade funicular structure. Poorly differentiated HCC was present in some parts of the tumor. The diagnosis was moderately to poorly differentiated HCC (T2N0M0, pStage Ⅱ UICC TNM classification). Peripheral portal vein invasion was detected (Figure 2). The postsurgical course was uneventful, and

Figure 1 An abdominal computed tomography angiographic scan, showing a diffuse high-density area. A: An abdominal computed tomography angiographic scan including a lesion previously treated by radiofrequency ablation in segment 8 of the right hepatic lobe, in the arterial phase (arrow); B: The area was washed out on late-phase images (arrow).

the patient was discharged on postoperative day 14. After the operation, the serum concentration of PIVKA-2 decreased to 439 mAU/mL, whereas the concentration of AFP fell to 25.5 ng/mL after the operation. Upper gastrointestinal endoscopy revealed the presence of mild esophageal varices without gastric varices (Li, Cw, F1, RC0, according to the General Rules for Recording Endoscopic Findings of Esophagogastric Varices[9]).

Six months later, serum concentrations of PIVKA-2 (507 mAU/mL) and AFP (189 ng/mL) rose again. Enhanced CT of the abdomen revealed a tumor (20 mm in diameter with slight enhancement) on the right side of the abdominal aorta (Figure 3). Magnetic resonance imaging of the abdomen revealed a high-intensity tumor in the right para-aortic region on T2-weighted images. Fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) revealed an increased standard uptake value (SUV) in this tumor (Figure 4). There was no evidence of recurrence in any other region. Upper-gastrointestinal endoscopy and colonoscopy showed no evidence of a malignant tumor in the gastrointestinal tract. The tumor was diagnosed as a para-aortic LN metastasis from HCC because of the increased tumor marker level and increased SUV on FDG-PET. We performed lymphadenectomy. At the time of the operation, the surface of the tumor was smooth and slightly adhesive. Histopathological examination showed that the tumor was largely necrotic, with poorly differentiated HCC

Figure 2 **Histopathological examination revealed the presence of considerable necrosis and fibrosis in the tumor.** The hepatocellular carcinoma (HCC) was graded as moderate-grade funicular type. A: Poorly differentiated HCC was present in some regions; B: Peripheral portal vein invasion was detected (hematoxylin and eosin, ×600).

Figure 3 **An enhanced computed tomographic scan of the abdomen revealed a tumor (20 mm in diameter, arrow) on the right side of the abdominal aorta.**

Figure 4 **Fluorine-18 fluorodeoxyglucose positron emission tomography revealed that the tumor had an elevated standard uptake value (arrow).**

Figure 5 **Histopathological examination revealed that the tumor consisted of a broad region of necrosis with poorly differentiated hepatocellular carcinoma on its surface (hematoxylin and eosin, ×600).**

on its surface (Figure 5). The invasion of lymphocytes was noted. The tumor was diagnosed as a para-aortic LN metastasis from HCC. Recovery was uneventful, and the patient was discharged on postoperative day 17. After 6 mo, the levels of tumor markers remained normal, with no evidence of recurrence.

DISCUSSION

LN metastasis is an important risk factor for the pro-gression and recurrence of many malignant tumors[10]. LN status is an established prognostic factor in oncologic surgery, with a major impact on long-term survival[7]. LN metastasis also plays an important role in the outcomes of patients with HCC, in whom its presence is related to poorer survival and a higher risk of tumor recurrence[7]. Even after lymphadenectomy, patients with LN metastases still have poorer disease-free survival and overall survival than those without LN metastases[11].

The most common site of LN metastases from HCC is the hepatic pedicle node, followed by the retropancreatic space and common hepatic artery station. These nodes appear to be key stations for lymphatic spread from liver tumors toward regional and more distant nodes[12]. However, solitary para-aortic LN metastasis from HCC is relatively rare. Some HCCs can lead to what has been termed "skip LN metastases", LN metastases at distant sites without metastases in the hepatoduodenal ligament[13-16]. Moreover, peritumoral vascular or lymphatic invasion, tumor multifocality, or distal obstruction of the lymphatics by tumor metastasis can change the direction of lymph flow to alternative routes[17]. In our patient, skipping LN metastasis might have been caused by liver cirrhosis or an operation altering the direction of lymph flow.

Previous studies have reported that patients with more advanced intrahepatic tumors or vessel invasion at initial diagnosis are more likely to have extrahepatic metastases[18]. A primary tumor size of > 5 cm has been associated with the presence of extrahepatic metastasis in HCC[19]. In our patient, the primary HCC was associated with peripheral portal vein invasion.

No standard treatment for extrahepatic metastases developing after hepatic resection of HCC is available[3]. Most patients with recurrent HCC have multiple extrahepatic metastases. The median survival time after the diagnosis of extrahepatic metastases is about 5 mo[20]. Patients with a solitary metastasis from a controlled intrahepatic tumor can be treated surgically, and good outcomes have been reported[8]. In our patient, the para-aortic LN metastasis was solitary, and there was no tumor in the residual liver or evidence of other extrahepatic metastases. We therefore resected the solitary LN metastasis.

A recent study reported that FDG-PET is a useful imaging technique for identifying extrahepatic metastases[19]. The sensitivity of FDG-PET for the detection of extrahepatic metastases was 79%. The detection rate was influenced by the size of metastatic lesions: it was 83% for metastatic lesions larger than 1 cm and 13% for lesions less than or equal to 1 cm[21]. FDG-PET is considered a very useful noninvasive imaging technique for diagnosis, staging and monitoring the treatment response in a variety of malignant tumors[22,23]. In patients with HCCs that produce AFP, an unexplained rise of the serum AFP level after treatment is an early sign of tumor recurrence or extrahepatic metastasis[24]. One study reported that tumor restaging by FDG-PET can detect and localize disease recurrence among patients with no or mild symptoms and elevated levels of tumor markers[25]. As well as being a useful tool for the preoperative staging of HCC, FDG-PET is also better than conventional diagnostic modalities for follow-up, especially staging and re-staging after hepatectomy[24]. In our patient, the serum AFP level increased after hepatectomy, and para-aortic LN metastasis was detected on FDG-PET. We therefore recommend FDG-PET for the screening of metastasis from HCC after hepatectomy.

In conclusion, our experience suggests that a solitary para-aortic LN metastasis from HCC can be treated surgically.

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LETTERS TO THE EDITOR

Effect of discounting on estimation of benefits determined by hepatitis C treatment

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Abstract

The combination of either boceprevir or telaprevir with ribavirin and interferon (triple therapy) has been shown to be more effective than ribavirin+interferon (dual therapy) for the treatment of genotype 1 hepatitis C. Since the benefit of these treatments takes place after years, simulation models are needed to predict long-term outcomes. In simulation models, the choice of different values of yearly discount rates (e.g., 6%, 3.5%, 2%, 1.5% or 0%) influences the results, but no studies have specifically addressed this issue. We examined this point by determining the long-term benefits under different conditions on the basis of standard modelling and using quality-adjusted life years (QALYs) to quantify the benefits. In our base case scenario, we compared the long-term benefit between patients given a treatment with a 40% sustained virologic response (SVR) (dual therapy) and patients given a treatment with a 70% SVR (triple therapy), and we then examined how these specific yearly discount rates influenced the incremental benefit. The gain between a 70% SVR and a 40% SVR decreased from 0.45 QALYs with a 0% discount rate to 0.22 QALYs with a 6% discount rate (ratio between the two values = 2.04).
Testing the other discounting assumptions confirmed that the discount rate has a marked impact on the magnitude of the model-estimated incremental benefit. In conclusion, the results of our analysis can be helpful to better interpret cost-effectiveness studies evaluating new treatment for hepatitis C.

TO THE EDITOR

The review by Tsubota *et al*[1] has examined the main options available for the treatment of hepatitis C, including two antiviral drugs that have recently been marketed in many countries. Focusing more thoroughly on these two innovative agents is worthwhile because boceprevir and telaprevir, along with other innovative agents, are thought to be an important advancement in the treatment of this disease[2], although at a high cost.

Hepatitis C virus (HCV) genotype 1, which accounts for 60% of all HCV-infected patients[3-5], is the target at which these two new agents are directed in combination with ribavirin + interferon. Considering that the combination of either boceprevor or telaprevir with ribavirin+interferon (triple therapy) has been shown

Table 1 Main characteristics of the Markov models[1]

Authors	Modelling details (base case)	Expected outcome		
		No treatment	Interferon monotherapy	Dual treatment
Bennett *et al*[16] 1997	Age = 35 yr; time horizon = lifetime; discount rate = 0% per year	36.2 LYs	37.7 LYs	NR
Bennett *et al*[16] 1997	Age = 35 yr; time horizon = lifetime; discount rate = 5% per year	16.2 LYs	16.4 LYs	NR
Bennett *et al*[16] 1997	Age = 35 yr; time horizon = lifetime; discount rate = 0% per year	28.0 QALYs	31.7 QALYs	NR
Shepherd *et al*[17] 2004	Age = 36 yr; time horizon = 30 yr; discount rate = 1.5% per year	21.464 QALYs	NR	23.417 QALYs
Shepherd *et al*[18] 2007	Age = 40 yr; time horizon = lifetime; discount rate = 1.5% per year	20.17 QALYs	NR	From 20.94 to 22.48 QALYs
Hartwell *et al*[19] 2011[2]	Age = 40 yr; time horizon = lifetime; discount rate = 3.5% per year, dual therapy with PEG-interferon alpha-2α	NR (naïve patients), 10.74 QALYs (previously treated patients)	NR	15.68 QALYs (naïve patients), 11.05 QALYs (previously treated patients)
Hartwell *et al*[19] 2011[2]	Age = 40 yr; time horizon = lifetime; discount rate = 3.5% per year, dual therapy with PEG-interferon alpha-2β	NR (naïve patients), 10.74 QALYs (previously treated patients)	NR	13.89 QALYs (naïve patients), 11.14 QALYs (previously treated patients)

LY: Life year; QALY: Quality-adjusted life year; PEG: Pegylated; NR: Not reported. [1]In a preliminary search on PubMed, we identified 47 articles describing a simulation model for hepatitis C; the complete references for these studies can be obtained from the authors upon request; [2]This study assessed also the shortened duration regimen of PEG-interferon + ribavirin, the data of which have not been reported in this table.

Figure 1 Estimation of the values of quality-adjusted life expectancy per patient under different modelling assumptions (time horizon = 30 years). The y-axis shows five different assumptions of yearly discount rate. QALYs: Quality-adjusted life years; SVR: Sustained virologic response.

to be more effective than ribavirin + interferon (dual therapy) in genotype 1[1-3], in the near future the dual therapy is expected to be replaced by the triple therapy in a certain proportion of these cases. The debate is still ongoing to set appropriate criteria to identify the best candidates for the triple therapy, and this selection will depend on a number of factors including pretreated *vs* naïve condition[3] and interleukin 28B polymorphism[6].

The economic impact of this new approach to HCV treatment can be very substantial since it has been estimated that around 120 million euros per year are needed in a country with 60 million inhabitants[5], and this figure seems to be confirmed by the recent sales in the United States where these "third" drugs have already been available[7].

The predicted expenditure for the "third" drug (irrespective of whether it is boceprevir or telaprevir) is likely to be at least 20 000 euros per patient[5]. Since this is also the typical expenditure for target therapies in oncologic

patients, decision-makers will have to face the competition for the same pharmaceutical budget between oncologic innovative treatments approved recently (e.g., ipilimumab for metastatic melanoma) and the triple therapy for genotype-1 hepatitis C.

The typical benefit of the latest oncologic treatments is a gain of 2-4 mo of survival per patient[8]; their pharmacoeconomic profile suggests an expenditure of 20 000 euros to gain up to a 4-mo survival, i.e., a cost-effectiveness ratio of 5000 euros per month or 60 000 euros per year.

Contrasting the cost-effectiveness between oncologic treatment and the triple therapy implies the need to compare the short-term benefits observed in oncologic patients (e.g., survival prolongation in metastatic melanoma from 6 mo without ipilimumab to 10 mo with ipilimumab) with the benefits in HCV patients that are instead known to take place at least 10 years after treatment.

The discount rate is the typical method employed in cost-effectiveness studies to convert future clinical benefits into their present value[9-14]. In the United States, rates around 5% or 6% per year were suggested nearly 20 years ago, but later various panels of experts revised this suggestion by proposing an annual rate of 3%[9,10]. In the United Kingdom, the National Institute of Clinical Excellence initially chose to use 3.5% per year[11], but in August 2011 this value was re-determined as 1.5% per year at least in some cases[15].

Several years ago, the pharmacoeconomic studies comparing dual therapy *vs* interferon alone led to the development of numerous models[16-19] based on the Markov technique that were aimed at predicting the natural history of the disease with or without achievement of post-treatment sustained virologic response (SVR). Although the number of simulation models for hepatitis C published in the past is exceedingly high, the systematic review by Hartwell *et al*[19] confirms that the models initially developed by Bennett *et al*[16] and by Shepherd *et al*[17,18] remain still valid to carry out a thorough comparative assessment of the new *vs* old treatments.

The choice of specific values of yearly discount rates

is the key factor influencing the model's outcome (Table 1). For this reason, we have summarized the different effects determined by the choice of different discount rates using a single simulation model among those reported in the literature.

The results of our analysis are shown in Figure 1. The values of quality-adjusted life years (QALYs) per patient have been calculated by examining five different assumptions of yearly discount rates (6%, 3.5%, 2%, 1.5% and 0%) and four SVR rates (0%, 40%, 70% and 100%). With regard to the SVR rates, the assumption of a 100% SVR has, of course, a purely hypothetical function, whereas the assumption of 0% SVR represent the option of no treatment. More importantly, the assumption of 40% SVR represents the typical outcome of dual treatment while 70% SVR is used to estimate the outcome of triple treatment, as well as other treatments that are currently under investigation, but will become available quite soon[2].

The information shown in Figure 1 clearly indicates that the effect of choosing different discount rates is very substantial. The gain between 100% SVR and 0% SVR (a purely hypothetical comparison) decreases from 4.47 QALYs with a 0% discount rate to 2.16 QALYs with a 6% discount rate (ratio between the two values =2.07). On the other hand, the gain between 70% SVR and 40% SVR decreases from 0.45 QALYs with a 0% discount rate to 0.22 QALYs with a 6% discount rate (ratio between the two values =2.04). These simulations have a general validity because they are only based on the clinical end-point of SVR, and therefore do not rely on specific assumptions in the patients whether are naive or pretreated. As shown in Figure 1, we could compute the value of QALYs per patient for any intermediate value of SVR (SVR$_{NN\%}$) in a range from 0% to 100% according to the equation: QALYs $_{SVRNN\%}$ = [QALY$_{SVR100\%}$ × NN + QALYs$_{SSVR0\%}$ × (100-NN)]/100. It should be noted that, in real practice as well as in model-based estimations, the favorable economic results of these treatments do not result only from the economic countervalue of the clinical benefit, but also from the savings derived from reduced morbidity. However, the latter factor was beyond the purposes of the present study.

In conclusion, our analysis has exclusively focused on the consequences of choosing different discount rates in estimating the magnitude of the clinical benefit of treatments for hepatitis C. Our results indicate that varying the discount rate within commonly accepted values can produce more than 2-fold variations in the estimates of the incremental benefit. This point should be kept in mind when regulatory agencies or third-part payers will be asked to determine the value-based price for the new treatments in this area.

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World Journal of Gastroenterology

MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166,
United States

April 20-22, 2012
Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

World Journal of Gastroenterology

INSTRUCTIONS TO AUTHORS

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The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

World Journal of Gastroenterology

Instructions to authors

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Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including t test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (n). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the P value (if it indicates statistical significance).

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AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/ study/…"; MATERIALS AND METHODS (no less than 140 words); RESULTS (no less than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, *P* < 0.001; CONCLUSION (no more than 26 words).

Key words
Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet. com/1007-9327/g_info_20100315215714.htm.

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Figures should be numbered as 1, 2, 3, *etc*., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520. pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http:// www.wjgnet.com/1007-9327/13/4986.pdf; http://www. wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc*. It is our principle to publish high resolution-figures for the printed and E-versions.

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1   **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224   DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2   **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorse-laar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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]

Volume with supplement

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Issue with no volume

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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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Contents

Contents

ABOUT COVER

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EDITORIAL

Kidneys in chronic liver diseases

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Abstract

Acute kidney injury (AKI), defined as an abrupt increase in the serum creatinine level by at least 0.3 mg/dL, occurs in about 20% of patients hospitalized for decompensating liver cirrhosis. Patients with cirrhosis are susceptible to developing AKI because of the progressive vasodilatory state, reduced effective blood volume and stimulation of vasoconstrictor hormones. The most common causes of AKI in cirrhosis are pre-renal azotemia, hepatorenal syndrome and acute tubular necrosis. Differential diagnosis is based on analysis of circumstances of AKI development, natriuresis, urine osmolality, response to withdrawal of diuretics and volume repletion, and rarely on renal biopsy. Chronic glomerulonephritis and obstructive uropathy are rare causes of azotemia in cirrhotic patients. AKI is one of the last events in the natural history of chronic liver disease, therefore, such patients should have an expedited referral for liver transplantation. Hepatorenal syndrome (HRS) is initiated by progressive portal hypertension, and may be prematurely triggered by bacterial infections, nonbacterial systemic inflammatory reactions, excessive diuresis, gastrointestinal hemorrhage, diarrhea or nephrotoxic agents. Each type of renal disease has a specific treatment approach ranging from repletion of the vascular system to renal replacement therapy. The treatment of choice in type 1 hepatorenal syndrome is a combination of vasoconstrictor with albumin infusion, which is effective in about 50% of patients. The second-line treatment of HRS involves a transjugular intrahepatic portosystemic shunt, renal vasoprotection or systems of artificial liver support.

INTRODUCTION

The best known cause of azotemia in patients with decompensated liver cirrhosis is functional vascular renal insufficiency, which is an indirect consequence of severe peripheral arterial vasodilatation with coexistent hyperstimulation of powerful vasoconstrictor systems. Acute kidney injury (AKI) linked to this mechanism may assume a prerenal form, hepatorenal syndrome (HRS) or acute tubular necrosis (ATN). Differentiation among these three main causes of AKI has important prognostic and therapeutic implications. The diagnosis may be, however, difficult because the clinical presentations are similar and one form may convert to another . Patients with cirrhosis may also have chronic renal failure resulting from

different mechanisms including renal hypoperfusion (type 2 HRS) and glomerulonephritis, related to immune or metabolic factors (Table 1).

This review covers the pathophysiological conditions leading to renal hypoperfusion in the setting of portal hypertension, and the entire spectrum of acute and chronic renal diseases in chronic liver injury.

DEFINITIONS OF AKI AND HRS

AKI is defined by the AKI Network as an abrupt (within 48 h) reduction in kidney function manifested by an absolute rise of serum creatinine of at least 0.3 mg/dL (26 μmol/L) or the equivalent to a percentage increase of 50% (1.5-fold) from baseline, or a urine output < 0.5 mL/kg per hour for > 6 h[1]. In patients with cirrhosis in whom serum creatinine levels are regularly decreased, the initial stages of AKI are commonly overlooked as increases in creatinine levels occur within the range of reference values.

HRS is a specific form of AKI that may be diagnosed only in a situation when hypercreatininemia is associated with decompensated cirrhosis (mostly in patients with diuretic-resistant ascites) or acute liver failure. Other necessary diagnostic criteria ("major criteria"), updated in 2007 by the International Ascites Club[2], include: (1) serum creatinine level > 1.5 mg/dL (133 μmol/L); (2) no improvement in serum creatinine level (decrease to 1.5 mg/dL or less) evaluated after at least 2 d under diuretic withdrawal and volume expansion with albumin given at a dose of 1 g/kg per day; maximum 100 g; (3) absence of parenchymal kidney disease as indicated by proteinuria > 500 mg/d and microhematuria > 50 red blood cells per high power field; (4) exclusion of urinary tract outflow disturbances (normal renal ultrasonography); (5) no current or recent treatment with nephrotoxic drugs or vasodilators; and (6) absence of septic or hemorrhagic shock. Less important and not regularly occurring diagnostic features of HRS ("minor criteria") are urine volume < 400 mL/d, low sodium concentration in the serum (< 130 mEq/L) and urine (< 10 mEq/L).

There are two types of HRS. In type 1, renal function deteriorates rapidly with doubling of the initial serum creatinine level to > 2.5 mg/dL (220 μmol/L) in < 2 wk. Development of type 1 HRS may be preceded by type 2 HRS, which is a chronic renal failure lasting several weeks to months, with serum creatinine levels in the range of 1.5-2.5 mg/dL. The annual risk of type 1 HRS development in patients with decompensated cirrhosis is about 20%, and within 5 years, it increases to 40%[3,4]. Some authors have singled out a type 3 HRS, which is an overlap of functional renal failure on an already existing chronic or acute intrinsic kidney disease. In differential diagnosis, it is important to consider that some kidney diseases are genetically linked with specific liver pathologies (e.g., autosomal dominant renal polycystic syndrome and oxalosis), and certain systemic diseases simultaneously affect the liver and kidneys (Table 2)[2].

Table 1 Causes of renal failure in chronic liver disease

Acute	Chronic
Hypovolemia (diuretics, hemorrhage, diarrhoea)	Hepatorenal syndrome - type 2
Hepatorenal syndrome - type 1	Glomerulonephritis (HCV infection)
Acute tubular necrosis	Glomerulonephritis (HBV infection)
Nephrotoxic agents (NSAIDs, aminoglycosides, radiological contrasts)	Immunoglobulin A nephropathy[1]
Sepsis	Diabetic nephropathy[2]

[1]Mainly in alcoholic cirrhosis; [2]Mainly combined with non-alcoholic steatohepatitis. NSAIDs: Non-steroidal anti-inflammatory drugs; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Clinical conditions leading to concomitant liver and renal injury

Drug-induced hepato-nephrotoxicity (acetaminophen, aspirin, NSAIDs)
Granulomatous diseases (e.g., sarcoidosis, leptospirosis)
Storage diseases (e.g., amyloidosis)
Systemic autoimmune diseases (e.g., lupus erythematosus)
Non-alcoholic fatty liver disease and diabetic nephropathy
Autosomal dominant polycystic kidney disease
Wilson's disease
Pregnancy-induced liver diseases (pre-eclampsia /HELLP syndrome)
Shock (cardiac failure, sepsis, hemorrhage, dehydration)
Alpha1-antitrypsin deficiency

NSAIDs: Non-steroidal anti-inflammatory drugs; HELLP: Hemolysis, elevated liver enzymes, low platelet count.

KIDNEY FUNCTIONING IN PORTAL HYPERTENSION

Renal blood perfusion and the glomerular filtration rate (GFR) depend on systemic and local factors such as cardiac output, systemic mean arterial pressure, basal muscular tone of renal arterioles, renal vascular autoregulation and intra-abdominal pressure. All these factors are considerably affected by end-stage liver cirrhosis as, in this disease, the mean arterial pressure is decreased, the renal adrenergic tone is enhanced, cardiac performance is diminished as a consequence of chronic heart overload, renal synthesis of vasoprotective peptides is reduced, and intra-abdominal pressure increases due to accumulating ascites.

Splanchnic vasodilatation

In patients with decompensated cirrhosis, arterial vasodilatation affects many vascular territories such as the skin, skeletal muscles, brain and lungs. However, it is most accentuated in the splanchnic area involving the intestines, pancreas and mesentery[5]. On one hand, the pathophysiological significance of splanchnic vasodilatation relies on the maintenance of a high portal pressure and, on the other hand, on disturbances of hemodynamic homeostasis in systemic circulation.

Splanchnic vasodilatation appears in the early stages of cirrhosis, however, it remains masked by increased

Figure 1 Pathogenesis of hepatorenal syndrome with possible triggering factors. SIRS: Systemic inflammatory response syndrome; NSAIDs: Nonsteroidal anti-inflammatory drugs.

cardiac output. At this point, the GFR is increased and glomeruli may be hypertrophied[5,6]. Splanchnic vasodilatation increases along with the progression of liver injury, portal hypertension and mesenteric angiogenesis. In advanced cirrhosis, the hyperkinetic cardiac function characterized by high stroke volume and tachycardia is no longer able to compensate for significant enlargement of the arterial vascular compartment. The disproportion between the blood volume and capacity of the arterial vascular tree leads to reduction of the effective blood volume and arterial pressure. In this setting, the baroreceptors located in the central circulation trigger hyperstimulation of the sympathetic system, renin-angiotensin-aldosterone axis and hypothalamic vasopressin secretion[7]. These adaptive mechanisms help to maintain arterial blood pressure at a safe level. Another mechanism contributing to this adaptation is renal retention of sodium and water. Endogenous vasoconstrictors have limited impact on the splanchnic arterial system, due to its intrinsic hyporeactivity[8]. By contrast, they strongly constrict renal arterioles initiating functional renal failure. The contributing mechanism to renal hypoperfusion is failure of renal vascular autoregulation, having its source in the impaired synthesis of vasodilators within the kidney (mainly prostaglandin E) and increased local release of angiotensin Ⅱ and endothelin[9,10]. The chronology of pathophysiological events underlying HRS is shown in Figure 1.

Substances most commonly incriminated in splanchnic vasodilatation are the intestinal and pancreatic vasoactive peptides, which reach the systemic circulation through portosystemic collaterals. Among compounds having myorelaxing properties are glucagon, vasoactive intestinal peptide, adrenomedulin, endogenous cannabinoid receptor agonists, substance P, natriuretic factor and carbon monoxide[11]. Major attention, however, has been focused on endothelial factors; that is, nitric oxide (NO) and prostacyclin. Results of many studies have provided evidence for increased production of NO in the arterial vascular system, as in cirrhosis, both the constitutive and inducible isoforms of NO synthetases are activated[12].

Portal cardiomyopathy
Cardiac failure in cirrhosis, named portal cardiomyopathy, has a functional background and disappears after liver transplantation. It comprises systolic and diastolic dysfunctions, mainly of the left heart chamber, and electromechanical abnormalities including a prolongation of the Q-T interval. Diastolic heart dysfunction precedes abnormalities of systolic cardiac performance. Left ventricular end-diastolic pressure is elevated in patients with decompensated cirrhosis[13]. Rapid hemodynamic changes occurring, for example, after a transjugular intrahepatic portosystemic shunt (TIPS), or liver transplantation may cause a striking increase in filling pressure favoring the development of congestive heart failure.

At rest, the portal cardiomyopathy is camouflaged by a low afterload (low peripheral vascular resistance), but limited cardiac reserve may be unveiled by hemodynamic stress (e.g., physical activity, TIPS, voluminous intravenous infusion or use of vasoconstrictor), which is followed by significantly lower increments in the stroke volume than in healthy persons[14].

Severe infections resulting in septic shock syndrome are known to induce cardiodepression due to the emergence of inflammatory mediators having negative inotropic effects. Reduction of cardiac output and inadequate cardiac contractile response to inflammatory stress is an important cause of HRS development in patients with end-stage liver cirrhosis. Ruiz-del-Arbol *et al*[15] have studied a group of 23 patients with spontaneous bacterial peritonitis (SBP) that was treated with antibiotics. The eight patients who developed HRS had significantly lower baseline cardiac output than the 15 patients without renal dysfunction. In the subgroup of patients with HRS, the cardiac output further declined with a concurrent drop in arterial pressure.

Ascites
Liver cirrhosis is associated with hypervolemia as a consequence of renal sodium retention. In conditions of splanchnic vasodilatation and increased cardiac output, the blood is preferentially pooled in the portal venous system. High hydrostatic pressure in this system accompanied by hypoalbuminemia is responsible for plasma escape from the vascular compartment to the peritoneal cavity. This phenomenon mainly occurs at the level of sinusoidal vessels and to a lesser degree at intestinal capillaries.

Ascites is responsible for a worsened quality of life, risk of SBP and renal failure[7]. The independent variables of mortality in ascitic patients are Child-Pugh class C,

Figure 2 Hemodynamic and neurohormonal consequences of progressive vasodilatation in splanchnic vascular territory. CO: Cardiac output; EAV: Effective arterial volemia; HR: Heart rate; MAP: Mean arterial pressure; GFR: Glomerular filtration rate; RAAS: Renin-angiotensin-aldosterone system; SNS: Sympathetic nervous system; HRS: Hepatorenal syndrome.

hyponatremia renal failure and, as has recently been suggested, β-blocker therapy[16].

Tense ascites causes an increase in intra-abdominal pressure, giving rise to abdominal compartment syndrome, which is a known risk factor for AKI developing in critically ill patients with acute abdominal disorders such as peritonitis, acute pancreatitis, ileus, trauma or after urgent abdominal surgeries[17]. Intra-abdominal pressure > 20 mmHg, measured in the urinary bladder, leads to severe impairment of renal venous blood flow and secondary disturbances in arterial perfusion of the kidneys[18].

Fluid removal in volume-overloaded patients with decompensated heart failure (refractory to intensive medical therapy) caused an improvement in renal function corresponding to a reduction of persistently elevated intra-abdominal pressure[19]. Knowledge of the actual influence of tense ascites on kidney function in cirrhosis is not complete. It has been shown in pilot studies that large-volume paracentesis (LVP) followed by intravenous administration of albumin ameliorates renal function in patients hospitalized for HRS or esophageal varices bleeding[20-22]. LVP should be followed by diuretics to prevent reaccumulation of fluid.

FACTORS TRIGGERING HRS

HRS develops spontaneously in about 50% of cases and it is triggered by identifiable factors dependent or independent of medical activities in the remaining cases. Pathogenesis of HRS with triggering factors is shown in Figure 2.

Bacterial infections
Patients with cirrhosis have a particular predisposition to bacterial infections that is mostly a result of impaired

reactivity of reticuloendothelial cells and neutrophils. Bacterial infections develop in 30%-60% of patients with cirrhosis, being responsible for about 25% of overall mortality in this disease[23]. The most common site of infection is the ascitic fluid, and less frequent are the respiratory system, urinary tract or subcutaneous tissue. Gastrointestinal bleeding, irrespective of its source, is associated with increased translocation of intestinal bacteria through ischemic mucosa and very high risk of SBP.

Bacterial infection induces HRS because of aggravation of splanchnic arterial vasodilatation and deterioration of liver function by endotoxemia and cytokine overproduction. Type 1 HRS commonly develops in patients with SBP, especially in those with serum bilirubin levels > 4 mg/dL (68 μmol/L) or serum creatinine levels > 1 mg/dL (88 μmol/L) - that is, in patients with pre-damaged kidneys by type 2 HRS or by other causes[24].

In a randomized trial comparing daily norfloxacin with placebo, it was demonstrated that a low content of protein in the ascitic fluid (< 1.5 g/dL) and severe liver disease indicated a beneficial effect of this antibiotic on the development of SBP and survival[25]. It seems reasonable to use norfloxacin (or trimethoprim/sulfamethoxazole) continuously in patients that meet these criteria[26].

Systemic inflammatory response syndrome
Inflammation developing in cirrhotic patients has been shown to favor serious complications specific for liver failure and portal hypertension, including HRS[27,28]. Systemic inflammatory response syndrome (SIRS) is mostly triggered by overt or occult bacterial infection but also may result from activation of the inflammatory pathways related to circulating endotoxins or proinflammatory mediators. Accordingly, SIRS occurs in many nonbacterial abdominal acute inflammatory diseases such as pancreatitis, alcoholic steatohepatitis or portal venous thrombosis. It is also speculated that SIRS may be induced by liver necrosis associated with a "spill over" of inflammatory mediators of hepatic origin. All these conditions generate production of proinflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α)], which enforce the endothelial cells to produce more NO, playing an important role in the pathogenesis of arterial vasodilatation.

According to the American Society of Critical Care Medicine Consensus Conference, SIRS is diagnosed if the patient fulfills at least two of the following criteria: (1) core temperature > 38 ℃ or < 36 ℃; (2) heart rate ≥ 90 beats per minute; (3) respiratory rate ≥ 20 breaths per minute; or (4) white blood cell count ≥ 12 G/mm^3 or ≤ 4 G/mm^3 with a differential count showing ≥ 10% immature polymorphonuclear neutrophils[29]. An important limitation for use of these criteria is the fact that many of them may be modified by cirrhosis itself.

Diarrhea and vomiting
Diarrhea and vomiting lead to dehydration and hypovolemia, which can aggravate the hemodynamic disturbances responsible for the development of HRS. Diarrhea is a common problem associated with alcoholism. More-

over, the diarrhea and vomiting occur as a consequence of infectious diseases but also may have an iatrogenic background. Diarrhea is a side effect of drugs commonly used in cirrhotic patients, such as lactulose or ursodeoxycholic acid. An increased number of bowel movements in patients with decompensated cirrhosis should prompt the withdrawal or dose reduction of these drugs.

Diuretics

Patients with peripheral edema tolerate aggressive diuresis because the fluid from the subcutaneous tissue is easily absorbed to the intravascular system. In such patients, the daily reductions of body weight may reach 2 kg. Using high doses of diuretics following the disappearance of peripheral edema frequently leads to hypovolemia, which initiates the development of HRS. In patients with ascites without peripheral edema, the urinary output should not be higher than 1100 mL/d, with diurnal reductions of the body weight not exceeding 0.5 kg. This principle originates from the fact that the diurnal ability of ascites to relocate from the peritoneal cavity to the vascular system is limited to 700-900 mL, and in the setting of postinflammatory peritoneal lesions, this limitation is even more severe. Diuretics should be discontinued if the serum creatinine is > 2.0 mg/dL (180 μmol/d) and serum sodium is < 120 mEq/L, despite fluid restriction, or in the case of encephalopathy[26,30]. Intravenous use of furosemide is not recommended as a dose of 80 mg has been shown to cause an acute reduction in renal blood flow and subsequent azotemia in patients with cirrhosis and ascites[26].

Gastrointestinal bleeding

Patients with cirrhosis carry a significant risk of bleeding from the upper digestive tract. The most likely sources of bleeding are esophageal/gastric varices, portal gastropathy or gastroduodenal peptic ulcers. A 2-year risk of bleeding from large esophageal varices in patients who never bled was 25%-30%, and in those who had at least one episode of bleeding in the past exceeded 60%[31]. Acute bleeding from the upper digestive tract leads to hypovolemia and a decrease of renal perfusion. The probability of HRS development in bleeding patients depends on the amount of blood loss, the functional reserve of the liver, and the presence of bacterial infection. In one study, hemorrhagic shock and HRS were independent risk factors of in-hospital mortality. If HRS developed in a bleeding patient, the mortality rate was 55% (3% in patients without renal failure)[32].

LVP

LVP is considered to be a relatively safe and effective procedure for the treatment of refractory ascites. However, removal of > 5 L of fluid without blood volume expansion may lead to paracentesis-induced circulatory dysfunction (PICD). Ascitic patients show increased heart rate and cardiac output and low diastolic arterial pressure, which are accentuated after LVP[33-35]. Mechanisms of

PICD are not entirely clear, but LVP causes further reduction of volemia in the central circulation with simultaneous stimulation of all vasoconstrictive systems, having a deleterious effect on renal function. According to earlier studies, HRS develops in about 10% of cirrhotic patients treated with LVP[36]. It has been recently shown that use of β-blockers may be associated with an increased risk of PICD in patients with cirrhosis and refractory ascites[37].

Recognition of PICD is based on finding a significant increase in plasma renin activity (> 50% of pretreatment values on days 5-7 after paracentesis). According to this definition, Nasr *et al*[38] recognized PICD in 73.3% of patients with tense ascites despite volume expansion (with dextran in 87.5% and with albumin in 38.5% of patients). In this study, PICD was a clinically silent complication with no clinical or laboratory predictive factor.

At present, salt-free human albumin is the plasma expander of choice, if at least 5 L of ascitic fluid is evacuated. The role of vasopressors in PICD escape is still uncertain. In the randomized study including 24 patients with decompensated cirrhosis, midodrine was not as effective as albumin in preventing PICD, because this syndrome developed in six patients in the midodrine group (60%) and in only four patients (31%) in the albumin group[39]. By contrast, in two randomized studies with 20 and 40 ascitic patients, terlipressin and midodrine were not less effective than albumin for prevention of PICD[40,41].

Nonsteroidal anti-inflammatory drugs

Renal perfusion in advanced cirrhosis greatly depends on vasoprotective mechanisms attributed to prostaglandin E2 and prostacyclin ("prostaglandin dependency"). Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit renal synthesis of prostaglandins, therefore, they impair vasoprotective mechanisms. Generally, NSAIDs should be avoided in patients with cirrhosis because in this population, they induce renal failure in 33% of patients (3%-5% in the population with an intact liver)[42]. Moreover, NSAIDs are responsible for the development of resistance to diuretics[43]. Renal complications are also observed after use of selective cyclo-oxygenase 2 antagonists such as celecoxib and rofecoxib. Even short-term therapy with celecoxib induces significant reduction in GFR in patients with decompensated cirrhosis[44].

Hypotensive drugs

In patients with advanced liver cirrhosis, the homeostasis of arterial pressure is highly dependent on adaptive neurohormonal mechanisms. Administration of drugs inhibiting these mechanisms; that is, inhibitors of angiotensin converting enzyme (ACE), angiotensin II receptor antagonists or clonidine, increases the risk of a significant reduction in arterial pressure, resulting in functional renal failure. These drugs may affect renal function irrespective of arterial hypotension. It has been shown that the administration of small doses of ACE inhibitors, having no effect on arterial pressure, cause a significant decline

in GFR[45]. However, not all studies have confirmed the harmful effects of anti-angiotensin drugs on the kidneys[46,47].

THERAPEUTIC STRATEGIES IN HRS

Treatment of renal failure in advanced liver cirrhosis aims to abate the most important pathophysiological factors responsible for the development of HRS. The first-line treatment is focused on reduction of portal hypertension and counteracting the arterial vasodilatation and effective hypovolemia, by vasoconstrictor and albumin infusion. The second-line treatment involves a reinforcement of renal vasoprotection, kidney replacement therapy or artificial liver support.

Repletion of the vascular system

Albumin is the preferred volume expander in patients with renal failure. Except for augmentation of oncotic pressure, the albumin binds endotoxins, bile acids, bilirubin, fatty acids and NO; therefore, it joins metabolic, immune and vasoconstrictor effects[48,49]. Albumin should be infused in high percentage solutions (20%-25% albumin) and the typical dosage is 1 g/kg (up to 100 g) on day 1, and 20-60 g/d thereafter.

Use of albumin may prevent the development of HRS. In a non-placebo-controlled study, the plasma volume expansion with albumin has been shown to reduce the incidence of renal failure in cirrhotic patients hospitalized for SBP as compared with antibiotic monotherapy[24]. Moreover, an unblinded randomized controlled trial in patients with new-onset ascites demonstrated that weekly 25-g infusions of albumin for 1 year followed by infusions every 2 wk improved survival compared to treatment with diuretics alone[50]. By contrast, treating HRS with albumin monotherapy is only minimally effective, but adding albumin to vasoconstrictors provides a supplementary benefit. In a recent study, 46 patients with HRS were randomly assigned to treatment with terlipressin plus albumin or to albumin monotherapy[51]. Renal function improved in 43.5% of patients treated with terlipressin plus albumin, and in only 8.7% of patients treated with albumin alone.

Elevation of peripheral vascular resistance

Many pharmaceutical agents, predominantly vasoconstrictors along with albumin infusion, have been studied in patients with type 1 HRS. These studies are based on controlled or uncontrolled short case series. Splanchnic vasoconstrictors improve effective arterial blood volume and paradoxically dilate renal vasculature. The optimal choice of vasoconstrictor, time for initiation and duration of therapy and most favorable dosage remains to be determined. Vasoconstrictor drugs should not be given to patients with ischemic disease of the heart, brain or lower extremities. Their use is also contraindicated in patients with heart failure, cardiac arrythmias, asthma or respiratory failure.

Terlipressin is a synthetic analog of vasopressin that is characterized by a longer half-time (i.v. administration every 4-6 h) and fewer side effects (ischemic complications occurring in 5%-12% of patients) in comparison with the parent drug[52-54]. Although terlipressin is only a partial agonist of renal vasopressin V2 receptors, an acute reduction in serum sodium concentration is a common finding during use of this drug, and is sometimes associated with reversible neurological complications[55].

Treatment with terlipressin and albumin leads to the normalization of renal function in 34%-65% of patients with type 1 HRS and the recurrence of HRS after treatment withdrawal occurs in 15%-22% of cases[52,55-60]. The most applicable predictor of HRS reversal seems to be baseline serum creatinine[61]. Terlipressin plus albumin therapy increases short-term survival by 34%-43%[56-58]. In a multivariate analysis, the predictors of improved survival at 6 wk were: a significant reduction in serum creatinine between baseline and day 4, a serum level of bilirubin < 10 mg/dL and a treatment-induced increase of the mean arterial pressure by > 5 mmHg[62]. However, not all studies have shown survival benefits for terlipressin. A European multicenter, randomized, controlled trial of terlipressin and albumin *vs* albumin monotherapy in 46 patients with both types of HRS demonstrated an improvement in renal function but no survival advantage at 3 mo[50]. Hence, liver transplantation is still the optimal therapy for HRS, and use of terlipressin is considered as a bridge to transplantation. According to recent recommendations of American Association for the Study of Liver Diseases, patients with type 1 HRS should have an expedited referral for liver transplantation[26].

Somatostatin is a hormone that, in pharmacological doses, decreases splanchnic arterial blood flow. This effect is not a result of intrinsic vasoconstrictive properties, but rather comes from inhibitory effects on the release of vasoactive intestinal and pancreatic peptides. Compared with terlipressin, somatostatin exerts a less beneficial effect on renal sodium excretion in patients with or without ascites[63]. Octreotide is a synthetic analog of somatostatin, with similar hemodynamic effects. Two studies, including one with randomization and a crossover design, demonstrated that octreotide alone is not effective for type 1 HRS[64,65]. Acute administration of octreotide to cirrhotic patients, with or without ascites, did not produce any change in the GFR or in the estimated renal plasma blood flow[66]. Unfortunately, octreotide significantly decreased the free water clearance and fractional excretion of filtered sodium[67]. Also chronic use of octreotide had no renoprotective effects, because improvement in renal blood flow was associated with a reduced GFR[68].

Midodrine is an oral α1-adrenergic agonist with vasoconstrictive properties. In three pilot studies involving 79 patients with type 1 HRS, short-term administration of midodrine in conjunction with octreotide caused significant changes in systemic hemodynamics and normalization of renal function in 49% of patients[69-71]. It is unknown whether these beneficial effects are attributable

to midodrine alone or also to the potentiating effects of octreotide. However, favorable renal effects of this regimen may disappear under chronic treatment. In 1 mo therapy of refractory ascites with midodrine, octreotide and albumin, significant reductions in the plasma renin and aldosterone concentrations were found, but renal function tests were not improved[72]. In a retrospective study involving 60 patients with type 1 HRS treated with midodrine, octreotide plus albumin and 21 nonrandomized albumin-treated controls, the 30-d mortality was reduced in the treatment group (43% *vs* 71%, respectively, $P < 0.05$)[69].

The regimen composed of midodrine, octreotide and albumin may be used outside of an intensive care unit. This is not the case for norepinephrine, which requires continuous intravenous infusion and hemodynamic monitoring. This drug is infrequently used in treatment of HRS, although efficacy of norepinephrine in reversal of renal failure may be not inferior to terlipressin[73].

Renal vasoprotection
Apart from systemic causes, the development of HRS in decompensated cirrhosis is dependent on the failure of local vasoprotective factors. Until now, no drugs enforcing renal vasoprotection have been used as routine treatment for HRS. Although earlier studies have suggested the reversal of HRS during treatment with misoprostol, a prostaglandin E1 analog[74], the high doses and side effects have prevented this drug from use in cirrhotic patients.

Pentoxifylline is an inhibitor of TNF-α, which plays a major role in the pathogenesis of alcoholic hepatitis and endotoxin-mediated liver injury[75]. Meta-analysis of five trials, with a total of 336 randomized participants with alcoholic hepatitis, showed a positive effect of pentoxifylline on mortality associated with HRS[76]. The usefulness of pentoxifylline in other liver diseases is unclear. In a recent French study, the chronic peroral use of pentoxifylline (400 mg, 3 times daily) in patients with advanced cirrhosis of a different etiology did not decrease short-term mortality, however, it reduced the risk of complications (including AKI) at 2 mo and 6 mo[77].

TIPS
Implantation of TIPS augments the return of blood to the right heart, thus it counteracts the central hypovolemia and neurohormonal activation. In several studies, the plasma renin activity, norepinephrine concentrations and excretion of sodium improved gradually after insertion of the TIPS[78-80]. In a large study involving 129 cirrhotic patients with varying degrees of baseline renal function, TIPS has been shown to improve renal function. Patients with pre-treatment serum creatinine levels between 1.2 and 1.9 mg/dL had reduced mean levels of creatinine, from 1.5 to 1.1 mg/dL, and those with pre-TIPS creatinine levels > 2.0 mg/dL showed a reduction from 2.8 to 1.5 mg/dL. This means that patients with poorer renal function benefit the most from TIPS[81]. A meta-analysis

including five randomized controlled trials (330 refractory ascites patients) showed that TIPS improved survival when compared with repetitive paracenteses[82].

Decision on insertion of TIPS must be balanced against the risk of complications, which include: (1) portal encephalopathy; (2) liver insufficiency; (3) loss of stent function; (4) cardiac failure; (5) bacteremia; (6) hemolysis; or (7) bacterial infection of the stent[83,84].

Renal replacement therapy
Continuous or repeated dialysis may be considered as rescue therapy for patients with type 1 HRS, in whom pharmacological treatment is ineffective and there are no contraindications for liver transplantation. Independent indications for use of dialysis are hypervolemia not responding to diuretics, metabolic acidosis and refractory hyperkalemia. Dialysis is a bridging therapy aimed at keeping the patient alive until receiving the graft. A more recent study has reported that eight of 30 patients with HRS survived 30 d with use of dialysis or continuous venovenous hemodialysis in the intensive care unit setting[85].

In patients with cirrhosis, the hypotensive reactions and blood clotting abnormalities are more frequent during dialysis than in patients with an intact liver. Peritoneal dialysis may be better tolerated by cirrhotic patients than hemodialysis, with no increase in the number of complications[86]. This procedure enables removal of the ascites fluid and does not expose patients to anticoagulants.

Patients who are on continuous renal replacement therapies require anticoagulation to prevent thrombosis. Different anticoagulants have been used to ensure the patency of hemodialysis catheters. In patients who are at high risk for bleeding, nonstandard preventive options are applied, such as minimum-dose or no-heparin regimens or regional citrate anticoagulation, which are associated with low risks of bleeding and severe dialyzer clotting[87]. Sodium citrate is metabolized by the liver and the body clearance of this compound has been shown to be significantly reduced in critically ill cirrhotic patients. In addition, citrate clearance cannot be predicted by standard liver function tests and is not influenced by renal function[88]. It is, therefore, advisable to minimize the dose of citrate anticoagulation and monitor serum ionized calcium level and blood pH in hemodialyzed cirrhotic patients.

Systems for artificial liver support
At present, there are a few systems of artificial liver support, such as: Molecular Adsorbent Recirculating System (MARS), Fractionated Plasma Separation, Adsorption and Dialysis System and Single-Pass Albumin Dialysis Extended. These systems are designed to remove from the blood toxins, which are soluble in water and are adsorbed on albumin molecules. It seems that this kind of therapy may only achieve a temporary improvement in metabolic abnormalities that result from liver and kidney insufficiencies.

Figure 3 Differential diagnosis of three forms of acute kidney injury in patients with decompensated cirrhosis. SBP: Spontaneous bacterial peritonitis; Cr: Creatinine; HRS-1: Type 1 hepatorenal syndrome; ATN: Acute tubular necrosis; U$_{Osm}$: Urine osmolality; U$_{Na}$: Urinary sodium.

In pilot studies, MARS caused a statistically significant decrease of serum bilirubin and creatinine levels in patients with type 1 HRS who were disqualified from other forms of treatment. In addition, in a randomized study, the use of MARS led to increases of arterial blood pressure and urinary sodium output[89]. However, in a recent small study comprising six patients with type 1 HRS not responding to vasoconstrictor treatment, MARS was shown to be ineffective regarding systemic hemodynamics and renal function[90]. Experience with other artificial liver support systems in HRS treatment is limited[91]. Patients undergoing treatment with adsorbent recirculating systems can experience declines in blood pressure, hypothermia, bradycardia, tissue hypoxia or blood clotting abnormalities associated with use of anticoagulants.

INTRINSIC RENAL DISEASES

Epidemiological data suggest that AKI in cirrhosis has a prerenal origin in 60% of cases, and only one third of these patients fulfills the criteria of type 1 HRS[92]. Intrinsic renal injury in the form of ATN may be responsible for about 40% of AKI in cirrhotic patients with ascites[93]. Type 2 HRS is the most common form of chronic renal disease. The Multidisciplinary Working Party has defined chronic renal disease as an estimated GFR < 60 mL/min for > 3 mo[94]. Chronic glomerular injury related to viral, immune and metabolic factors is usually an asymptomatic disease with mild proteinuria and hematuria. Occurrence of arterial hypertension in liver cirrhosis is a rare finding indicating a possibility of chronic glomerulonephritis. Among hospitalized patients with decompensated cirrhosis, chronic glomerulonephritis is responsible for about 1% of all causes of azotemia[92].

ATN

ATN should be suspected in all cases of AKI preceded by septic or posthemorrhagic shock, prolonged dehydration, severe pancreatitis, exposure to nephrotoxic substances (aminoglycosides, contrast agents) or major surgical interventions[95].

In clinical practice, the distinction between ATN and type 1 HRS may be difficult (Figure 3). Usually, in patients with HRS, the urinary concentration of sodium does not exceed 10 mEq/L (preserved tubular ability for sodium reabsorption and urine concentration), whereas in patients with ATN, it is > 20 mEq/L with urine osmolality < 350 mOsm/kg. However, these criteria may be misleading in certain cases. Another finding favoring a diagnosis of ATN is the presence of bile-stained granular or epithelial casts in the urine, but this finding is also not definitive[95]. It is expected that a positive response to a vasoconstrictor plus albumin should rule out a diagnosis of ATN, however, a recent study has demonstrated some benefit of terlipressin in patients with cirrhosis and ATN[55]. Studies on using urine biomarkers for ATN are underway and the most promising candidates are interleukin-18, urinary kidney injury molecule 1 and urinary neutrophil gelatinase-associated lipocalin[96,97].

Reduction in GFR in ischemic ATN results from injury to the tubular epithelium and impaired glomerular capillary pressure. An overlapping factor may be tubular obstruction from casts composed of detached epithelial cells and cellular debris as well as hemoglobin and myoglobin deposits[98]. ATN caused by ischemia lasts 7-21 d[99] and, in most patients, renal function returns to normal or near normal levels following regeneration of the tubular epithelium. Regenerating cells derive from the stem cells that have survived the ischemic insult. It is also likely that migrating bone marrow stem cells contribute to this process[100,101]. During ATN-related renal failure, patients should be treated with renal replacement therapy, which may last 6-8 wk if tubular damage is caused by toxic agents[92].

Nephrotoxic agents

Aminoglycoside antibiotics still constitute an important therapeutic alternative against germs that are insensitive to other antibiotics[102]. Nephrotoxicity is the most important therapeutic limitation of aminoglycoside antibiotics, especially gentamicin. The typical manifestation of aminoglycoside toxicity is nonoliguric or even polyuric renal dysfunction accompanied by proteinuria, hypercalciuria, hypermagnesemia, aminoaciduria, glycosuria, and serum electrolyte alterations[103]. Despite rigorous patient monitoring, kidney injury appears in 10%-25% of therapeutic courses. Traditionally, aminoglycoside nephrotoxicity has been considered to result mainly from tubular damage, but recently the role of reduced glomerular filtration has also been raised[104].

For patients with chronic liver diseases, the use of gentamicin or tobramycin considerably enhances the risk of damaging the tubules and onset of acute renal insufficiency[103,105]. The particular sensitivity of cirrhotic patients to aminoglycosides may be related to sodium depletion,

concomitant use of diuretics and reduced renal function. Other drugs that can lead to AKI are amphotericin, acyclovir, penicillin and cytostatics.

Use of contrast media is a well-known cause of tubular injury, particularly in the presence of predisposing conditions like dehydration or diabetic nephropathy. Mechanisms of contrast-induced nephropathy (CIN) are complex and not fully explained. It has been shown that renal blood flow remains 45% below baseline level for at least 4 h following contrast administration[106]. Renal vasoconstriction with medullary hypoxia may be mediated by high osmolality and viscosity of contrast agents, as well as the release of endothelin and adenosine[107]. The cytotoxic effects including the generation of oxygen free radicals are also considered. Currently used iodinated contrasts are either ionic and hyperosmotic (1400-1800 mosmol/kg) or nonionic iso- or low-osmolal, characterized by lower nephrotoxicity[108]. Application of contrast media in cirrhotic patients with hypercreatininemia is not recommended, because it is associated with a high risk of AKI. This applies to use of contrast in both diagnostic and therapeutic procedures[109]. Patients with cirrhosis and normal renal function do not seem to be hypersensitive to CIN. In a retrospective case-control study including 72 patients with cirrhosis and 72 patients without cirrhosis, the intravenous administration of 100-150 mL of low osmolality radiocontrast medium induced AKI in two patients with cirrhosis and one patient in the control group (difference not significant). The cirrhotic patients who developed CIN had received high-dose diuretics and were hypovolemic[110].

Chronic glomerulonephritis

The most common form of glomerular disease found in patients with chronic hepatitis C is membranoproliferative glomerulonephritis (MPGN)[111,112], followed by membranous nephropathy, focal glomerulosclerosis and immunoglobulin A (IgA) nephropathy[113-115]. In autopsy studies, the glomerular pathology was detected in more than half of hepatitis C patients. Histopathological features of MPGN were present in 11.2%, membranous nephropathy in 2.7%, mesangioproliferative glomerulonephritis in 17.6% and mesangial expansion without proliferation in 23.4% of deceased hepatitis C patients. Cirrhosis was associated with a higher percentage of inflammatory changes in glomeruli compared with the precirrhotic stage of liver disease (59.2% *vs* 32.3%)[116]. MPGN is associated with mixed essential (type Ⅱ) cryoglobulinemia. Renal biopsy revealed the presence of cryoglobulins in about 70% of hepatitis C patients with MPGN[117]. This finding strongly indicated an etiological relationship between cryoglobulinemia and MPGN.

Successful treatment of noncirrhotic hepatitis C patients with interferon-α is probably paralleled with a favorable effect on MPGN, because a positive response to treatment was associated with a significant reduction in serum levels of cryoglobulin and creatinine[118]. One study has shown the efficacy of rituximab in the treatment of type-Ⅱ-cryoglobulinemia-related MPGN in patients with hepatitis C[119].

MPGN, membranous nephropathy and polyarteritis nodosa create a wide spectrum of glomerular lesions found in patients infected with hepatitis B virus (HBV). The prevalence of glomerulonephritis among patients with chronic hepatitis B is significantly higher in endemic regions, where the hepatitis B surface antigen carrier state is common[120,121]. Chronic carriage of HBV leads to the development of nephrotic syndrome in some individuals (particularly children), with a strong male predominance. Studies showing expression of HBV viral antigens in kidney tissue confirm the role of the virus in the development of glomerulonephritis[120,122].

In children, the membranous nephropathy often retreats spontaneously, whereas in adults it may show rapid progression[120]. The main symptom of membranous nephropathy is nephrotic syndrome, with the cause not revealed until a renal biopsy is done. Retrospective analysis of clinical studies involving small groups of hepatitis B patients with membranous nephropathy or MPGN has indicated a beneficial role of antiviral therapy. Positive virological responses to treatment with interferon-α2-A or lamivudine were associated with significant improvement in renal function[123,124].

IgA nephropathy

IgA nephropathy is the most common form of primary glomerulonephritis in developed countries[125,126]. The disease may appear at any age with the highest prevalence in the second and third decade of life. The incidence of mesangial IgA deposition in apparently healthy individuals ranges from 3% to 16%[127]. Usually, IgA nephropathy is recognized on routine urine screening with asymptomatic hematuria and/or proteinuria. The diagnosis may be established only by renal biopsy that discloses globular IgA deposits in the mesangium, and to a lesser degree in the glomerular capillary walls (immunofluorescence microscopy)[125,128].

Class A immunoglobulins (with or without small quantities of other immunoglobulins and C3 complement) are found in glomeruli of 35%-90% of patients with liver cirrhosis[129]. In these patients, IgA nephropathy is usually subclinical and the features of nephrotic syndrome occur in about 1.5% of cases. Hematuria is found less frequently than in the primary form of IgA nephropathy. This type of nephropathy may be particularly common in patients with alcoholic cirrhosis, in whom IgA serum levels are regularly increased[130].

Isoform IgA2 is cleared from circulation with the participation of the hepatic asialoglycoprotein receptor, whereas IgA1 only partially uses this elimination pathway[131]. The pathogenesis of IgA nephropathy coexisting with liver cirrhosis is unknown, however, a hypothesis of reduced hepatic clearance of immunological complexes, due to impaired asialoglycoprotein receptor function and phagocytosis by Kupffer cells, is very likely. The direct influence of immunological mechanisms should also be

considered, as they play an important role, not only in autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis, but also in hepatitis B and C, alcoholic liver disease, primary hemochromatosis and α-1 antitrypsin deficiency[132-136].

Diabetic nephropathy

Diabetic nephropathy (DN) occurs in types 1 and 2 diabetes mellitus. The epidemiology of DN has been studied in type 1 diabetes. Twenty to thirty percent of this population presents with microalbuminuria 15 years after diabetes diagnosis and almost half of those patients will progress to overt nephropathy[137]. Development of DN is strongly dependent on the control of glycemia and arterial blood pressure. The aggressive treatment of arterial hypertension and use of ACE inhibitors have been shown to reduce the rate of DN progression[138]. In Caucasians with type 2 diabetes, the prevalence of progressive renal disease seems to be lower than in type 1[139], however, there are also some data suggesting that the risk of renal injury is currently equivalent in both types of diabetes[140,141].

The mesangial expansion, thickening of glomerular basement membrane and glomerular sclerosis belong to major histological changes of DN. The late abnormality is deposition of hyaline in the glomerular arterioles, which may assume a nodular appearance[142]. DN usually leads to arterial hypertension, renal arteriosclerosis, proteinuria and fluid retention. Patients with DN may tolerate less spironolactone because of hyperkalemia.

Among patients with cirrhosis related to HCV infection, diabetes occurs in about 25% of cases. Even more frequent is the occurrence of diabetes in patients with cirrhosis related to nonalcoholic steatohepatitis, which like diabetes, is a feature of the metabolic syndrome. The percentage of glucose intolerance in the population of patients with liver cirrhosis of different etiology approaches 60%[143,144]. For these reasons, DN is one of the most common causes of chronic renal failure in patients with cirrhosis. Diabetes promotes vascular pathology, hence, advanced atherosclerosis of the coronary arteries is recognized in 20%-25% of candidates for liver transplantation[145].

POSTRENAL INSUFFICIENCY

An obstruction in urine outflow as the cause of renal failure is found in < 1% of patients with liver cirrhosis. Postrenal causes of renal insufficiency are similar to those occurring in the general population. They include nephrolithiasis, benign prostatic hyperplasia in men, ureter infiltration by tumors of the reproductive organs in women, kidney tumors, or neurogenic urinary bladder. Patients with alcoholic liver disease seem to have an increased risk of renal papillary necrosis that, after sequestration, obstructs urine outflow[146]. The diagnosis of postrenal failure is based on imaging studies (i.e., ultrasound and computed tomography), which show urine retention or hydronephrosis. The aim of the treatment is removal or bypassing of the obstacle.

CLINICAL SIGNIFICANCE OF SERUM CREATININE

Parameters of renal function are strong predictors of survival in liver cirrhosis, because vascular-mediated renal failure is one of the latest events in the natural history of chronic liver disease. The median survival time in patients with type 2 HRS is about 5 mo, but with type 1 of this syndrome, is as short as 2-4 wk[25].

Generally, the creatinine level is not a perfect indicator of renal function as it is influenced by many nonrenal factors (e.g., body weight, age, sex, race and blood volume), shows a nonlinear relationship with GFR and does not distinguish between functional and organic renal diseases. Still, a less reliable indicator of renal function is the serum level of blood urea nitrogen, which is strongly modified by the diet or the presence of blood within the digestive tract.

The creatinine levels in patients with end-stage cirrhosis regularly overestimate the actual GFR because of decreased hepatic production of creatine and muscle wasting[24]. An additional factor responsible for low serum creatinine in decompensated cirrhosis is hypervolemia, and accordingly, the increased volume of distribution for this substance. For these reasons, the early stages of AKI usually remain unrecognized in cirrhotic patients. Moreover, in jaundiced patients, the measurement of creatinine may provide erroneous results due to chromogenic interference with bilirubin in a spectrophotometric assay[147]. Recently, it has been shown that, in cirrhotic patients, the serum concentration of creatinine being higher than 0.97 mg/dL (88 μmol/L) is tantamount to impairment in renal function, as this level is equivalent to the GFR of 50 mL/min[148].

Mathematical models based upon creatinine levels, such as Cockroft-Gault or modification of diet in renal disease, introduce a systematic error to calculations of the GFR. It particularly regards the Cockroft-Gault formula that estimates GFR from serum creatinine level and body mass, which in cirrhotic patients is highly dependent on body water content. On the basis of existing studies, the significance of cystatin C in the measurement of renal function in cirrhosis cannot be definitely determined.

Serum creatinine is one of three variables that form the model of end stage liver disease (MELD) score that predicts 3-mo survival in patients with end-stage cirrhosis. Therefore, MELD score is an important tool to set up the priority for access to the graft among patients waiting for a liver transplant. In the MELD score, creatinine has been assigned a much higher, and probably excessive, weight than bilirubin[149].

Total muscle mass is one of the factors influencing the serum creatinine concentration. It is the reason why men and women having the same GFR may differ with respect to serum creatinine levels. Therefore, it can be assumed that equal MELD values in both sexes may mean a worse prognosis for women. This assumption has been confirmed by finding a higher mortality rate among women listed for liver transplant in comparison with the

pre-MELD era[27]. This finding challenges the belief that MELD realizes the principle that "the sickest goes first".

RENAL FAILURE AND LIVER TRANS-PLANTATION

Development of type 1 HRS is an unfavorable moment to conduct a liver transplantation because an elevated creatinine level is a predictor of perioperative complications and death. It also predicts the duration of stay in the intensive care unit and probability of renal replacement therapy[150,151]. Therefore, improvement of renal function should be the goal before liver transplantation. Nonetheless, 3-year survival of patients undergoing liver transplantation during HRS is approximately 60%[150], thus clearly worse than in patients with normal renal function, but still much better than in those without transplantation.

Development of ATN before liver transplantation denotes maintenance of renal failure after this procedure. Diagnosis of ATN before the scheduled hepatic transplantation may enforce the decision on simultaneous liver and kidney transplantation (SLKT). A reliable diagnosis of ATN in such circumstances requires renal biopsy, preferably in a transcaval way, because coagulopathy or large ascites precludes percutaneous biopsy. Indications for SLKT in patients with end-stage liver cirrhosis are: (1) AKI (including HRS) with persisting hypercreatininemia ≥ 2 mg/dL for a period of at least 8 wk (despite renal replacement therapy); (2) chronic renal insufficiency with GFR < 30 mL/min; and (3) chronic renal failure with histopathological evidence of sclerosis or fibrosis of > 30% of glomeruli[86].

From 1984 to 2008, SLKT was reported in 3536 patients. Apart from liver cirrhosis and chronic or acute renal failure, the main indications were oxalosis and polycystic liver and kidney disease. The cumulative 5-year survival of both organs was 60.9%, and patient survival was 42.6%[152].

With improvements in operative techniques, patient survival following liver transplantation has substantially increased with more common occurrence of chronic renal disease resulting from nephrotoxicity of immunosuppressive drugs, rendering kidney transplantation inevitable. After 10 years from liver transplantation, renal failure occurs in about 20% of patients[153].

CONCLUSION

Hypercreatinemia is a common finding in patients with end-stage liver disease and it may be a form of AKI or chronic renal failure; for example, DN or glomerular nephritis associated with nonalcoholic fatty liver disease and viral hepatitis, respectively. Knowledge of the nature of renal disease is essential for therapeutic decisions, especially in patients eligible for liver transplantation. AKI is an ominous manifestation of circulatory dysfunction in patients with late cirrhosis, generally identified with HRS.

However, HRS may be only part of the pathophysiological continuum of renal hypoperfusion-related diseases, ranging from prerenal insufficiency to ischemic kidney damage. Moreover, HRS is probably not the most common form of renal disease associated with end-stage cirrhosis. At present, the diagnosis of HRS is based on exclusion of contact with nephrotoxic agents and the spectrum of factors leading to hypovolemia. In the near future, the diagnosis of HRS will probably require the absence of urinary markers specific for injury to tubular epithelial cells.

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TOPIC HIGHLIGHT

Uday Ghoshal, Professor, *Series Editor*

Pathogenesis of achalasia cardia

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Abstract

Achalasia cardia is one of the common causes of motor dysphagia. Though the disease was first described more than 300 years ago, exact pathogenesis of this condition still remains enigmatic. Pathophysiologically, achalasia cardia is caused by loss of inhibitory ganglion in the myenteric plexus of the esophagus. In the initial stage, degeneration of inhibitory nerves in the esophagus results in unopposed action of excitatory neurotransmitters such as acetylcholine, resulting in high amplitude non-peristaltic contractions (vigorous achalasia); progressive loss of cholinergic neurons over time results in dilation and low amplitude simultaneous contractions in the esophageal body (classic achalasia). Since the initial description, several studies have attempted to explore initiating agents that may cause the disease, such as viral infection, other environmental factors, autoimmunity, and genetic factors. Though Chagas disease, which mimics achalasia, is caused by an infective agent, available evidence suggests that infection may not be an independent cause of primary achalasia. A genetic basis for achalasia is supported by reports showing occurrence of disease in monozygotic twins, siblings and other first-degree relatives and occurrence in association with other genetic diseases such as Down's syndrome and Parkinson's disease. Polymorphisms in genes encoding for nitric oxide synthase, receptors for vasoactive intestinal peptide, interleukin 23 and the *ALADIN* gene have been reported. However, studies on larger numbers of patients and controls from different ethnic groups are needed before definite conclusions can be obtained. Currently, the disease is believed to be multi-factorial, with autoimmune mechanisms triggered by infection in a genetically predisposed individual leading to degeneration of inhibitory ganglia in the wall of the esophagus.

INTRODUCTION

Achalasia is an esophageal motor disorder characterized by aperistalsis of the esophageal body and lack of relaxation of the lower sphincter in response to swallows. It affects both sexes and all age groups[1,2]. Achalasia was first described by Willis[3] in 1674 as "food blockage in

esophagus". He treated these patients successfully with a dilator made of whale bone and sponge[3]. The term "achalasia" was first coined by Hurst[4] in 1927. He had observed such patients since 1914 and suggested that their disorder might be due to absence of normal relaxation of the sphincter, possibly resulting from organic changes in Auerbach's plexus[5]. Achalasia is a Greek word that means "failure of relaxation". Achalasia can be primary (idiopathic) or secondary. In secondary achalasia, the cause for the degeneration of esophageal nerve fibers is known. Pathophysiologically, achalasia is caused by loss of inhibitory ganglion cells in the myenteric plexus. Since the initial description, several studies have attempted to explore initiating agents that may cause the disease such as viral infection, other environmental factors, autoimmunity, and genetic factors. However, the exact pathogenesis of primary achalasia is still not known. In this paper, the literature regarding pathogenesis of primary achalasia is reviewed.

PATHOPHYSIOLOGICAL ISSUES

The pathophysiology of achalasia is outlined in a simplified manner in Figure 1. In healthy esophagus, progressive delay in contractility of the lower esophageal muscles results from the presence of inhibitory neurotransmitters such as nitric oxide and its receptors in the lower esophagus (Figure 1)[6,7]. In the initial stage of the disease, degeneration of inhibitory nerve fibers in the esophagus results in unopposed action of excitatory neurotransmitter such as acetylcholine, which leads to high amplitude non-peristaltic contractions (not progressively delayed or simultaneous)[8]. This stage of achalasia is known as vigorous achalasia (average amplitude of contractions in lower esophagus > 40 mmHg)[9]. Progressive loss of cholinergic neurons results in dilation and low amplitude simultaneous contractions in the esophageal body; this stage of achalasia is called classic achalasia. Studies demonstrating reduction in number of ganglion cells in the esophageal body at autopsy of patients with achalasia and an inverse correlation between number of ganglion cells and duration of disease support their involvement in the disease process[8]. In experimental studies published long ago, a muscles strip obtained from the esophageal body of patients with esophageal achalasia failed to contract on addition of the ganglion stimulant nicotine though it contracted in response to acetylcholine, which is a direct muscle stimulant. A muscle strip from the lower esophageal sphincter (LES) of patients with achalasia contracted in response to acetylcholine though it failed to relax in response to nicotine[10]. These findings demonstrate that in patients with achalasia, there is degeneration of ganglia though the muscles remain contractile in response to acetylcholine. Degeneration of inhibitory control of the LES has also been demonstrated by studies that showed cholecystokinin, which reduces LES pressure in healthy subjects, increases pressure in patients with achalasia[8]; esophageal distension failed to cause relaxation of LES in these patients[11] and gastric distension failed

to induce transient LES relaxation[12]. All the above data suggest that achalasia results from degeneration of the esophageal nerve plexus, particularly the inhibitory fibers. However, neural degeneration might not be confined to the esophagus alone as evidenced by demonstration of Wallerian degeneration in the vagus nerve on electron microscopy[13], improvement in some abnormalities with stimulation of vagus nerve in animal models[14,15], presence of Lewy bodies in the brainstem of patients with achalasia[16], and delayed gastric emptying[17] and abnormal gastric secretory response to insulin-induced hypoglycemia in a subset of patients[18]. It is, however, not clear why some people develop neural degeneration causing achalasia. The various environmental, autoimmune and genetic factors incriminated in pathogenesis are reviewed below.

A study in an animal model showed that the nitric oxide inhibitor, recombinant human hemoglobin, caused incomplete LES relaxation and high amplitude simultaneous contractions in the body of the esophagus[19]. Similar results have been reported in humans[20]. Another study in an animal model failed to show an association of intramuscular interstitial cells of Cajal in the nitrenergic pathway and dysfunction of LES found in achalasia[21].

Infection
A number of studies implicating viral agents in the pathogenesis of achalasia showed conflicting results. An initial report by Jones *et al*[22] showed a statistically significant increase in antibody titer against measles virus (MV) in patients with achalasia compared with controls (14 of 21 achalasia patients *vs* 7 of 21 controls, P < 0.05). This difference persisted only at a titer of 1/32 or more. However, there was similar frequency of infection in both groups as was evident by similar antibody detection rates at low titer[22].

Robertson *et al*[23] reported an association between Varicella zoster virus (VZV) infection in patients with achalasia by demonstrating viral DNA in esophageal tissue. They demonstrated VZV particles by DNA hybridization techniques in three of nine esophageal myotomy specimens from achalasia patients, but in none from 20 control subjects. DNA probes for cytomegalovirus (CMV) and herpes simplex virus type 1 (HSV-1) were negative in both achalasia patients and controls[23]. This study provided an attractive hypothesis, but failed to establish a causal association. We have reported one patient who presented with motor dysphagia due to esophageal hypomotility and also developed gastroparesis following infection with VZV[24].

A few studies, however, failed to show evidence of infection as a cause for achalasia[25,26]. Niwamoto *et al*[25] used polymerase chain reaction to detect human herpes virus DNA (HSV-1 and 2, CMV, VZV, Epstein-Barr virus and human herpes virus-6) or MV RNA in the esophageal muscle of 12 patients with achalasia and six with upper esophageal carcinoma. Only HSV-1 and 2 were detected in all samples including patients and controls. Other viruses were not detected. Similarly, another study failed

Figure 1 Schematic diagram outlining possible pathogenesis of achalasia cardia. A: Diagram showing distribution of nitric oxide (NO) and acetycholine (Ach) neurons in the esophagus with normal motility pattern and barium esophagogram; B: Loss of NO in esophagus in early stage of the disease resulting in high amplitude simultaneous contractions in body (called vigorous achalasia). In this stage esophagus is not dilated in barium esophagogram; C: Further degeneration of inhibitory and additional degeneration of excitatory neurons causes low amplitude simultaneous contractions in esophageal body (called classic achalasia). In this stage esophagus is dilated in barium esophagogram. NANC: Non-adrenergic, non-cholinergic.

to detect human herpes virus, MV and human papilloma virus sequences both in achalasia or control specimens[26]. Two studies proposed an association between MV and VZV infection in patients with achalasia[22,23]. However, VZV particles were found only in a third of achalasia patients[23]. Moreover, not all patients with VZV and MV infection develop achalasia. It has been hypothesized that most patients might have cleared virus or there could be sampling error. However, most of these data suggest that VZV may not be an important cause of achalasia.

Considering the above possibilities, a recent study tried to demonstrate infection in the absence of direct evidence of virus in esophageal tissue. Facco *et al*[27] reported oligoclonal selection of T cells in achalasia patients by flow cytometry and CDR3 length spectra typing analysis of lymphocytes. They also demonstrated increased proliferation of T cells and Th-1 type cytokine release in response to HSV-1 antigen. In conclusion, available evidence suggests that infection may not be a definite cause for esophageal achalasia. One strong piece of evidence in favor of infection in the pathogenesis of achalasia, however, is the fact that Chagas disease, caused by *Trypanosoma cruzi*, very closely mimics the pathophysiology of primary achalasia[28].

Immunological factors

An autoimmune etiology for achalasia has been considered because of the presence of neural inflammation in absence of conclusive evidence of infection. Studies have demonstrated inflammatory cell infiltrate of the myenteric plexus in 90%-100% of esophageal specimens from achalasia patients[29,30]. This hypothesis has been further supported by the presence of autoantibody in sera of patients with achalasia (Table 1) and an association with major histocompatibility complex class II antigen (Table 2). Immune activation and inflammation is known to be associated with altered gastrointestinal motility due to neural dysfunction in the gut[31]. Taking the analogy of other gastrointestinal motility disorders such as post-infectious irritable bowel syndrome, intestinal pseudo-obstruction and ileus, it has been postulated that achalasia may have a similar pathophysiological basis[31]. However, more studies are needed on this issue.

Autoantibodies in achalasia: Several studies showed a higher prevalence of autoantibodies in achalasia patients compared to controls. Storch *et al*[32] suggested a role of autoimmunity by demonstrating a higher prevalence of anti-myenteric autoantibody in achalasia patients (64%)

Table 1 Studies comparing autoantibody levels between achalasia patients and control subjects

Authors	Tests	Autoantibody, *n*/N (%)		*P* value
		Achalasia	Control	
Storch *et al*[32]	Indirect immunofluorescence	37/58 (64)	4/54 (7) in healthy	< 0.0001
			Absent in 12 of Hirschsprung's disease	
			Absent in 12 esophageal cancer	
Verne *et al*[33]	Double-label Indirect immunofluorescence	7/18 (40)	Absent in 22 healthy	< 0.05
			Absent in 9 GERD	
Ruiz-de-León *et al*[34]	Indirect immunofluorescence	50/92 (54.3)	3/40 (7.5)	< 0.001
Moses *et al*[36]	Immunohistochemistry	23/45 (51)	2/22 (9) in healthy control	< 0.001
			8/16 (50) of GERD	NS

GERD: Gastroesophageal reflux disease; NS: Not significant.

Table 2 Studies showing association between achalasia and human leukocyte antigen

Authors	Allele	Allele frequency, *n*/N (%)		*P*c value	Autoantibody in achalasia, *n*/N (%)		*P* value
		Achalasia	Control		AM positive	AM negative	
Ruiz-de-León *et al*[34]	DQA1*0103	27/92 (29.3)	40/275 (14.5)	< 0.02	22/50 (44)	5/42 (20)	< 0.02
	DQB1*0603	23/92 (25)	33/275 (12)	0.05	19/50 (38)	4/42 (10)	0.05
Latiano *et al*[40]	DQB1*0502	(10.2)	(4.1)	0.016	Antineuronal antibodies:		
	DQB1*0601	(5.93)	(0.51)	0.001	10/41 (24.4) in achalasia		
	DQA1	-	-	NS	None in controls		
					Both HLA risk allele and		
					antibody: in one patient		
					None in controls		

AM: Anti-myenteric; HLA: Human leukocyte antigen; NS: Not significant.

compared to healthy controls (7%). They also showed absence of anti-myenteric autoantibody in Hirschsprung's disease as well as in esophageal cancer patients, and in 8%-9% of patients with peptic esophagitis and myasthenia gravis[32]. This hypothesis was further supported by subsequent studies[33,34].

Another interesting study demonstrated that patients with Chagasic achalasia more often had autoantibodies against muscarinic acetylcholine receptors [M(2) mAchR] as compared to patients with achalasia not resulting from Chagas disease[35].

Verne *et al*[33] tried to demonstrate the presence of regional and cellular specific antibody in achalasia patients. Staining of the esophageal and intestinal sections of rat with sera from achalasia patients showed binding of antibody to neurons in the esophageal as well as intestinal sections, though none of the sera of patients with gastroesophageal reflux disease (GERD) or controls showed staining[33]. Since idiopathic achalasia is primarily a disorder of esophageal smooth muscles, binding on intestinal sections may suggest non-specific binding of this antibody and hence, may not suggest a causal association.

A study by Moses *et al*[36] demonstrated a similar degree of immune-reactivity in the myenteric plexus of the esophagus and ileum of guinea-pig and mouse when immune-stained with sera of achalasia and **GERD patients;** however, both the groups had higher immune-reactivity when compared with normal individuals. Western blotting analysis failed to reveal specific myenteric neuronal

proteins that could be targeted by antibodies in achalasia or GERD serum[35]. These observations do not support anti-neuronal antibodies to be causative in achalasia. These antibodies may perhaps be an epiphenomenon.

Human leukocyte antigen association: An association between achalasia and **human leukocyte antigen (HLA)** class Ⅱ histocompatibility antigens was proposed for the first time by Wong *et al*[37]. They performed HLA typing on 40 achalasia patients and found that Caucasians and black populations with DQw1 had 4.2 and 3.6 times higher risk of developing achalasia, respectively[37]. **Subsequent** studies[38,39] demonstrated associations between achalasia and HLA-DQA1*0101 allele, DQB1*0602 allele and DRB1*15 allele, and confirmed the findings reported by Wong *et al*[37]. Another study showed higher frequency of DQA1*0103, DQB1*0603 and DQA1*0103-DQB1*0603 heterodimer in patients with achalasia compared to controls. They also demonstrated that achalasia patients with DQA1*0103 and DQB1*0603 alleles had significantly higher prevalence of anti-myenteric antibody[34]. In contrast, Latiano *et al*[40] failed to show any correlation among HLA alleles and anti-neuronal antibodies. **However, acha-lasia** patients had higher frequency of DQB1*0502 and DQB1*0601 alleles. All these reports might suggest an autoimmune etiology of achalasia; however, not all patients with achalasia had predisposing HLA while not all people with the specific HLA had the disease.

In conclusion, all these data are not sufficient to con-

clude that achalasia is an autoimmune disease. To define a disease as autoimmune in nature, the disease should provide the following three features: (1) direct evidence based on transfer of disease by antibodies; (2) indirect evidence based on reproduction of autoimmune disease in an animal or genetic model; and (3) detection of auto-reactive T cells in the target organ of disease[41]. To date, all the evidence to support an autoimmune etiology of achalasia has not been substantiated. More studies are needed on this issue.

Genetic factors: Some evidence supports a genetic basis for achalasia. The disease has been reported in monozygotic twins[42] and siblings[43-45]. A few reports described familial occurrence of achalasia cardia[46]. Studies showed genetic correlations of the *ALADIN* gene in achalasia associated with Allgrove syndrome[47,48]. Additionally, the disease has also been reported in association with other genetic diseases such as Down's syndrome and Parkinson disease[49,50]. The genetic basis for achalasia might have been underestimated, in contrast to that for Hirschsprung's disease, though both these enteric neuropathies have several features in common[51]. Hirschsprung's disease, like achalasia, is also known to have familial occurrence and the former has been reported quite commonly in different syndromes with a genetic basis. Both the conditions have altered motor function with loss of inhibitory innervation[51]. We wish to review some studies on genetic polymorphisms in certain genes in patients with achalasia cardia.

Nitric oxide synthase polymorphism: Nitric oxide is a necessary inhibitory neurotransmitter of the esophageal myenteric plexus involved in esophageal sphincter relaxation. Nitric oxide synthase (NOS) synthesizes nitric oxide from L-arginin. Reported studies showed that in patients with achalasic cardia, the LES has less NOS compared with controls[52,53]. At present, three different NOS isozymes, neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3) have been reported. *NOS1*, *NOS2* and *NOS3* genes are located on human chromosomes 12q24.2, 17q11.2-q12 and 7q36, respectively. A microsatellite (CA repeat) polymorphism is found within the 3'-untranslated region of exon 29 of the *NOS1* gene. The *NOS2* gene has two biallelic polymorphisms in exons 16 and 22 representing C/T and G/A transitions, respectively, and *NOS3* shows a 27-bp variable number of tandem repeat polymorphisms in intron 4. Published studies have failed to explain a strong role for these functional polymorphisms in the susceptibility for achalasia[54,55].

***VIPR1* gene polymorphism:** The *VIPR1* gene is located on human chromosome 3p22[56]. Vasoactive intestinal peptide is a small neuropeptide, which acts as a neurotransmitter with anti-inflammatory properties; it is present in the myenteric plexus to modulate relaxation of the distal esophageal wall and LES[57-59]. Reported stud-

ies have suggested a role for this neuropeptide as chief element in the maintenance of neuro-endocrine immune system communication that activates signal transduction through specific receptors present on immune cells[60]. Receptor of vasoactive intestinal polypeptide (VIPR1) belongs to the secretin receptor family, a group of G-protein coupled receptors expressed by different immune cells, including T cells, macrophages and dendritic cells[56]. VIPR1 is present on myenteric neurons of the distal esophagus and LES; continued inflammation leads to the impairment of VIPR1 signaling, which alters the effect of VIP on myenteric neurons that progresses to ganglion cell loss and nerve fiber fibrosis[8,29,32-34,36,53]. A few further studies among patients with rheumatic diseases showed the receptor down-regulation of the *VIPR1* gene resulting in decreased signaling[61-64]. Paladini *et al*[64,65] reported five single nucleotide polymorphisms in the *VIPR1* gene in patients with achalasia cardia which included (rs421558) Intron-1, (rs437876) Intron-4, (rs417387) Intron-6, rs896 and rs9677 (3'UTR) and they found that the presence of A allele at (rs421558) Intron-1 and C allele at rs896 (3' UTR) was associated with a less efficient down-regulation of the receptor. Hence, AC haplotype may protect from the disease by down-regulation of VIPR1 receptor during inflammation.

Interleukin-23 receptor polymorphism: The interleukin-23 receptor (*IL-23R*) gene, located on chromosome Ip31, encodes for heterodimeric subunits of the IL-23R complex[66]. IL-23R is a type I trans-membrane protein expressed on Th1 derived T-cells which produce IL-17, designated as Th17 cells. Previous data support a role for Th17 cells and these cytokines in inflammatory and autoimmune processes[67]. Activated JAK-STAT signaling pathway by IL-23 binds with the IL-23R/IL-12β1 receptor, which influences the number of downstream immune responses[66]. Reported studies showed that IL-23R is associated with inflammatory as well as chronic autoimmune disorders[68-74]. In a single nucleotide polymorphism of the *IL-23R* gene, arginine replaces glutamine at codon 381. One study reported *IL-23R* coding variant 381Gln was a protective allele, whereas another study found this variant to be a risk factor. de León *et al*[75] reported coding variant 381Gln of *IL-23R* was significantly more common in patients with achalasia as compared with healthy controls. Hence, this 381Gln variant of *IL-23R* polymorphism could be a risk factor for achalasia cardia.

Protein tyrosine phosphatase non-receptor 22 gene polymorphism: The protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene is located on chromosome 1p13.3-p13, a region associated with autoimmune disease[75,76]. It encodes a lymphoid specific phosphatase known as Lyp. Lyp is an intracellular protein tyrosine phosphatase and an important down-regulator of T-cell activation[77]. The *PTPN22* gene exhibits a single nucleotide polymorphism at position 1858C/T, which leads to a replacement in codon 620 of Arg (R) to Trp (W). A study

showed that Lyp-W620 had more phosphatase activity, which could slow down T-cell signaling more effectively than Lyp-R620. In a different population it has already been reported that the *PTPN22* T allele is a risk factor for autoimmune diseases[78-83]. Santiago *et al*[84] reported that *PTPN22* C1858T polymorphism increased risk of achalasia in a Spanish population.

CONCLUSION

Achalasia is caused by loss of inhibitory ganglion in the myenteric plexus in the esophagus. Gradual progression of neuronal degeneration is associated with progression of the disease from vigorous to classic achalasia. Though several studies have attempted to explore initiating agents that may cause the disease, the exact factors responsible for the degeneration of ganglion cells in the myenteric plexus are poorly understood. The disease is likely to be multi-factorial involving host genetic factors, autoimmunity, and environmental factors such as infections. More studies are needed to explore the exact cause of this enigmatic disease.

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REVIEW

Ductal adenocarcinoma of the pancreatic head: A focus on current diagnostic and surgical concepts

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Abstract

Complete surgical resection still remains the only possibility of curing pancreatic cancer, however, only 10% of patients undergo curative surgery. Pancreatic resection currently remains the only method of curing patients, and has a 5-year overall survival rate between 7%-34% compared to a median survival of 3-11 mo for unresected cancer. Pancreatic surgery is a technically demanding procedure requiring highly standardized surgical techniques. Nevertheless, even in experienced hands, perioperative morbidity rates (delayed gastric emptying, pancreatic fistula *etc.*) are as high as 50%. Different strategies to reduce postoperative morbidity, such as different techniques of gastroenteric reconstruction (pancreatico-jejunostomy *vs* pancreatico-gastrostomy), intraoperative placement of a pancreatic main duct stent or temporary sealing of the main pancreatic duct with fibrin glue have not led to a significant improvement in clinical outcome. The perioperative application of somatostatin or its analogues may decrease the incidence of pancreatic fistulas in cases with soft pancreatic tissue and a small main pancreatic duct (< 3 mm). The positive effects of external pancreatic main duct drainage and antecolic gastrointestinal reconstruction have been observed to decrease the rate of pancreatic fistulas and delayed gastric emptying, respectively. Currently, the concept of extended radical lymphadenectomy has been found to be associated with higher perioperative morbidity, but without any positive impact on overall survival. However, there is growing evidence that portal vein resections can be performed with acceptable low perioperative morbidity and mortality but does not achieve a cure.

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INTRODUCTION

Since many aspects of the pathogenesis and optimal man-

agement of ductal pancreatic adenocarcinoma (DPAC) remain unclear, this tumor entity continues to be the fourth leading cause of cancer related death in the Western world[1]. Even with the widespread use and refinements of diagnostic tools (e.g., contrast-enhanced transabdominal ultrasound (US), thin-sliced contrast-enhanced helical computer tomography (CT), contrast-enhanced magnetic resonance imaging (MRI), positron emission tomography (PET-CT), transduodenal ultrasound and fine-needle biopsy (FNB), early diagnosis of pancreatic cancer remains rare, since most patients (about 80% to 90%) at the time of diagnosis are found to have locally or even systemically advanced disease. Therefore, only 10% of patients with DPAC can undergo curative resection, which remains the only possibility of achieving long-term survival. Unfortunately, only 20% of resected patients remain free of any tumor recurrence five years postoperatively[2]. A national survey in France showed a relevant decrease in postoperative mortality after pancreaticoduodenectomy (PD) for DPAC from 11% to 3.3% between 1991 and 2010[3,4]. During the same period of observation, the overall survival of resected patients increased from 11% five years postoperatively to 25% after resection[3-5]. To date, there is insufficient solid data available regarding the exact role of neoadjuvant therapies, however, in the case of locally advanced disease, neoadjuvant chemo/radio-therapy has been reported to increase the number of patients who undergo curative surgery[6]. This review focuses on the clinical value of preoperative diagnostic and interventional techniques, results of different types of pancreatic head resection, the role of extended radical lymphadenectomy, vascular resections and perioperative medical and surgical approaches to decrease perioperative morbidity.

DIAGNOSIS AND PREOPERATIVE STAGING

Transabdominal US and contrast-enhanced US

The clinical finding of painless jaundice in an appropriately aged patient (fifth to sixth decade of life), must be considered pancreatic cancer until proven otherwise. Transabdominal US is rapid, non-invasive and inexpensive and is usually the first step in radiological evaluation. The sensitivity of US in diagnosing pancreatic cancer has a wide reported range. As a direct radiological sign, a hypoechogenic lesion can be visualized in about 55%-90% of patients[7-9]. Major limitations of US are the detection of small tumors (< 2 cm of diameter), lesions that are mainly located in the left side of the pancreatic gland, multifocal pancreatic lesions and obesity as the latter is a risk factor for pancreatic cancer[10]. Indirect radiological signs of pancreatic cancer such as dilatation of the main pancreatic duct (> 2 mm in combination with upstream areas of atrophied pancreatic gland), biliary tree, pseudocystic lesions, peripancreatic lymphadenopathy, ascites, pleural effusion and metastatic tumor deposits to the liver should strongly suggest pancreatic cancer. The great

operator dependability of US with its above-mentioned diagnostic limitations has recently led to the introduction of contrast-enhanced ultrasonography (CEUS). In a very recently published multicenter study, CEUS was reported to diagnose DPAC with an accuracy of 87% in patients with an already visualized pancreatic mass by conventional US[11]. Such findings were also confirmed by other groups[12,13]. Although some experts in the field of CEUS propose its use as an additional work-up examination for pancreatic pathologies, CEUS is currently not considered a diagnostic standard.

Thin-sliced, intravenous contrast-enhanced CT

Thin-sliced, intravenous contrast-enhanced computer tomography (CECT) has become the imaging modality of choice to evaluate patients with pancreatic cancer. The overall sensitivity and specificity of CECT has been reported to be around 90% in experienced centers[14-18]. CECT with timed sequences to capture arterial and venous phases is able to demonstrate a hypodense pancreatic tumoral lesion in 80% to 95% of cases[14-16,19] (Figure 1). Dilatation of the biliary tree or the main pancreatic duct can be found in 86% and 88% of cases, respectively (Figure 1). Tumoral obstruction of the main pancreatic duct with upstream atrophy of the pancreatic parenchyma or pseudocystic lesions are present in 82% and 10% of patients[14,20] (Figure 1). The finding of a tumor that surrounds the entire circumference of a vessel is generally recognized as unresectable tumor encasement[14,21]. CECT criteria have been developed to indicate the probability of vascular involvement based on the relationship of tumor to adjacent vessels. A prospective case series by Lu *et al*[22] introduced a new classification based on tumor involvement of the portal and superior mesenteric veins and the celiac, hepatic and superior mesenteric arteries which was graded on a scale 0-4 scale based on circumferential contiguity of tumor to vessel by CECT (Grade 0, no contiguity of tumor to vessel; Grade 1, tumor contiguous to less than one quarter circumference; Grade 2, between one-quarter and one-half circumference; Grade 3, between one-half and three-quarters circumference; and Grade 4, greater than three-quarters circumferential involvement or any vessel constriction). A cut-off between Grade 2 and Grade 3 showed the lowest number of false-negatives and an acceptable number of false-positives for unresectability. Furthermore, such a cut-off level was reported to have a sensitivity of 84%, a specificity of 98%, a positive predictive value of 95%, and a negative predictive value of 93% for unresectability of the vessels[22]. In general, typical reports in the literature regarding the accuracy of CECT using the classification by Lu for predicting vascular invasion range from 62% to 92% with a somewhat higher sensitivity for arterial infiltration[17,23] (Figure 2). Positive overall predictive values for local surgical unresectability have been reported to be excellent (89% and 100%)[14,15,19,21]. CECT has a reported sensitivity of 75%-87% in diagnosing liver metastases[24,25]. In many cases, hepatic metastatic lesions missed by CECT are small, but originate from an already

Figure 1 Ductal dilation, computer tomography 3-phase contrast-enhanced thin-slice helical scan. A: Heterogenous tumor of the pancreatic head with consecutive extra- and intra-hepatic bile duct dilatation (arrow); B: "Double duct sign" due to a tumor of the papilla of vater (arrow); C: Tumor of the pancreatic neck with an upstream dilatation of the pancreatic duct and parenchymal atrophy of the pancreatic gland. Presence of a cavernoma due to tumor thrombosis of the portal vein (arrow); D: Classic radiological presentation of a pancreatic neck tumor with a less pronounced enhancement compared to the normal pancreatic parenchyma (arrow).

Figure 2 Vascular tumor extension, computer tomography 3-phase contrast-enhanced thin-slice helical scan, sagittal section and 3D reconstruction. A, B: Sheathing and thrombosis of the celiac trunk (asterisk) and superior mesenteric artery (arrow) with collateral blood flow *via* the inferior mesenteric vessels; C: Tumor of the pancreas (arrow) in contact with the superior mesenteric artery and infiltration of the portal vein; D: Tumor sheathing or the origin of the superior mesenteric artery (arrow) with irregularities as a sign of arterial invasion.

larger pancreatic tumor (> 3 cm)[26-28] and are therefore retrospectively not unexpected. The identification of lymphatic nodal involvement and peritoneal disease is difficult with all currently available imaging modalities. On cross-sectional imaging, size (> 1 cm) is the criterion for identifying nodal metastases, and therefore the accuracy of CECT remains limited at 54%[17].

MRI, MRI-cholangiopancreaticography

To diagnose and stage pancreatic cancer, the systematic use of MRI is still questioned by many clinicians. However, MRI has been found to offer several benefits in imaging the pancreatic gland. It inherently offers better soft-tissue contrast than CECT before the administration of an i.v. contrast agent, and images can be obtained

Figure 3 Magnetic resonance imaging appearance. A: T1 sequence showing an adenocarcinoma of the pancreas with a hypo-intense signal (arrow), whereas normal pancreatic tissue appears hyper-intense; B: T1 sequence with fat saturation injection: after injection of gadolinium, the pancreatic adenocarcinoma is hypo-enhanced (arrow) compared to the healthy parenchyma; C: A sequence of diffusion: hyper-intensity (arrow) signal due to the hyper-cellularity of the tumor; D: Sequence 3D-magnetic resonance cholangiopancreatography: stenosis of the main pancreatic duct (arrow) with upstream dilatation due to a tumor of the pancreatic isthmus.

in multiple planes. MRI can be performed in patients with a history of allergy to iodinated contrast agents and in those with renal insufficiency. Today, MRI has been shown to have high diagnostic value in cases where a clear diagnosis remains unclear even after CECT has been performed. Such a situation is mostly found in cases with a suspected tumor of the pancreatic head which is isodense on CECT and/or small lesions (< 2 cm). In such situations, MRI is superior to CECT at detecting or excluding a pancreatic tumor. The greatest advantage of MRI is found in patients in whom CECT demonstrates enlargement of the pancreatic head without clear definition of a pancreatic tumor. The overall sensitivity and specificity of MRI in diagnosing pancreatic cancer has currently been reported to be around 90% and 80%, respectively[29]. Magnetic resonance cholangiopancreatography (MRCP) is a special type of MRI exam that produces detailed images of the hepatobiliary and pancreatic systems, including the liver, gallbladder, bile ducts, pancreas and pancreatic duct. Additionally, MRCP has a clear advantage over ERCP in detecting pancreatic carcinoma since MRCP prevents inappropriate explorations of the pancreatic and common bile duct[30]. MRCP is a reliable and reproducible method of evaluating intraductal papillary mucinous neoplasms (IPMN), particularly in patients being followed non-operatively or in those who require surveillance of the pancreatic remnant after PD[31]. In a study comparing MRI with CECT, MRI had an accuracy of 93.5% for the detection of liver metastases compared with 87% for CECT[25]. However, a recently published meta-analysis showed equal overall capabilities of MRI and CECT to diagnose and stage pancreatic cancer[32-34]. Even the evaluation of vascular tumor infiltration can be

evaluated by CECT or MRI with equal results[35]. MRI has lower diagnostic power to detect peritoneal carcinomatosis and/or local lymphadenopathy compared to CECT. MRI also has the potential to assess fat content which may be helpful in assessing the risk of pancreatic fistula (PF) following resection[36] (Figure 3).

Endoscopic US

When endoscopic ultrasound (EUS) was introduced, initial reports indicated a sensitivity higher than 90% for the identification of pancreatic tumors[9]. The superiority of EUS over classical CT was most evident for pancreatic lesions smaller than 3 cm in diameter. Therefore, EUS was considered the gold standard for diagnosing and staging pancreatic cancer. However, with the introduction of thin-sliced, intravenous CECT, the sensitivity and specificity of CECT for lesions smaller than 2 cm in diameter were reported to be as high as 77% and 100%, respectively[37]. Currently, EUS and CECT are considered to be equal in the diagnostic work-up of patients with suspected pancreatic cancer. However, EUS is still reported to be superior in assessing local tumor extension in the case of periampullary cancer compared to CECT and MRI (EUS: 78%, CECT: 24%, MRI: 46%)[38], however, due to the limited penetration depth of EUS, it is clearly inferior in detecting liver metastasis. In the case of suspected vascular infiltration (loss of interface between the tumor and the vessel wall; a tumor within the vessel lumen; collateral circulation; irregular vessel wall), sensitivity (85%-100%) and accuracy (55%-90%)[39] for EUS are reported to be equivocal compared to CECT/MRI. However, since these signs for vascular involvement are mainly indirect signs, these findings need careful interpretation, especially

Table 1 Summary of preoperative evaluation of pancreatic adenocarcinoma

Painless jaundice in an appropriately aged patient is highly suspicious for pancreatic cancer
Contrast-enhanced computer tomography is the diagnostic standard
  High overall diagnostic sensitivity and specificity
  Highly accurate in determining local respectability
  Less adequate in identifying small hepatic metastases, extent of local lymphadenopathy and peritoneal tumor deposits
Magnetic resonance imaging gives additional information on small isodense or atypical pancreatic lesions
  More accurate than contrast-enhanced computer tomography in detecting smaller hepatic metastases
Enhanced ultrasonography/fine-needle biopsy are reserved for the work-up of small lesions (< 2 cm), or in cases where a fine-needle biopsy is required
before palliative or neoadjuvant therapy is initiated

in pancreatitis, IPMN or after biliary drainage (BD), not to exclude potentially resectable patients from curative surgery. Nowadays, EUS is used more selectively, mainly in cases of small pancreatic head tumors (< 2 cm), in which CECT and MRI findings remain equivocal. Furthermore, patients with locally unresectable or already distant metastatic disease, EUS guided transduodenal FNB is mandatory for diagnostic purposes before the initiation of neoadjuvant or palliative treatment.

18-F FDG PET-CT

18-F FDG PET-CT is mainly used in cases of preoperatively suspected distant metastatic disease or to investigate the response to neoadjuvant treatment. Currently, PET-CT is not considered a preoperative diagnostic standard and its routine use is only reported by some centers. Moreover, some studies found a comparable reliability rate of CECT and PET-CT in detecting distant metastasis[40-42]. Nevertheless, the preoperative routine use of PET-CT was found to change the management in 16% of patients who were deemed resectable based on standard staging examinations and was reported to be cost saving[43]. More recently, contrast-enhanced PET-CT has been shown to be a highly accurate staging tool as a 1-stop-shop procedure[43]. It is very likely that the use of this strategy will increase in the near future.

Preoperative FNB

Preoperative FNB is only required in cases of locally unresectable or already distant metastatic disease before non-surgical treatment (e.g., radio- and/or chemotherapy) is planned. Furthermore, FNB is required if there is any doubt about the underlying disease. If a FNB is planned, this should, whenever possible, be performed by the endoscopic route (transgastric/transduodenal) under endosonographic guidance with multiple biopsies taken to improve the diagnostic sensitivity (Table 1).

PERIOPERATIVE MANAGEMENT

Preoperative biliary drainage

In a recently published meta-analysis by the Cochrane Library, a statistically significant increased number of perioperative infectious complications, increased length of hospital stay, and higher overall hospital costs were reported in patients who had undergone preoperative

BD[44]. These findings were confirmed in a prospective, randomized multicenter study. In addition, a significant increased risk of sustaining severe perioperative infectious complications (39% vs 74%) and a greater number of patients requiring hospital readmission (12% vs 33%) were also observed in drained patients[45]. As a relative indication for BD, in selected cases, patients suffering from severe malnutrition might benefit from BD and delayed surgery. Infection of the biliary tree is constantly (sub- or clinically) present after any drainage procedure of the biliary tree[46-48], and a peri-interventional antibiotic treatment is justified in all cases. Treatment with amoxicillin and clavulanic acid has been shown to be more efficient in decreasing septic complications than the use of second generation cephalosporins[4] (Table 2).

Perioperative supportive medical care «fast-track surgery» was not only applied for colorectal surgery

The concept of fast-track surgery is nowadays widely accepted by clinicians and has been shown to significantly enhance recovery leading to decreased hospital stay with a reduction in medical morbidity, but unaltered surgery-specific morbidity in a variety of procedures[49]. However, most data on fast-track surgery were generated by analyzing patients who underwent colorectal surgery - fewer data are available on pancreatic surgery. Nevertheless, fast-track surgery in patients undergoing major pancreatic surgery has been shown to be feasible and safe with a low readmission rate (3.5%-6.2%), in-hospital postoperative mortality (2%) and morbidity rates (35%), associated with improvements in delayed gastric emptying, earlier hospital discharge (10 d), but without compromising patient outcome[50,51]. Therefore, patients undergoing pancreatic surgery should not be excluded from the general principles of enhanced perioperative recovery programs.

INTRAOPERATIVE MANAGEMENT

Prevention of PF

The most frequent complication after pancreatic surgery is PF. The incidence of this complication varies widely between 5% and 30% depending on the different reported series[52]. However, this wide reported range is mainly based on the fact that there was, until recently, no uniform definition available for this complication. More recently, a uniform definition on the presence and

Table 2 Indications for preoperative biliary drainage
Total bilirubin > 250 mmol/L
Acute cholangitis
Severe malnutrition and delayed surgery scheduled (relative indication)
Patients who require neo-adjuvant chemotherapy
Perioperative antibiotic treatment with penicillin in cases with evident infection of the biliary tree and in all patients undergoing biliary drainage

Table 3 Prevention of pancreatic fistula
There is currently no favored pancreatico-digestive anastomotic technique with regard to decreased pancreatic fistula rates
The routine use of octreotide can only be recommended in the case of:
Friable pancreatic tissue
Small diameter of the main pancreatic duct (< 3 mm)
Trans-anastomotic, percutaneously placed drainage of the main pancreatic duct decreases the risk of pancreatic fistula formation

severity of postoperative PF has been proposed by the International Study Group on PF. A PF is a drain output of any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than 3 times the serum amylase activity. The severity of PF is graded as follows: Grade A: PF managed medically; Grade B: PF requires endoscopic or radiological intervention; Grade C: reoperation[52]. In the case of a Grade C fistula, an increased mortality of 40% was found in a recently published French multicenter study of more than 680 consecutive patients[53]. Friable pancreatic tissue, a main pancreatic duct (Wirsung) smaller than 3 mm in diameter and low volume pancreatic surgeons are reported to be risk factors for the development of PF[54]. To decrease the incidence of PF, several different technical and medical strategies have been proposed: (1) internal or external perioperative drainage of the main pancreatic duct; (2) temporary fibrin glue sealing (TFGS) of the main pancreatic duct; (3) the perioperative systematic use of somatostatin or its analogues; and (4) the role of different types of pancreatic-enteric reconstruction [pancreatico-jejunostomy (PJ) *vs* pancreatico-gastrostomy (PG)] (Table 3).

Drainage of the main pancreatic duct (Wirsung)

A prospective randomized trial from the Johns Hopkins University failed to demonstrate any benefit of an intraoperatively placed internal main pancreatic duct stent regarding the incidence and/or severity of PFs[55]. In contrast, external drainage of the main pancreatic duct, especially in the case of soft or friable pancreatic parenchyma, significantly reduced the number of perioperative PFs. In a prospective, randomized trial, the effect of external pancreatic main duct drainage during duodenopancreatectomy was found to be associated with a significantly lower incidence of PFs (6.8% *vs* 29.3%; $P < 0.007$) compared to the group of patients without drainage[56]. This finding has been further supported by a prospective, randomized study which not only showed a significantly lower incidence of PFs (20% *vs* 6.7%; $P = 0.032$) but also a decreased length of hospital stay (23 d *vs* 17 d; $P = 0.039$) for the drained group[57]. Analogue findings were also reported in a recently published French multicenter study[58].

TFGS of the main pancreatic duct

Several studies have investigated the possible value of TFGS of the main pancreatic duct to decrease the number and/or severity of clinically evident PFs. One in particular is a multicenter study of patients who underwent pancreatic resection with the formation of a pancreatico-jejunal anastomosis. Patients in group 1 ($n = 80$) received TFGS, and the control group 2 ($n = 102$) underwent standard PJ without fibrin glue sealing. The incidence of PF was found to be equal in the two groups (17% *vs* 15%) with no significant difference in the incidence of intra-abdominal septic complications (15% *vs* 24%) and postoperative mortality (9% *vs* 6%)[59]. Based on the currently available data in the medical literature, TFGS does not decrease the incidence or the severity of PF, therefore, can not be recommended in daily routine practice.

Routine post-operative administration of somatostatin or its analogues

The systematic application of somatostatin or its analogues, which are known to decrease the secretory capacity of the endo- and exocrine pancreatic gland, has been assumed to have a protective effect against the formation and/or severity of PF.

If somatostatin or its analogues are used, they should be started before surgery[60]. In a meta-analysis of seven studies including a total of 1359 patients having undergone pancreatic surgery, the perioperative application of somatostatin or its analogues was found to be associated with a significant reduction in the incidence of PF after elective pancreatic surgery. However, this risk reduction was not associated with a significant difference in postoperative mortality. Another meta-analysis of 1918 patients found that somatostatin or its analogues did not reduce mortality after pancreatic surgery, but reduced overall morbidity as well as the incidence of biochemical fistula but not that of clinical anastomotic disruption[61]. However, there are also data showing that the routine use of somatostatin or its analogues is not beneficial in all patients and should be limited to certain situations with an increased risk for PF formation such as: low volume pancreas centers with a high PF rate > 10%, a small main pancreatic duct (< 3 mm) and a friable pancreatic gland[62,63].

PJ vs PG

There is an ongoing debate regarding the optimal pancreatico-enteric reconstruction technique after PD. When comparing PJ with PG, several clinical trials reported a decreased incidence of PFs after PG[64-66]. In contrast to these data, three prospective randomized trials comparing PJ and PG found equal outcomes for both tech-

niques[67-69]. In a meta-analysis published in 2007 by Wente *et al*[70], no difference was found between PJ and PG by analyzing prospective randomized trials, whereas observational clinical studies favored the use of PG with a reduced incidence of PF and postoperative mortality rates. The authors concluded, that there was a possible risk of publication bias in observational clinical trials and all randomized controlled trials failed to show an advantage of a specific type of reconstruction. Therefore, PG and PJ can be considered to be equally safe[70]. Theoretically, PG might lead to decreased activity of pancreatic enzymes due to inactivation by gastric acid which would result in an increased incidence of postoperative exocrine pancreatic insufficiency. However, this issue was refuted in a study by Lemaire *et al*[71] who found no difference in pancreatic exocrine insufficiency between PG and PJ.

Pylorus preserving PD or classic Kausch-Whipple

Proponents of pylorus preservation argue that the gastroduodenal physiology is better maintained and therefore, especially postoperative quality of life, is superior to the classic Kausch-Whipple (CKW) technique. In contrast, proponents of the CKW technique state that preservation of the pylorus does not follow the rules of radical tumor surgery with inadequate clearance of lymphatic nodes, inadequate tumor staging, and increased risk of tumor recurrence and impaired overall survival. In the most recently published meta-analysis by Fitzmaurice *et al*[72], 43 studies [6 randomized controlled trials, 12 prospective studies and 25 retrospectives studies; pylorus preserving pancreaticoduodenectomy (PPPD): *n* = 1870; CKW: *n* = 1923] were analyzed. To investigate the postoperative overall survival, a total of 26 studies with only surgery for pancreatic cancer patients were analyzed. The overall postoperative survival was found to be equal following PPPD and CKW. However, by only analyzing those studies of higher scientific quality, a significantly longer overall survival was found in patients who had undergone PPPD.

Thirty-three studies were eligible for analyzing postoperative mortality. The authors reported no significant difference between the two procedures. As far as the quality of life is concerned, the studies are difficult to compare since a large variety of different quality of life scores (if used at all) and parameters were used[72]. Another recently published meta-analysis has shown that PPPD reduced the operation time and reduced blood loss[73,74]. Therefore, the CKW operation should only be performed in situations where tumor spread towards the stomach cannot be ruled out or when lymph node metastases are suspected. Irrespective of whether PPPD or CKW is performed, antecolic reconstruction is preferred to decrease the incidence of postoperative delayed gastric emptying[75].

Is there a role for routine intra-peritoneal drainage?

The theoretical advantage of routine intraoperatively placed abdominal drainage is to drain the pancreatic juice in the case of PF formation which avoids the negative sequelae of free pancreatic juice in the abdominal cavity. The concept of the routine use of intra-peritoneal drainage (IPD) is still in the mind of many surgeons. In contrast to such paradigms, Conlon *et al*[76] found in their prospective, randomized study of patients having undergone pancreatic resections that the routine use of a closed IPD resulted in a higher number of patients suffering from local septic complications and an increased rate of PFs (22% *vs* 9%, *P* < 0.02). In another recently published trial, short-term abdominal drainage (< 3 d) in patients with a low risk of PF formation did not show any benefit in the routine use of an IPD. To date, there is a lack of evidence for the routine use of IPD in pancreatic surgery[77].

LYMPH NODE DISSECTION AND PATHOLOGICAL WORK-UP

Radicality of pancreatic resection

A strict surgical technique and a high quality pathological work-up of the surgical specimen are of utmost importance. To improve the number of R0 resections, transsection of the main bile duct is performed just below the biliary confluence in a monobloc technique including the gallbladder - preparation is carried out in close contact with the right border of the superior mesenteric artery to achieve maximum retroperitoneal tumor clearance. Intraoperative frozen section analysis of the resection margins is mandatory - especially, as the pancreatic resection margin shows microscopic tumor infiltration in 10%-20% of cases[78].

Lymphadenectomy during pylorus PPPD/CKW

As for any other cancer type, the lymph node status is of major clinical and prognostic value. However, some controversies remain regarding how these should be reported (total number or lymph node ratio) and on the impact of an extended lymphatic clearance. Standard lymphadenectomy for PPPD/CKW includes the lymph nodes of the hepato-duodenal ligament, along the common hepatic artery, portal vein, cranial portion of the superior mesenteric vein as well as the right border along the superior mesenteric artery and celiac trunk. Extended lymphadenectomy includes in addition to the lymphatic reservoir of the interaortocaval space, the left-side of the celiac trunk as well as the left side along the superior mesenteric artery. In a study of 517 pancreatic cancer patients, no prognostic difference was found between peripancreatic lymph node metastases and second level lymphatic nodes N2 (along the common hepatic artery, portal vein, cranial portion of the superior mesenteric vein as well as the right border along the mesenteric superior artery and celiac trunk). Furthermore, in patients with one positive lymph node metastasis (N1), overall survival was similar to nodal negative (N0) patients. A poorer prognosis was reported with two or more positive lymphatic nodes (> N1), irrespective of the total number of affected lymph nodes[79]. The lymph node ratio has been introduced to

Table 4 Improvement of radicality of resection	
Resection	**Exclusion of resection**
Standard lymph node clearance for PPPD/CKW include the regional peripancreatic lymph nodes, hepato-duodenal ligament, common hepatic artery, portal vein, cranial portion of the superior mesenteric vein, right border along the mesenteric superior artery and celiac trunk	Extended lymphadenectomy can not be recommended
Vascular resection of the portal vein or superior mesenteric vein is feasible and safe and should not be an exclusion criterion in curative surgery	Thrombosis of the mesenteric-portal vein or tumoral infiltration > 180° of these vascular structures are contraindications in attempting curative resection

PPPD: Pylorus preserving pancreaticoduodenectomy; CKW: Classic Kausch-Whipple.

characterize lymphatic tumor load and to create a prognostic parameter independent of the rough estimation N0 *vs* N1 or the overall number of affected lymph nodes[80,81]. There is still some debate about the exact cut-off level of the lymph node ratio which indicates poorer survival. In a study of 4000 patients, a cut-off of 0.2 was reported as a strong predictor of poor survival[82]. Currently, a minimum of 10-12 lymph nodes need to be cleared during PPPD/CKW[83]. The para-aortic lymph nodes are generally considered as metastatic disease (M1). However, some confusion exists whether clearance of these nodes improves survival. In a review by Glanemann *et al*[84], patients with para-aortal positive lymph nodes showed a poor survival. The authors concluded that such patients should not undergo resection. The role of extended lymph node dissection has been extensively investigated. No benefit was found for this approach[85,86]. Since extended lymphadenectomy increases perioperative morbidity and impairs quality of life, this procedure should not be performed routinely.

Resection margins

Surgical resection margin is a major prognostic factor. Any incomplete resection (R1) must be considered as palliative[87,88]. However, there are also data on long-term survival after R1 resections[88,89]. A possible explanation for such conflicting data is most likely due to the heterogeneity between the study populations and different pathological work-up standards of the surgical specimens. Indeed, the number of patients with a positive resection margin was found to be between 14% and 85%[90,91]. In fact, a standardized examination of the resected specimens showed intraoperative coloration of the retroperitoneal resection margin using India ink and in a higher number of paraffin-embedded thin-sliced sections. With this technique, more than two-thirds of patients were found to be R1 resected in the retroperitoneal margin[91]. The incidence of R1 resections was correlated with the number of thin-sliced sections performed[90]. A retroperitoneal margin of 1.5 mm was classified as a R0 resection. This, however, is unfortunately rarely achievable[92].

Management of vascular infiltration

Major arterial resection such as the superior mesenteric artery is technically feasible, major arterial resection during duodenopancreatectomy is currently not established and there are insufficient data to perform such a proce-

dure[4]. In contrast, venous involvement is not a contraindication for excluding patients from undergoing curative surgery. Venous resection, partial or even circumferential with an adequate technique of reconstruction is associated with a survival similar to those groups of patients having undergone PD for adenocarcinoma[89]. However, if the tumoral infiltration of the portal vein is 50% or more of the vascular circumference, survival rates of such patients undergoing duodenopancreatectomy and venous resection are inferior compared to patients having undergone duodenopancreatectomy alone[93]. Unfortunately, the exact extent of venous tumoral infiltration is difficult to estimate preoperatively, and the definitive extent of vascular infiltration is only made by pathological examination of the resected specimen[89,94-97]. However, the impact of portal vein resection during PD remains unclear. The number of patients who undergo a R1 resection varies between 38% and 59%[97-101]. In a recently published review of 1600 patients having undergone pancreatic resection in combination with venous resection, the number of patients who finally had a R1 resection was 40%[102]. Several series have reported a similar survival after PD with or without venous resection[97,98,99-101]. In a review of 1646 patients having undergone portal/superior mesenteric vein resection, the long-term survival at 1-, 3- and 5-years was 50%, 16% and 7%, respectively[102]. Since PD and mesenteric or portal vein resection have the same reported morbidity and mortality as patients who have undergone PD without vascular resection, and the tumor involvement of such venous structures is a consequence of the tumor location rather than a reflection of highly aggressive tumoral behavior, venous resection during duodenopancreatectomy has become a standard procedure. However, vascular infiltration has been reported as a risk factor for local tumor recurrence[97]. In addition, the results remain disappointing since the reported median survival after duodenopancreatectomy and venous resection was only 13 mo[4,102] with a high number of patients (40%) not free of tumor (R1)[89] (Table 4).

CONCLUSION

The survival of patients with pancreatic cancer has only slightly improved over the last few years. An increase in median survival from 16 mo in the eighties to 20 mo nowadays was reported by the French Surgical Association in 2010. This achievement is poor compared to the

progress made in other cancer types (e.g., rectal cancer). Radical surgery so far remains the only chance of long-term cure. However, new molecular markers for early diagnosis[103-105], a deeper understanding of the molecular alterations during the genesis and progression of pancreatic cancer, specifically designed new neoadjuvant and/or adjuvant therapies which directly interact with the molecular cancer cascade need to be developed in the future. Without such progress, the prognosis of pancreatic cancer remains catastrophic.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Function of chloride intracellular channel 1 in gastric cancer cells

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Abstract

AIM: To investigate the effect of chloride intracellular channel 1 (CLIC1) on the cell proliferation, apoptosis, migration and invasion of gastric cancer cells.

METHODS: CLIC1 expression was evaluated in human gastric cancer cell lines SGC-7901 and MGC-803 by real time polymerase chain reaction (RT-PCR). Four segments of small interference RNA (siRNA) targeting CLIC1 mRNA and a no-sense control segment were designed by bioinformatics technology. CLIC1 siRNA was selected using Lipofectamine 2000 and transfected transiently into human gastric cancer SGC-7901 and MGC-803 cells. The transfected efficiency was observed under fluorescence microscope. After transfection, mRNA expression of CLIC1 was detected with RT-PCR and Western blotting was used to detect the protein expression. Proliferation was examined by methyl thiazolyl tetrazolium and apoptosis was detected with flow cytometry. Polycarbonate membrane transwell chamber and Matrigel were used for the detection of the changes of invasion and migration of the two cell lines.

RESULTS: In gastric cancer cell lines SGC-7901 and MGC-803, CLIC1 was obviously expressed and CLIC1 siRNA could effectively suppress the expression of CLIC1 protein and mRNA. Proliferation of cells transfected with CLIC1 siRNA3 was enhanced notably, and the highest proliferation rate was 23.3% (P = 0.002) in SGC-7901 and 35.55% (P = 0.001) in MGC-803 cells at 48 h. The G2/M phase proportion increased, while G0/G1 and S phase proportions decreased. The apoptotic rate of the CLIC1 siRNA3 group obviously decreased in both SGC-7901 cells (62.24%, P = 0.000) and MGC-803 cells (52.67%, P = 0.004). Down-regulation of CLIC1 led to the inhibition of invasion and migration by 54.31% (P = 0.000) and 33.62% (P = 0.001) in SGC-7901 and 40.74% (P = 0.000) and 29.26% (P = 0.002) in MGC-803. However, there was no significant difference between the mock group cells and the negative control group cells.

CONCLUSION: High CLIC1 expression can efficiently inhibit proliferation and enhance apoptosis, migration and invasion of gastric cancer cells *in vitro*. CLIC1 might be a promising target for the treatment of gastric cancer.

Key words: Chloride intracellular channel 1; Gastric carcinoma; Small interference RNA; Apoptosis; Invasion; Migration

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INTRODUCTION

Gastric cancer represents the second most common cause of cancer-related deaths in the world, and the incidence is higher in Asia than in other geographical areas[1,2]. Although progress is made in early detection and adjuvant therapy, the precise mechanism underlying gastric cancer remains unclear.

Recently, the roles of ion transporters have been studied in cancer cells[3,4] and various types of ion transporters have been found in cancers of digestive organs. Chloride intracellular channel (CLIC) proteins are components or regulators of novel intracellular anion channels in mammalian cells. To date, seven distinct members of CLICs have been identified: CLIC1, CLIC2, CLIC3, CLIC4, CLIC5, p64 and parchorin[5]. These genes have a high degree of homology at their carboxyl termini. In terms of their molecular function *in vitro*, it is clear that all CLIC proteins and the invertebrate CLIC-like proteins exist as both soluble globular proteins and integral membrane proteins that possess ion channel activity. The transition between these two forms is influenced by pH and redox conditions under most instances[6]. CLIC1 is the first cloned human member of the CLIC family, and is a 241 amino acid ion channel protein. Like other members of the family, CLIC1 is highly conserved across a wide range of species and its relatives can be identified in the genome of all vertebrates so far sequenced. CLIC1 was initially found to localize in the cell nucleus and intracellular vesicles[7]. Ulmasov *et al*[8] found that CLIC1 was expressed in the apical domains of several simple columnar epithelia, including glandular stomach, small intestine, colon, bile ducts, pancreatic ducts, airway, the tail of the epididymis and renal proximal tubule. Chen *et al*[9] found that a trace amount of CLIC1 was expressed in normal gastric tissues, but it was overexpressed in gastric cancer. Elevated CLIC1 expression was strongly correlated with lymph node metastasis, lymphatic invasion, perineural invasion and pathological staging, which suggested that it was a potential prognostic marker[9]. However, its role in gastric cancer deserves further studies.

In the present study, we used the RNA interference (RNAi) technology, with a Lipofectamine 2000, in order to deliver small interference RNA (siRNA) molecules that target CLIC1 gene of the gastric cancer cells. RNAi is a highly specific, homology dependent suppression of gene expression by small double-stranded RNA (dsRNA)[10]. Long dsRNAs are cleaved by the endoribonuclease Dicer into short dsRNA duplexes or siRNA. siRNAs are loaded onto RNA-induced silencing complex (RISC)[11]. RISC contains argonaute 2 (Ago-2) which cleaves and releases one strand from the dsRNA, resulting in an activated form of RISC with a single-strand RNA (guide siRNA) that directs the specificity of the target mRNA recognition through complementary base pairing[12]. Ago-2 then cleaves the target mRNA between bases 10 and 11 related to the 5' end of the siRNA antisense strand, thereby causing mRNA degradation and gene silencing[12,13], which occurs at a post-transcriptional level[14]. Here, we employed CLIC1 siRNA to knockdown the expression of CLIC1, and investigated its effects on cell proliferation, cycle, apoptosis, migration, invasion and the mechanisms involved.

MATERIALS AND METHODS

Cell cultures

Human gastric cancer cell lines SGC-7901 (adenocarcinomas) and MGC-803 (adenocarcinomas) were obtained from Shanghai cell bank (http://www.cellbank.org.cn), Chinese Academy of Sciences (Shanghai, China). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, United States) , supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone, Logan City), penicillin G (100 units/mL) (Sigma, United States) and streptomycin (100 μg/mL) (Sigma, United States), which was termed complete medium. Cells were grown in monolayer culture at 37 ℃ in humidified air with 5% CO_2.

Real-time polymerase chain reaction assays

Total RNA was extracted from human gastric cancer cell lines SGC-7901 and MGC-803 using TRIzol reagent (Invitrogen, CA). Total RNA (1-2 μg) was reversely transcribed using the Fermentas Kit (MBI, United State). Primers were based on sequences reported at Genebank (NM_001288.4). CLIC1 sense sequence was 5'-AATCAAACCCAGCACT-CAATG-3' and anti-sense sequence was 5'-CAGCACTG-GTTTCATCCACTT-3'. The expected product size of CLIC1 cDNA was 114 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense sequence was 5'-CAAC-GACCCCTTCATTGACC-3' and anti-sense sequence was 5'-CGCCAGTAGACTCCACGACAT-3'. The expected product size of GAPDH cDNA was 203 bp. Polymerase chain reaction (PCR) amplification was performed in 50 μL reaction volumes containing 0.2 mmol/L each dNTP, 0.1 RM of each oligonucleotide primer, and 1.25 U Tag polymerase in PCR buffer. cDNA was amplified on a PCR thermal controller with an initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 20 s, 59 ℃ for 30 sec, and 68 ℃ for 30 s and a final extension step of 72 ℃ for 10 min. The amount of starting cDNA was adjusted using GAPDH intensity.

siRNA synthesis and cell transfection assays

This study included four human siRNAs (GenePharma, Shanghai, China) designed against CLIC1 (GenBank NM_001288.4). One negative random siRNA (GenePharma, Shanghai, China) exhibiting no significant sequence similarity to human, mouse or rat gene sequence, served

as a negative control. The sequences for CLIC1 siRNAs and control siRNA were: siRNA1: CLIC1-Homo-131 (sense: 5'-GAGCUUGUGUUGUGCUGAATT-3' antisense: 5'-UUCAGCACAACACAAGCUCTT-3'); siRNA2: CLIC1-Homo-630 (sense: 5'-CAGCACUCAAUGACAAUCUTT-3' antisense: 5'-AGAUUGUCAUUGAGUGCUGTT-3'); siRNA3: CLIC1-Homo-805 (sense: 5'-GCCAAAGUUA-CACAUAGUATT-3' antisense: 5'-UACUAUGUGUAA-CUUUGGCTT-3'); siRNA4: CLIC1-Homo-1195 (sense: 5'-GGACAACAUAUUUCAGUAATT-3' antisense: 5'-UUACUGAAAUAUGUUGUCCTT-3'); and the negative random siRNA 5 (sense: 5'-UUCUCCGAACGUGUCAC-GUTT-3' anti-sense: 5-ACGUGACACGUUCGGAGA-ATT-3'). The cells were split into seven groups, including the mock group supplemented with only the transfection reagent, the negative control group added with a non-targeting control siRNA and the transfection reagent, and the other five groups supplemented with different CLIC1 siRNA and the transfection reagents. For cell transfection, cells were plated on 96-well (5×10^3 cells for SGC-7901 and 3×10^3 cells for MGC-803) and 6-well plates (5×10^5 cells for SGC-7901 and 3×10^5 cells for MGC-803) in DMEM with 10% FBS and were allowed to attach for 24 h, and then treated with 5 pmol and 100 pmol siRNA per well. Equimolar amounts of siRNAs were incubated with Lipofectamine 2000 Transfection Reagent from Invitrogen (Madison, WI, United States) according to the manufacturer's instructions. Transfected cells were grown at 37 ℃ for 6 h, followed by incubation with complete medium. Cells were maintained for 24 h, 48 h and 72 h before experiments, unless otherwise described.

Quantitative real-time PCR assays

Total RNA was extracted from the human gastric cancer cell lines SGC-7901 and MGC-803 using TRIzol reagent (Invitrogen, United States). After reverse transcription of the total RNA, the first-strand cDNA was then used as template for detection of CLIC1 expression using quantitative real-time PCR with the SYBR Green Master Mix (Roche, Germany). β-actin was used as control. The primers of CLIC1 sense sequence was 5'-AATCAAACCCAGCACTCAATG-3' and CLIC1 anti-sense sequence 5'-CAGCACTGGTTTCATCCACTT-3' (product size of 114 base pairs). β-actin sense sequence was 5'-ACACTGTGCCCATCTACG-3' and anti-sense sequence 5'-TGTCACGCACGATTTCC-3' (product size of 153 base pairs). The cycling conditions included a holding step at 95 ℃ for 10 min, and 42 cycles of 95 ℃ for 20 s, 59 ℃ for 30 s and 68 ℃ for 30 s. A dissociation protocol was added to verify that the primer pair produced only a single product at 95 ℃ for 15 s, 60 ℃ for 1min, and 95 ℃ for 31s. Melting curves also showed a single sharp peak indicating one PCR product. Quantitative real-time PCR analysis was performed using an ABI 7500 Sequence Detector (ABI, Warrington, United Kingdom) according to the manufacturer's protocol, and the results were analyzed by the $2^{-\Delta\Delta ct}$. These experiments were performed in triplicate and repeated in three independent experiments.

Western blotting assays

Total protein extracts were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (20 mg per lane), and transferred onto a polyvinylidene fluoride membrane. After blocking with 5% bovine serum albumin, the membrane was then incubated with antibodies specific for CLIC1 (Sigma; 1:500), and β-actin (Abmart; 1:5000) at 4 ℃ overnight. The secondary antibody (KPL; 1:5000) was infrared at room temperature for 1 h. The density of the bands was quantified by densitometric analysis using the two-color infrared laser imaging analyzer system of Odyssey (United States). The inhibitory rate of CLIC1 protein expression was calculated as follows: inhibitory rate = [1-(siRNA CLIC1 density/siRNA β-actin density)/(untransfected CLIC1 density/untransfected β-actin density)] × 100%.

Cell growth and viability assay

The effect of CLIC1 specific siRNA on the viability of cells was determined by the methyl thiazolyl tetrazolium (MTT) assay. Briefly, cells were plated in 96-well microtitre plates. Cell viability was determined after transfection for 24 h, 48 h and 72 h. Then 20 μL MTT (10 mg/mL in PBS stock, diluted to a working concentration of 1 mg/mL with media) was added to each well and incubated for 4 h. After careful removal of the medium, 200 μL dimethyl sulfoxide was added to each well and shaken carefully for 10 min. The absorbance was recorded on the iMark Microplate Reader (Bio-Rad, United Kingdom) at a 570 nm wavelength. The effect of CLIC1 siRNA on cell growth inhibition was assessed as percentage cell viability where vehicle-treated cells were taken as 100% viable.

Cell cycle analysis and annexin V staining assays

For flow cytometric cell cycle analysis, the cells treated with siRNA were collected, washed with PBS, fixed in cold 70% ethanol, and stored at 4 ℃ until staining. After fixation, the cells were washed with PBS and incubated with 100 μL RNaseA (Sigma, United States) for 30 min at 37 ℃, before staining with 400 μL propidium iodide (Sigma, United States). Apoptotic cells in early and late stages were detected using an annexin V-FITC Apoptosis Detection Kit from BioVision (Mountain View, CA, United States). In brief, culture media and cells were collected and centrifuged. After washing, cells were resuspended in 500 μL annexin V binding buffer, followed by the addition of 5 μL annexin V-FITC and 5 μL propidium iodide. The samples were incubated in the dark for 5 min at room temperature and analyzed using flow cytometry (FCM).

Cell migration assay

The ability of cells to migrate through filters was measured using Polycarbonate Membrane Transwell Inserts (Corning, United State). At 24 h after transfection, cells

were trypsinised. Cell culture inserts with an 8 μm pore size polycarbonate membrane were used according to the protocol of the manufacturer. The bottom chamber included medium (0.5 mL) containing 5% FBS, whereas mock, negative control or CLIC1 siRNA transfected cells (1.0×10^6 per mL suspended in 0.1 mL of medium containing 0.5% FBS) were seeded into the upper chamber and incubated 24 h at 37 ℃ in a humidified atmosphere containing 5% CO_2. The remaining cells on the upper surface were mechanically removed. Membranes were then washed, fixed, and stained by Methyl Violet (Medion Diagnostics, Germany). The migration ability of the cells was determined by counting the cells that had migrated to the lower side of the filter with a microscope. Experiments were performed in triplicate, and 3 fields were counted in each experiment.

Matrigel invasion assay

Matrigel invasion assay was performed using polycarbonate membrane transwell (Corning, United States) coated with the matrigel (BD Biosciences, San Jose, CA, United States). The density of cells that were seeded into the upper chamber was 3.0×10^5/mL for SGC-7901 and 1.5×10^5/mL for MGC-803. Other treatments were the same with the cell migration assay.

Statistical analysis

Data were analyzed using SPSS16.0 software. All data were expressed as mean ± SD, and analyzed by one-way analysis of variance and post-hoc testing comparing means by Student-Newman-Keuls' test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of CLIC1 in cultured gastric cancer cells

We first evaluated the endogenous expression of CLIC1 in the human gastric cancer cell lines SGC-7901 and MGC-803 by RT-PCR, and found that there was CLIC1 mRNA in the human gastric cancer cell lines SGC-7901 and MGC-803 (Figure 1).

Downregulation of CLIC1 expression effectively suppresses CLIC1 protein and mRNA expression in gastric cancer cells

To examine the possible roles of CLIC1 in gastric cancer cells, we knocked down the expression of CLIC1 using siRNA. Western blotting and quantitative real-time PCR were performed to examine the effect of siRNA transfection on CLIC1 protein and mRNA expression levels in SGC-7901 and MGC-803 cells. When SGC-7901 and MGC-803 cells were transfected with CLIC1 siRNA, the CLIC1 mRNA and protein levels were decreased in different degrees in siRNA1, siRNA2, siRNA3 and siRNA4 transfected cells as compared with siRNA5, mock and negative control cells at different time points ($P < 0.05$). There were no significant differences among siRNA5, mock and negative control cells ($P > 0.05$) (Figure 2). The

Figure 1 Expression of chloride intracellular channel 1 in human gastric cancer cell lines SGC-7901 and MGC-803. Specific reverse transcription polymerase chain reaction analysis for chloride intracellular channel 1 (CLIC1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.

results showed that these siRNAs designed for CLIC1, especially the CLIC1 siRNA3, successfully exerted a silencing effect for CLIC1 expression *in vitro*. Therefore, the CLIC1 siRNA3 was chosen for the subsequent *in vitro* experiments.

Downregulation of CLIC1 expression enhances growth of gastric cancer cells in vitro

We investigated whether CLIC1 expression could decrease the survival of gastric cancer cells. Downregulation of CLIC1 expression enhanced notably proliferation of gastric cancer cells in a time-dependent manner, and the highest growth rates were 25.60% ($P = 0.002$) in SGC 7901 for CLIC1 siRNA3 at 24h and 35.55% ($P = 0.001$) in MGC-803 cells for CLIC1 siRNA3 at 48 h. The cells transfected with CLIC1 siRNA3 survived at increased rates compared with the mock group cells and the negative control group cells. Our findings demonstrated that downregulation of CLIC1 expression enhances growth of SGC-7901 and MGC-803 cells *in vitro* (Figure 3).

Downregulation of CLIC1 expression induces cell cycle arrest in gastric cancer cells

The cell cycle assay indicated that the inhibition of CLIC1 expression by specific CLIC1 siRNA3 significantly changed the proportions of the G0/G1, S and G2/M phases at 48 h after transfection with CLIC1 siRNA3. The percentage of the G2/M phase proportion increased notably in after transfection with CLIC1 siRNA3 in SGC-7901 (2.66-fold) and MGC-803 (1.28-fold), respectively, while there was decrease in the proportion of G0/G1 and S phases (Figure 4).

Downregulation of CLIC1 expression inhibits gastric cancer cell apoptosis in vitro

We investigated whether CLIC1 could induce human gastric cancer SGC-7901 and MGC-803 cell apoptosis. Cells were harvested and disposed by the Annexin V assay with indicated treatment at 48 h after transfection in SGC-7901 and MGC-803 cells. Then apoptosis was examined by FCM. The rates of apoptosis were obviously decreased in both SGC-7901 and MGC-803 cells (Figure 5). The CLICI siRNA3 showed the least apoptosis rate, suggesting that CLIC1 expression can increase apoptosis of gastric cancer cells *in vitro*.

Figure 2 Effect of chloride intracellular channel 1 knockdown in SGC-7901 and MGC-803 cells. Chloride intracellular channel 1 (CLIC1) protein expression was detected by Western blotting using a rat polyclonal CLIC1 antibody (1:500). β-actin was used as loading control. CLIC1 mRNA expression was detected by quantitative real-time polymerase chain reaction. β-actin was used as loading control. The figure represents one of the three experiments performed independently with similar results. A: CLIC1 protein expression in SGC-7901 by Western blotting; B: CLIC1 protein expression in MGC-803 by Western blotting; C: Relative CLIC1 protein expression level in SGC-7901; D: Relative CLIC1 protein expression level in MGC-803; E: Relative CLIC1 mRNA expression level in SGC-7901; F: Relative CLIC1 mRNA expression level in MGC-803. The experiment was done in triplicate and the value obtained from the control (CTRL) group set as 100%. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* CTRL and mock.

Downregulation of CLIC1 expression inhibits gastric cancer cell migration and invasion *in vitro*

We determined the effect of decreased CLIC1 expression on gastric cancer cell invasion, and examined the role of CLIC1 in gastric cancer cell migration. There was a sig-

nificant decrease in CLIC1 siRNA3 transfected cells in the Matrigel invasion assay (Figure 6A and B). Decreased CLIC1 expression led to the inhibition of invasion by 54.31% ($P = 0.000$) in SGC-7901 and 40.74 % ($P = 0.000$) in MGC-803 cells. And cancer cell migration was also

Figure 3 Effects of chloride intracellular channel 1 knockdown on cell growth in gastric cancer cell lines. Basal growth was determined in complete medium by the methyl thiazolyl tetrazolium assay after 0, 24, 48 and 72 h. A: Data obtained from SGC-7901 cells; B: Data obtained from MGC-803 cells. Results were shown as mean absorbance of the respective SGC7901 and MGC-803 cell lines. The experiment was done in triplicate. [a]$P < 0.05$, [b]$P < 0.05$ *vs* control (CTRL) and mock.

Figure 4 Measurement of the cell-cycle distribution by flow cytometry. Downregulation of chloride intracellular channel 1 (CLIC) expression by small interference RNA (siRNA) 3 transfection changed cell cycle distribution in SGC-7901 and MGC-803 cells. At 48 h post-transfection, the cell cycle distribution of these cells was analyzed using propidium iodide staining on flow cytometry. The experiment was done in triplicate. The figure represented one of the 3 experiments performed in duplicate. A: The figure was obtained from SGC-7901 cells; B: The figure was obtained from MGC-803; C: The data was obtained from SGC-7901 cells; D: The data was obtained from MGC-803 cells. [a]$P < 0.05$ *vs* control (CTRL) and mock.

Figure 5 Chloride intracellular channel 1 knockdown reduces apoptosis in gastric cancer cell lines. Apoptosis detection by flow cytometry. For each sample, 10 000 cells were counted, and the apoptotic cells were summarized in the right panel. A: Figure was obtained from SGC-7901 cells; B: Figure was obtained from MGC-803 cells; C: The apoptotic rate of the chloride intracellular channel 1 (CLIC1) specific small interference RNA (siRNA) 3 group obviously decreased in both SGC-7901 cells (62.24%, P = 0.000) and MGC-803 cells (52.67%, P = 0.000), compared with the control (CTRL) group; while there were no significant difference of cell apoptosis rate between the negative control group cells and the mock group cells. The experiment was done in triplicate. The figure represented one of the 3 experiments performed in duplicate. [a]P < 0.05, [b]P < 0.05 *vs* CTRL and mock.

D

Mock   siRNA3   CTRL

Figure 6 Effects of chloride intracellular channel 1 knockdown on cell migration and invasion in SGC-7901 and MGC-803 cell lines at 48 h. A: The figure was obtained from the invasion assay of SGC-7901 cells; B: The figure was obtained from the invasion assay of MGC-803 cell; C: The figure was obtained from the migration assay of SGC-7901 cells; D: The figure was obtained from the invasion assay of MGC-803 cell; E: The data was obtained from the invasion assay; F: The data was obtained from the migration assay. The experiment was done in triplicate. The figure represented one of the 3 experiments performed in duplicate. Cell invasion and migration were evaluated after two days SGC-7901 and MGC-803 cells were transfected *in vitro*. Representative microscopic images were presented in the upper panel of each assay graph. The number of cells that invaded or migrated through the filter was counted. Data are expressed as average number of migrated or invaded cells. The cells transfected with chloride intracellular channel 1 (CLIC1) specific small interference RNA (siRNA) 3 were significantly less able to invade or migrate than the mock cells or the negative control cells in both SGC-7901 and MGC-803 cells. $^{a}P < 0.05$, $^{b}P < 0.05$ *vs* control (CTRL) and mock.

significantly reduced by CLIC1 siRNA3 in the migration assay (Figure 6C and D). Decreased CLIC1 expression inhibited the cell migration by 33.62% ($P = 0.001$) in SGC-7901 and 29.26% ($P = 0.002$) in MGC-803. Taken together, these results clearly indicate that suppression of CLIC1 inhibits the invasion and migration ability of SGC-7901 and MGC-803 cells.

DISCUSSION

Gastric cancer is a highly aggressive and lethal malignancy. It accounts for 8.6% of all new cancer cases worldwide, and is the second leading cause of cancer-related deaths[15]. Surgery is the standard treatment for localized gastric cancer, but advanced patients with distant metastasis or recurrence remain incurable[16]. Identification of key regulatory molecules in metastasis is crucial for understanding the mechanism for tumor dissemination as well as development of novel interventions. However, molecular events promoting invasiveness of gastric carcinoma cells remain unknown and routine biomarkers for metastastic gastric carcinoma are not yet available[17].

CLIC1 (also called nuclear chloride channel-27 or NCC27), the first identified human member of a growing family of organelle ion channels, is a transmembrane protein sufficient to form a functional ion channel as a tetrameric assembly of subunits. Both human and murine *CLIC1* genes are located within the major histocompatibility complex class Ⅲ region, one of the most conserved and important regions in the genome. In humans, it is located at 6p21.3[18]. The structure of the soluble form of CLIC1 is a typical soluble glutathione S-transferase superfamily protein, but contains a glutaredoxin-like active site[19]. Some recent studies have suggested that CLIC1 plays an important role in tumor metastasis[10,20].

Chen *et al*[9] reported that CLIC1 expression was significantly up-regulated in 67.9% of gastric cancer patients. The CLIC1 expression in tumor tissues increased 1.95-fold (range: 0.01-6.19-fold) compared with that expressed by adjacent noncancerous mucosa. Elevated CLIC1 expression was strongly correlated with lymph node metastasis, lymphatic and perineural invasion, pathological staging and poor survival. Additionally, the 5-year survival rate of the low CLIC1 expression group was higher than that

of the high CLIC1 expression group. But further investigations are needed to observe this phenomenon *in vitro* and elucidate the mechanisms of CLIC1's involvement in tumor metastasis. In this study, we used RNAi technology to inhibit the expression of CLIC1 in gastric cancer cell lines SGC-7901 and MGC-803, and explored the possible mechanism by observing the biological behavior changes (such as invasion, migration, *etc.*) after inhibition.

We designed four pairs of siRNA against the *CLIC1* gene in this study to guarantee that there was at least one CLIC1 siRNA that could inhibit gene expression effectively. We used the four CLIC1 siRNAs to downregulate the expression of CLIC1 in human gastric cancer cell lines SGC-7901 and MGC-803. Quantitative real-time PCR and Western blotting assay showed that CLIC1 mRNA and protein expression were down-regulated by CLIC1 siRNA. Generally, the inhibition rate of CLIC1 siRNA3 was higher than siRNA1, siRNA2 and siRNA4, but there were no significant differences among siRNA5 group, mock group and CTRL group cells at all the time points in both cell lines ($P > 0.05$). And 24, 48 and 72 h after transfection with siRNA3, the expression of mRNA and protein was down-regulated significantly in both SGC-7901 (inhibition rates of mRNA: 98.35%, 94.15% and 95.38%; inhibition rates of protein: 70.00%, 76.39% and 87.90%) and MGC-803 cells (inhibition rates of mRNA: 78.33%, 86.81% and 84.65%; inhibition rates of protein: 72.63%, 81.97% and 64.06%). These results showed that CLIC1 siRNA3 can efficiently inhibit CLIC1 expression. Therefore, CLIC1 siRNA3 was chosen for the subsequent experiments *in vitro*. However, the decreased level of mRNA and protein expressions after RNAi are inconsistent in SGC-7901 but in MGC-803, the specific mechanism needs to be further explored. In addition, we noted that the inhibition rates in the two cell lines were different, *i.e.*, the inhibition rate was higher in SGC-7901 than in MGC-803 cells. The possible reasons for the difference were different cell growth characteristics and tissue source between the two cell lines. SGC-7901 originates from metastatic lymph node of gastric carcinoma, but MGC-803 comes from poorly differentiated adenocarcinoma of gastric mucous cells. Wang *et al*[21] found that the ability of cell growth, proliferation, migration and invasion of MGC-803 was much stronger than SGC-7901. In addition, MGC-803 has the characteristic of short growth cycle, unstable adhesion and high sensitivity to external stimuli. After successfully transfected with siRNA, MGC-803 exfoliated easily due to its vulnerable characteristics. Therefore, the inhibition rate of MGC-803 was lower than SGC-7901 under the same transfection conditions.

In this study, the *A* value increased significantly by the silencing of CLIC1 in the cell growth assay, suggesting that downregulation of CLIC1 by siRNA3 promoted the proliferation of gastric cancer cells. That is to say, *CLIC1* gene itself could inhibit the proliferation of gastric cancer cells. Some scholars observed the same phenomenon in their studies[20,22,23].

It has been found that CLIC1 expression in the cell membrane only occurred at G2/M phase, and located in the cytoplasm in G1/S phase. Some researchers utilized CLIC1 blocker and found Chinese hamster ovary (CHO) cell cycle arrest of CHO cell was in the G2/M phase, suggesting that CLIC1 was involved in cell cycle[24]. Unlike typical membrane protein, the soluble CLICl presented firstly in the cytoplasm after synthesized, and then transferred to the plasma membrane to play a role of an ion channel[25]. Using the technology of RNAi, we inhibited the expression of CLIC1 in gastric cancer cell lines SGC-7901 and MGC-803, and detected the cells with the FCM. As a result, we found that cell cycle arrest in the G2/M phase was increased notably. Along with previous studies, we speculated that the expression of CLIC1 in this phase was enhanced significantly, and then played a role of an ion channel, such as promotion of cell growth. The findings proved that CLIC1 participated in regulation of cell cycle in the G2/M phase, and played an important role in the process of cell division. In addition, the apoptosis rate was decreased markedly in the CLIC1 siRNA3 group. Compared with control group, the apoptosis rates in SCC-7901 and MGC-803 were reduced by 62.24% and 52.67%, respectively. This suggests that the gene itself can promote gastric cancer cell apoptosis. And some studies also found this function in colon cancer, mouth cavity squamous cell carcinoma, melanoma and mouse hepatocellular carcinoma[20,23,26].

Some scholars[20,27,28] found that overexpression of CLIC1 significantly increased cell motility, and knockdown of CLIC1 markedly inhibited cell migration and invasion. But the role of CLIC1 in gastric cancer metastasis remains largely unclear. We studied the CLIC1 functions with a Transwell assay in gastric cancer cells, and the results demonstrated that down-regulation of CLIC1 expression by siRNA3 obviously suppressed the invasion and migratory capacity of SGC-7901 and MGC-803 cells *in vitro*. These results were consistent with previous experiments. A recent study to explain how CLIC1 promotes the movement of cancer cells found that CLIC1 has been implicated in modifying cell adhesion[24,29]. Tung *et al*[27] reported that reduced CLIC1 expression caused a reduction in endothelial migration, branching morphogenesis, capillary-like network formation, and capillary-like sprouting. FACS analysis showed that reducing CLIC1 expression increased integrins of β1, α3 and αvβ3 expression, but decreased the αvβ5 expression. Integrins are essential for invasion and metastasis of carcinoma cells and are a major family of cell adhesion molecules，which mediate cell-cell adhesion or cell-extracellular matrix adhesion and affect signal transduction, cell proliferation, differentiation, survival and apoptosis[30]. By increasing the surface expression of β1, α3 and αvβ3, downregulation of CLIC1 expression can increase endothelial cell adhesion to the extracellular matrix and inhibit motility by preventing the cell from breaking its contact with the extracellular matrix. These shifts in integrin expression also provide a possible explanation for the cell growth and viability defects[31]. Additionally, studies confirmed that invasion and migration of gastric cancer was closely associated with integrins[32-34], therefore, we speculated that invasion and migration inhibition of gastric cancer cells after knock-

down of CLIC1 was associated with the up-regulation of β1, α3 and αvβ3. However, the specific mechanism needs to be further studied.

In summary, our findings show that CLIC1 siRNA3 can efficiently inhibit CLIC1 expression, and suppress gastric cancer migration and invasion *in vitro*. However, downregulation of CLIC1 expression promotes the proliferation of gastric cancer and reduces gastric cancer cellular apoptosis. The molecular mechanism remains unclear. Future studies need to focus on the precise mechanism of tumorigenesis. In addition, animal models need to be established. In short, CLIC1 plays an important role in regulating the biological behavior of gastric cancer cells. Our research lays a solid foundation for future studies.

COMMENTS

Background
Gastric cancer is a highly aggressive and lethal malignancy. Metastasis or recurrence is the main obstacle to improvement of the treatment efficacy of gastric cancer. Identification of key regulatory molecules in metastasis is crucial for understanding tumor dissemination and for development of novel interventions. However, molecular events promoting invasiveness of gastric carcinoma cells are still hardly known and routine biomarkers for metastastic gastric carcinoma are not yet available.

Research frontiers
Some researchers found that chloride intracellular channel 1 (CLIC1) was overexpressed in gastric cancer and was a potential prognostic marker, and elevated CLIC1 expression was strongly correlated with lymph node metastasis, lymphatic invasion, perineural invasion, and pathological staging. However, its role in gastric cancer deserves further studies.

Innovations and breakthroughs
Previous studies mainly focused on the phenomenon that the level of CLIC1 expression was associated with clinicopathologic features of gastric cancer in vivo. However, whether it is consistent with the phenomenon *in vitro*, and the mechanisms of CLIC1's involvement in tumor metastasis remain unclear. In this study, the authors used RNA interference (RNAi) technology to inhibit the expression of CLIC1 in gastric cancer cell line SGC-7901 and MGC-803, and explored the possible mechanism by observing the biological behavior changes (cell proliferation, apoptosis, migration and invasion) after inhibition.

Applications
High CLIC1 expression can efficiently inhibit proliferation and enhance apoptosis, migration and invasion of gastric cancer cells *in vitro*. CLIC1 can be used as a promising target for the treatment of gastric cancer.

Terminology
CLIC1 is the first cloned human member of the CLIC family, and is a 241 amino acid ion channel protein. Like other members of the family, CLIC1 is highly conserved across a wide range of species and its relatives can be identified in the genome of all vertebrates so far sequenced. CLIC1 was initially found to localize to the cell nucleus and intracellular vesicles. RNAi is the highly specific, homology dependent suppression of gene expression by small double-stranded RNA (dsRNA). Long dsRNAs are cleaved by the endoribonuclease Dicer into short dsRNA duplexes or small interference RNA (siRNA). siRNA are loaded onto RNA-induced silencing complex (RISC). RISC contains argonaute 2 (Ago-2) which cleaves and releases one strand from the dsRNA, resulting in an activated form of RISC with a single-strand RNA (guide siRNA) that directs the specificity of the target mRNA recognition through complementary base pairing. Ago-2 then cleaves the target mRNA between bases 10 and 11 related to the 5' end of the siRNA antisense strand, thereby causing mRNA degradation and gene silencing, which occurs at a post-transcriptional level.

Peer review
This is a good study in which the authors employed CLIC1 siRNA to knockdown the expression of CLIC1, investigated its effects on gastric cancer cell lines SGC-7901 and MGC-803, and then explored the possible mechanisms. The manuscript is well prepared, study design is reasonable, statistical methods are appropriate, and conclusions are based on the convincing statistical analysis.

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ORIGINAL ARTICLE

Pseudomyxoma peritonei of 92 Chinese patients: Clinical characteristics, pathological classification and prognostic factors

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Abstract

AIM: To assess the clinicopathologic features and its relationship with prognosis of pseudomyxoma peritonei (PMP) in Chinese patients.

METHODS: The clinicopathologic features and follow-up data of 92 patients with PMP were reviewed and retrospectively analyzed. The cases were categorized into three groups: disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis (PMCA), and peritoneal mucinous carcinomatosis with intermediate or discordant features (PMCA-I/D). The log-rank test was used to analyze survival for each group and various clinicopathological parameters. Multivariate Cox proportional-hazard models were constructed to determine the important factors associated with survival.

RESULTS: The median age at diagnosis was 51.9 years (range: 22-76 years). The median follow up was 124 mo. The 3-, 5- and 10-year survival rates were 74.0%, 67.4% and 49.1%, respectively. There were 49 (53.2%) patients with DPAM, 26 (28.3%) with PMCA-I and 17 (18.5%) with PMCA. Patients with DPAM, PMCA-I/D and PMCA exhibited statistically significant difference in survival (P = 0.001). The 3 year survival for DPAM, PMCAI/D and PMCA was 97.0%, 80.0% and 67.0%, respectively; the 5 year survival was 80.0%, 67.0% and 50.0%, respectively; and the 10 year survival was 65.0%, 28.0% and 14.0%, respectively. Survival rate was significantly lowest in patients < 40 age years of age (P = 0.011). Appendiceal tumor and extra-ovarian parenchymal organ involvement were significantly related to overall survival. Patients with appendiceal mucinous adenocarcinoma (MACA) showed the significantly poorer prognosis (P = 0.011). Multivariate analysis showed that pathological classification, age, appendiceal tumor were significant related to overall survival.

CONCLUSION: The clinical process "PMP" should be pathologically classified into DPAM, PMCA and PMCA-I/D. Pathological classification, age, appendiceal MACA are survival independent predictors in Chinese patients with PMP.

Key words: Pseudomyxoma peritonei; Pathologic; Clinical; Classification; Prognosis

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INTRODUCTION

Pseudomyxoma peritonei (PMP) is a rare clinical entity that is characterized by grossly disseminated intraperitoneal mucinous tumors, often with gelatinous ascites and usually secondary to an appendiceal mucinous tumor[1-8]. Occasionally, mucinous tumors in other sites are the culprit, such as colon[9,10], ovarian[11-13], pancrease[14] and so on[15]. Currently, the pathologic classification and biological behavior of PMP has been plagued with controversy and confusing terminology. There is considerable variability in the criteria and terminology used by different pathologists to diagnose patients with "PMP". For lesions with the same morphology, the diagnosis may be described as "ruptured appendix mucinous adenoma with PMP" by some pathologists, or "well-differentiated mucinous adenocarcinoma (MACA)" by others. Inconsistent pathological diagnostic criteria and designations may lead to confusion and uncertainty in survival analyses and clinical treatment. In recent years, the classification and nomenclature of PMP has been discussed in some English literatures[16-23]; However the conclusion were not consistent completely. In addition currently, clinicians and pathologists still lack sufficient understanding of this disease in China. In 2007 we reported a clinicopathologic analysis of 40 patients with PMP[24], but the results was not very satisfied. Taking into account that the number of cases was small and the statistical results may be influenced, we added 52 new cases treated in our hospital in the past 4 years into the present analysis. We aimed to further clarify the nomenclature and prognostic factors of PMP, and to provide guidance for clinical treatment and prognosis decision.

MATERIALS AND METHODS

Patient selection

We reviewed the demographic and clinicopathological data of 101 patients with PMP who received treatment at our hospital. All of the pathological data from each patient were reviewed as far as possible, including lesions of peritoneal, appendix and other involved organs. All patients were followed up by letters and telephone calls. 9 patients (four males and five females) were excluded: suboptimal (R2/3) cytoreduction (n = 7), biopsy (n = 2). Eventually, 92 patients were selected for the present analysis. Some of these patients were reported previously.

Assessment of peritoneal lesions

The following items were evaluated for the peritoneal lesions[16,17]: Architecture was evaluated for (1) simple nonstratified strips or cluster of epithelium, or focal proliferative strips of cells with stratification; (2) extensive proliferative strips of cells wtih extensive stratification; and (3) individual signet ring cells, clusters of cells, or complex glands, arranged in a cribriform pattern consistant with caicinoma.

Cytologic atypia was assessed based on the most proliferative area and was graded as follows: (1) "minimal" cytologic atypia, histologically benign or mildly atypical; (2) "moderate" cytologic atypia, moderately enlarged hyperchromatic or vesicular nuclei with or without prominent nucleoli and some nuclear irregularity; and (3) "marked" cytologic atypia, enlarged, irregular, hyperchromatic or vesicular pleomorphic nuclei with prominent nucleoli or signet ring cells.

Mitotic activity was assessed by scanning multiple high-power fields (approximately 10 at × 400) in the most active areas according to the following system: Rare (0-2 mitotic figures), occasional (approximately 3-5 mitotic figures), and abundant (> 5 mitotic figures).

Assessment of appendiceal tumors

Appendiceal tumors were classified into two groups based upon their architectural complexity and degree of cytologic atypia. Tumors that demonstrated lowgrade cytologic atypia (nucleomegaly, nuclear stratification, rare mitotic figures and single cell necrosis) and minimal architectural complexity (villiform, flat epithelial proliferation and small papillary excrescences) were classified as low-grade appendiceal mucinous neoplasms (LAMNs). Appendiceal tumors were classified as MACAs if they demonstrated any of the following: destructive invasion of the appendiceal wall; high-grade cytologic atypia (extensive full-thickness nuclear stratification, vesicular nuclei, marked nuclear membrane irregularities, prominent nucleoli and brisk mitotic activity); or complex epithelial proliferation (complex papillary fronds and cribriform glandular spaces)[4].

Parenchymal organs invasion was defined as mucin and epithelium within the parenchymal of an organ. Involvement of only the peritoneal surface of an organ was not considered invasion.

Assignment of pathological category

We classified peritoneal lesions into three groups, disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis (PMCA), and peritoneal mucinous carcinomatosis with intermediate or discordant features (PMCA-I/D)[1,3]. Cases were categorized as DPAM (Figure 1) if the peritoneal lesions were characterized by relatively scant strips of simple or focally proliferative mucinous epithelium with minimal to moderated cytologic atypia and no significant mitotic activity in abundant extracellular mucin, with or without appendiceal LAMN. Cases were categorized as PMCA (Figure 2), if the peritoneal lesions were characterized by more abundant proliferative mucinous epithelium, glands, nests, or individual cells, including signet ring cells, consistent with carcinoma and demonstrating marked mitotic activity, accompanied by MACAs of the appendix. Cases with PMCA-I/D (Figure 3) including those which the appendiceal tumor was a LAMN with significant atypia and those in which the peritoneal lesions displayed a spectrum of patterns from histologically benign to carcinomatous epithelium, were also characterized as DPAM if the peritoneal tumors displayed at least focal areas consistent with mucinous car-

Figure 1 Peritoneal lesions consist of scant strips (A) and gland (B) of histologically bland mucinous epithelium associated with abundant extracellular mucin and fibrousis in disseminated peritoneal adenomucinosis (HE, × 40).

Figure 2 Markedly atypical epithelial fragments are suspended in pools of extracellular mucin in peritoneal mucinous carcinomatosis (HE, × 100).

Figure 3 The mucinous epithelium displays nuclear stratification and cytologic atypia in peritoneal mucinous carcinomatosis-I/D (HE, × 40).

cinoma similar to the other carcinoma cases, although some areas had the appearance of the peritoneal lesions seen in the DPAM cases.

Statistical analysis

Retrospective analysis was performed in combination with follow-up data. Survival time was calculated from the date of first visit to our hospital to the date of death from PMP. Surviving patients were censored as of the last date on which they were known to be still alive. Survival estimates were calculated by using the Kaplan-Meier method. The log-rank test was used to analyze survival among the patients with between different pathological category, gender, age, operations times, appendix tumor, the architecture, cytologic atypia and mitotic activity of the peritoneal lesions, and organ involvement. Multivariate Cox proportional-hazard models were constructed to determine the important factors associated with survival. All analyses were performed using SPSS13.0. The significance level α was set at 0.05.

RESULTS

Clinicopathologic characteristics

Characteristics of clinicopathology are shown in Table 1. A total of 92 patients, 45 male, 47 female, median age 51.9 years (range: 22-76 years) were identified. The most common presenting symptoms were abdominal distension and enlargement (94.6%, 87/92), abdominal mass (67.4%, 62/92), abdominal pain (62.0%, 57/92) and weight loss (16.3%, 15/92).

During surgery, a large volume of jelly-like mucous substances was seen in the abdomen. Multiple mucous lesions could be observed on the surface of the peritoneum and visceral organs, and complete cytoreduction (R0 61, R1 31) was performed. Ninety patients underwent appendectomy. Among them 48 (52.2%) with LAMN, 29 (31.5%) with MACA, 10 (10.9%) with unknown pathologic diagnosis, and 3 (3.1%) with appendicitis. All the 3 patients with appendicitis had undergone appendectomy at other hospitals before they presented at our hospital, and the

appendiceal slides were not available, no mucinous tumor in other organs were found.

Patients received an average of 2.1 operations (range: 1-6 operations), and 30.4% (28/92) patients underwent 3 or more operations. 63.0% (58/92) patients had hyperthermic intraperitoneal chemotherapy.

In all the patients, the intra-abdominal masses consisted of multiple nodules or grape-like masses; most of which had a smooth and shiny surface. Upon sectioning, the nodules were full of a jelly-like mucous substance. Microscopically some mucous glandular structures were floating in a large number of mucous lakes, whose epithelium showed different architecture, cytologic atypia and mitotic activity. Ninety-two patients were designated into three groups, DPAM in 53.2% (49/92), PMCA-I/D in 28.3% (26/92), and PMCA in 18.5% (17/92). There was a slight preponderance of women (16/26) in the PMCA-I/D group, but there were more men than women in the DPAM and the PMCA group. The number of operations was not significantly different among the three groups.

The disease was accompanied by ovarian mucinous tumors in 68.1% (32/47) female patients. In 15 cases (17.5%), a total of 19 other intra-abdominal parenchymal organs were involved (Table 1). In the PMCA group, the lesions penetrated the diaphragm and involved the pleura in two cases (2.2%).

Table 1 Clinicopathologic finding of 92 cases of pseudomyxoma peritonei *n* (%)

Index	DPAM	PMCA-I/D	PMCA	Total
No. of cases	49 (53.2)	26 (28.3)	17 (18.5)	92 (100.0)
Gender				
Male	27	10	10	45 (48.9)
Female	22	16	7	47 (51.1)
Age (range, yr)	55.2 (22-79)	51.9 (24-76)	50.9 (31-75)	53.4 (22-79)
Operative times				
⩽ 2	34	18	12	64 (69.6)
> 2	15	8	5	28 (30.4)
Appendix				
UR	1	0	1	2 (2.2)
UP	6	4	0	10 (10.9)
Appendicitis	1	1	1	3 (3.1)
LAMN	31	11	6	48 (52.2)
MACA	10	10	9	29 (31.5)
Architecture				
Simple	15	0	0	15 (16.3)
FP	20	16	1	37 (40.2)
EP	4	10	4	18 (19.6)
Carcinomatous	0	0	12	12 (13.1)
Cytologic atypia				
Minimal	45	12	1	58 (63.0)
Moderate	4	14	4	22 (23.9)
Marked	0	0	12	12 (13.1)
Mitosis				
Rare	48	13	1	52 (56.5)
Occasional	1	10	7	18 (19.6)
Abundant	0	3	9	12 (13.1)
Organs involvment	2	3	10	15 (16.3)
Small intestine	1[1]	1	5[2,3]	7 (7.6)
Colon	0	1	3[3]	4 (4.3)
Rectum	0	0	1[4]	1 (1.1)
Urinary bladder	1[1]	0	0	1 (1.1)
Liver	1	0	0	1 (1.1)
Uterus	0	1	1[4]	2 (2.2)
Fallopian tube	0	0	1	1 (1.1)
Pleura	0	0	2[2]	2 (2.2)
Ovarian (*n* = 47)	13	12	7	32 (68.1)

[1,2,3,4]Represents one case had two organ involvement. LAMN: Low-grade appendiceal mucinous neoplasm; MACA: Mucinous adenocarcinoma; UP: Unknown pathology; UR: Unresected; FP: Focally proliferation; EP: Extensively proliferation; DPAM: Disseminated peritoneal adenomucinosis; PMCA-I/D: Peritoneal mucinous carcinomatosis with intermediate or discordant features.

Outcome and overall survival

Among the 92 patients, 15 died of intestinal obstruction, 5 died of septicemia secondary to postoperative wound infection, and 2 died of complications of tumor invasion into the thoracic cavity. Fifty-six patients were still alive. The longest survival was 350 mo. Fourteen patients were lost to follow-up at 1 to 146 mo after surgery. The median survival of the whole patient population was 124 mo (range: 1-350 mo). Using the Kaplan Meier survival curve method, the overall 3-, 5-, and 10-year survival rates were 74.0%, 67.4% and 49.1%, respectively.

Survival analysis

The survival time was compared among three groups, 16.3% patients (8/49) died in the DPAM group with the median survival time 312.9 mo. Twenty-three point one percent of patients (6/26) died in the PMCA-I/D group

Table 2 Stepwise multivariate Cox proportional hazards regression analysis

Variable	β	Se (β)	Wald, χ^2	*P*	RR	95% CI
Age	-0.943	0.473	3.984	0.046	0.39	0.154, 0.983
Pathologic classification	0.778	0.311	6.255	0.012	2.18	1.183, 4.006
Appendiceal tumor	0.723	0.331	4.765	0.029	2.06	1.077, 3.941

RR: Risk ratio; CI: Confidence interval.

with the median survival time 84.0 mo, and 47.1% patients (8/17) died in the PMCA group with the median survival time 31.7 mo. The 3 year survival for DPAM, PMCA-I/D and PMCA was 97.0%, 80.0% and 67.0%, respectively; the 5 year survival was 80.0%, 67.0% and 50.0%, respectively; and the 10 year survival was 65.0%, 28.0% and 14.0%, respectively. A significant difference among three groups in overall survival was seen (*P* = 0.001) (Figure 4A). Pairwise comparison showed the survival characteristics are significantly different between the DPAM and PMCA-I/D groups (*P* = 0.005), the PMCA-I/D and PMCA groups (*P* = 0.003). The prognosis was best of DPAM and worst of PMCA.

Association analysis of age and survival time using age as a continuous variable showed that age was associated with the survival time; the younger the patient, the shorter the survival (*P* = 0.020). The mean age was 51.9 years. So we separated the patients into three age groups, < 40 years, 40-59 years and ⩾ 60 years, to analyze. The results showed that the survival was significantly different among the three groups (*P* = 0.011) (Figure 4B). The prognosis was poorest in patients < 40 years, in whom the 3- and 5-year survival rates were 83.0% and 48.0%, respectively. The longest survival time was 96 mo (the survival rate was 24.0%).

Because of its poor reliability and the limited number of cases we combined the 3 patients with "appendicitis" and 2 patients without appendectomy into the "pathology-unknown" group and excluded them for analysis. Survival time was significantly different between patients with LAMN and MACA (*P* = 0.008). The prognosis of patients with MACA of the appendix was poorer than those with LAMN (Figure 4C).

In addition, ovarian involvement did not appear to influence the female patients' outcome (*P* = 0.897). But extra-ovarian parenchymal organ involvement were significantly associated with the survival time of patients (*P* = 0.005). Gender and operation times were not associated with the survival (*P* = 0.547 and 0.692, respectively).

Stepwise multivariate Cox proportional-hazard regression analysis indicated that age (*P* = 0.046), pathological classification (*P* = 0.012) and appendiceal MACA (*P* = 0.029) were significant related to survival and predicted for death (Table 2).

DISCUSSION

PMP is a rare clinical disease characterized by a large amount of gelatinous mucus in the abdominal cavity, and

Figure 4 Survival related to histological classification, age and appendiceal mucinous adenocarcinoma. A: Overall survival of the three histologic groups: Disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis with intermediate or discordant features (PMCA-I/D) and peritoneal mucinous carcinomatosis (PMCA) (*P* = 0.001); B: Overall survival of the three age groups: < 40, 40-59 and ⩾ 60 years (*P* = 0.011); C: Overall survival of the two appendiceal tumor groups: low-grade appendiceal mucinous neoplasm and mucinous adenocarcinoma (*P* = 0.008).

wide peritoneal and omental dissemination. Its incidence is approximately 2/10 000 patients undergoing lapa-

rotomy[25]. Clinically, it is characterized by non-remitting abdominal pain, abdominal swelling, and mucoid ascites. Ultimately, the tumor occupies most space in the abdominal cavity, showing manifestations of "jelly belly" syndrome. It is very difficult to have a definite diagnosis before surgery, and most cases are diagnosed after laparotomy. Pathological examination shows that the abdominal tumor consists of a large amount of gelatinous mucinous substance, some of which may have an appearance of multiple mucinous nodules. It can be seen under the microscope that varying amounts of the mucinous epithelial component is floating in the mucous lake, and in most cases, the morphology of the epithelial component is benign. In recent years, most scholars believe that PMP has originated from appendiceal mucinous disease[7,8] and occasionally, mucinous tumors of other places have been implicated[9-15].

Naming and pathological diagnosis of PMP
As the chemical nature of the gelatinous substance of PMP is different from that of mucous protein, it is called pseudo-mucous protein, and accordingly, the condition was named "PMP". After it was first proposed in 1884, the name of "PMP" has been in use to date. However, strictly speaking, PMP reflects only the clinical process and characteristics of the disease, and cannot be used for pathological diagnosis. Therefore, its pathological classification and naming have long been debated. Some scholars believe that it can be diagnosed as cancer as long as the epithelial component is found outside of the appendiceal wall; however, some believe that in some cases, the survival time of patients is very long although PMP tends to be characterized by multiple recurrences; therefore, it should be considered benign or a low-grade malignancy. Pathological diagnosis directly affects the postoperative treatment. The difference in the pathological diagnosis of PMP results in confusion and uncertainty in clinical treatment.

In recent years, investigators have obtained different results. In 1995, Ronnett *et al*[16] analyzed the pathological conditions in 109 cases of PMP, and they first proposed that PMP should be divided into three categories: DPAM, PMCA-I/D and PMCA. Since then, they followed that group of patients for many years, and carried out a prognostic analysis in 2001. They grouped patients with PMCA-I/D and PMCA together and compared them with the DPAM group. They found that the 5- and 10-year survival rates (26% and 9%) were significantly worse than that of the DPAM group (75% and 68%)[17]. Miner *et al*[22] thought that PMP should be divided into low-grade and high-grade peritoneal MACA. However, in 2006 Bradley *et al*[20] analyzed 101 cases of PMP and found that the survival rate was not significantly different between the DPAM and PMCA-I/D groups. Therefore, they combined the two groups into low-grade mucinous carcinoma peritonei (MCP-L), and these patients classified into PMCA according to Ronnett *et al*[16,17] were renamed high grade mucinous carcinoma peritonei (MCP-H). The

prognosis of MCP-L was significantly better than that of MCP-H with the 3 - and 5-year survival rates 72.8% and 62.5%, 37.7% and 37.7%, respectively[20]. However, the recent study of Bruin *et al*[9] supported Ronnett's conclusion that the prognosis was not significantly different in PMCA-I/D and PMCA, and therefore, these two groups should be combined.

Referring to the study of Ronnett *et al*[16,17] in 2007, we divided 40 cases of PMP into three groups, DPAM, PM-CA-I/D and PMCA, and compared them[24]. The pair-wise comparison showed no significant differences between the PMCA-I/D and PMCA groups. Therefore, PMCA-I/D and PMCA were combined into one group, and re-analysis showed the 3-, 5- and 10-year survival rates were 58.9%, 47.1% and 35.3%, which were significantly different from those in the DPAM group.

Taking into account small cases for the previous analysis the statistical results may be influenced, now we added 52 new cases treated in the past 4 years into the analysis and the survival rate of the three groups were compared. There were 49 (53.2%) patients with DPAM, 26 (28.3%) with PMCA-I and 17 (18.5%) with PMCA. Patients with DPAM, PMCA-I/D and PMCA exhibited statistically significant difference in survival. The 3 year survival for DPAM, PMCAI/D and PMCA was 97.0%, 80.0% and 67.0%, respectively; the 5 year survival was 80.0%, 67.0% and 50.0%, respectively; and the 10 year survival was 65.0%, 28.0% and 14.0%, respectively. But Pair-wise comparison showed the survival characteristics are significantly different between the DPAM and PMCA-I/D groups, the PMCA-I/D and PMCA groups. The prognosis was best in the DPAM group and worst in the PMCA group. The results supported the morphological classification of PMP into three categories, and they should be named DPAM, PMCA-I/D and PMCA, respectively to further guide postoperative clinical treatment.

Biological behaviors, prognosis and treatment of PMP

Although the progression of PMP is slow, and the pathological morphology is benign or low-grade malignancy in the majority of cases, its biological behavior is malignant and it can not be removed completely during surgery because of its extensiveness and invasiveness. Relapse and adhesions may occur easily, and the patient may have cachexia of chronic disease. Postoperative recurrence occurs in 60% to 76% of patients, and Multiple surgical resections are often required. Although this disease may progress slowly, it is often fatal. Common causes of death are systemic infection secondary to wound infection, bowel obstruction, hernia and pleural pseudomyxoma caused by tumor passing through the diaphragm[26,27].

It is reported that the median survival time of patients with PMP is 5.9 years, and the 3-, 5- and 10-year survival rates were 81.1%-83%, 50.0%-81%, and 18.2%-32%, respectively. Prognostic factors of PMP are the location, primary tumor and the histopathological grade of the peritoneal lesions[22,27]. In this study the survival time ranged from 1 to 350 mo with the median 124 mo. The

3-year, 5-year and 10-year survival rates were 74.0%, 67.4% and 49.1%, respectively. The 10-year survival rate was higher than that reported in the literature. Thirty point four percent of (28/92) patients underwent 3 or more operations. Fifteen died of intestinal obstruction, 5 died of septicemia secondary to postoperative wound infection, and 2 died of complications of tumor invasion into the thoracic cavity. Among the clinicopathological characteristics we found age, the pathological classification of peritoneal tumor, appendiceal tumor, extra-ovarian parenchymal organ involvement were significantly related to the survival time. Stepwise multivariate Cox proportional-hazard regression analysis indicated that pathological classification, age, appendiceal MACA are survival independent predictors.

It has been reported that prognosis of PMP is related to age. The prognosis of patients > 50 years was significantly poorer than that of patients < 50 years[13]; However, the statistical analysis of this study showed that the younger the patient, the worse the prognosis. The prognosis of patients < 40 years was significantly worse than that of patients > 40 years.

Complete cytoreduction, supplemented by hyperthermic intraperitoneal and systemic chemotherapy was considered as the gold standard treatment of PMP. Complete cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy could prolongs long-term survival[28-36].

On conclusion we suggest that the name of PMP should be avoided as far as possible in the pathological diagnosis, and DPAM, PMCA-I/D, and PMCA should be used to indicate the degree of differentiation of the tumors. Therefore, additionally, the appendix should be explored as far as possible during gross examination, and sampling and serial sections should be made in order to determine and report the severity of the appendiceal lesions. Attention should be paid to examine the involvement of parenchymal organs in order to provide more information for clinical treatment. For patients with PMP, clinicians should determine the prognosis according to age on the basis of a complete pathology report.

COMMENTS

Background
Pseudomyxoma peritonei (PMP) is a rare clinical entity that is characterized by grossly disseminated intraperitoneal mucinous tumors, often with gelatinous ascites and usually secondary to an appendiceal mucinous tumor. Occasionally, mucinous tumors in other sites are the culprit, such as colon, ovarian, pancrease and so on. Currently, the pathologic classification and biological behavior of PMP has been plagued with controversy and confusing terminology. There is considerable variability in the criteria and terminology used by different pathologists to diagnose patients with "PMP" for lesions with the same morphology.

Research frontiers
In recent years, the classification and prognosis of PMP has been discussed in some English literatures. However the conclusions were not consistent completely. In addition currently, clinicians and pathologists still lack sufficient understanding of this disease in China. In this study, the authors demonstrate pathological classification, age,(< 40 years), appendiceal appendiceal mucinous adenocarcinoma (MACA) are survival independent predictors of Chinese patients with PMP. They think the clinical process "PMP" should be pathologically

classified into disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis with intermediate or discordant features (PMCA-I/D), and peritoneal mucinous carcinomatosis (PMCA).

Innovations and breakthroughs

Recent reports have hignlighted the classification and prognosis of PMP. But the conclusions were not consistent completely. Unfortunately, few reports have observed the classification and prognosis of PMP in Chinese patients. And currently, clinicians and pathologists still lack sufficient understanding of this disease in China. This study is the first large sample study on the pathological classification and prognosis of PMP in Chinese patients.

Applications

By understanding the survival predictors in Chinese patients with PMP, the name of PMP should be avoided as far as possible in the pathological diagnosis, and DPAM, PMCA-I/D and PMCA should be used to indicate the degree of differentiation of the tumors. Therefore, additionally, the appendix should be explored as far as possible during gross examination, and sampling and serial sections should be made in order to determine and report the severity of the appendiceal lesions. Attention should be paid to examine the involvement of parenchymal organs in order to provide more information for clinical treatment. For patients with PMP, clinicians should determine the prognosis according to age on the basis of a complete pathology report.

Terminology

PMP is a disease with a large volume of extensively implanted gelatinous mucous substance on the surface of the peritoneum. The terminology is came from the jelly-like substance, whose chemical properties are different from those of the mucous proteins and called pseudo-mucin.

Peer review

This is a nice review of the histologic features of "PMP" in Chinese patients and and suggest that the clinical process "PMP" should be pathologically classified into DPAM, PMCA and PMCA-I/D. Pathological classification, age and appendiceal MACA are survival independent predictors in Chinese patients with PMP, nicely addresses the challenges with the pathologic diagnosis.

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ORIGINAL ARTICLE

Effects of anesthetic methods on preserving anti-tumor T-helper polarization following hepatectomy

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Abstract

AIM: To investigate the impact of different anesthetic techniques on T-helper (Th) cell subsets in hepatocellular carcinoma (HCC) patients undergoing hepatectomy.

METHODS: Sixty-one HCC patients who received hepatectomies were randomized into an epidural combined general anesthesia (G + E; n = 31) or a general anesthesia (G; n = 30) group. Blood samples were obtained the morning before the operation (d0), and on the second (d2) and seventh (d7) day after the operation. Th cell contents were evaluated using flow cytometry, real-time reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay.

RESULTS: In all 61 patients, Th1 and Th2 cell frequencies, and interferon-γ (IFN-γ) mRNA expression markedly increased on d2, compared to d0. They recovered slightly on d7, and the Th1/Th2 ratio increased markedly on d7, compared with d2. In contrast, Th17, regulatory T cell (Treg), and interleukin-17 (IL-17) levels and *FOXP3* mRNA expression showed no significant change on d2, and then markedly decreased on d7. Similarly, plasma IFN-γ concentration on d2 was much higher than that on d0, and then partly recovered on d7. As compared with the G group, in the G + E group, Th1 cell frequencies and the Th1/Th2 ratio were slightly higher on d2 and significantly higher on d7, while Th2, Th17, and Treg cell frequencies were slightly lower on d2, and significantly lower on d7. Consistently, on d7, IFN-γ mRNA and protein levels and the IFN-γ/IL-4 ratio in the G + E group were higher than those in the G group. In contrast, the IL-17 mRNA level, and IL-17 and transforming growth factor-β1 concentrations in the G + E group were lower than those in the G group.

CONCLUSION: G + E is superior to G in shifting the Th1/Th2 balance towards Th1, while decreasing Th17 and Treg, potentially benefiting HCC patients by promoting anti-tumor Th polarization.

INTRODUCTION

The overall survival of patients with hepatocellular carcinoma (HCC) remains poor despite improved diagnostic and treatment strategies[1]. Although surgical resection is still one of the first priorities[1], surgery may inevitably induce profound systemic neuroendocrine, metabolic, inflammatory, and immunological stress[2,3]. In particular, alterations occur in every component of the immune response, including T-helper (Th) cells[4]. As a consequence, the stress response could lead to significant changes in post-operative recurrence rates and patient survival[5,6].

Th cells are sub-groups of lymphocytes that play a central role in orchestrating host immune responses through their capacity to help other cells in the immune system[7]. In the scenario of cancer, Th1 cells mediate anti-tumor reactivity, by producing interferon-γ (IFN-γ), resulting in tumor regression[8]. In contrast, most studies have shown that Th2, Th17, and regulatory T cell (Treg) cells may inhibit anti-tumor efficacy[9,10]. The Th subsets are known for their altered frequencies, distribution and balance in cancer-bearing patients. More importantly, recent research has revealed that the balance of Th subsets determines the direction of anti-tumor immune responses and hence patient clinical outcomes[11,12]. Proper peri-operative management, including selection of suitable anesthetic methods, may help recover the disturbed balances of Th subsets or even maintain the balance of anti-tumor responses.

Epidural anesthesia is known to prevent or attenuate an excessive stress response during or after surgery, which prevents noxious afferent input from reaching the central nervous system[13]. Both preclinical and clinical studies have suggested that the addition of spinal blockade to general anesthesia attenuates the metastasis-promoting effect of surgery in the tumor-bearing host[14,15]. Currently, general anesthesia alone, and epidural blockade combined with general anesthesia are the two most commonly used methods in hepatectomy. However, there is a lack of clinical studies evaluating the effects of hepatectomy itself, as well as the choice of anesthetic technique on peri-operative Th cell subset balances.

In this study, we investigate Th cell subset balances in the peripheral blood of HCC patients before surgery, and on 2 and 7 d after hepatectomy, to elucidate the changes in Th frequencies induced by surgery. In particular, we compared the differences in Th cell subsets between the epidural anesthesia combined with general anesthesia (G + E) group and the general anesthesia (G) group at multiple peri-operative time points to determine whether anesthetic methods have an impact on postoperative Th subset balances.

MATERIALS AND METHODS

Patients and groups

Sixty-one patients with HCC who had undergone hepatectomies between October 2009 and April 2010 at the Liver Cancer Institute, Zhongshan Hospital of Fudan University (Shanghai, China) were enrolled. We randomized the patients between 18-65 years old, American Society of Anesthesiologists (ASA) scores Ⅰ-Ⅱ, with normal leukocyte and lymphocyte counts, who had an imaging diagnosis of HCC with the intention of curative surgery and without distant metastasis or any prior anti-cancer treatments into two groups, that is, the G + E group and G group. We then excluded patients who were not pathologically diagnosed as HCC or who were given a blood transfusion during or after surgery. We finally chose 61 out of 70 patients, 31 in the G + E group and 30 in the G group. Fourteen cases received right lobectomy, 11 cases left hemihepatectomy and 36 cases segmentectomy. The study was approved by the Research Ethics Committee of Zhongshan Hospital, and informed consent was obtained from all patients.

Anesthesia and analgesia

All patients were free of preoperative medication and had fasted for over 8 h. Epidural puncture for patients in the G + E group was carried out at T8 and T9 into the epidural space, with patients in a left-lateral position. After successful puncture, epidural catheters were placed toward the head for 3-4 cm. We fixed the catheters and gave 2% lidocaine (3 mL) through the catheters. After 5 min, upon confirming no spinal analgesia and that the epidural block was successful, we gave the patients 0.375% bupivacaine liquor (8 mL), then 4 mL at each 50-min interval.

Induction and maintenance of general anesthesia: All patients were induced with 5 μg/kg fentanyl, 2.0 mg/kg propofol and 0.9 mg/kg rocuronium and then incubated for approximately 90 s. Anesthesia was maintained with sevoflurane (1.5%-3.5%) based on a bispectral index monitor.

Postoperative analgesia: Patients in the G + E group used postoperative patient-controlled epidural analgesia, made up of 0.125% bupivacaine + morphine (30 μg/mL). The patients in the G group used postoperative patient-controlled intravenous analgesia containing morphine (0.4 mg/mL) administered *via* a jugular vein catheter. Patient-controlled analgesia was administered *via* an ambulatory infusion pump (AutoMed, Acemedical AM3400) and lasted for about 48 h. All patients were followed up at the end of analgesia using a visual analogue scale (VAS) for evaluation of their pain levels, and the corresponding drug dosage used was recorded.

Blood samples

Blood samples (10 mL) were obtained from all patients in the recumbent position with a 20-gauge needle for a

clean veni-puncture of an antecubital vein at three time points: early morning on the operation day (d0), and the second (d2) and seventh day (d7) after the operation. The samples were collected into tubes containing 0.2 mL sodium heparin. Peripheral blood mononuclear cells (PBMCs) were prepared using a Ficoll density gradient, for flow cytometry and real-time polymerase-chain-reaction analyses. Plasma was obtained after centrifugation and stored at -80 ℃ for measurement of cytokines.

Preparation for flow cytometry analysis

For analyses of Th1, Th2 and Th17, PBMCs were suspended at a density of 2×10^6 cells/mL in complete culture medium (RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol glutamine and 10% heat-inactivated fetal calf serum). The cell suspension was transferred to individual wells of 24-well plates. Cultures were stimulated with phorbol myristate acetate (50 ng/mL), ionomycin (1 μmol) and Brefeldin A (10 μg/mL, all from Sigma-Aldrich, St. Louis, MO, United States) for 4 h. The cultures were incubated at 37 ℃, in a 5% CO_2 environment. After 4 h of culture, the contents of the well were transferred to 1.5 mL sterile centrifuge tubes. To analyze Tregs, PBMCs were aliquoted into tubes for further staining.

Surface and intracellular staining

For Th1, Th2 and Th17 analyses, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 at 4 ℃ for 20 min. For Treg analysis, the cells were incubated with FITC-conjugated anti-human CD4 and allophycocyanin (APC)-conjugated anti-human CD25 monoclonal antibodies (mAbs). After surface staining, the cells were stained with R-phycoerythrin (PE)-conjugated anti-human IFN-γ, APC-conjugated anti-human interleukin (IL)-4 and IL-17 mAbs for detection of Th1, Th2 and Th17, respectively; or PE-conjugated anti-human FOXP3 mAb for Treg detection after fixation and permeabilization, according to the manufacturer's instructions. Isotype controls were given to enable correct compensation and confirm antibody specificity. All antibodies were purchased from eBioscience (San Diego, CA, United States). Stained cells were analyzed using a FACScan cytometer equipped with CellQuest software (BD Bioscience, San Jose, CA, United States).

Real-time reverse transcription-polymerase chain reaction analysis

Total RNA was extracted with TRIzol regent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. First-strand cDNA was synthesized using random hexamer primers and RNase H-reverse transcriptase (Fermentas, Glen Burnie, MD, United States). TaqMan primers and probes for human IFN-γ, IL-4, IL-17 and FoxP3 were purchased from TAKARA Bio Inc. (Osaka, Japan), and samples were analyzed utilizing the Opticon Monitor 3 System (Bio-Rad, Hercules, CA, United States, formerly MJ Research). The following primer

sets were used: IFN-γ: F: 5'-TGCAGGACCCATATG-TAAAAGA-3', R: 5'-TCAAAATGCCTAAGAAAAG-3'; IL-4: F: 5'-TACAGCCACCATGAGAAGGACA-3', R: 5'-GCCAGGCCCCAGAGGTT-3'; IL-17: F: 5'-CGCTGATGGGAACGTGGACTAC-3', R: 5'-GGTGGACAATCGGGGTGACA-3'; FoxP3: F: 5'-CGCTGATGGGAACGTGGACTAC-3', R: 5'-GGTGGACAATCGGGGTGACA-3'; and β-actin: F: 5'-CAACTGGGACGACATGGAGAAAAT-3', R: 5'-CCAGAGGCGTACAGGGATAGCAC-3'. For each sample, the mRNA expression level was normalized to the level of β-actin.

Enzyme-linked immunosorbent assay analysis

The plasma levels of IFN-γ, IL-4, IL-17, IL-10 and transforming growth factor-β1 (TGF-β1) were measured by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (R and D Systems, Minneapolis, MN, United States). Intra-assay and inter-assay coefficients of variation for all ELISAs were < 5% and < 10%, respectively. All samples were measured using three independent experiments, in duplicate.

Statistical analysis

Statistical analyses were performed with SPSS 16.0 software (SPSS, Chicago, IL, United States). Values are expressed as the mean ± SE of the mean. Student's *t* tests were used to compare quantitative variables, and Fisher's exact tests were used for categorical variables. Two-tailed $P < 0.05$ was judged to be significant.

RESULTS

Patient characteristics

There were no significant differences in age, gender, clinicopathologic factors, blood loss, anesthesia time and VAS scores between patients in the G + E group and G group. Because of the different anesthetic methods, the dosage of opioids (fentanyl and morphine) and inhalation anesthetics (sevoflurane) were obviously different between the two groups (Table 1).

Circulating Th1, Th2, Th17 and Treg frequencies

In all 61 patients, the frequencies of Th1 ($CD4^+IFN$-$γ^+CD4^+$ T cells) and Th2 ($CD4^+IL$-4^+CD4^+ T cells) were markedly increased on d2 ($P < 0.001$ and $P < 0.001$, respectively), as compared with d0. The frequencies partly recovered on d7 ($P = 0.015$ and $P < 0.001$, respectively), while the ratio of Th1/Th2 showed no differences between d0 and d2, but increased significantly on d7 ($P < 0.001$, Figure 1 and Table 2). In contrast, the frequencies of Th17 ($CD4^+IL$-17^+CD4^+ T cells) and Treg ($CD4^+CD25^+FoxP3^+CD4^+$ T cells) on d2 showed no significant differences compared to those on d0 ($P = 0.269$ and $P = 0.06$, respectively), but markedly decreased on d7 ($P < 0.001$ and $P = 0.021$, respectively, Figure 1 and Table 2).

Further comparisons were made between the G + E

Table 1 Patient characteristics

Characteristics	G + E group (*n* = 31)	G group (*n* = 30)	*P*
Age (yr)	50.7 ± 1.8	46.9 ± 1.5	0.416[1]
Gender (male/female)	4/27	8/22	0.211[2]
Hepatitis history (yes/no)	5/26	6/24	0.749[2]
AFP (≤ 20 ng/mL/> 20 ng/mL)	13/18	14/16	0.799[2]
Liver cirrhosis (Yes/no)	10/21	7/23	0.570[2]
ALT (≤ 40 U/L/> 40 U/L)	18/13	11/19	0.795[2]
Child-Pugh Score (A/B)	2/29	2/28	1.000[2]
Tumor differentiation (Ⅰ-Ⅱ/Ⅲ-Ⅳ)	7/24	9/21	0.570[2]
Tumor size (≤ 5 cm/> 5 cm)	8/23	7/23	1.000[2]
Tumor number (single/multiple)	8/23	5/25	0.534[2]
Tumor encapsulation (complete/none)	12/19	15/15	0.444[2]
Vascular invasion (no/yes)	6/25	8/22	0.554[2]
Blood loss (≤ 200 mL/> 200 mL)	1/30	4/26	0.195[2]
Anesthesia time (≤ 3 h/> 3 h)	18/13	9/21	0.426[2]
VAS score	3.58 ± 0.32	3.67 ± 0.38	0.863[1]
Dose of fentanyl (μg)	0.30 ± 0.01	0.51 ± 0.01	< 0.001[1]
Dose of morphine (mg)	2.26 ± 0.08	95.30 ± 1.17	< 0.001[1]
Dose of sevoflurane (mL)	21.74 ± 0.70	37.90 ± 1.60	< 0.001[1]

[1]Student's *t* test; [2]Fisher's exact test. G: General anesthesia; E: Epidural block; AFP: Alpha-fetoprotein; ALT: Alanine transaminase; VAS: Visual analogue scale.

group and G group. As expected, Th1, Th2, Th17 and Treg cell frequencies showed no differences on d0 between the G + E group and G group (Table 3). On d2, only Th2 cell frequencies were lower in patients in the G + E group, compared with patients in the G group ($P = 0.028$), while Th1, Th17, and Treg frequencies, as well as the Th1/Th2 ratio showed no significant differences (Figure 2 and Table 3). Notably, circulating Th1 cell frequencies were considerably higher, but Th2 frequencies showed no significant change in patients in the G + E group when compared with patients in the G group on d7 ($P < 0.01$ and $P = 0.231$, respectively). Consequently, on d7, the Th1/Th2 ratio of the G + E group was significantly higher than that in the G group ($P < 0.001$). Conversely, Th17 and Treg cell frequencies were significantly lower in patients in the G + E group than in patients in the G group on d7 ($P = 0.047$ and $P = 0.003$, respectively, Figure 2 and Table 3).

mRNA expression of IFN-γ, IL-4, IL-17 and FoxP3 in PBMCs

IFN-γ, IL-4, and IL-17 are the cytokines typically produced by Th1, Th2 and Th17 cells, respectively[16]. *FOXP3* is the master transcription factor in Treg cells[9]. We thus investigated the mRNA expression profile of IFN-γ, IL-4, IL-17 and *FOXP3* in PBMCs of HCC patients.

In all 61 patients, the mRNA expression levels of IFN-γ on d2 were increased compared to that on d0 ($P < 0.01$), then partly recovered on d7 ($P = 0.042$). IL-4, IL-17 and *FOXP3* mRNA expression levels showed no significant differences from d0 to d2, whereas mRNA levels of IL-17 and *FOXP3* clearly decreased from d2 to

d7 ($P = 0.032$ and $P < 0.01$, respectively, Figure 3A).

Similar to Th cell frequencies, IFN-γ, IL-4, IL-17 and *FOXP3* mRNA expression levels on d0 showed no significant differences between the two groups (Table 4). On d2, the IL-4 mRNA level in the G + E group was significantly lower as compared with that in the G group ($P < 0.01$). On d7, the IFN-γ mRNA level in the G + E group and the IFN-γ/IL-4 ratio were significantly higher than that in the G group ($P = 0.029$ and $P = 0.003$, respectively); IL-17 mRNA level in the G + E group was significantly lower than that in the G group ($P = 0.042$); *FOXP3* mRNA expression level in the G + E group was relatively lower than that in the G group, but this did not reach statistical significance ($P = 0.109$, Table 4). The results derived from mRNA expression analyses of Th-cell markers were generally in line with the results of flow cytometry analyses of Th-cell frequencies.

Plasma concentrations of IFN-γ, IL-4, IL-17, IL-10 and TGF-β1

IFN-γ, IL-4 and IL-17 are the cytokines typically produced by Th1, Th2 and Th17 cells, respectively. IL-10 and TGF-β1 are the cytokines produced by Treg cells. In all 61 patients, the plasma IFN-γ, IL-4, IL-17 and TGF-β1 concentrations on d2 were higher than on d0 ($P < 0.01$, $P < 0.01$, $P = 0.011$ and $P = 0.021$, respectively). Subsequently, IFN-γ and IL-17 levels recovered toward baseline levels on d7 ($P = 0.008$, $P < 0.001$, respectively), while IL-4, IL-10 and TGF-β1 showed no obvious decrease (Figure 3B).

The levels of all plasma cytokines on d0 showed no significant differences between the G + E and G groups. On d2, IFN-γ and IL-4 concentrations in the G + E group were significantly lower than those in the G group ($P = 0.018$ and $P = 0.013$, respectively). On d7, the IFN-γ/IL-4 ratio in the G + E group was obviously higher than that in the G group ($P < 0.001$). However, IL-17 and TGF-β1 concentrations in the G + E group were significantly lower than those in the G group ($P = 0.009$ and $P = 0.031$, respectively, Table 5). These data were highly consistent with the conclusions from flow cytometry and mRNA expression analyses.

DISCUSSION

The Th1/Th2 balance, as well as Th17 and Treg levels are key indices of immune function, playing an important role in HCC metastasis and recurrence[10,17]. To assess whether this balance was disturbed during anesthesia and to determine which anesthetic method was better at restoring the balance toward anti-tumor responses in HCC patients after hepatectomy, we examined Th1, Th2, Th17 and Treg levels through the analyses of cell frequencies, mRNA expression of Th-cell markers, and protein levels of typical Th-cell cytokines.

Effects of hepatectomy on Th cell balances

Surgical trauma is generally considered to have a major

Figure 1 Circulating T-helper 1 and T-helper 2 frequencies increased on d2 *vs* d0, and then recovered on d7, while T-helper 17 and regulatory T cell frequencies showed no difference on d2 *vs* d0, but decreased on d7. A and B: FACS pictures showing T-helper (Th)1/Th2/Th17/Treg frequencies from a representative patient (d0 *vs* d2, [b]$P < 0.01$ for both) (Th1: d0 *vs* d7, [b]$P < 0.01$; d2 *vs* d7, [a]$P < 0.05$; Th2: d2 *vs* d7, [b]$P < 0.01$) (Th17: d2 *vs* d7, [b]$P < 0.01$; Treg: d2 *vs* d7, [a]$P < 0.05$); C: Statistical analyses of results from flow cytometry ($n = 61$).

Figure 2 Representative FACS pictures of T-helper-cell subsets in the G + E and G group. A: Circulating T-helper (Th) 1 frequencies were higher in the G + E group than those in the G group on d7; B: Th2 frequencies were lower in the G + E group than those in the G group on d2; C and D: Th17 and Treg frequencies were lower in the G + E group than those in the G group on d7. G: General anesthesia; E: Epidural block.

Table 2 T-helper 1, T-helper 2, T-helper 17 and regulatory T cell frequencies in patients

Variables	d0 (%)	d2 (%)	d7 (%)	*P* (d0/d2)	*P* (d0/d7)	*P* (d2/d7)
Th1	7.21 ± 1.01	18.82 ± 1.93	13.40 ± 0.79	< 0.001	< 0.001	0.015
Th2	1.12 ± 0.09	3.32 ± 0.31	0.93 ± 0.10	< 0.001	0.180	< 0.001
Th17	2.57 ± 0.22	2.95 ± 0.26	1.23 ± 0.10	0.269	< 0.001	< 0.001
Treg	6.02 ± 0.27	6.93 ± 0.38	5.45 ± 0.52	0.160	0.329	0.021
Th1/Th2	8.12 ± 1.24	8.50 ± 1.14	14.87 ± 1.26	0.823	< 0.001	< 0.001

Paired student's *t* test. Th: T-helper; Treg: Regulatory T cell.

Table 3 Comparison of T-helper 1, T-helper 2, T-helper 17, regulatory T cell frequencies and T-helper 1/T-helper 2 cell ratio between G + E and G anesthesia groups

Variables	G + E group (%)	G group (%)	*P*
Th1			
d0	7.44 ± 1.54	7.38 ± 1.35	0.87
d2	14.66 ± 1.96	20.42 ± 2.77	0.098
d7	16.57 ± 0.96	10.37 ± 0.84	< 0.001
Th2			
d0	1.08 ± 0.14	1.14 ± 0.12	0.745
d2	2.64 ± 0.30	3.98 ± 0.49	0.028
d7	0.73 ± 0.07	1.14 ± 0.17	0.231
Th17			
d0	2.80 ± 0.26	2.65 ± 0.36	0.424
d2	2.56 ± 0.30	3.01 ± 0.38	0.374
d7	1.08 ± 0.10	1.45 ± 0.15	0.047
Treg			
d0	5.44 ± 0.26	6.62 ± 0.45	0.372
d2	7.42 ± 0.60	6.43 ± 0.46	0.194
d7	4.51 ± 0.56	6.19 ± 0.80	0.003
Th1/Th2 ratio			
d0	8.63 ± 1.76	7.62 ± 1.32	0.688
d2	9.67 ± 1.94	7.40 ± 1.26	0.253
d7	14.64 ± 0.56	6.03 ± 0.83	< 0.001

Student's *t* test. G: General anesthesia; E: Epidural block; Th: T-helper; Treg: Regulatory T cell.

Table 4 Comparison of interferon-γ, interleukin-4, interleukin-17, FoxP3 mRNA levels and interferon-γ/interleukin-4 mRNA ratio between G + E and G groups

Variables	G + E group ($\times 10^5$)	G group ($\times 10^5$)	*P*
IFN-γ			
d0	21.84 ± 2.99	16.49 ± 0.84	0.585
d2	50.91 ± 1.46	62.87 ± 2.71	0.096
d7	41.05 ± 1.05	29.36 ± 0.73	0.029
IL-4			
d0	47.12 ± 28.65	59.97 ± 15.17	0.651
d2	66.63 ± 18.45	110.12 ± 24.11	< 0.001
d7	31.80 ± 14.59	58.21 ± 5.74	0.224
IL-17			
d0	351.29 ± 55.29	386.64 ± 61.88	0.768
d2	406.68 ± 68.21	677.80 ± 133.07	0.171
d7	226.20 ± 18.37	429.94 ± 11.90	0.042
FoxP3			
d0	430.38 ± 48.95	384.52 ± 42.19	0.408
d2	468.85 ± 89.81	432.97 ± 44.45	0.338
d7	248.68 ± 58.71	361.01 ± 46.75	0.109
IFN-γ/IL-4 ratio			
d0	0.44 ± 0.03	0.39 ± 0.04	0.883
d2	0.80 ± 0.10	0.58 ± 0.03	0.198
d7	1.14 ± 0.08	0.56 ± 0.01	0.003

Student's *t* test. G: General anesthesia; E: Epidural block; IFN-γ: Interferon-γ; IL: Interleukin.

A

B

Figure 3 Statistics of T-helper-cell contents as measured by mRNA expression and cytokine levels. A: mRNA expression of interferon (IFN)-γ, interleukin (IL)-4, IL-17 and *FoxP3* on d0, d2 and d7; The mRNA expression levels of IFN-γ on d2 were increased compared to that on d0 (d0 *vs* d2, [b]$P < 0.01$), then partly recovered on d7 (d2 *vs* d7, [c]$P <0.05$). IL-4, IL-17 and *FOXP3* mRNA expression levels showed no significant differences from d0 to d2, whereas mRNA levels of IL-17 and *FOXP3* clearly decreased from d2 to d7 (IL-17: d2 *vs* d7, [c]$P < 0.05$; Treg: d2 *vs* d7, [d]$P < 0.01$); B: Cytokine concentrations of IFN-γ, IL-4, IL-17, IL-10 and transforming growth factor (TGF)-β1 on d0, d2 and d7 (d0 *vs* d2: [a]$P < 0.05$, [b]$P < 0.01$, respectively).

Table 5 Plasma interferon-γ, interleukin-4, interleukin-17, interleukin-10, transforming growth factor-β1 concentrations between G + E and G groups

Variables	G + E group (pg/mL)	G group (pg/mL)	*P*
IFN-γ			
d0	7.56 ± 0.44	6.67 ± 0.70	0.653
d2	11.17 ± 0.97	19.94 ± 1.37	0.018
d7	8.67 ± 0.74	5.76 ± 0.53	0.165
IL-4			
d0	10.31 ± 1.78	10.53 ± 1.28	0.892
d2	21.11 ± 3.76	36.69 ± 6.97	0.013
d7	13.00 ± 1.88	25.50 ± 2.98	0.065
IL-17			
d0	20.26 ± 5.82	18.83 ± 3.95	0.304
d2	27.26 ± 10.30	40.10 ± 10.56	0.239
d7	9.12 ± 1.67	16.66 ± 2.38	0.009
IL-10			
d0	3.67 ± 0.35	3.37 ± 0.31	0.528
d2	7.17 ± 0.86	5.97 ± 0.48	0.218
d7	4.09 ± 0.29	5.65 ± 0.72	0.06
TGF-β1			
d0	6565 ± 1667	5312 ± 1102	0.539
d2	15680 ± 4254	8375 ± 1395	0.12
d7	7555 ± 1555	10691 ± 2571	0.311
IFN-γ/ IL-4			
d0	0.74 ± 0.10	0.65 ± 0.13	0.618
d2	0.67 ± 0.08	0.56 ± 0.05	0.309
d7	1.10 ± 0.14	0.35 ± 0.08	< 0.001

Student's *t* test. G: General anesthesia; E: Epidural block; IFN-γ: Interferon-γ; IL: Interleukin; TGF: Transforming growth factor.

role in altering the immune response[3,6]. We had thus expected that surgical trauma would have a significant impact on the Th1/Th2 ratio, as well as Th17 and Treg levels after hepatectomy. In this study, we found that, on d2 after hepatectomy, all Th cells showed an increased trend, to varying degrees. By d7 after surgery, all Th cells recovered towards the baseline. These results revealed that surgical trauma increased Th cell numbers, but the stimulation did not last a significantly long time. There was no obvious change in the Th1/Th2 balance on d2 after surgery. However, there was a detectable shift towards Th1 dominance on d7 after surgery. In addition, Treg and Th17 frequencies decreased significantly compared with d0. These results support the conclusion that removal of the tumor leads to the relief of systemic immune suppression. As a result, host responses are reprogrammed towards a direction that benefits patient outcome. Therefore, it is likely that the change in Th1/Th2 balance, as well as Th17 and Treg frequencies in patients undergoing hepatectomy are not only a consequence, but may also

play an important role in the defense mechanism against tumor cells. Supporting this idea, it has been reported that surgical removal of primary tumors could reverse tumor-induced immunosuppression, even in the presence of metastatic disease[18,19]. On the other hand, the increase in Th cell numbers after hepatectomy may be due to stress caused by surgery. It is well recognized that inflammation typically peaks at d2 after surgery, and gradually dissipates by d7 after surgery. Consistently, Th cell frequencies reach their plateau on d2 and then decrease significantly on d7.

Anesthetic technique impacts Th1/Th2 balance, and Th17, Treg frequencies

Anesthetics *per se* are associated with suppressed immunity during perioperative periods because of their direct suppressive effects on cellular and humoral immunity[4]. As such, anesthetics and anesthetic methods should be selected with careful consideration. In the current study, on d7, the Th1/Th2 balance in the G + E group was profoundly shifted towards Th1 dominance, compared to the G group. In contrast, Th17 and Treg frequencies in the G + E group decreased significantly, indicating the benefit of combined G + E anesthesia against G anesthesia alone in HCC patients. In addition, on d2, Th1, Th2 and Th17 frequencies in the G + E group were relatively lower than those in the G group. This could also be attributed to the hypothesis that epidural anesthesia combined with general anesthesia was better than gen-

eral anesthesia in reducing or eliminating surgery-related stress responses, which peaked on d2 after surgery.

Several major differences between the two anesthetic methods may be responsible for the distinct patterns in Th1/Th2 balance, as well as Th17 and Treg frequencies between the two groups. First, regional anesthesia substantially attenuates surgery-induced stress responses, including increases in levels of corticosteroid hormone and catecholamine[13]. Second, opioids inhibit both cellular and humoral immune function in humans[20]. In the G + E group, opioids were administered epidurally, whereas morphine was administered systemically in the G group. Animal experiments have shown that morphine suppresses the lymphocyte proliferative response to mitogens when given systemically, but not when given intrathecally[21]. Similarly, patients receiving an epidural mixture of opioids and local anesthetics exhibited better preservation of lymphocyte proliferation and cytokine production than those receiving intravenous opioids alone. Third, intravenous and inhalation anesthetics are associated with elevated serum concentrations of catecholamines and cortisol[22,23]. Glucocorticoids and catecholamines can heavily influence immunomodulation, including decreases in Th1/Th2 cytokine production and an increase in *FOXP3* mRNA expression[24]. Consequently, it is not surprising that G anesthesia used alone suppressed the surgical stress-induced immune response more profoundly than combined G + E anesthesia.

Apart from the surgery and anesthesia, there are still many factors that could influence the Th1/Th2 balance, as well as Th17, and Treg frequencies, including HCC itself, hepatitis virus infection, liver cirrhosis, blood transfusion, and pain levels[25,26]. To eliminate these confounding factors, we prospectively and randomly assigned patients into the G + E or G group, and hence there were no significant differences in these factors. In addition, the results from flow cytometry and cytokine expression analyses were highly consistent with each other, indicating the reproducibility and reliability of the current study.

Anesthesia methods and oncologic prognosis

Oncologic prognosis has been reported to be associated with tumor bionomics, as well as trauma related to surgery, but not anesthesia. Recently however, the effect of anesthetic technique on tumor prognosis has received widespread attention[27]. In rat models, the addition of a spinal block to halothane general anesthesia markedly attenuated the promotion of pulmonary metastasis induced by surgery[28]. Furthermore, the reduction in tumor metastasis by the addition of a spinal block to sevoflurane general anesthesia accompanying surgery was ascribed to preserving the Th1/Th2 balance in mice[29]. Importantly, there are several retrospective clinical studies that are consistent with this hypothesis. Paravertebral anesthesia and analgesia for breast cancer surgery have been associated with an approximately four-fold reduced risk of recurrence or metastasis[30]. Similar results were observed in melanoma, prostate and colon cancer, where epidural an-

esthesia or epidural supplementation was associated with enhanced survival and reduced tumor recurrence[14,15,31]. The above results indicate that a regional block combined with general anesthesia is a better choice for cancer patients, compared to general anesthesia alone. Considering that there was postoperative Th1 dominance, and observed decreases in Th17 and Treg were more prominent in the G + E group than the G group, we assume that G + E may be superior to G alone for HCC patients in terms of achieving better patient clinical outcomes.

Hepatectomy resulted in the shifting of the Th1/Th2 balance towards Th1, with concomitant decreases in Th17 and Treg frequencies. Epidural anesthesia combined with general anesthesia is superior to general anesthesia alone in preserving the Th1/Th2 balance, and reducing Th17 and Treg numbers after surgery. We thus propose that epidural anesthesia combined with general anesthesia might be an optimal choice for HCC patients undergoing hepatectomies. However, animal experiments and prospective trials evaluating the effects of the Th1/Th2 balance, as well as Th17 and Treg on tumor prognosis are warranted.

COMMENTS

Background
Surgical resection is one of the first priorities for hepatocellular carcinoma (HCC), but may inevitably induce immunological stress. T-helper (Th) cells are subgroups of lymphocytes that play a central role in orchestrating host immune responses through their capacity to help other cells in the immune system. Whether proper peri-operative management, including selection of suitable anesthetic methods, may help to recover the disturbed balances of Th subsets is still questionable. In particular, they compared the differences in Th cell subsets between an epidural anesthesia combined with general anesthesia (G + E) group and a general anesthesia (G) group at multiple peri-operative time points to determine whether anesthetic methods have an impact on postoperative Th subset balances.

Research frontiers
Epidural anesthesia is known to prevent or attenuate an excessive stress response during or after surgery, which prevents noxious afferent input from reaching the central nervous system. Both preclinical and clinical studies have suggested that the addition of spinal blockade to general anesthesia attenuated the metastasis-promoting effect of surgery in a tumor-bearing host. The research hotspot is how epidural anesthesia affects peri-operative Th cell subset balances.

Innovations and breakthroughs
The Th1/Th2 balance, as well as Th17, and Treg numbers are key indices of immune function in many types of carcinoma. They also play an important role in HCC metastasis and recurrence. There are rare reports of surgery or anesthetic methods affecting the Th subset balance. The innovative approach in this study was to assess whether the balance was disturbed and which anesthetic method was better at restoring the balance in anti-tumor responses in HCC patients after hepatectomy.

Applications
Epidural anesthesia combined with general anesthesia is superior to general anesthesia alone in preserving the Th1/Th2 balance, and reducing Th17 and Treg numbers after surgery. They thus propose that epidural anesthesia combined with general anesthesia might be an optimal choice for HCC patients undergoing hepatectomy.

Terminology
Epidural anesthesia is most commonly placed in the lower back (lumbar region). This technique may also be accomplished in the mid-back (thoracic region) for surgery in the chest area. Th cells are a sub-group of lymphocytes that play an important role in the immune system, particularly in the adaptive

immune system. They are essential in B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages.

Peer review

This is a study in which authors investigated the influence of the different anesthesia modalities on Th cells. They randomized 61 HCC patients into G + E group and G group. The cell frequency of Th1, Th2, Th17, Treg and mRNA level of interferon-γ, interleukin (IL)-17, IL-4 and *FoxP3* in the sequentially collected blood samples (d0, d2 and d7 after surgery) were evaluated.

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BRIEF ARTICLE

Changes of smooth muscle contractile filaments in small bowel atresia

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Abstract

AIM: To investigate morphological changes of intestinal smooth muscle contractile fibres in small bowel atresia patients.

METHODS: Resected small bowel specimens from small bowel atresia patients (n = 12) were divided into three sections (proximal, atretic and distal). Standard histology hematoxylin-eosin staining and enzyme immunohistochemistry was performed to visualize smooth muscle contractile markers α-smooth muscle actin (SMA) and desmin using conventional paraffin sections of the proximal and distal bowel. Small bowel from age-matched patients (n = 2) undergoing Meckel's diverticulum resection served as controls.

RESULTS: The smooth muscle coat in the proximal bowel of small bowel atresia patients was thickened compared with control tissue, but the distal bowel was unchanged. Expression of smooth muscle contractile fibres SMA and desmin within the proximal bowel was slightly reduced compared with the distal bowel and control tissue. There were no major differences in the architecture of the smooth muscle within the proximal bowel and the distal bowel. The proximal and distal bowel in small bowel atresia patients revealed only minimal differences regarding smooth muscle morphology and the presence of smooth muscle contractile filament markers.

CONCLUSION: Changes in smooth muscle contractile filaments do not appear to play a major role in postoperative motility disorders in small bowel atresia.

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INTRODUCTION

Small bowel atresia is a congenital disorder of unknown pathogenesis, which carries significant morbidity[1-8]. Because of the severity of the dilatation of the proximal bowel and the hypoplasia of the distal bowel, various postoperative gastrointestinal motility problems might occur. The postoperative course can be complicated by a prolonged adynamic ileus (11%) and need for total parenteral nutrition (30%-70%)[9]. Although the underly-

ing cause of this postoperative intestinal motility disorder is unclear, it has been clearly shown that normal gastrointestinal motility depends on the coordinated function of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs)[10]. Consequently, previous publications have examined these factors for changes in small bowel atresia patients, especially within the atretic and adjacent proximal and distal bowel. Hypertrophy of the small bowel muscle proximal to the atresia has been found in clinical and experimental studies on small bowel atresia. Furthermore, various changes have been reported within the ENS in small bowel atresia[11]. We have recently shown differential changes of the ENS and the ICCs within the proximal bowel in small bowel atresia[12]. However, the smooth muscle of small bowel atresia patients has not been studied in detail. Therefore, the aim of this study was to investigate specific contractile filaments of the smooth muscle cells in resected specimens of small bowel atresia.

MATERIALS AND METHODS

Patients and tissues
Resected small bowel specimens (ileum) from term newborn small bowel atresia patients (*n* = 12) were included in the investigation after obtaining parental consent. The resected ileal specimens were divided into three segments (proximal, atretic and distal). Bowel specimens of two age-matched patients (who underwent surgery for Meckel's diverticulum) served as control tissue.

Tissue processing
The specimens were fixed in 4% paraformaldehyde, embedded in paraffin blocks and sectioned at 2-4 μm (Leica SM 2000 R) followed by drying overnight at 37 ℃. Before immunohistochemical staining, the paraffin sections were dewaxed for 10 min in xylene, 10 min in acetone and 10 min in acetone/Tris-buffered saline (TBS: 1:1). After treatment, the slides were washed in TBS.

Antigen demasking
When antigen retrieval by heat was required, dewaxed paraffin sections were placed in microwave-proof tubes containing target retrieval solution (Dako). The slides were treated in the tubes for 5 min at 600 W in a microwave (SS 566H; Bosch, Munich, Germany). The evaporated volume was replaced with distilled water, and the procedure was repeated twice. After microwave treatment, the slides were cooled and washed in TBS.

Histology and immunohistochemistry
Standard HE histology was performed. For immunohistochemistry, an alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining kit (Dako Real™ Detection System, APAAP, Mouse) using anti-smooth muscle actin (SMA, polyclonal, Dako, 1:500) and anti-desmin (Desmin, polyclonal, Dako, 1:25) antibodies was used. A nonsense

mAb (clone: MR 12/53) served as the negative control.

Evaluation
Immunohistochemical analysis focused on the proximal and distal parts of the resected ileum. The sections were evaluated by two independent investigators using light microscopy (magnification: 40 ×). HE staining was used to visualize the overall histology of the specimens. The distribution and density of immunoreactive SMA-positive and desmin-positive muscle filaments were studied in each part of the resected bowel (proximal, atretic and distal). Because immunohistochemical staining cannot be quantified, semi-quantitative scoring was performed as follows: - no expression, + low expression, ++ moderate expression, +++ high expression.

RESULTS

Patients
The study included resected ileal segments from 12 term newborn small bowel atresia patients (gestational age: 38-40 wk). Eleven patients presented with type Ⅲ a ileal atresia and one patient presented with multiple ileal atresia. All patients were operated during the first or second day of life.

Standard hematoxylin-eosin staining
Standard HE staining revealed normal muscle components within the bowel wall. The muscle layers of the proximal bowel appeared to be slightly thicker. However, these findings were not consistent in all specimens (Figure 1).

Smooth muscle actin immunohistochemistry
The gross histology of the affected (proximal) bowel remained unchanged. There was a variable increase in muscle layer thickness.

SMA expression in filaments was moderately decreased within the proximal bowel (moderate expression: ++) of small bowel atresia patients compared with control tissues (high expression: +++) (Figure 2A and C).

SMA positive filaments were uniformly less expressed in the tunica muscularis mucosa compared with the tunica muscularis propria (circular muscle, longitudinal muscle).

The distal bowel had normal expression of SMA-positive filaments (high expression: +++) within the smooth muscle of the tunica muscularis propria and the tunica muscularis mucosa compared to controls (high expression: +++) (Figure 2B and D).

Smooth muscle desmin immunohistochemistry
The gross histology of the proximal and distal small bowel was unchanged, as observed by desmin immunohistochemistry. The expression of desmin-positive filaments was moderately reduced within all muscle layers of the affected proximal bowel (moderate expression: ++) compared with controls (high expression: +++). There

Figure 1 Hematoxylin-eosin staining of the proximal (A) and distal (B) bowel. CM: Circular muscle; LM: Longitudinal muscle.

Figure 2 Smooth muscle actin immunohistochemistry of the proximal (A, C) and distal (B, D) bowel. CM: Circular muscle; LM: Longitudinal muscle; MM: Muscularis mucosa.

were no differences in desmin expression between the tunica muscularis propria (circular and longitudinal muscle) and the tunica muscularis mucosa within the proximal bowel (Figure 3A and C).

The distal bowel showed normal expression of desmin-positive filaments (high expression: +++) within the smooth muscle of the tunica muscularis propria and the tunica muscularis mucosa compared to controls (high expression: +++) (Figure 3B and D).

DISCUSSION

The extent of damage to the smooth muscle in small bowel atresia has not been well characterized. Previ-

ous studies of histological and ultrastructural changes of the affected bowel have investigated the ENS and the ICCs[13-20]. In these studies, differential changes of the ENS and ICCs were especially found in the proximal dilated bowel in small bowel atresia. Our present study revealed only moderate changes in the morphology of the of the smooth muscle and contractile filaments of the resected bowel proximal to the small bowel atresia. Furthermore, varying degrees of smooth muscle layer thickening was evident. The correlation between the degree of smooth muscle layer thickening and the duration of small bowel obstruction could not be evaluated since no data on the prenatal onset of the true obstruction were available.

Figure 3 Desmin immunohistochemistry of the proximal (A, C) and distal (B, D) bowel. CM: Circular muscle; LM: Longitudinal muscle.

A previous animal study creating a partial obstruction in dog ileum tissue revealed that the ganglion cells increased in size and the smooth muscle of the dilated bowel became thicker[21].

Another study used a murine partial small bowel obstruction model and revealed that 2 wk following the onset of a partial obstruction, the bowel increased in diameter, and hypertrophy of the tunica muscularis occurred oral to the obstruction site[22]. ICC networks were disrupted orally to the obstruction, and this disruption was accompanied by the loss of electrical slow waves and responses to enteric nerve stimulation. These defects were not observed aboral to the obstruction. Furthermore, it was shown that the removal of the obstruction led to the redevelopment of ICC networks and the recovery of slow wave activity within 30 d. Neural responses were partially restored in 30 d[22]. Similar repair mechanisms may occur after surgical correction of small bowel atresia. It seems obvious that decreases in ICCs in small bowel atresia and their restoration after removal of the obstruction contributes to the regulation of gastrointestinal motility.

Masumoto *et al*[11] showed that the circular muscle of the proximal small bowel is hypertrophied and expresses less SMA. This study showed that the ultrastructure and distribution pattern of the SMA-positive smooth muscle cells remained similar in the affected bowel compared with controls.

In another study, Masumoto *et al*[23] showed muscular alterations, such as abnormal smooth muscle bundles within the proximal segment of small bowel atresia. We could not confirm the existence of these abnormal smooth muscle bundles in our investigations. A case report recently revealed long lasting chronological changes within the ENS, muscle components and ICCs in small bowel atresia[24]. Interestingly, in this case, SMA-positive areas were found in both the circular and longitudinal musculature, and an increase in the proximal segment was observed at two different time points (newborn and 6 mo of age) compared with controls. The expression of SMA was similar in the distal segments compared with controls. Again, no clear changes were seen using SMA in the proximal bowel compared with the distal bowel and controls.

Ozguner *et al*[25] reported that the proximal segment of the atretic intestine showed structural deficits. Abnormal ganglia cells and defects in the intestinal musculature were prominent, but the intestinal mucosa remained intact. They found that abnormalities on both the antimesenteric and mesenteric sides, and their interpretation supported a vascular accident as a causative factor. Nevertheless, our study was not able to show muscular disruptions within the proximal bowel.

Previous studies have clearly shown that the ENS and ICCs are altered in the proximal and dilated bowel in small bowel atresia[12]. The innervation pattern of the proximal bowel resembles intestinal neuronal dysplasia[12]. These changes might be the result of a long-lasting bowel obstruction and bowel content stasis. Surprisingly, the smooth muscle appears hypertrophied, but substan-

tial changes within its ultrastructure were not observed. Therefore, we believe that the moderate histological changes within the smooth muscle do not contribute to the pathogenesis of small bowel atresia. Furthermore, the moderately altered smooth muscle does not seem to play a major role in the postoperative gastrointestinal motility of the affected patients.

In conclusion **the possible restoration of ICCs and** the moderate changes within the contractile filaments of smooth muscle components in small bowel atresia suggest that changes within the ENS are responsible for the postoperative motility problems. Furthermore, we speculate that extensive resection of the dilated proximal bowel is necessary to restore passage in adequate time.

COMMENTS

Background

Small bowel atresia is a congenital anomaly of unknown cause. Despite early corrective surgery, patients carry a substantial morbidity because of postoperative gastrointestinal motility problems. Normal gastrointestinal motility is generated by the complex interaction of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs). Alterations in the ENS and ICCs may contribute to the motility problems in patients with small bowel atresia after surgery. It has not yet been investigated whether changes in the smooth muscle occur in small bowel atresia and whether these possible changes influence the postoperative course.

Research frontiers

The relationship between the macroscopic and histological changes of the affected bowel and the postoperative motility disorder are still under investigation. Furthermore, the role of the smooth muscle and its contractile filaments in small bowel atresia needs to be further elucidated.

Innovations and breakthroughs

This study showed that the smooth muscle contractile filaments are only moderately altered in the proximal and dilated bowel in small bowel atresia. These results suggest that extensive resections of dilated proximal is not necessary in affected patients.

Applications

Previously shown changes within the ENS and the ICCs may influence postoperative gastrointestinal motility in affected patients. The moderate variations of smooth muscle contractile filaments in small bowel atresia do not seem to play a role in the postoperative course.

Terminology

Bowel atresia is a congenital defect in the continuity of the bowel. The incidence of small bowel atresia is higher than that of large bowel atresia and varies between 1:300 and 1:3000.

Peer review

The manuscript is well written and addresses an important concept of a clinical problem.

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BRIEF ARTICLE

Alcohol consumption in patients with primary sclerosing cholangitis

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Abstract

AIM: To assess the alcohol drinking patterns in a cohort of primary sclerosing cholangitis (PSC) patients and the possible influence on the development of fibrosis.

METHODS: Ninety-six patients with PSC were evaluated with a validated questionnaire about a patient's lifetime drinking habits: the lifetime drinking history (LDH) questionnaire. In addition, clinical status, transient elastography and biochemistry values were analysed and registered. Patients were defined as having either significant or non-significant fibrosis. Significant fibrosis was defined as either an elastography value of $\geq$ 17.3 kPa or the presence of clinical signs of cirrhosis. Patients were divided into two groups depending on their alcohol consumption patterns; no/low alcohol consumption (one drink or unit/d) and moderate/high alcohol consumption ($\geq$ 1 drink or unit/d). LDH data were calculated to estimate lifetime alcohol intake (LAI), current alcohol intake, drinks per year before and after diagnosis of PSC. We also calculated the number of episodes of binge-drinking (defined as consuming $\geq$ 5 drinks per occasion) in total, before and after the diagnosis of PSC.

RESULTS: The mean LAI was 3882 units of alcohol, giving a mean intake after onset of alcohol consumption of 2.6 units per week. Only 9% of patients consumed alcohol equal to or more than one unit per day. Current alcohol intake in patients with significant fibrosis (n = 26) was less than in patients without significant fibrosis (n = 70), as shown by lower values of phosphatidylethanol (B-PEth) (0.1 μmol/L *vs* 0.33 μmol/L, respectively, P = 0.002) and carbohydrate-deficient transferrin (CDT) (0.88% *vs* 1.06%, respectively, P = 0.02). Self-reported LAI was similar between the two groups. Patients with significant fibrosis reduced their alcohol intake after diagnosis from 103 to 88 units per year whereas patients without fibrosis increased their alcohol intake after PSC diagnosis from 111 to 151 units/year. There were no correlations between elastography values and intake of alcohol (units/year) (r = -0.036).

CONCLUSION: PSC patients have low alcohol consumption. The lack of correlation between fibrosis and alcohol intake indicates that a low alcohol intake is safe in these patients.

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INTRODUCTION

Little is known about risk factors for progression of fibrosis in primary sclerosing cholangitis (PSC) except for duration of disease and presence of symptoms[1-4]. Excessive consumption of alcohol causes liver disease[5-7], and a high intake of alcohol acts as a co-factor for progression of other chronic liver diseases, such as non-alcoholic steatohepatitis, hereditary hemochromatosis and hepatitis C (HCV). For instance, heavy episodic drinking has been shown to be associated with progression of fibrosis in non-alcoholic fatty liver disease[8], alcohol consumption of more than 60 g per day increases the risk for cirrhosis 9-fold in patients with hereditary hemochromatosis[9], and an alcohol intake of more than 210 g per week in patients with HCV has been shown to increase fibrosis[10-13]. The threshold for a safe intake of alcohol with regard to development of fibrosis in patients with concomitant liver disease is unclear and most patients with a chronic liver disease are advised to keep their alcohol intake to a minimum. The evidence for giving such advice to patients with chronic liver diseases in general is scarce. There is no evidence that alcohol in low amounts influences disease progression and there are data suggesting that a low alcohol intake, on the contrary, may have a protective effect against fibrosis. A recent study by Cheung *et al*[14] indicated no increased risk for fibrosis in HCV patients with alcohol intake less than 210 g per week and in a study by Moriya *et al*[15], low consumption of alcohol seemed to protect against non-alcoholic fatty liver disease in healthy individuals.

The impact of alcohol on progression of fibrosis in PSC has not been previously studied, although alcohol consumption has been reported to be associated with development of cholangiocarcinoma[16]. One of the most common questions from patients with PSC is: what amount of alcohol can be considered to be a safe intake? "Safe" amounts of alcohol (in liver-healthy individuals) are usually considered to be less than 210 g (approximately 18 units or drinks) of alcohol per week[7]. The purpose of our study was to describe the alcohol consumption patterns, and to evaluate whether lifetime alcohol consumption correlates to the fibrosis stage, in patients with PSC.

MATERIALS AND METHODS

Patients

All patients diagnosed with PSC at the Department of Gastroenterology and Hepatology, Karolinska University Hospital are recorded in a local PSC register.

Eligible for this study were 141 patients with PSC who were identified as currently living in the Stockholm area and who were having their regular follow-ups at our clinic.

The diagnosis of PSC were made according to accepted criteria; *i.e.*, typical cholangiographic findings of bile duct irregularities, strictures and dilatations or histological findings of cholangitis, or signs of small-duct PSC in combination with biochemical and clinical findings[4,17].

Figure 1 Flow chart for inclusion of patients. [1]Exclusion due to: Not Swedish speaking, presence of Down's disease, dementia, current pregnancy, severe psychiatric disease, co-existing hepatic disease (hepatitis B or C, hemochromatosis, recent diagnosis of liver cancer) or patients who had moved to other parts of the country or declined to participate (*n* = 4).

We excluded 32 patients with a recent diagnosis of cancer, not Swedish speaking, presence of Down's syndrome, dementia, current pregnancy, severe psychiatric disease (e.g., psychosis, bipolar disease), co-existing liver diseases (e.g., hepatitis B and C or hereditary hemochromatosis). Four patients declined to participate. The study cohort and patient selection are summarized in Figure 1.

Data collection

From our registry and patient charts, the following data were registered: duration of disease, age, sex, co-existing inflammatory bowel disease (IBD, diagnosed through endoscopy and histology), symptoms, smoking and body mass index (BMI). The patients were interviewed with a structured protocol for confirmation and validation of the data collected from the registry and for current symptoms and alcohol habits, including the question as to whether the patients had reduced their alcohol intake after the diagnosis of PSC was established.

At the interview, patients received the lifetime drinking history (LDH) questionnaire, a detailed and validated questionnaire about the patient's lifetime drinking habits[18,19]. This questionnaire allows the calculation of the total number of units during the patient's lifetime, with the possibility of calculating changes in drinking habits during life. It also allows measurement of total number of binge drinking episodes, defined as drinking 5 or more units of alcohol at one occasion. One unit of alcohol is equivalent to 12 g of alcohol. Patients were thoroughly informed about the questionnaire and later filled it out at home. When data were missing, the patient was contacted by telephone and information was supplemented through a telephone interview. Six of 105 patients (5.7%) did not return the LDH questionnaire despite being reminded and were excluded. No/low alcohol consumers were defined as drinking less than one drink per day, and moderate/high alcohol consumer as drinking equal to or more than one drink per day.

Transient elastography

Transient elastography with FibroScan (EchoSens, Paris, France) was performed on all patients on the same occasion as the interview. The cut-off values for significant

fibrosis were adopted from Corpechot *et al*[20] and the threshold for fibrosis stage 4 according to Ludwig (cirrhosis) was set to $\geq$ 17.3 kPa. At least 10 measurements were made, and only the scans where more than 60% of measurements were valid were accepted. We divided the population into two subgroups: patients with significant and non-significant fibrosis. Significant fibrosis was defined as either elastography values $\geq$ 17.3 kPa or a clinical diagnosis of cirrhosis diagnosed with histology[21] or typical radiological and biochemical findings of cirrhosis (such as irregular hepatic parenchyma, splenomegaly, oesophageal varices, presence of intraabdominal collaterals) or manifestation of decompensation. In nine patients elastography failed, most often due to overweight. In six of these, presence of significant fibrosis was evident from clinical data and they were included into the "significant fibrosis" group. The three patients with no available information on fibrosis from either elastography or clinical data were excluded. Twenty-six patients were found to have significant fibrosis and 70 patients had non-significant fibrosis.

Biochemistry

Biochemical data including blood count, sodium, potassium, creatinine, alkaline phosphatase, serum transaminases, total bilirubin, PK-INR, albumin, carbohydrate-deficient transferrin (CDT) and phosphatidylethanol (B-PEth, measured by liquid chromatography-mass spectrometry) in plasma were collected and analysed at the routine biochemistry laboratory at Karolinska University Hospital.

Statistical analysis

Continuous variables were analyzed using the Mann-Whitney *U*-test or the Wilcoxon Signed Rank Test where appropriate. For comparison of categorical data the χ^2 analysis was used or, in the case of small expected frequencies, *F* test. For correlation tests of linear data, the Pearson *r* test was used. We controlled the results for duration of disease using co-variance analysis of variance. Statistical data were analyzed using the Statistica® 9.1 software (StatSoft Inc., Tulsa OK) and SAS 9.2 software (SAS Institute Inc., Cary NC).

Ethical considerations

The local ethics committee at Karolinska University Hospital approved this study, registry No: 2009/1894-31/1. Written informed consent was obtained from all participating subjects.

RESULTS

Clinical characteristics

Clinical characteristics and data on lifetime alcohol consumption for the 96 patients are presented in Table 1. There were 66% men, mean age was 47 $\pm$ 13 years (range: 22-75 years) and 73 patients (76%) were diagnosed with concomitant IBD. Mean elastography value was 11.1 $\pm$ 8.2 kPa (range: 2.8-48 kPa). Seven patients (7.3%) had been diagnosed with PSC before they first started drinking al-

Table 1 Clinical characteristics and drinking habits of 96 patients with primary sclerosing cholangitis

	Mean (range)
Age at inclusion (yr)	47 (22-75)
Male sex	63/96
Duration of primary sclerosing cholangitis (yr)	12 (0-30)
Age at primary sclerosing cholangitis diagnosis (yr)	35 (11-65)
Fibroscan value (kPa)	11.1 (2.8-48)
Undergone orthotopic liver transplantation, *n* (%)	12 (12.5)
Bilirubin (μmol/L)	15.3 (3-82)
Alkaline phosphatase (μkat/L)	2.66 (0.5-12.4)
Phosphatidylethanol (μmol/L)	0.28 (0.1-8.4)
Carbohydrate-deficient transferrin (%)	1.01 (0.5-3)
Body mass index (kg/m²)	24.1 (17.2-34.3)
Inflammatory bowel disease (%)	73/96 (76)
Smoking-current user, *n* (%)	4 (4.2)
Smoking-ex user, *n* (%)	16 (16.7)
Smoking-never used, *n* (%)	76 (79.2)
Lifetime drinking habits	
Age at first alcohol intake (yr)	17 (12-28)
Lifetime alcohol intake (unit)	3882 (0-20 270)
Yearly alcohol intake, total (unit)	137 (0-1180)
Yearly alcohol intake, before diagnosis (unit)	109 (0-674)
Yearly alcohol intake, after diagnosis (unit)	134 (0-1110)
Total occasions of binge-drinking	294 (0-4054)
Yearly occasions of binge-drinking, total	11.3 (0-165)
Yearly occasions of binge-drinking, before diagnosis	12.0 (0-295)
Yearly occasions of binge-drinking, after diagnosis	8.0 (0-83)

Normal range, laboratory values. Bilirubin: < 26 μmol/L; Alkaline phosphatase: < 1.9 μkat/L; Phosphatidylethanol: < 1.0 μmol/L; Carbohydrate-deficient transferrin: < 2.0%.

cohol. There were no cases of patients with Child-Pugh score of 10 (*i.e.*, class C) or higher.

Alcohol consumption

Mean age at onset of alcohol consumption was 17 $\pm$ 3 years (range: 12-28 years). The mean lifetime alcohol intake (LAI) was 3882 units of alcohol (median: 2275 units, range: 0-20 270 units), giving a yearly mean alcohol intake of 137 units per drinking year and a mean number of 2.6 units per week during the years of alcohol consumption. Nine percent (9/96) drank equal to or more than one unit per day, and one percent (1/96) had a mean consumption of more than two units per day. The mean number of total episodes of binge drinking was 294 (median: 96, range: 0-4054), equalling eleven binges per year or 0.2 binges per week on average.

Twenty-eight percent (24/87) of no/low alcohol consumers (< 1 unit per day) had significant fibrosis compared to 22% of consumers with moderate/high alcohol intake (> 1 unit per day, *P* = 0.57). Moderate/high consumers had significantly more episodes of binge drinking. There were no significant differences in biochemical data or BMI (data not shown) between the moderate/high and the no/low alcohol consumption groups.

Comparison of patients with and without significant fibrosis

There were no significant differences in mean units of alcohol consumed per year between patients with significant and non-significant fibrosis, as shown in Figure 2.

Figure 2 Comparison between the total mean units of alcohol per year for the significant and non-significant fibrosis group.

Figure 3 Correlation between elastography values and units per year. Patients with clinically significant fibrosis but not a valid elastogram were given the value of 17.3 kPa.

There was no correlation between yearly alcohol intake (units/year) and elastography values (Figure 3). Thirty-eight percent of all patients (36/96) reported a decreased alcohol intake after the diagnosis of PSC. This figure was similar in patients with and without significant fibrosis (39% *vs* 37%). To further evaluate if the drinking habits changed after diagnosis, the LDH data were compared before and after PSC diagnosis. A total increase from 109 to 134 units per year, and a decrease in binge drinking of twelve to eight binges per year was found in all patients (not signitficant). Among patients with non-significant fibrosis, we found an increase in total alcohol consumption after PSC diagnosis (111 units per year *vs* 151 units per year, $P = 0.07$) whereas a decrease in total alcohol consumption after PSC diagnosis (103 units per year *vs* 88 units per year, $P = 0.59$) was found in the significant fibrosis group. Binge-drinking before and after PSC diagnosis was 14.9 binges per year *vs* 9.6 binges per year ($P = 0.24$) in the non-significant fibrosis group and 4.3 binges per year *vs* 3.6 binges per year ($P = 0.5$) in the significant fibrosis group. The significant fibrosis group had higher bilirubin values (24.8 μmol/L *vs* 11.8 μmol/L, $P = 0.015$),

lower CDT values (0.88% *vs* 1.06%, $P = 0.02$) and lower PEth values (0.1 *vs* 0.33, $P = 0.0016$) than the non-significant fibrosis group. Comparison of clinical variables and alcohol consumption between patients with significant and non-significant fibrosis are summarized in Table 2. We also performed a similar analysis with different cut-off values; 12.5 and 14.5 kPa respectively, which are the suggested cut-off values for cirrhosis (stage 4 fibrosis) in HCV[22,23], with similar results as when the cut-off level of ≥ 17.3 kPa was used (data not shown).

DISCUSSION

This study describes for the first time the alcohol consumption patterns in a large cohort of PSC patients before and after PSC diagnosis. The majority of the PSC patients were shown to have low alcohol consumption. The mean LAI was 3882 units of alcohol, giving a mean intake after onset of alcohol consumption of 2.6 units per week, and only 9% drank more than one unit per day and 1% more than two units per day. In comparison, to develop alcohol-induced liver cirrhosis, subjects need to drink at least 30 g of alcohol per day, equaling around 3 drinks per day[5-7] over several years.

The lifetime alcohol consumption did not correlate with the presence of significant fibrosis, although the current alcohol intake in fibrotic patients was less than in patients without significant fibrosis, shown by lower values of PEth and CDT. There was also a trend that patients with significant fibrosis had reduced their alcohol intake after the diagnosis of PSC whereas patients without significant cirrhosis actually increased their consumption after diagnosis. This is consistent with findings from studies of the effect of alcohol on other chronic liver diseases implicating that a low intake of alcohol seems to be harmless[14,15]. One may speculate whether or not low alcohol consumption actually protects against more progressive development of cirrhosis. Although no such conclusions can be drawn from the present study, our data support that a low consumption is harmless for fibrosis progression. The alcohol consumption among our PSC patients was lower than we expected, which has influenced our ability to evaluate the impact of more marked alcohol consumption for the progression of fibrosis. We were unable to evaluate whether a moderate/high consumption was harmful or safe since the number of patients with this pattern of consumption was too low.

CDT values can be affected by factors other than alcohol, such as end-stage liver disease (Child-Pugh score ≥ 10)[24,25]. None of our patients had a Child-Turcotte-Pugh score of more than 10 and patients with significant fibrosis had lower CDT values than patients with non-significant fibrosis. CDT has not been validated in patients with PSC; however, it has been studied in primary biliary cirrhosis and not been implicated to produce false-positive results[26]. B-PEth[27] measured by mass spectrometry is an even more sensitive marker than CDT for detecting alcohol consumption over the previous 1-2 wk. It has been reported to be stable in patients with

Table 2 Comparison of clinical variables and alcohol consumption between patients with and without significant fibrosis

Variable	Non-significant fibrosis (*n* = 70)	Significant fibrosis (*n* = 26)	*P* value
Duration of disease (yr)	10.1 ± 6.8	15.6 ± 7.9	0.019
Age at primary sclerosing cholangitis diagnosis (yr)	36 ± 14	34 ± 13	0.29
Lifetime alcohol intake (unit)	3896 ± 4441	3845 ± 4457	0.44
Yearly alcohol intake, mean (unit)	144 ± 178	117 ± 135	0.27
Total occasions of binge-drinking	331 ± 692	194 ± 341	0.23
Yearly occasions of binge-drinking	12.7 ± 24.7	7.1 ± 13	0.41
Yearly alcohol intake, before diagnosis (unit)	111 ± 129	103 ± 141	0.24
Yearly alcohol intake, after diagnosis (unit)	151 ± 200	88 ± 86	0.26
Yearly binge drinking episodes, before diagnosis	14.9 ± 38.1	4.3 ± 7.3	0.07
Yearly binge drinking episodes, after diagnosis	9.6 ± 18.6	3.6 ± 4.7	0.23
Bilirubin (μmol/L)	11.8 ± 6.8	24.8 ± 23.1	0.015
Alkaline phosphatase (μkat/L)	2.66 ± 2.65	2.66 ± 2.22	0.45
Phosphatidyl ethanol (μmol/L)	0.33 ± 1	0.1 ± 0	0.0016
Carbohydrate deficient transferrin (%)	1.06 ± 0.38	0.88 ± 0.2	0.02
Body mass index (kg/m²)	24.12 ± 3.12	24.02 ± 4.24	0.43

Normal range, laboratory values. Bilirubin: < 26 μmol/L; Alkaline phosphatase: < 1.9 μkat/L; Phosphatidylethanol: < 1.0 μmol/L; Carbohydrate-deficient transferrin: < 2.0%.

concomitant liver disease[28], but has not been studied in detail in patients with PSC.

The low alcohol consumption seen among our patients may be an effect of the general advice these patients are given in clinical practice, which is to keep alcohol intake at a minimum level. Patients with significant fibrosis also had higher bilirubin levels indicating a more severe disease, which in itself inhibits alcohol consumption. Thus, the knowledge of significant fibrosis in a patient contributes to decreased alcohol consumption. It is well known that smoking is associated with high alcohol consumption[29]; PSC patients have a low smoking frequency[30], also seen in this study. The correlation between a low smoking frequency and small total alcohol consumption in this cohort further validates our results.

Binge drinking decreased in both patients with significant and non-significant fibrosis after PSC diagnosis. This finding may reflect a change in drinking pattern with age, rather than the presence of PSC. Also, there is a general trend in Sweden towards less binge drinking[31]. The total alcohol consumption in Sweden has increased by approximately 15% since the mid 1990s[31,32]. Thirty-eight percent of all our patients reported that they had reduced their alcohol intake after the PSC diagnosis; however, the trend when looking at the LDH data was an increase in the total yearly alcohol intake. The perception of having reduced the consumption may be related to a reduction in the occasions of binge drinking episodes, which was confirmed in the questionnaire.

One limitation of the present study is the retrospective self-reported alcohol intake, which may be impaired by recollection bias. However, the LDH questionnaire is well validated and has a high test-retest correlation[19,33]. We also had a high response rate to the LDH questionnaire which is why we believe that our data are reliable. In addition, there is a risk that we have underestimated the presence of significant fibrosis since we did not perform liver biopsies to measure fibrosis. Liver biopsy is

not mandatory for the diagnosis of PSC and we chose to refrain from biopsies for ethical reasons due to risk of complications. The role of transient elastography in cholestatic liver disease is not well established, although it has been shown to be a good alternative to histology for evaluating fibrosis, mainly in HCV, but also in other chronic liver diseases[20,34-36].

Chalasani *et al*[16] reported in 2000 that alcohol was a risk factor for developing cholangiocarcinoma (CCA) in PSC; however, they were unable to quantify the amount of alcohol consumed. Our data can at present not evaluate whether alcohol intake is important for CCA since none of our patients have developed CCA. However, we have obtained solid data regarding alcohol consumption in a large cohort of PSC patients which is being prospectively followed. This allows future studies exploring the role of alcohol as a risk factor for developing CCA in this cohort.

In conclusion, patients with PSC have low alcohol consumption. Only 9% consumed an amount of alcohol equal to or more than one unit per day. There was a trend towards increased alcohol consumption after the PSC diagnosis in patients without significant fibrosis, and these patients have significantly increased plasma levels of CDT and PEth as compared to those having significant fibrosis or cirrhosis. We found no correlation between alcohol consumption and significant fibrosis. In summary, our results indicate that low alcohol consumption is safe in patients with PSC.

ACKNOWLEDGMENTS

COMMENTS

Background
Fibrosis progression in primary sclerosing cholangitis (PSC) is a heterogeneous process with large individual variations before significant fibrosis develops.

Research frontiers
Alcohol, in high amounts, is known to be a risk factor for progression of fibrosis in other chronic liver diseases. The role of alcohol intake for progression of fibrosis has not previously been studied in PSC.

Innovations and breakthroughs
This is the first study of the alcohol consumption pattern in a large cohort of PSC patients and they aimed to correlate this to the occurrence and degree of fibrosis.

Applications
By increasing their knowledge of risk factors for progression of fibrosis in PSC, this study can help give doctors relevant information to patients regarding their alcohol habits.

Peer review
The paper is an interesting paper assessing the lifetime drinking history. The paper is quite well written but the reader needs reason for using certain biochemical variables in this context and the statistical analysis has to be better explained. Also most readers of this paper do not know what is the amount of drinks used by patients with alcohol dependency and alcoholic cirrhosis.

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BRIEF ARTICLE

Decreased blood riboflavin levels are correlated with defective expression of *RFT2* gene in gastric cancer

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Abstract

AIM: To investigate the relationship between blood riboflavin levels and riboflavin transporter 2 (*RFT2*) gene expression in gastric carcinoma (GC) development.

METHODS: High-performance liquid chromatography was used to detect blood riboflavin levels in patients with GC. Real-time fluorogenic quantitative polymerase chain reaction and immunohistochemistry were used to analyze the expression of RFT2 mRNA and protein in samples from 60 GC patients consisting of both tumor and normal tissue.

RESULTS: A significant decrease in the RFT2 mRNA levels was detected in GC samples compared with those in the normal mucous membrane (0.398 ± 0.149 *vs* 1.479 ± 0.587; $P = 0.040$). Tumors exhibited low RFT2 protein expression (75%, 16.7%, 8.3% and 0% for no RFT2 staining, weak staining, medium staining and strong staining, respectively), which was significantly lower than that in the normal mucous membrane (10%, 16.7%, 26.7% and 46.7% for no RFT2 staining, weak staining, medium staining and strong staining, respectively; $P < 0.05$). Tumors with low RFT2 expression were significantly associated with tumor stage and histological grade. Moreover, a significantly decrease in Uyghur patients was observed compared with Han patients. However, other parameters-gender, tumor location and lymph node metastasis-showed no significant relationship with RFT2 expression. Blood riboflavin levels were reverse correlated with development of GC ($1.2000 \pm 0.97\,569$ ng/mL in high tumor stage patients *vs* $2.5980 \pm 1.31\,129$ ng/mL in low tumor stage patients; $P < 0.05$). A positive correlation of plasma riboflavin levels with defective expression of RFT2 protein was found in GC patients ($\chi^2 = 2.619$; $P = 0.019$).

CONCLUSION: Defective expression of RFT2 is associated with the development of GC and this may represent a mechanism underlying the decreased plasma riboflavin levels in GC.

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INTRODUCTION

Gastric carcinoma (GC) is one of the most common cancers and the second leading cause of cancer deaths worldwide. In China, the incidence and mortality rates for gastric cancer in 2006 were 35.02 and 26.08 per 100 000 persons, respectively. The survival rate for gastric cancer is generally low, with a 5-year survival rate not exceeding 30%[1]. *Helicobacter pylori* infection of the gastric mucosa is associated with GC, infecting the gastric mucosa of 50% or more of the world's population. However, the relatively low GC risk suggests that other factors[2] such as lifestyle factors-including diet and genetic predisposition-may play an etiologic role[3,4]. Nitrosamines have been suspected in the etiology of GC in the high incidence area of China; however, riboflavin deficiencies and other micronutrients may also be involved[5,6].

Riboflavin is a water-soluble vitamin that is a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which participate in various redox reactions, some of which are absolutely essential to the function of aerobic cells. Milk and dairy products, meats, fatty fish, certain fruit as well as vegetables are good sources of riboflavin. However, riboflavin deficiency and poor riboflavin status seem to be of most concern for the elderly and adolescents, despite the diversity of available riboflavin-rich foods[7]. Thus, whether other factors exist that may influence dietary riboflavin absorption or that may change the status of riboflavin needs to be determined.

Indeed, a recent study showed that riboflavin transporter 2 (RFT2) was found to transport riboflavin that is highly expressed in the small intestine and may be involved in such riboflavin absorption[8]. Additionally, Fujimura *et al*[9] demonstrated that RFT2 is a transporter involved in the epithelial uptake of riboflavin in the small intestine for its nutritional utilization. Because RFT2 is responsible for transporting riboflavin and riboflavin deficiency has been reported as a risk factor for GC, and our previous genome-wide association studies showed that genetic variations at 20p13 for RFT2 (or C20 or f54) contribute significantly to the risk for cutaneous squamous cell carcinoma and GC in Chinese Han and Uyghur populations[10], we propose that human RFT2 is likely to have an important role in gastric carcinogenesis that involves modulating riboflavin absorption. However, most previous studies on riboflavin and RFT2 were relatively small and investigated only the functional roles of riboflavin and RFT2[11-13].

To clarify these issues, in the present study, we examined blood riboflavin levels and their tissue riboflavin transporter gene statuses. Additionally, we further analyzed the relationship between RFT2 and riboflavin in the development of GC.

MATERIALS AND METHODS

Clinical samples

A total of 120 paraffin-embedded, formalin-fixed tissue specimens were used in this study. Sixty patients with GC who underwent surgery at the Department of General Surgery of the first affiliate Hospital of Xinjiang Medical University in 2011 were enrolled in the study. Additionally, 60 matched cases with normal mucous membranes were involved. The patient population consisted of 39 men and 21 women, with a mean age of 54.9 years (range: 39-76 years). Peripheral blood samples also collected into EDTA Vacutainer Tubes (Becton Dickinson) and immediately placed on ice. Next, the remaining samples were centrifuged (10 min at 2000 g and 4 ℃) and collected plasma was stored at -80 ℃ until use. Control samples were obtained from subjects who underwent routine health checks, were recruited in the same area, and were matched with GC patients regarding age and gender. The selection criteria included individuals who were free from certain diseases, including neoplasms, cardiovascular diseases, hepatic diseases, renal diseases or inflammatory diseases. All patients were enrolled with written informed consent, and the study was approved by the Ethical Committee of the Medical University of Xinjiang. Patients had not received preoperative chemotherapy and/or radiotherapy. Tumor stage was determined according to the tumor node metastasis classification system of the International Union against Cancer. In addition, 60 frozen biopsies that included 30 GC and 30 matched normal mucous epithelia (5 cm away from the tumor), which were collected within 30 min after resection and kept at -80 ℃, were subjected to real-time reverse transcription-polymerase chain reaction (RT-PCR) for detection of RFT2 mRNA expression. Histological diagnosis was confirmed for each specimen.

Determination of riboflavin levels in plasma

Blood plasma was analyzed for its concentration of riboflavin by high-performance liquid chromatography (HPLC) as described previously[14]. The HPLC system used was a Waters 2695 liquid chromatograph and Waters 2475 fluorescence detector with the autosampler set at 28 ℃ and configured for a 96-well microtiter plate. Water was generated using a Milli-Q water system. All chemicals were of analytical grade. For quality control, we used three Clin Chek serum controls, reconstituted and stored at -80 ℃. Aliquots of aqueous (0.3860 g/L $C_2H_7NO_4$) flavin stock solutions (5 mmol/L) were stored at -20 ℃ in the dark. An excitation wavelength of 450 nm was

used and riboflavin was detected at an emission wavelength of 520 nm. The peak area was measured and used for quantification.

RNA extraction and real-time RT-PCR

RFT2 mRNA expression was detected by quantitative real-time RT-PCR. Total RNA was extracted from fresh frozen tissue using Trizol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions and treated with TURBO DNA-free™ DNase (Ambion, Austin, TX, United States) to remove the genomic DNA. Reverse transcription was performed using a reverse transcription system kit (Takara Bio, Tokyo, Japan) as previously described[15]. Real-time PCR for RFT2 was performed in a 10-μL reaction volume using the Platinum SYBR Green q PCR Super Mix-UDG (Invitrogen Life Technologies, Carlsbad, CA, United States) and the Light Cycler 480 system (Roche Diagnostics, Penzberg, Germany). The following hRFT2 and β-actin (as a reference) primers were used for RT-PCR: hRFT2 forward primer: AATCTAGAGCACTTGGACCTTTCC; hRFT2 reverse primer GGGTTCAGGGACAGGTCTAAAGA; β-actin forward primer: GGCACCCAGCACAATGAAG; β-actin reverse primer: CCGATCCACACGGAGTACTTG. The thermal cycle conditions were 95 ℃ for 10 s for one cycle, followed by 40 cycles of amplification at 95 ℃ for 5 s, and 60 ℃ for 45 s. The expression level of RFT2 mRNA was obtained using the $2^{-\Delta\Delta CT}$ calculation method. All PCR products were analyzed on a 2% agarose gel with ethidium bromide staining.

Immunohistochemical studies

Immunohistochemistry (IHC) was performed using Histostain-SP kits (Zhongshan Golden Bridge, Beijing, China) according to the manufacturer's recommendations and as described previously[16]. Sections of paraffin embedded tissue with a thickness of 3 μm were deparaffined in xylene, and then rehydrated in graded concentrations of ethyl alcohol (100%, 95%, 80% and 70%), and samples were pretreated using a microwave for 15 min on high mode in Tris/EDTA buffer (pH 9.0). After cooling and rinsing in distilled water, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature. Subsequently, the slides were pretreated with 1% bovine serum albumin in phosphate-buffered saline (PBS; pH 7.4) for 10 min. Samples were then preincubated with a protein blocking solution for 15 min and incubated with an RFT2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States; dilution: 1:300) diluted in PBS at 4 ℃ overnight in a humid chamber. Slides were washed three times in PBS, and then incubated with a secondary biotinylated antibody for 15 min at room temperature. Thereafter, sections were washed with PBS, and treated with peroxidase-conjugated streptavidin for 15 min. Finally, the sections were lightly stained with hematoxylin, and PBS was used instead of the primary antibody as a negative control. All immunostained sections were coded and independently examined by two investigators using light microscopy. Results were scored for each antibody separately and semi-quantitatively by assessing the staining intensity and the percentage of stained cells in the tumors. Staining intensity was scored as 0 (no staining), 1+ (weak), 2+ (medium), or 3+ (strong). The percentage of stained cells was categorized as follows: (1) 0% to 10% stained cells; (2) 11% to 50% stained cells; and (3) 50% stained cells or greater. The final score was obtained by multiplying the two scores. Cases with a score of 0 to 4 were classified as negative, and those with a final score of 5 to 9 were classified as positive.

Statistical analysis

SPSS 15.0 (SPSS, Inc., Chicago, IL, United States) was used for statistical analysis. The Pearson χ^2 test or Fisher exact test was used to compare qualitative variables. The Wilcoxon two-sample test was used to compare the mean RFT2 expression in fresh frozen GC tissues with that in normal samples, as determined by real-time quantitative RT-PCR. Results were presented as mean ± SE. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

RFT2 protein expression in GC and its association with GC clinicopathologic characteristics

IHC staining of 60 primary GC lesions and samples adjacent to the tumor was performed using RFT2 antibodies (Table 1). Representative staining patterns for RFT2 are shown in Figure 1. IHC staining demonstrated that RFT2 was localized to the cytoplasm. Positive staining for RFT2 was generally observed within normal gastric epithelium adjacent to the tumor, but weak or no RFT2 staining was detected in GC cells. RFT2 expression was undetectable (75%) in gastric cancer tissue. By contrast, no strong IHC staining (3+) was detected in gastric cancer tissue. The positive rates (strong staining, medium staining and weak staining) of RFT2 expression in the normal gastric surface epithelium were 46.7%, 26.7% and 16.7%, respectively. These values sharply decreased to 0%, 8.3% and 16.7%, respectively, in GC lesions (*P* < 0.001, compared with the positive rate in healthy tissue). We also evaluated the possible relationship between the expression of RFT2 in tumor cells and the clinicopathologic characteristics of GC, including tumor stage, histological grade, lymph node metastasis, and other parameters such as gender, tumor location, and ethnic groups. RFT2 expression was significantly decreased in GC with poor differentiation and high tumor stage. Defective expression of RFT2 in tumor cells was significantly associated with poor differentiation and high tumor stage. However, other parameters such as gender, tumor location, and lymph node metastasis had no significant relationship with RFT2 expression (Table 1). Additionally, lower expression was observed in Uyghur patients compared with Han patients, but the difference was not statistically significant.

Table 1 Statistical analysis of riboflavin transporter 2 expression and clinicopathologic factors in gastric cancer _n_ (%)

Characteristics	_n_	RFT2 expression				_Z_	_P_ value
		−	+	++	+++		
Normal mucous epithelia	60	6 (10.0)	10 (16.7)	16 (26.7)	28 (46.7)	-7.937	< 0.001
Gastric cancer	60	45 (75.0)	10 (16.7)	5 (8.3)	0		
Gender							
Male	39	31 (79.5)	6 (15.4)	3 (7.7)	0	-0.621	0.534
Female	21	14 (66.7)	4 (19.0)	2 (9.5)	0		
Ethnic groups							
Han	32	21 (55.0)	8 (25.0)	3 (20.0)	0	-1.943	0.052
Uyghur	28	24 (81.82)	2 (13.6)	2 (4.5)	0		
Tumor location							
Cardia of stomach	10	2 (20.0)	4 (40.0)	4 (40.0)	0	-3.127	0.209
Body of stomach	11	8 (72.1)	2 (18.2)	1 (8.7)	0		
Antrum of stomach	39	35 (89.7)	4 (10.3)	0	0		
Differentiation							
Moderate/well	19	10 (52.6)	5 (26.3)	4 (21.1)	0	-2.834	0.005
Poor	41	35 (85.4)	5 (12.2)	1 (2.3)	0		
L/N metastasis							
Negative	10	9 (90.0)	1 (10.0)	0	0	-1.245	0.28
Positive	50	36 (72.0)	9 (18.0)	5 (10.0)			
Stage							
Ⅱ and Ⅲa	41	27 (65.9)	9 (22.0)	5 (12.1)	0	-2.414	0.019
Ⅲb and Ⅳ	19	18 (94.7)	1 (5.3)	0	0		

RFT2: Riboflavin transporter 2; L/N metastasis: Lymph node metastasis. -: Negative; +: Weak positive; ++: Medium positive; +++: Strong positive.

Table 2 Statistical analysis of riboflavin transporter 2 expression and blood riboflavin levels in gastric cancer (mean ± SD)

RFT2 protein expression	Riboflavin level	χ^2	_P_ value
−	0.7012 ± 0.68 778	-2.619	0.019
+	1.6425 ± 1.01 783		
++	2.9691 ± 1.30 345		

RFT2: Riboflavin transporter 2; -: Negative; +: Weak positive; ++: Medium positive.

RFT2 mRNA expression in gastric carcinoma and normal controls

To confirm the IHC results, RFT2 mRNA expression in gastric biopsies was detected by RT-PCR (Figure 2). Similar to the IHC results, the RFT2 mRNA expression levels were significantly lower in GC than in normal counterpart tissue (0.398 ± 0.149 ng/mL *vs* 1.479 ± 0.587 ng/mL; $P = 0.040$) (Figure 2).

Relationship between riboflavin level and RFT2 expression in gastric carcinoma

Riboflavin concentrations were determined in samples obtained from gastric cancer patients who were not taking vitamin supplements. The average blood concentration of riboflavin was 1.7632 ± 0.9836 ng/mL in GC patients. The average blood riboflavin level was 1.2000 ± 0.97 569 ng/mL in high tumor stage patients, which was significantly lower than in patients with low tumor stage (2.5980 ± 1.31 129 ng/mL; $P = 0.019$). Riboflavin chromatograms of plasma samples from a low tumor stage patient and a high tumor stage patient are shown in Figure 3. A tendency of decreasing blood riboflavin level

was found to be associated with GC development. However, no significant difference was observed among other parameters such as gender, tumor location and ethnic groups and patients with GC. We also analyzed the relationship between the blood riboflavin level and expression of the *RFT2* gene and development of GC. A positive association was found between changes in the blood riboflavin levels and changes in RFT2 protein expression as well as development of malignant GC (Table 2).

DISCUSSION

In the present study, we investigated the association between plasma levels of riboflavin and the RFT2 mRNA and protein expression status in patients with gastric adenocarcinoma. The results showed a tendency for an inverse association between riboflavin levels and GC risk, and an inverse association between RFT2 expression status and GC risk. Additionally, a positive association was found between riboflavin levels and RFT2 with GC risk. The results indicate that RFT2 likely plays an important role in gastric carcinogenesis that involves modulating riboflavin absorption.

Previous studies have reported that dietary habits and intake of nutrients play an important role in the prevention and causation of GC. High consumption of dark-green vegetables, fruits, and milk and dairy products (riboflavin is a water soluble vitamin present in various foods) has also been suggested to decrease the risk of GC. Several epidemiological studies have reported[17,18] that riboflavin deficiency is linked to an increased risk of GC because riboflavin is involved in essential oxidation-reduction reactions and its deficiency leads to skin and

Figure 1 **Immunohistochemical staining of gastric lesions using riboflavin transporter 2 gene-specific monoclonal antibodies.** A: Expression of riboflavin transporter 2 (RFT2) in normal gastric epithelium with strong staining; B: Moderate expression of RFT2 protein in gastric cancer (GC) tissue; C: Weak expression of RFT2 protein in GC tissue (original magnification, × 400).

Figure 2 **mRNA expression of riboflavin transporter 2 in gastric cancer tissue and control tissue.** Panels 1 to 4 and 9 are from normal gastric epithelium. Panels 5 to 8 and 10 are from gastric cancer tissues. Panels 1 to 8 show expression of riboflavin transporter 2 mRNA. Panels 9 and 10 show expression of β-actin.

of GC, and such characteristics of the riboflavin transporter gene were also consistent with those of the carrier-mediated riboflavin transport system in the Caco-2 cell line as an intestinal epithelial model. Thus, it is likely that hRFT2 is the molecular entity of the riboflavin transport system in the Caco-2 cell line[20].

Riboflavin (vitamin B_2) has been confirmed to serve as a co-factor in fat, amino acid, carbohydrate and vitamin metabolism as well as to reduce oxidative stress, affect cell proliferation, and affect angiogenesis[21,22]. Few epidemiological studies have investigated plasma concentrations of riboflavin in relation to GC risk[23]. The present study observed riboflavin concentrations in samples obtained from GC patients by HPLC, and the results showed a tendency of decreasing blood riboflavin level with development of GC. Use of HPLC as a convenient method for separation and measurement of vitamins in plasma has been reported in many medical institutions[14,24].

Riboflavin is essential for synthesis of FAD and FMN, which function as cofactors for several biological processes involved in energy metabolism. The most important dietary sources of riboflavin are milk and dairy products. In Xinjiang, a multi-ethnic residential area in China, the Chinese Han and Uyghur populations are the main ethnic groups and they have different dietary habits. The Uyghur population tends to consume milk, dairy products, and meat as well as fruits and vegetables. They should not lack intake of riboflavin. Therefore, riboflavin deficiency in blood is expected to be related to a disturbance in riboflavin absorption. If inadequate intake of riboflavin exists, disturbances in the steps in intermediary metabolism may occur.

In conclusion, we have identified down-regulated expression of RFT2 mRNA and protein as being closely related to the progression of GC lesions and also found a positive relationship between blood riboflavin levels and RFT2 protein expression as well as between blood riboflavin levels and development of GC. The *RFT2* gene may be the key target of environmental and genetic factors in the development of GC.

COMMENTS

Background

Epidemiological and etiological research has confirmed that environmental

mucosal disorders. Therefore, measurement of plasma riboflavin can be used to assess vitamin B_2 status in individuals at high risk for GC. Although riboflavin supplements can significantly reduce the risk of GC, different individual intervention effects were observed after dietary supplementation with riboflavin[19]. Therefore, RFT2 may be the key target of environmental and genetic factors because the *RFT2* gene also has been reported as a susceptibility gene for GC using a genome-wide association study approach[10].

RFT2 is a transmembrane protein, which may function biologically as a transporter of riboflavin in the small intestine, and the role of riboflavin in cellular homeostasis has been well documented. Therefore, we speculate that mutation of the riboflavin transporter gene might cause riboflavin deficiency, resulting in an increased risk

Figure 3 Chromatographic profiles of a low tumor stage patient and a high tumor stage patient.

and genetic factors play an important role in gastric carcinogenesis. The riboflavin transporter 2 (*RFT2*) gene as a susceptibility gene for gastric carcinoma (GC) is likely to have an important role in gastric carcinogenesis that involves transporting riboflavin and modulating riboflavin absorption. However, riboflavin deficiency has been reported as a risk factor for GC.

Research frontiers

Riboflavin deficiency of is a risk factor for GC, and riboflavin supplements can significantly reduce the risk of GC. However, different individual intervention effects have been observed after dietary supplementation with riboflavin. In the present study, defective expression of RFT2 was associated with development of GC and may be a potential mechanism underlying the decreased plasma riboflavin levels in GC.

Innovations and breakthroughs

Recent reports have highlighted that genetic susceptibility combined with exposure to environmental risk factors contributes to high rates of cancer. In particular in GC, RFT2 is down-regulated. This is the first study to report that RFT2 is down-regulated in GC. Furthermore, their studies suggest that this protein may be the cause of decreased plasma riboflavin levels in GC.

Applications

By understanding how RFT2 affects the absorption of riboflavin and by enhancing its expression, this study may offer a future strategy for the treatment of patients with GC.

Terminology

RFT2 is a transmembrane protein that functions biologically as a transporter of riboflavin and in maintenance of cellular homeostasis. Riboflavin is a water-soluble vitamin that participates in various redox reactions, some of which are essential for the function of aerobic cells.

Peer review

The authors performed a study on riboflavin levels as well as on *RFT2* gene mRNA levels in esophageal squamous cell carcinoma samples and normal counterpart tissue. The level of RFT2 was found to be inversely related to tumor stage. Down-regulation and loss of RFT2 protein expression were important in the pathogenesis of gastric cancer. This study was well designed and the molecular experiments were well done and interpreted. The data seem convincing.

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BRIEF ARTICLE

Relationship between *HLA-DR* gene polymorphisms and outcomes of hepatitis B viral infections: A meta-analysis

Ze-Hui Yan, Yi Fan, Xiao-Hong Wang, Qing Mao, Guo-Hong Deng, Yu-Ming Wang

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Abstract

AIM: To assess the rigorous relationship between human leukocyte antigens (HLA)-DR alleles and outcomes of hepatitis B virus (HBV) infections by means of meta-analysis.

METHODS: Medline/PubMed, EMBASE, CNKI and VIP were searched to identify relevant studies. Study quality was evaluated using the Newcastle-Ottawa Scale. Odds ratios (OR) and 95% confidence interval (95% CI) were pooled using Stata 11.0. Subgroup analyses were performed by ethnicity. Heterogeneity and publication bias analyses were performed to validate the credibility.

RESULTS: A total of 2609 patients with chronic hepatitis B and 2606 controls spontaneously recovering from prior HBV infection were included. Meta-analysis showed that HLA-DR*04 (OR = 0.72, 95% CI: 0.60-0.85) and DR*13 (OR = 0.27, 95% CI: 0.19-0.37) alleles were significantly associated with HBV clearance while patients carrying HLA-DR*03 (OR = 1.47, 95% CI: 1.16-1.87) or DR*07 (OR = 1.59, 95% CI: 1.24-2.03) alleles had a significantly increased risk of chronic HBV persistence. For the HLA-DR*01 polymorphism, a significantly association with HBV clearance was found in Chinese Han group (OR = 0.48, 95% CI: 0.26-0.86), but not found in other ethnic groups (*P* = 0.191). For other polymorphisms, no association with the HBV infection outcome was found.

CONCLUSION: HLA-DR*04 and DR*13 alleles may be the protective factors for HBV clearance and HLA-DR*03, and DR*07 alleles may be the risk factors for HBV persistence.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem affecting more than 400 million people worldwide. The clinical features of HBV infection vary from clearance of the virus to fulminate hepatitis. Although some HBV carriers spontaneously eliminate the virus, 2%-10% of the individuals with chronic hepatitis B

(CHB) are estimated to develop liver cirrhosis, and a subset of these individuals eventually suffer from liver failure or hepatocellular carcinoma[1]. A complex combination of environmental, pathogenic and host genetic factors plays a role in determining both susceptibility to HBV and the course of the infection[2]. Several epidemiological factors, such as age at infection, sex, chronic alcohol abuse and co-infection with other hepatitis viruses, were suspected to affect viral persistence. In addition, host immunological factors and genetic background were also considered to influence the susceptibility to and outcome of persistent HBV infection[3]. Family studies in China provide some evidences that the host genetic factors affect viral persistence, as concordance rates of HBeAg persistence were higher in identical twins than in non-identical twins[4].

Although genetic variants in *IFNG*[5], *TNF*[6], *VDR*[7], *MBP*[8], *ESR1*[9], *CXCL10*[10], *IL-10*[11] and several human leukocyte antigen (HLA) loci[12,13] have been shown to associate with CHB, none of the associations has been proven to be conclusive. The mechanism of susceptibility to chronic persistent HBV infection is not well clarified. Since the outcome of HBV infection mainly depends on the host immune response, and HLA, an integral component of the immune response, plays an important role in immunological reaction to HBV infection[14], the highly polymorphic *HLA* gene has been considered as an appropriated biological candidate susceptibility gene that is associated with the development and the progression of chronic HBV infection. Indeed, previous studies have highlighted that *HLA-DR* polymorphisms influence individual immune responses, thus affecting the outcome of diseases, and that many different *HLA* alleles play a role in HBV infection[15]; however, this relationship between *HLA-DR* polymorphisms and HBV infection is not universal for all investigated populations. As for Caucasians[16,17] and Koreans[18], *HLA-DRB1*1301-02* has been found to be associated with acute self-limited hepatitis B. For Taiwanese people, *HLA-DRB1*0406* is associated with recovery from HBV infection in the Han Chinese, as is *HLA-DRB1*4001* in indigenous Taiwanese people[19]. Han Chinese with HLA-DR12 (especially one of its alleles, *DRB1*1201*) or *HLA-DRB1*1101/1104* are able to resist HBV infection, while those with *HLA-DR9*, *DQ9*, *HLA-DRB1*0301*, *HLA-DQB1*0301*[20,21], and *DRB1*10* are susceptible to chronic HBV infection[22].

In spite of the pivotal role that the polymorphic HLA antigens play in immune surveillance and immune response and the multitude of studies performed, there is still a lack of conclusive evidence of the association between polymorphisms and the outcomes of HBV infection; the relationship between them is not universal for all the investigated populations. In this meta-analysis, the identification of common *HLA-DR* alleles causing CHB susceptibility was examined through a systematic review of the literature followed by a meta-analysis of all case-control studies. Meta-analysis is a powerful method for quantitatively summarizing the results from different studies. One of the advantages is to enhance the statistical power of outcomes in ethnically- and ancestrally-rich populations and to enlarge the sample sizes, which may reduce the possibility of producing false-positive or false-negative association by random error[23].

MATERIALS AND METHODS

Literature search strategy

A systematic search was conducted by two investigators independently (Yan ZH and Fan Y). All articles were retrieved from four main databases: Medline/PubMed, EMBASE, CNKI (China National Knowledge Infrastructure) and VIP database (Chinese Journal of Science and Technology of VIP). The latest search was updated on April 20, 2011, using the search terms: "hepatitis B" or "HBV", "polymorphism", "human leukocyte antigen" or "*HLA*" or "Major Histocompatibility Complex" or "MHC". The search was not confined to articles written in a certain language, but focused on studies conducted on human subjects. All searched studies were retrieved and the relevant publications in the bibliographies were checked simultaneously. Abstracts and unpublished reports were not considered; only published studies with full-text articles were included. When the same patient population was included in several publications, only the most recent or complete study was used in this meta-analysis. When a study reported results of different subpopulations, we treated each subpopulation as a separate comparison in the meta-analysis. This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by University Hospital Birmingham Trust (LREC 2002/166). All patients provided informed written consent.

Inclusion and exclusion criteria

The inclusion and exclusion criteria were drawn up on the basis of discussion. The articles to be included in this meta-analysis should meet these inclusion criteria: (1) the full text was available for the published study; (2) the study was designed as an unrelated case-control study; (3) the study provided the number of persistent chronic HBV infection cases and controls; (4) the study provided the number of individual *HLA-DR* genotypes in cases and controls; (5) the study provided enough information for odd ratio (OR) and 95% confidence interval (95% CI) calculation; and (6) the diagnosis for CHB was based on the following criteria: seropositive for HBsAg for at least 6 mo; serum HBV DNA $> 1 \times 10^5$ copies/mL for HBeAg-positive and $> 1 \times 10^4$ copies/mL for HBeAg-negative patients; and persistent or intermittent elevation in alanine aminotransferase (ALT) level, or liver biopsy showing chronic hepatitis with moderate or severe necroinflammation. The criteria for spontaneous recovery from infection were: positive for both anti-HBs and anti-HBc antibodies, negative for HBsAg, normal liver function tests and no history of dominant hepatitis B and HBV vaccination.

The exclusion criteria were: (1) the study was not a case-control study; (2) the control group was only with

healthy patients and without those who recovered from prior HBV infection; (3) the study did not fit the diagnosis criteria; (4) the individuals included in the case-control study had serologic evidence for co-infection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus (HIV); and (5) the studies were duplicated (only the most recent or complete study was used in this meta-analysis when the same patient population was included in several publications[24]). In addition, interim analyses and comparisons of laboratory methods were all excluded.

Data extraction and validity assessment
In order to retrieve articles as completely and correctly as possible, two investigators extracted data independently using a standardized form and they reached a consensus on all items. Disagreement was resolved by discussion among the whole study groups. For each study, the following data were extracted: name of the first author, year of publication, ethnicity, ethnic origin of the studied population, number of patients with persistent HBV infection (including asymptomatic carriers and patients with chronic liver diseases), number of spontaneously recovered controls, number of individual genotypes, source of controls, genotype frequency of cases and controls, genotyping methods, statistical methods and the results of the study.

The quality of the primary studies was evaluated using the Newcastle-Ottawa Scale (NOS)[25]. This scale judges the study on three broad perspectives: the selection of the study groups; the comparability of the groups; and the ascertainment of either the exposure or outcome of interest for case-control.

Statistical analysis
We compared persistent HBV infection cases with spontaneously recovered controls to assess the relationship between *HLA-DR* polymorphisms and HBV infection clearance. Subgroup analyses were mainly performed by ethnicity. Ethnic groups were mainly defined as Chinese Han and other ethnic group. Statistical analysis was conducted using the Stata 11.0 (StataCorp, College Station, TX, United States). The association between *HLA-DR* polymorphisms and the CHB risk was measured by OR with 95% CI. The pooled OR was determined by the Z test and statistical significance was set at $P < 0.05$. All P values were two-sided. To ensure the reliability and the accuracy of the results, two investigators uploaded the data into the statistic software programs independently and obtained the same results. In our study, two models of meta-analysis for dichotomous outcomes were constructed: the random-effects model and the fixed-effects model. The random-effects model was constructed using the DerSimonian and Laird's method[26], which assumed that studies were conducted in populations with varying effect sizes and calculated the study weights from both in-study and between-study variances. The fixed-effects model was constructed using the Mantel-Haenszel's method[27], which assumed that studies were conducted in populations with

the same effect sizes and made an adjustment to the study weights according to the in-study variances.

To assess the between-study heterogeneity more precisely, both the χ^2-based Cochran's Q-statistic[28] (to test the heterogeneity) and the Higgins (I^2) test[29] (to quantify the proportion of the total variation due to heterogeneity) were calculated. Because of the low power of Cochran's Q statistic, heterogeneity was considered significant at $P_h < 0.10$, and the random-effects model was used to pool the results. When the P value of Cochran's Q statistic was greater than 0.10, the fixed-effects model was used to pool the results. Besides, the Galbraith plot was used to spot the outliers as the possible major sources of heterogeneity[30]. To better investigate the possible sources of between-study heterogeneity, meta-regression analysis was also applied to both general analyses and subgroup analyses, when heterogeneity was observed. To confirm the effect of clinical heterogeneity between studies on the conclusions of the meta-analysis, subgroup analysis was conducted based on races. To validate the credibility of outcomes in this meta-analysis, a sensitivity analysis was performed by sequential omission of individual studies or by omitting studies plotted by the Galbraith plot method as the possible major source of heterogeneity. The potential publication bias was estimated by funnel plot, in which the standard error of logOR of each study was plotted against its logOR. An asymmetric plot suggests a possible publication bias. Visual inspection of asymmetry in funnel plots was conducted to assess the potential for publication bias. In addition, Begg's rank correlation method and the Egger weighted regression method were also used to statistically assess the publication bias (publication bias with a $P \leqslant 0.05$ was considered statistically significant)[31].

RESULTS
Characteristics of studies
A flow diagram of the study selection process is shown in Figure 1. We identified a total of 313 potentially relevant articles to our search criteria. After carefully reviewing the titles, abstracts and full text of the literature, we identified 72 articles with analysis on the association between *HLA-DR* polymorphisms and persistent HBV infection. However, according to the exclusion criteria, 54 publications were excluded including 21 articles involving the same study subjects as other included articles, nine papers not about case-control study, 22 publications without spontaneously recovered patients in the control group, and two papers in which the individuals included in the case-control study had serologic evidence for co-infection with HIV. Finally, 18 articles evaluating *HLA-DR* polymorphisms met the inclusion criteria and were chosen for this systematic review and meta-analysis. All the selected studies presented original data on independent samples.

The characteristics of each study are presented in Table 1. These 18 articles were published from 1995

Figure 1 Results of literature search. This figure describes the whole process of searching for articles for inclusion in this systematic review and meta-analysis. HLA: Human leukocyte antigens; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus.

to 2011 with 15 in English[16,18-21,32-41] and three in Chinese[42-44]. Of them, nine studies were on Han Chinese populations[19-21,34-36,42-44] and 10 studies on other race (Gambia[32], Caucasian[16], Korean[18,37,39], Iran[33,40], Thai[38] and Turkish[41]). Besides, one publication contained two individual case-control studies from two different ethnic populations (Chinese Han and Taiwanese aborigines[19]). All studies used the individuals who spontaneous recovered from prior HBV infection as control subjects. The sample size in the individual case-control studies ranged from 60 to 638. A total of 2609 patients with CHB and 606 spontaneously recovered controls prior to HBV infection, were included from 19 studies (cases and controls were counted only once for each study). Genotyping methods used in the studies included PCR-restriction fragment length polymorphism, PCR-sequence specific primer, PCR-sequence specific oligonucleotide probe and sequence-specific oligonucleotide probe hybridization.

The NOS results showed that the median overall score was 7 (range, 5-8), which indicated that the methodological quality was generally good. We defined studies that scored a 7 or above as having high methodological quality, and judged that 2 out of the 19 studies to be of low quality (one study[44] scored 5 and another study[43] scored 6) primarily due to either no description of case selection, no definition of control, a lack of adjusted analysis, or no description of the method of ascertainments for case-controls.

Meta-analysis of relationship between HLA-DR gene polymorphisms and clearance of persistent HBV infection

Table 2 lists the main results of meta-analysis of the relationship between *HLA-DR* polymorphisms and the clearance of persistent HBV infection. We found no evidence of associations between the clearance of persistent HBV infection and *HLA-DR*08, DR*09, DR*10, DR*11, DR*12, DR*14, DR*15, DR*16* alleles ($P_{OR} > 0.05$). For these negatively associated alleles, the results of subgroup analyses by ethnicity also showed no associations in either Chinese Han group or other ethnic group ($P_{OR} > 0.05$).

For the *HLA-DR*01* polymorphism, the fixed-effects model was used to pool the meta-analysis result, as the between-study heterogeneity was not significant ($I^2 = 0\%$, $P_H = 0.826$) when all eligible studies were pooled into meta-analysis. The results of pooling all 13 eligible studies showed that *HLA-DR*01* allele was associated with an increased clearance rate of HBV infection ($P_{OR} = 0.016$, $OR_{fixed\text{-}effects} = 0.69$, 95% CI: 0.52-0.93). In the subgroup analyses by ethnicity, the association between the *DR*01* allele and HBV clearance was found in Chinese Han group ($P_{OR} = 0.014$, $OR_{fixed\text{-}effects} = 0.48$, 95% CI: 0.26-0.886), but not found in other ethnic groups ($P_{OR} = 0.191$) (Figure 2A).

For the *HLA-DR*03* polymorphism, the between-study heterogeneity was also not significant when all 12 eligible studies were pooled into meta-analysis ($I^2 = 16.0\%$, $P_H = 0.287$). The results of the fixed-effects model meta-analysis which pools all studies showed that the *HLA-DR*03* allele was associated with a significantly increased risk of chronic HBV persistence ($P_{OR} = 0.002$, $OR_{fixed\text{-}effects} = 1.47$, 95% CI: 1.16-1.87). The subgroup analyses by ethnicity showed that the *DR*03* allele was associated with a significantly increased risk of chronic HBV persistence in both Chinese Han group ($P_{OR} = 0.020$, $OR_{fixed\text{-}effects} = 1.57$, 95% CI: 1.07-2.30) and other ethnic groups ($P_{OR} = 0.029$, $OR_{fixed\text{-}effectss} = 1.41$, 95% CI: 1.03-1.91) (Figure 2B).

For the *HLA-DR*04* polymorphism, the between-study heterogeneity was also not significant when all 16 studies were pooled into meta-analysis ($I^2 = 0\%$, $P_H = 0.757$). The results of pooling all studies using the fixed-effects model meta-analysis showed that the *HLA-DR*04* allele was associated with the clearance of HBV infection ($P_{OR} = 0.000$, $OR_{fixed\text{-}effects} = 0.72$, 95% CI: 0.60-0.85). The subgroup analyses by ethnicity showed that the *DR*04* allele was associated with an increased clearance rate of HBV infection in both Chinese Han group ($P_{OR} = 0.002$, $OR_{fixed\text{-}effects} = 0.63$, 95% CI: 0.48-0.84) and other ethnic groups ($P_{OR} = 0.016$, $OR_{fixed\text{-}effectss} = 0.77$, 95% CI: 0.62-0.95) (Figure 2C).

For the *HLA-DR*07* polymorphism, the fixed-effects model was used to pool the meta-analysis result, since the between-study heterogeneity was not significant ($I^2 = 14.0\%$, $P_H = 0.304$) when all 13 studies were pooled into

Table 1 Main characteristics of case-control studies included in this systematic review and meta-analysis

No.	Study[ref.]	Yr	Language	Ethnicity	Genotyping	Spontaneously recovered control		Chronic hepatitis B case		NOS score
						n	Characteristics	*n*	Characteristics	
1	Thursz *et al*[32]	1995	English	Gambia	PCR-RFLP	413	218 children; 195 adults	225	185 children; 40 adults	7
2	Hohler *et al*[16]	1997	English	Caucasian	PCR-SSP	24	NA	70	Outpatients	7
3	Ahn *et al*[18]	2000	English	Korean	PCR-SSP	243	156 males, mean age: 41 yr	83	53 males, mean age: 35 yr	8
4	Chen *et al*[42]	2002	Chinese	Chinese Han	PCR-SSP	56	43 males, mean age: 47.6 yr	30	22 males, mean age: 38.7 yr	7
5	Akcam *et al*[33]	2002	English	Iran	PCR-SSP	30	20 males, mean age: 35.9 yr	30	7 males, mean age: 31.0 yr	7
6	Jiang *et al*[21]	2003	English	Chinese Han	PCR-SSP	30	24 males, mean age: 33.2 yr	52	43 males, mean age: 33.46 yr	7
7	Meng *et al*[20]	2003	English	Chinese Han	PCR-SSP	56	22 males, mean age: 38.7 yr	30	43 males, mean age: 47.6 yr	7
8	Wu *et al*[19]	2004	English	Chinese Han	SSOPH	324	169 males, mean age: 39.1 yr	98	66 males, mean age: 50.9 yr	8
9	Wu *et al*[19]	2004	English	Taiwanese aborigines	SSOPH	229	90 males, mean age: 52.1 yr	138	60 males, mean age: 45.7 yr	8
10	Lu *et al*[43]	2006	Chinese	Chinese Han	PCR-SSP	148	70 males, mean age: 37.75 yr	417	289 males; 207 CHB, 212ASC ASC	6
11	Zhang *et al*[34]	2006	English	Chinese Han	PCR-SSP	32	20 males, mean age: 38.4 yr	61	40 males, mean age: 38.4 yr	7
14	Yang *et al*[35]	2007	English	Chinese Han	PCR-SSP	108	Age and sex matched	108	90 males, 58 LC, 24 HCC	7
15	Song *et al*[44]	2007	Chinese	Chinese Han	PCR-SSP	102	63 males, mean age: 33.9 yr	276	168 males, 77 LC, 106 ASC	5
14	Zhu *et al*[36]	2007	English	Chinese Han	PCR-SSP	133	63 males, mean age: 37 yr	151	120 males, mean age: 40 yr	7
15	Hwang *et al*[37]	2007	English	Korean	PCR-SSP	438	286 man, mean age: 39 yr	198	148 males, 76 LC, 28 HCC	7
16	Kummee *et al*[38]	2007	English	Thai	PCR-SSP	100	48 males, mean age: 51.0 yr	150	80 males, mean age: 30.9 yr	8
17	Cho *et al*[39]	2008	English	Korean	PCR-SSP	80	60 males, mean age: 47.9 yr	384	283 males, mean age: 41.0 yr	8
18	Remezani *et al*[40]	2008	English	Iran	PCR-SSP	30	17 males, mean age: 32.2 yr	33	20 males, mean age: 38 yr	8
19	Albayrak *et al*[41]	2011	English	Turkish	PCR-SSP	30	15 males, mean age: 33.9 yr	75	48 males, mean age: 33.0 yr	8

NA: Relative data were not available in original studies; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; PCR-SSP: Polymerase chain reaction sequence specific primers; PCR-SSO: Polymerase chain reaction sequence specific oligonucleotide probe; SSOPH: Sequence-specific oligonucleotide probe hybridization: CHB: Chronic hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ASC: Asymptomatic hepatitis B virus carriers; NOS: Newcastle-Ottawa Scale.

meta-analysis. The results showed that *HLA-DR*07* allele was associated with a significantly increased risk of chronic HBV persistence ($P_{OR} = 0.000$, $OR_{fixed-effects} = 1.59$, 95% CI: 1.24-2.03). In the subgroup analyses by ethnicity, the results showed that the *DR*07* allele was associated with a significantly increased risk of chronic HBV persistence among Chinese Han group ($P_{OR} = 0.017$, $OR_{fixed-effects} = 1.50$, 95% CI: 1.07-2.10) and other ethnic groups ($P_{OR} = 0.005$, $OR_{fixed-effects} = 1.69$, 95% CI: 1.17-2.44) (Figure 2D).

For the *HLA-DR*13* polymorphism, the fixed-effects model was used to pool the meta-analysis result, since the between-study heterogeneity was not significant ($I^2 = 0.00\%$, $P_H = 0.699$). The results showed that *HLA-DR*13* allele was associated with an increased clearance rate of HBV infection ($P_{OR} = 0.000$, $OR_{fixed-effects} = 0.27$, 95% CI: 0.19-0.37). In the subgroup analyses by ethnicity, the results showed that the *DR*13* allele was associated with HBV clearance among Chinese Han group ($P_{OR} = 0.003$, $OR_{fixed-effects} = 0.36$, 95% CI: 0.18-0.70) and other ethnic groups ($P_{OR} = 0.000$, $OR_{fixed-effects} = 0.24$, 95% CI: 0.17-0.35) (Figure 2E).

Heterogeneity analysis and sensitivity analysis

The heterogeneity was calculated among all studies using the Q-statistic and the I^2 test. As shown in Table 2, for the *HLA-DR*11* polymorphism, the between-study heterogeneity was significant ($I^2 = 41.3\%$, $P_H = 0.053$) when all 14 studies were pooled into meta-analysis. In the subgroup analyses by ethnicity for this allele, the between-study heterogeneity was also significant in Chinese Han subgroups ($I^2 = 59.1\%$, $P_H = 0.023$), while heterogeneity

was not found in other ethnic groups ($I^2 = 20.0\%$, $P_H = 0.277$). Except for the meta-analysis for DR*13* allele, heterogeneity in the all meta-analysis for other *HLA-DR* alleles was not found ($P_H > 0.10$).

Univariate analysis of meta-regression suggested that the publishing year and language were not important sources of between-study heterogeneity in both generate analyses and subgroup analyses. We carried out a sensitivity analysis for each *HLA-DR* allele interaction analysis by sequential omission of individual studies. A single study involved in the meta-analysis was deleted each time to investigate the influence of the individual dataset on the pooled ORs. The corresponding pooled ORs were not materially altered, indicating that our results were statistically robust.

Publication bias

Begg's funnel plot and Egger's test were performed to assess the publication bias of the literature in this meta-analysis. All the P values of Begg's test (P_{beggs}) and Egger's tests (P_{Eggers}) are shown in Table 2. The P_{Eggers} with more than 0.05 was considered as statistical evidence of the funnel plots' asymmetry. The Egger's test results suggested that publication bias in our meta-analyses of *HLA-DR* alleles was not remarkable except for the three subgroup meta-analyses. As shown in Table 2, the publication bias was borderline significant in the analysis of HLA-DR*10 allele ($P_{Eggers} = 0.040$) and Chinese Han subgroups analysis of HLA-DR*16 ($P_{Eggers} = 0.042$). The publication bias was evident in other ethnic group analysis of *HLA-DR*13* allele ($P_{Eggers} = 0.017$). Then, the Duval and Tweedie non-parametric "trim and fill" method was used to adjust

A HLA-DR*01 allele

Study	OR (95% CI)	Weight (%)
Non-Chinese Han		
Thursz *et al*[32] (1995)	1.05 (0.56, 1.99)	17.38
Hohler *et al*[16] (1997)	0.61 (0.21, 1.75)	7.90
Ahn *et al*[18] (2000)	0.75 (0.29, 1.90)	10.29
Hwang *et al*[37] (2007)	0.45 (0.17, 1.19)	13.80
Cho *et al*[39] (2008)	1.15 (0.49, 2.67)	9.89
Remezani *et al*[40] (2008)	0.32 (0.06, 1.81)	4.66
Albayrak *et al*[41] (2011)	1.00 (0.29, 3.47)	4.69
Subtotal ($I^2 = 0.0\%$, $P = 0.643$)	0.79 (0.56, 1.12)	68.62
Chinese Han		
Chen *et al*[42] (2002)	0.19 (0.01, 3.67)	2.96
Jiang *et al*[21] (2003)	0.57 (0.03, 9.44)	1.18
Meng *et al*[20] (2003)	0.19 (0.01, 3.67)	2.96
Zhang *et al*[34] (2006)	0.49 (0.11, 2.11)	4.65
Yang *et al*[35] (2007)	0.74 (0.25, 2.20)	7.16
Zhu *et al*[36] (2007)	0.45 (0.17, 1.16)	12.49
Subtotal ($I^2 = 0.0\%$, $P = 0.928$)	0.48 (0.26, 0.86)	31.38
Overall ($I^2 = 0.0\%$, $P = 0.826$)	0.69 (0.52, 0.93)	100.00

B HLA-DR*03 allele

Study	OR (95% CI)	Weight (%)
Non-Chinese Han		
Thursz *et al*[32] (1995)	0.96 (0.55, 1.67)	23.39
Hohler *et al*[16] (1997)	1.22 (0.40, 3.76)	5.16
Ahn *et al*[18] (2000)	1.62 (0.69, 3.78)	7.06
Akcam *et al*[33] (2002)	8.83 (1.01, 76.96)	0.70
Thio *et al*[17] (2003)	1.71 (1.01, 2.91)	18.47
Cho *et al*[39] (2008)	2.14 (0.49, 9.36)	2.87
Remezami *et al*[40] (2008)	0.69 (0.17, 2.85)	4.21
Subtotal ($I^2 = 8.6\%$, $P = 0.363$)	1.41 (1.03, 1.91)	61.85
Chinese Han		
Jiang *et al*[21] (2003)	2.12 (0.73, 6.12)	4.55
Wu *et al*[19] (2004)	1.18 (0.64, 2.16)	17.20
Zhang *et al*[34] (2006)	0.50 (0.09, 2.63)	3.42
Yang *et al*[35] (2007)	3.38 (1.44, 7.95)	5.76
Zhu *et al*[36] (2007)	1.23 (0.48, 3.15)	7.21
Subtotal ($I^2 = 37.0\%$, $P = 0.174$)	1.57 (1.07, 2.30)	38.15
Overall ($I^2 = 16.0\%$, $P = 0.287$)	1.47 (1.16, 1.87)	100.00

C HLA-DR*04 allele

Study	OR (95% CI)	Weight (%)
Non-Chinese Han		
Thursz *et al*[32] (1995)	0.53 (0.28, 1.00)	8.93
Hohler *et al*[16] (1997)	0.78 (0.18, 3.28)	1.26
Ahn *et al*[18] (2000)	1.12 (0.67, 1.86)	8.74
Akcam *et al*[33] (2002)	0.20 (0.04, 1.02)	2.33
Wu *et al*[19] (2004)	0.95 (0.54, 1.69)	7.51
Hwang *et al*[37] (2007)	0.76 (0.42, 1.18)	15.77
Cho *et al*[39] (2008)	0.71 (0.17, 2.85)	10.49
Remezami *et al*[40] (2008)	0.47 (0.15, 1.42)	2.84
Albayrak *et al*[41] (2011)	0.97 (0.40, 2.34)	3.14
Subtotal ($I^2 = 0.0\%$, $P = 0.459$)	0.77 (0.62, 0.95)	61.01
Chinese Han		
Chen *et al*[42] (2002)	0.63 (0.22, 1.81)	2.79
Jiang *et al*[21] (2003)	1.10 (0.38, 3.14)	2.08
Meng *et al*[20] (2003)	0.63 (0.22, 1.81)	2.79
Wu *et al*[19] (2004)	0.54 (0.31, 0.93)	12.02
Zhang *et al*[34] (2006)	0.70 (0.24, 2.06)	2.40
Yang *et al*[35] (2007)	0.53 (0.29, 0.99)	8.71
Zhu *et al*[36] (2007)	0.75 (0.42, 1.34)	8.19
Subtotal ($I^2 = 0.0\%$, $P = 0.920$)	0.63 (0.48, 0.84)	38.99
Overall ($I^2 = 0.0\%$, $P = 0.757$)	0.72 (0.60, 0.85)	100.00

D HLA-DR*07 allele

Study	OR (95% CI)	Weight (%)
Non-Chinese Han		
Hohler *et al*[16] (1997)	1.60 (0.41, 6.17)	3.62
Ahn *et al*[18] (2000)	0.89 (0.39, 2.05)	11.89
Hwang *et al*[37] (2007)	2.36 (1.25, 4.42)	11.62
Cho *et al*[39] (2008)	2.28 (1.01, 5.18)	9.45
Remezani *et al*[40] (2008)	5.18 (0.57, 47.16)	0.88
Albayrak *et al*[41] (2011)	0.76 (0.26, 2.26)	7.16
Subtotal (I^2 = 27.7%, P = 0.227)	1.69 (1.17, 2.44)	44.61
Chinese Han		
Chen *et al*[42] (2002)	1.90 (0.11, 31.44)	0.67
Jiang *et al*[21] (2003)	1.18 (0.32, 4.31)	4.27
Meng *et al*[20] (2003)	1.90 (0.11, 31.44)	0.67
Wu *et al*[19] (2004)	0.48 (0.14, 1.65)	8.95
Zhang *et al*[34] (2006)	2.70 (0.82, 8.87)	3.76
Yang *et al*[35] (2007)	2.39 (1.28, 4.47)	12.88
Zhu *et al*[36] (2007)	1.26 (0.74, 2.14)	24.19
Subtotal (I^2 = 13.6%, P = 0.326)	1.50 (1.07, 2.10)	55.39
Overall (I^2 = 14.0%, P = 0.304)	1.59 (1.24, 2.03)	100.00

(axis: -1, 1, 10)

E HLA-DR*13 allele

Study	OR (95% CI)	Weight (%)
Non-Chinese Han		
Thursz *et al*[32] (1995)	0.46 (0.19, 1.16)	9.17
Hohler *et al*[16] (1997)	0.13 (0.04, 0.44)	7.23
Ahn *et al*[18] (2000)	0.14 (0.03, 0.60)	10.39
Akcam *et al*[33] (2002)	0.17 (0.03, 0.85)	4.88
Hwang *et al*[37] (2007)	0.40 (0.18, 0.86)	14.57
Kummee *et al*[38] (2007)	0.07 (0.01, 0.54)	6.23
Cho *et al*[39] (2008)	0.27 (0.14, 0.52)	15.23
Remezani *et al*[40] (2008)	0.10 (0.01, 0.89)	4.13
Albayrak *et al*[41] (2011)	0.15 (0.05, 0.46)	8.39
Subtotal (I^2 = 1.3%, P = 0.424)	0.24 (0.17, 0.35)	80.21
Chinese Han		
Chen *et al*[42] (2002)	0.59 (0.06, 5.92)	1.20
Jiang *et al*[21] (2003)	0.56 (0.07, 4.20)	1.42
Meng *et al*[20] (2003)	0.45 (0.05, 4.20)	1.57
Wu *et al*[19] (2004)	0.41 (0.12, 1.41)	6.01
Zhang *et al*[34] (2006)	1.05 (0.09, 12.05)	0.74
Yang *et al*[35] (2007)	0.24 (0.05, 1.14)	4.56
Zhu *et al*[36] (2007)	0.12 (0.01, 0.99)	4.29
Subtotal (I^2 = 0.0%, P = 0.867)	0.36 (0.18, 0.70)	19.79
Overall (I^2 = 0.0%, P = 0.699)	0.27 (0.19, 0.37)	100.00

(axis: -1, 1, 10)

Figure 2 Forest plots of pooled odd ratio with 95% confidence interval for associations between human leukocyte antigens-*DR* polymorphisms and clearance of chronic hepatitis B virus infection. The fixed-effects model was constructed using the Mantel-Haenszel's method. Subgroup meta-analysis was conducted based on ethnicity (Chinese Han group and other ethnic groups). The squares and horizontal lines correspond to the study-specific odd ratio (OR) and 95% confidence interval (95% CI); the box size is proportional to the meta-analysis study weight, the diamonds represent the pooled OR and 95% CI. HLA: Human leukocyte antigens.

publication bias[45]. Meta-analysis with and without using the "trim and fill" method did not draw different conclusions, indicating that our results were statistically robust.

DISCUSSION

It is believed that the host genetic factors involved in genetic polymorphisms are responsible for the susceptibility to and clinical outcomes of infectious diseases. In recent years, genetic susceptibility to chronic HBV infection has been a research focus, and it has been identified that the polymorphisms of a number of immune-response-associated genes, including *HLA* loci, affected the susceptibility to and clearance of persistent chronic HBV infection among different populations. HLA plays an essential role in the pathogenesis of virus-associated hepatitis. The HLA class Ⅱ molecules are expressed as cell surface glycol-proteins that bind short peptide epitope to CD4+ T cells. HLA-DR, a subtype of HLA class Ⅱ molecule, has a particular binding motif that dictates

Table 2 Meta-analysis of relationship between human leukocyte antigens-DR gene polymorphism and clearance of persistent hepatitis B virus infection

Allele	Present *vs* null	Studies No.	SRC (*n*/N)	CHB (*n*/N)	Heterogeneity		M	OR		Publication bias	
					I^2 (%)	PH		OR (95% CI)	POR	Pbeggr's	PEgger's
DR*01	Total studies	13	109/1500	132/1673	0	0.826	F	0.69 (0.52-0.93)	0.016	0.143	0.221
	Chinese Han group	6	18/432	34/415	0	0.928	F	0.48 (0.26-0.86)	0.014	0.348	0.219
	Other ethnic groups	7	91/1068	98/1258	0	0.643	F	0.79 (0.56-1.12)	0.191	0.133	0.104
DR*03	Total studies	12	180/1489	177/1789	16.0	0.287	F	1.47 (1.16-1.87)	0.002	0.945	0.572
	Chinese Han group	5	72/470	74/627	37.0	0.174	F	1.57 (1.07-2.30)	0.020	0.806	0.838
	Other ethnic groups	7	108/1019	103/1162	8.6	0.363	F	1.41 (1.03-1.91)	0.029	0.548	0.444
DR*04	Total studies	16	358/1766	549/2256	0	0.757	F	0.72 (0.60-0.85)	0.000	0.207	0.255
	Chinese Han group	7	104/530	215/739	0	0.920	F	0.63 (0.48-0.84)	0.002	0.453	0.299
	Other ethnic groups	9	254/1236	334/1517	0	0.459	F	0.77 (0.62-0.95)	0.016	0.175	0.178
DR*07	Total studies	13	239/1373	146/1584	14	0.304	F	1.59 (1.24-2.03)	0.000	0.625	0.634
	Chinese Han group	7	111/530	82/739	13.6	0.326	F	1.50 (1.07-2.10)	0.017	0.881	0.601
	Other ethnic groups	6	128/843	64/845	27.7	0.227	F	1.69 (1.17-2.44)	0.005	0.452	0.924
DR*08	Total studies	15	290/1736	301/2226	0	0.541	F	1.19 (0.98-1.44)	0.083	0.255	0.250
	Chinese Han group	7	105/530	124/739	0	0.850	F	1.30 (0.96-1.77)	0.086	0.293	0.188
	Other ethnic groups	8	185/1206	177/1487	27.7	0.207	F	1.11 (0.86-1.43)	0.409	0.711	0.447
DR*09	Total studies	14	344/1703	385/2196	33.3	0.108	F	1.17 (0.98-1.40)	0.075	0.661	0.740
	Chinese Han group	7	157/530	206/739	32.3	0.182	F	1.10 (0.85-1.42)	0.467	0.230	0.241
	Other ethnic groups	7	187/1173	179/1457	40.7	0.120	F	1.25 (0.98-1.59)	0.077	0.764	0.786
DR*10	Total studies	11	62/955	96/1447	0	0.921	F	1.08 (0.76-1.53)	0.682	0.815	0.343
	Chinese Han group	6	9/469	24/707	0	0.871	F	0.68 (0.29-1.49)	0.314	0.039	0.040
	Other ethnic groups	5	51/1206	70/1487	0	0.860	F	1.22 (0.82-1.81)	0.325	0.806	0.687
DR*11	Total studies	14	221/1459	229/1630	41.3	0.053	R	1.03 (0.75-1.42)	0.839	0.477	0.915
	Chinese Han group	7	73/530	107/739	59.1	0.023	R	1.08 (0.59-1.97)	0.806	0.652	0.848
	Other ethnic groups	7	148/929	122/891	20.0	0.277	F	1.00 (0.74-1.35)	0.993	0.548	0.953
DR*12	Total studies	12	336/1478	360/1590	30.1	0.152	F	1.08 (0.90-1.31)	0.406	0.945	0.948
	Chinese Han group	6	142/500	173/683	33.7	0.183	F	1.23 (0.93-1.62)	0.149	0.707	0.642
	Other ethnic groups	6	194/978	187/978	27.2	0.231	F	0.98 (0.76-1.26)	0.845	1.000	0.709
DR*13	Total studies	16	69/1779	211/2127	0	0.699	F	0.27 (0.19-0.37)	0.000	0.964	0.473
	Chinese Han group	7	12/531	48/739	0	0.867	F	0.36 (0.18-0.70)	0.003	0.368	0.510
	Other ethnic groups	9	57/1248	163/1388	1.3	0.424	F	0.24 (0.17-0.35)	0.000	0.175	0.017
DR*14	Total studies	14	281/1511	335/739	12.2	0.319	F	1.17 (0.94-1.46)	0.151	0.071	0.073
	Chinese Han group	7	73/530	97/739	0.2	0.422	F	1.11 (0.79-1.57)	0.552	0.176	0.108
	Other ethnic groups	7	208/981	238/1074	29.7	0.201	F	1.22 (0.92-1.62)	0.172	0.022	0.076
DR*15	Total studies	12	248/1230	236/1132	0	0.525	F	0.84 (0.67-1.05)	0.127	0.075	0.052
	Chinese Han group	7	113/530	161/739	13.2	0.329	F	0.92 (0.69-1.22)	0.556	0.293	0.155
	Other ethnic groups	5	135/700	75/393	0	0.717	F	0.73 (0.51-1.04)	0.084	0.221	0.133
DR*16	Total studies	12	41/876	78/1082	22.4	0.224	F	0.71 (0.47-1.06)	0.097	0.170	0.140
	Chinese Han group	7	24/530	59/739	12.7	0.333	F	0.60 (0.42-1.13)	0.137	0.652	0.042
	Other ethnic groups	5	17/346	19/343	44.8	0.124	F	0.76 (0.37-1.54)	0.447	0.221	0.764

M: Model of meta-analysis; F: Fixed-effects model; R: Random-effects model; PH: The *P* value for heterogeneity test; POR: The *P* value for OR test; Pbeggr's: The *P* value for Egger's test; PEgger's: The *P* value for Egger's test; CHB: Chronic hepatitis B; SRC: Spontaneously recovered control; OR: Odds ratio.

a specific range of peptides that can physically bind in a groove on the surface of the HLA molecule[14].

Wide variations have been documented in the frequencies of *HLA-DR* gene polymorphisms which have been most widely investigated in healthy populations and been demonstrated to influence TNF-α expression. The association between *HLA-DR* polymorphisms and outcome of HBV infection has been investigated by several research groups. However, the previous studies have yielded conflicting results, and included no more than a few hundred CHB cases, which are too few to assess the genetic effects reliably. Meta-analysis has been recognized as an important tool to precisely assess the effect of the selected genetic polymorphisms on the risk of diseases and to identify potentially important sources of between-study heterogeneity. In our present meta-analysis, a total of 2609 patients with CHB and 2606 controls spontane-

ously recovering prior to HBV infection were included from 19 case-control studies which were evaluated using the NOS. It could provide the most comprehensive assessment to draw reliable conclusions.

Heterogeneity is a potential problem when interpreting the results of all meta-analyses, and finding the sources of heterogeneity is one of the most important goals of meta-analysis[46]. In this present meta-analysis, we assessed the between-study heterogeneity by different methods, including the χ^2 based *Q* statistic test (Cochran's *Q* statistic)[28] (to test for heterogeneity) and the I^2 statistic (to quantify the between-study heterogeneity)[29]. For the meta-analyses comparing persistent HBV infection cases with spontaneously recovering controls, there was significant between-study heterogeneity in pooled meta-analyses of total eligible studies on *HLA-DR*11* allele, which suggested obvious consistency of effects across those

included studies. Subgroup analyses by ethnicity showed that the heterogeneity was still significant in subgroup analyses in Chinese Han populations for *HLA-DR*11* polymorphism. Univariate analysis of meta-regression suggested that the publishing year and language were not important causes of between-study heterogeneity in both general analyses and subgroup analyses. We presumed that the quality of the primary studies would be the main cause of heterogeneity. Interesting, we found no significant between-study heterogeneity in pooled meta-analyses of total eligible studies and subgroup analyses by ethnicity for other alleles.

Some limitations still exist in this meta-analysis. First, meta-analysis essentially remains with observational study that was subject to the methodological deficiencies of the included studies. Since only published studies written in English and Chinese were included in the meta-analysis, publication bias may occur, even though it was not found by statistical tests. Second, the associations were investigated in all kinds of cases (asymptomatic carriers, patients with CHB, patients with liver cirrhosis), and there may be specific genetic effects among these cases, but we could not obtain enough information to further estimate these effects. It is necessary to conduct large trials using standardized unbiased methods on homogeneous CHB patients and well matched controls, with the assessors blinded to the data. Third, our results were based on unadjusted estimates. A more precise analysis should be conducted with individual data, which would allow the adjustment by other co-varieties including age, ethnicity, family history, environmental factors and lifestyle. Finally, gene-gene and gene-environment interactions were not addressed in this meta-analysis due to the lack of sufficient data. For instance, the major genotypes of HBV in Chinese are B and C, but most of the studies did not analyze them separately, which could not be solved because of the methodological limitations of the meta-analysis.

Despite these limitations, this meta-analysis suggests that *HLA-DR*04* and *DR*13* alleles may be the protective factors for HBV clearance, and *HLA-DR*03* and *DR*07* alleles may be the risk factors for HBV persistence. For the *HLA-DR*01* polymorphism, a significantly association with HBV clearance was found in Chinese Han group, but not found in other ethnic groups. In summary, ethnicity may play an important role in HBV infection outcome, leading to conflicting results. More studies on individuals from various ethnic groups and large and carefully designed case-control studies will be necessary to determine the role of *HLA-DR* polymorphisms in the outcome of HBV infection.

COMMENTS

Background
Chronic hepatitis B virus (HBV) infection is a serious public health problem worldwide. Host genetic factors play a role in determining both susceptibility to HBV and the outcome of the infection. A large number of studies on the association between human leukocyte antigens (*HLA*)-*DR* gene polymorphisms and the risk of chronic hepatitis B (CHB) have been conducted, but their conclusions are different or even contradictory.

Research frontiers
The polymorphic HLA antigens play an important role in immune surveillance and immune response. However, the relationships HLA-DR polymorphisms and the outcomes of HBV infection are not universal for all the investigated populations and no meta-analysis has been conducted.

Innovations and breakthroughs
This meta-analysis systemically assessed the associations of *HLA-DR* gene polymorphisms with the outcomes of HBV infections, and concluded that *HLA-DR*03*, *DR*04*, *DR*07* and *DR*13* alleles have significant associations with HBV clearance in both Chinese Han population and other ethnic groups.

Applications
The results of meta-analysis in this study show that *HLA-DR*04* and *DR*13* alleles may be the protective factors for HBV clearance, while *HLA-DR*03* and *DR*07* alleles may be the risk factors for HBV persistence, which may benefit early prevention and treatment of CHB.

Peer review
This meta-analysis of *HLA-DR* gene polymorphisms and HBV infection outcomes is well written, addressed an important in the field. However, there are still some problems.

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BRIEF ARTICLE

Knockdown of liver-intestine cadherin decreases BGC823 cell invasiveness and metastasis *in vivo*

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Abstract

AIM: To assess BGC823 gastric cancer (GC) cell metastasis after knockdown of liver-intestine cadherin (CDH17) and the therapeutic value of CDH17-RNAi-lentivirus *in vivo*.

METHODS: We evaluated primary tumor growth and assessed local infiltration and systemic tumor dissemination using an orthotopic implantation technique. The therapeutic value of CDH17 knockdown was examined by intratumoral administration of CDH17-RNA interference (RNAi)-lentivirus in an established GC tumor xenograft mouse model. Furthermore, a comparative proteomic approach was utilized to identify differentially expressed proteins in BGC823 and lenti-CDH17-miR-neg cells following CDH17 knockdown.

RESULTS: Metastases in the liver and lung appeared earlier and more frequently in animals with tumors derived from BGC823 or lenti-CDH17-miR-neg cells than in tumors derived from lenti-CDH17-miR-B cells. Average tumor weight and volume in the CDH17-RNAi-lentivirus-treated group were significantly lower than those in the control group (tumor volume: 0.89 ± 0.04 cm^3 *vs* 1.16 ± 0.06 cm^3, $P < 0.05$; tumor weight: 1.15 ± 0.58 g *vs* 2.09 ± 0.08 g, $P < 0.05$). Fifteen differentially expressed proteins were identified after CDH17 silencing in BGC823 cells, including a variety of cytoskeletal and chaperone proteins as well as proteins involved in metabolism, immunity/defense, cell proliferation and differentiation, cell cycle, and signal transduction.

CONCLUSION: Our data establish a foundation for future studies of the comprehensive protein expression patterns and effects of CDH17 in GC.

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INTRODUCTION

Gastric cancer (GC) is one of the most prevalent and lethal malignancies worldwide owing to difficulties in early detection and high postsurgical recurrence rates[1]. Currently, there are no effective drugs for curing GC. Gastro-carcinogenesis is a complex process in which the altered proliferation and migration properties of cancer cells play important roles in tumor growth and metastasis[2]. Identi-

fication of specific markers for predicting recurrence will permit detection of early dissemination and perhaps even eradication.

Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell adhesion and that are strongly implicated in tumorigenesis[3-5]. Liver-intestine cadherin (CDH17) has been identified as a novel member of the cadherin superfamily, which is distinguished from classic cadherins by unique structural and functional features[6,7]. Previous studies have demonstrated the clinicopathological significance and prognostic influence of CDH17 expression in GC. For example, CDH17 has been proposed as a marker of gastric metaplasia and neoplasia[8-11]. In our earlier study, we found that downregulation of CDH17 inhibits proliferation and adherence of the poorly differentiated GC cells, BGC823, *in vitro* and induces cell cycle arrest[12]. Using an *in vivo* tumor growth assay with human tumor xenografts grown subcutaneously in nude mice, we also confirmed that CDH17 silencing slows the growth of GC derived from BGC823 cells. Although human tumor xenografts, which grow subcutaneously in nude mice, closely resemble the original tumors morphologically, biologically, and biochemically, these tumors do not metastasize[13]. Orthotopic tumor models are regarded as more suitable for mimicking human tumor disease because they encompass the entire process of primary tumor growth, local tumor infiltration, and subsequent distant metastasis[14,15]. Thus, in our current study, we evaluated primary tumor growth and assessed local infiltration and tumor dissemination using an orthotopic implantation technique.

The therapeutic value of lentivirus and adeno-associated virus has been identified using RNA interference (RNAi) technology[16-19]. Gene therapy for GC is a rationalized strategy because various genes are associated with this disease[20]. Previous research has proposed CDH17 as an *in vivo* target for liver cancer therapy[21]. In our current research, we further examined the therapeutic value of CDH17-RNAi-lentivirus in established GC xenografts in nude mice.

Proteomic technologies have been used to identify proteins involved in various pathways altered in disease[22,23]. Global analysis of protein expression complements and in some cases has certain advantages over genomic analyses. Given the cumulative data indicating possible roles for CDH17 in cancer development and/or outcomes[24-26], the important function of CDH17 in GC has elicited the need to further investigate the underlying mechanism. Thus, we also used 2-dimensional polyacrylamide gel electrophoresis (2-DE) followed by tandem mass spectrometry (MS) to perform proteome-wide profiling of GC cells in which CDH17 expression had been knocked down to identify proteins whose cellular levels are affected by with CDH17 expression.

MATERIALS AND METHODS

Chemicals

Dithiothreitol, urea, agarose, glycerol, bromophenol blue,

3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS), Immobiline DryStrips (4-7 L), Dry-Strip cover fluid, and PhastGel Blue R (Coomassie Brilliant Blue R-350 stain) were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Acrylamide, Bis, tri(hydroxymethyl)aminomethane (Tris), glycine, sodium dodecyl sulfate (SDS), ammonium persulfate, and tetramethylethylenediamine were from Bio-Rad (Hercules, CA, United States). Iodoacetamide, ammonium bicarbonate, and acetic acid were from Sigma (St. Louis, MO, United States). Acetonitrile and methanol were from Fisher (Fair Lawn, NJ, United States). Trifluoroacetic acid was from Merck (Darmstadt, Germany). Other chemicals are domestic products (analytical grade). All buffers were prepared with Milli-Q water.

Cell culture and lentivirus

The poorly differentiated human GC cell line BGC823 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, United States) at 37 ℃ in a humidified incubator containing 5% CO_2.

The stably transfected cell line in which CDH17 had been knocked down by RNAi (lenti-CDH17-miR-B), the empty vector-transfected control cells (lenti-CDH17-miR-neg), and the CDH17-RNAi-lentivirus and green fluorescent protein (GFP)-lentivirus were obtained as described[12]. Stably transfected cells were cultured at 37 ℃ in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 3 mg/mL blasticidin (Invitrogen, Carlsbad, CA, United States) in a 5% CO_2 environment.

Laboratory animals and orthotopic implantation technique

All nude mice in this study were treated following the experimental animal ethics rules of Wuhan University (Wuhan, China). BALB/c-nu mice (5-6 wk old) weighing 18-20 g were obtained from the Central Laboratory of Animal Science at Wuhan University. The animals were housed in laminar-flow cabinets and kept at a constant humidity (50%-70%) and temperature (25-28 ℃) according to standard guidelines under an approved protocol of Wuhan University. Donor nude mice were anesthetized with isoflurane (Abbott, Wiesbaden, Germany) inhalation. Then, 1×10^6 BGC823, lenti-CDH17-miR-neg, or lenti-CDH17-miR-B cells were injected subcutaneously at a single site each animal's flank. The mice were killed at the same time after several weeks when the largest diameter of the subcutaneous tumors had reached a size of 1 cm. The donor tumors were harvested and minced with a scalpel (No. 11) into small (1 mm^3) pieces. To avoid necrotic tissue from the central tumor areas, only macroscopically viable tumor tissue from the outer part of the donor tumors was used for orthotopic implantation.

Thirty-six tumor recipient nude mice were anes-

thetized with an intraperitoneal injection of xylazine hydrochloride (Rompun, 12 mg/kg BW; Bayer, Leverkusen, Germany). Each animal's abdomen was aseptically opened with a midline incision, and the stomach was gently exteriorized. One small tissue pocket was prepared in the submucosa of the distal stomach. One donor tumor fragment was placed into the gastric tissue pocket and fixed with one drop of tissue adhesive (Histoacryl, B Braun, Tuttlingen, Germany). The stomach was relocated into the abdominal cavity, which was then closed with two layers of 4-0 absorbable sutures (Ethicon, Norderstedt, Germany).

Observation period, assessment of primary tumor size, and dissemination

At 4 and 6 wk after transplantation, animals with non transfected BGC823 cells, empty vector-transfected cells (lenti-CDH17-miR-neg), and CDH17 microRNA-transfected cells (lenti-CDH17-miR-B) ($n = 6$ per cell line) were sacrificed, and an autopsy was done to examine tumor growth. The perpendicular diameters of the primary orthotopic tumors were measured with calipers, and the volume was calculated using the following formula: volume = (shortest diameter)^2 × (longest diameter)/2. Local infiltration was determined in the liver, lung, spleen, and diaphragmatic muscle. Parts of the tumor tissue and the organ were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Serial sections were cut at 3 μm thickness, stained with hematoxylin and eosin, and reviewed to confirm the findings of the macroscopic dissemination as described previously[11].

Treatment of GC with CDH17-RNAi-lentivirus in established GC xenografts in nude mice

To investigate whether CDH17-RNAi-lentivirus could serve as a therapeutic agent against GC, subcutaneous tumors were induced in nude mice using BGC823 cells as above. Once tumors reached approximately 0.5-0.6 cm^3, animals in the treated group were intratumorally injected with 1×10^9 copies of CDH17-RNAi-lentivirus, and those in the control group were treated with 1×10^9 copies of GFP-lentivirus, twice weekly for 2 wk. Tumor formation and growth were monitored daily. Tumors were harvested from mice 1 wk after the end of treatment and evaluated with hematoxylin and eosin. CDH17 expression was examined by immunohistochemistry[11].

2-DE and MS

In agreement with our previous research[12], we found no differences between BGC823 and lenti-CDH17-miR-neg cells with respect to proliferation, adherence, or invasive ability in our current study. Therefore, we compared the protein expression between BGC823 and lenti-CDH17-miR-B cells in our current study. Briefly, cells were suspended in 1 mL lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 65 mmol/L dithiothreitol, 2% IPG buffer (pH 4-7), and 1 mmol/L phenylmethylsulfonyl fluoride. After extraction, the protein concentration was quantified using Bradford's method. Five samples per

cell strain were prepared from individual cell cultures. All samples were stored at -80 ℃ prior to electrophoresis.

For each sample, 1 mg protein was mixed with a rehydration solution containing 8 mol/L urea, 2% CHAPS, 0.5% IPG buffer, pH 4-7 (nonlinear), 18 mmol/L dithiothreitol, and a trace of bromophenol blue, to a total volume of 250 μL, and applied to IPG dry strips. After rehydration for 12 h, isoelectric focusing was performed using ReadyStripTM IPG strips (17 cm, PH 3-10 NL, Bio-Rad) on a PROTEAN IEF system (Bio-Rad) until a total of 80 kVh was reached. Following isoelectric focusing separation, the gel strips were equilibrated two times for 15 min each in equilibration buffer (50 mmol/L Tris-HCl, pH 8.0, 6 mol/L urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue) and directly applied to a 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresi (SDS-PAGE) vertical slab gel for electrophoresis using a PROTEAN Ⅱ xi Cell system (Bio-Rad) until the bromophenol blue dye marker reached the bottom of the gel. Dithiothreitol (1%) was added to the first equilibration buffer and was replaced with 2.5% Iodoacetamide in the second equilibration buffer. The gels were visualized with Coomassie Blue G250 (Bio-Rad) staining after electrophoresis. Spot detection, quantification, and matching were performed using PDQuest Advanced 8.0.1 software (Bio-Rad). Five analytical gels for each cell strain, resulting from triplicate runs of individual samples, were completed. Quantitative analysis was performed using the Student's *t*-test between BGC823 and lenti-CDH17-miR-B gels. The confidence level was 95%.

In-gel digestion and MS analysis

The separated proteins in SDS-PAGE gels were excised, in-gel digested, and extracted according to the manufacturer's instructions (Promega, Madison, WI, United States). Briefly, protein spots of interest were destained with 100 μL 50% v/v acetonitrile in 25 mmol/L ammonium bicarbonate for 1 h. In-gel digestion was performed with 10 ng/μL trypsin (Promega) in 40 mmol/L ammonium bicarbonate for 15 h at 37 ℃. The resulting peptides were extracted with 5% trifluoroacetic acid in 50% acetonitrile for 1 h at 37 ℃ and dried completely with centrifugal lyophilization.

Peptide mixtures were redissolved in 0.5% trifluoroacetic acid, and 1.5 μL of the peptide elute was mixed with an identical volume of α-cyano-4-hydroxycinnamic acid matrix on the stainless matrix-assisted laser desorption ionization (MALDI) target until dry (i.e., air dried). All mass spectra were obtained on an Autoflex MALDI-time of flight (TOF) mass spectrometer (Bruker, Bremen, Germany) to generate peptide mass fingerprints. The analyses of peptides were performed in the reflectron mode with a 337-nm nitrogen laser with an acceleration voltage of 20 kV and a reflected voltage of 23 kV. Spectra were accumulated until a satisfactory signal/noise ratio had been obtained from a range of 800 MHz to 4000 MHz. After MS acquisition, 10 ions of maximum intensity were selected automatically for MS/MS analysis. Trypsin autolysis products and keratin-derived precur-

A

B

Figure 1 Tumorigenicity is inhibited by knockdown of liver-intestine cadherin using Lenti-microRNA in BGC823 cells. A: Knockdown of liver-intestine cadherin (CDH17) in lenti-CDH17-miR-B cells was confirmed by western blotting; B: The primary orthotopic tumor volume in the lenti-CDH17-miR-B group was much lower than that of the BGC823 and lenti-CDH17-miR-neg groups during the observation periods ([a]$P < 0.05$). Data represent the mean ± SD (n = 6). *P*-values for the indicated comparisons were determined with one-way analysis of variance.

sor ions were automatically excluded. The collision voltage was varied depending on the mass of the precursor. MS/MS data were processed with the search algorithm MASCOT (Version 2.2; Matrix Science, London, United Kingdom) against the SWISS-PROT protein database (UniProt_SP sprot_84; 230,133 sequences). Confident identification had a statistically significant ($P \leqslant 0.05$) protein score (based on combined MS spectra) and best ion score (based on MS/MS spectra).

Statistical analysis

All quantitative data were recorded as the mean ± SD. Comparisons between two groups were performed with the Student's *t*-test. Comparisons among multiple groups were performed with one-way analysis of variance. $P < 0.05$ was considered statistically significant. Computations were performed using the SPSS version 13.0 software package (Chicago, IL, United States).

RESULTS

CDH17 knockdown decreases the invasive and metastatic ability of BGC823 cells in nude mice with orthotopic implanted GC tumors

Downregulation of CDH17 in stably transfected lenti-CDH17-miR-B cells was confirmed by Western blotting (Figure 1A). We found that subcutaneous tumors reached a size of 1 cm after approximately 4 wk in BGC823- and

lenti-CDH17-miR-neg-treated groups, whereas 5 wk was required after subcutaneous injection with lenti-CDH17-miR-B cells, indicating that proliferation of GC cells was inhibited after CDH17 knockdown. These results were consistent with our previous report[12].

To demonstrate the progress of tumor growth, the animals were randomized into two groups and killed at 4 or 6 wk after tumor implantation. We found that the primary orthotopic tumor volume in the lenti-CDH17-miR-B group was much smaller than that of the BGC823 and lenti-CDH17-miR-neg groups (Figures 1B, 2A and B) ($P <$ 0.05). In addition, nude mice in the lenti-CDH17-miR-neg and BGC823 groups developed liver and lung metastases after 4 wk (lung 1/6, liver 1/6, in the lenti-CDH17-miR-neg group; lung 1/6, liver 1/6, in the BGC823 group), whereas neither type of metastasis was found in the lenti-CDH17-miR-B group at that time. Moreover, 6 wk after transplantation, we found that mice transplanted with lenti-CDH17-miR-B cells developed fewer metastases (lung 1/6, liver 1/6) than animals in the BGC823 group (lung 2/6, liver 3/6) and animals in the lenti-CDH17-miR-neg group (lung 2/6, liver 3/6) (Figure 2C-F). In addition, nude mice injected with BGC823 or lenti-CDH17-miR-neg cells also developed diaphragmatic muscle (1/6) (Figure 2G) and spleen (1/6) (Figure 2H) infiltration, neither of which was found in the lenti-CDH17-miR-B group. These results indicated that the cell line lenti-CDH17-miR-B displayed a less aggressive phenotype regarding tumor dissemination than the other two cell lines and suggested that CDH17 knockdown in GC cells downregulated the invasive and metastatic ability *in vivo*.

Intratumoral administration of CDH17-RNAi-lentivirus causes significant tumor regression in the established GC tumor xenograft mouse model

All 12 mice that were injected subcutaneously with 1×10^6 BGC823 cells developed detectable tumors at the termination of the experiment. The mice treated with CDH17-RNAi-lentivirus showed significantly suppressed tumor growth compared with those treated with GFP-lentivirus (Figure 3A-F). The average tumor volume (0.89 ± 0.04 cm^3) in the former group was significantly lower ($P < 0.05$) than that (1.16 ± 0.06 cm^3) in the latter group. The average tumor weight (1.15 ± 0.58 g) was also much lower ($P < 0.05$) than that in the control group (2.09 ± 0.08 g). Furthermore, we found that intratumoral injection of CDH17-RNAi-lentivirus reduced CDH17 levels (Figure 3G-H). These results indicated that CDH17 knockdown may be an effective means of slowing GC tumor growth *in vivo*.

Quantitative proteome alterations between lenti-miR-CDH17-B and BGC823 cells

Proteins extracted from lenti-CDH17-miR-B or BGC823 cells were subjected to 2-DE and stained with Coomassie, which revealed a match rate of 80.5% and showed an average of 1032 ± 31 and 1279 ± 2 spots, respectively (Figure 4A and B). A total of 26 differentially expressed

Figure 2 Tumors in which liver-intestine cadherin was knocked down show fewer invasive characteristics *in vivo*. Hematoxylin-eosin staining revealed that primary orthotopic tumors (A, B) derived from BGC823 or lenti-liver-intestine cadherin (CDH17)-miR-neg cells spread into the liver (C, D) and lung (E, F) more frequently than those derived from lenti-CDH17-miR-B cells. Nude mice injected with these two types of tumors also showed invasion of the diaphragmatic muscle (G) and spleen (H).

protein spots were defined as statistically significant based on two criteria: (1) intensity alterations > 3-fold; and (2) recurrence four or more times in the five pairs examined (Figure 4C). To eliminate the redundancy of proteins appearing in the database under different names or accession numbers, the protein member belonging to *Homo sapiens* with the highest MASCOT score was selected[27]. Fifteen different proteins from 2-DE gels were successfully identified using MALDI-TOF tandem MS (Table 1). Several protein spots could not be identified, possibly owing to

Table 1 Altered proteins in lenti-liver-intestine cadherin-miR-B compared with BGC823 cells

Protein description	Gene name	Accession no.[1]	MW/pI (theo)[1]	Sequence coverage[2] (%)	Mascot score[3]	MS/MS peptide sequence[4]	Cellular location
Clathrin light chain A	CLCA	P09496	27 174/4.43	15	59	K.AIKELEEWYAR.Q	Cytoplasmic vesicle membrane; peripheral membrane; cytoplasm
Nucleophosmin	NPM	Q96AT6	32 726/4.64	23	56	K.MSVQPTVSLGGFEITPPVVLR.L	Nucleus; nucleoplasm
Tropomyosin α-3 chain	TPM3	P06753	32 856/4.68	7	56	R.KLVIIEGDLER.T	Cytoplasm; cytoskeleton
Tubulin beta-2C chain	TBB2C	P68371	50 255/4.79	19	61	R.AVLVDLEPGTMDSVR.S	Cytoplasm
Glutathione S-transferase P	GSTP1	P09211	23 569/5.43	43	298	M.PPYTVVYFPVR.G	Nucleus; cytoplasm
Activator of 90 kDa heat shock protein ATPase homolog 1	AHSA1	O95433	38 421/5.41	30	143	K.ETFLTSPEELYR.V	Cytoplasm; endoplasmic reticulum
Interleukin-1 α	IL1A	P01583	30 758/5.04	23	64	K.LTFKESMVVVATNGK.V	Secreted
Heat shock 70 kDa protein 1A/1B	HSP71	P08107	70 294/5.48	27	239	K.DAGVIAGLNVLR.I	Cytoplasm
78 kDa glucose-regulated protein	GRP78	P11021	72 402/5.07	18	100	R.ITPSYVAFTPEGER.L	Endoplasmic reticulum lumen; melanosome
Actin, cytoplasmic 1	ACTB	Q96HG5	42 052/5.29	36	210	R.AVFPSIVGRPR.H	Cytoplasm; cytoskeleton
Pyruvate kinase isozymes M1/M2	KPYM	Q9BWB5	58 470/7.96	30	161	R.LDIDSPPITAR.N	Cytoplasm; nucleus
Mitochondrial 28S ribosomal protein S22	RT22	P82650	41 425/7.70	21	131	K.YVFTDISYSIPHR.E	Mitochondria
NADH dehydrogenase (ubiquinone) iron-sulfur protein 2, mitochondrial	NDUS2	O75306	52 911/7.21	25	86	K.TYLQALPYFDR.L	Mitochondrial inner membrane; peripheral membrane; matrix side
Vinculin	VINC	P18206	124 292/5.50	26	183	R.EAFQPQEPDFPPPPPDLEQLR.L	Cytoplasm; cytoskeleton; cell membrane; peripheral membrane
α-enolase	ENOA	Q6GMP2	47 481/7.01	29	134	R.AAVPSGASTGIYEALELR.D	Cytoplasm; cell membrane; myofibril; sarcomere; m-band

[1]Accession Number. Theoretical molecular weight (MW, kDa) and pI were derived from the SWISS-PROT protein database; [2]Sequence coverage of the matched peptides in the protein; [3]Protein score (based on combined mass and mass/mass spectra) were from MALDI-TOF/TOF identification; [4]Each spot corresponding to one protein had at least one of the shown peptides identified.

Figure 3 Local injection of liver-intestine cadherin-RNA interference-lentivirus suppresses the growth of established tumors in nude mice. A, B: Representative photographs of one mouse in each group 1 wk after the final injection of green fluorescent protein (GFP)-lentivirus or liver-intestine cadherin (CDH17)-RNA interference (RNAi)-lentivirus; C, D: Representative photographs of tumors isolated from one mouse in each group 1 wk after the final injection of GFP-lentivirus or CDH17-RNAi-lentivirus; E, F: Hematoxylin-eosinstaining of xenotransplanted tumors: Ccontrol group (E); CDH17-RNAi-lentivirus-treated group (F); G, H: Immunohistochemistry showed knockdown of CDH17 by intratumoral injection of CDH17-RNAi-lentivirus (SP × 200).

a lower amount of protein as revealed by a retrospective analysis of the spot intensities. Differences between the experimental MW/pI and the theoretical value were observed for some of the proteins, which may have been a consequence of post-translational modifications such as truncation and/or phosphorylation[28].

As listed in Table 1, we found that the altered proteins after CDH17 knockdown were mainly located in the cytoplasm (30.3%), nucleus (12.12%), or peripheral membrane (9.09%) (Figure 4D). In brief, these differentially expressed proteins between lenti-CDH17-miR-B and BGC823 cells play important and fundamental roles in

Figure 4 **Protein profile differences between lenti-liver-intestine cadherin-miR-B and BGC823 cells.** A, B: Comparison of protein profiles by 2-dimensional gel electrophoresis between BGC823 and lenti-liver-intestine cadherin (CDH17)-miR-B cells; C: Close-up image of differential expression of protein spots with intensity alterations > 3-fold and recurrence four or more times in independent experiments. The average intensity of each spot was determined using PDQuest 2-DE software; D: Cellular location of the 15 altered proteins identified by mass spectrometry; E: Molecular functions of the 15 altered proteins identified by mass spectrometry.

DISCUSSION

Using an orthotopic implantation technique, we examined the growth and metastatic potential of BGC823 cells in which CDH17 had been knocked down. Our results showed that BGC823 or lenti-CDH17-miR-neg cells produced more extensive local tumor growth during the observation period. Moreover, local infiltration into the liver and lung appeared much earlier and more frequently in animals with tumors derived from BGC823 or lenti-CDH17-miR-neg cells than in those derived from lenti-CDH17-miR-B cells, suggesting the less aggressive behavior of GC after CDH17 knockdown. However, the effects of CDH17 in gastric carcinogenesis should be examined in other human GC cell lines.

To investigate whether CDH17-RNAi-lentivirus could serve as a therapeutic agent against GC, BALB/c-nu mice with induced tumors of BGC823 cells were treated with CDH17-RNAi-lentivirus. The results showed that both the average tumor weight and volume were significantly lower in the CDH17-RNAi-lentivirus-treated group compared with the control group treated with GFP-lentivirus. Thus, intratumoral administration of CDH17-RNAi-lentivirus, which reduced CDH17 levels, significantly slowed tumor growth. However, our results also demonstrated that CDH17 knockdown did not eliminate the tumor completely, suggesting that CDH17 is not the only protease involved in invasion and growth of GC.

Although the *CDH17* gene was identified as a biomarker or a potential diagnostic and therapeutic target for GC using gene expression profiling[29,30], the molecular mechanisms of CDH17 remain largely undefined due to limited knowledge of CDH17 target recognition. Although 2-DE and MS-based proteomic strategies have certain limitations, these strategies provide high-throughput simultaneous identification of hundreds of proteins and are considered powerful approaches for analyzing alterations in protein expression in complex biological systems[31-34]. Using the 2-DE/MS approach, we identified 15 proteins in BGC823 cells whose expression levels are affected by CDH17.

Among these proteins, six were upregulated, and nine were downregulated after CDH17 knockdown. We found that these 15 proteins play important roles in metabolism, immunity/defense, cell structure and motility, intracellular protein trafficking, protein modification, cell cycle, signal transduction, cell proliferation and differentiation, electron transport, and muscle contraction, suggesting extensive roles for CDH17 in biological processes. These altered proteins may be potentially involved in the effect(s) of CDH17 in BGC823 cells and represent candidate proteins that may be regulated by CDH17. These diverse proteins may thus serve as focused targets for future studies to determine their potential genetic and/or physical interactions with CDH17 and their func-

tional relevance in GC. Although previous studies have demonstrated possible interactions between CDH17 and galectin-3, metal-responsive transcription factor-1, and placental growth factor in ductal adenocarcinoma of the pancreas and GC[11,24,26], these proteins were not identified in our current study. Thus, further validation experiments should be carried out to confirm our results. In-depth and detailed investigation of the interactions between CDH17 and other proteins may enhance our understanding of the mechanisms by which CDH17 affects carcinogenesis in humans. The mechanisms by which downregulation of CDH17 inhibits proliferation and invasion of BGC823 cells need to be explored in further studies.

COMMENTS

Background

Gastrocarcinogenesis is a complex process in which alterations in proliferation and migration properties of cancer cells have an important role in cancer infiltrative growth and metastasis. If specific markers related to cancer invasion were to be identified, dissemination could be detected early and perhaps even eradicated.

Research frontiers

Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell adhesion and have strong implications in tumorigenesis. Liver-intestine cadherin (CDH17), also known as LI-cadherin, has been identified as a novel member of the cadherin superfamily, which is distinguished from classic cadherins by its unique structural and functional features. Therefore, the invasive and metastasis ability of gastric cancer (GC) cells after CDH17 silencing were investigated.

Innovations and breakthroughs

Their results presented further evidence that CDH17 was closely correlated to GC invasion. The invasive and metastasis ability of GC cells was found to be significantly reduced *in vivo* after the downregulation of CDH17. Furthermore, protein profiles in GC cells were also significantly altered after CDH17 knockdown, which suggested the interactions of CDH17 with many other important proteins.

Applications

CDH17 could be recognized as an important marker of cancer invasion. Knockdown of CDH17 may become a way to downregulate the invasive ability of GC cells.

Peer review

The authors investigated the metastatic ability of the GC cells BGC823 with CDH17 silencing and the therapeutic value of CDH17-RNA interference-lentivirus. They demonstrated that knockdown of CDH17 in BGC823 cells downregulated invasive and metastatic ability *in vivo*. The work is well written. The methods are adequate and the results obtained justify the conclusions drawn.

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BRIEF ARTICLE

Comparison of total splenic artery embolization and partial splenic embolization for hypersplenism

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Abstract

AIM: To evaluate whether total splenic artery embolization (TSAE) for patients with hypersplenism delivers better long-term outcomes than partial splenic embolization (PSE).

METHODS: Sixty-one patients with hypersplenism eligible for TSAE (n = 27, group A) or PSE (n = 34, group B) were enrolled into the trial, which included clinical and computed tomography follow-up. Data on technical success, length of hospital stay, white blood cell (WBC) and platelet (PLT) counts, splenic volume and complications were collected at 2 wk, 6 mo, and 1, 2, 3, 4 years postoperatively.

RESULTS: Both TSAE and PSE were technically successful in all patients. Complications were significantly fewer (P = 0.001), and hospital stay significantly shorter (P = 0.007), in group A than in group B. Post-procedure WBC and PLT counts in group A were significantly higher than those in group B from 6 mo to 4 years (P = 0.001), and post-procedure residual splenic volume in group A was significantly less than that observed in group B at 1, 2, 3 and 4 years post-procedure (P = 0.001). No significant differences were observed in red blood cell counts and liver function parameters between the two groups following the procedure.

CONCLUSION: Our results indicate that TSAE for patients with hypersplenism not only delivers a better long-term outcome, but is also associated with lower complication rates and a shorter hospital stay than PSE.

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Key words: Embolization; Hypersplenism; Complications; White cell counts; Platelet counts

INTRODUCTION

To date, partial splenic embolization (PSE), which was first performed by Spigos *et al*[1] in 1979, has been considered first-line therapy for hypersplenism in many institutions, and has been proposed as an effective alternative to splenectomy for improving peripheral blood cell counts[2-4]. However, PSE is associated with many com-

plications, including intermittent fever, abdominal pain, nausea, vomiting, postembolization syndrome, splenic abscess, splenic rupture, pneumonia, refractory ascites, pleural effusion and gastrointestinal bleeding[3-6]. To ensure a sustained and long-term increase in platelet (PLT) and leucocyte counts, the splenic infarction rate needs to be greater than 50%[6]. Thus, severe complications can ensue[1-6].

Today, total splenic artery embolization (TSAE) is widely used and has been shown to be a safe and effective method for the treatment of splenic artery aneurysms[7-9]. In addition, stainless steel spring coils are used to embolize the main branch of the splenic artery in order to improve PLT counts pre-splenectomy[10]. We have initiated a new approach, that of TSAE for the treatment of patients with hypersplenism secondary to cirrhosis of the liver, and our preliminary results demonstrate the safety and feasibility of the approach[11]. In order to determine whether this approach can ensure a sustained and long-term increase in PLT and leucocyte counts, as well as a reduction in the rate of severe complications, we undertook this nonrandomized prospective trial of endovascular treatment of hypersplenism with TSAE or PSE, with the aim of comparing clinical outcomes of both methods.

MATERIALS AND METHODS

Patients

The protocol was approved by the institutional ethics committee, and all patients provided written informed consent. Between January 2006 and June 2011, consecutive patients with hypersplenism caused by cirrhosis referred for treatment with TSAE (group A) or PSE (group B) were screened for enrollment into this non-randomized, prospective, two-institution trial. They subsequently underwent computed tomography (CT) follow-up at both hospitals. The diagnosis of hypersplenism was established by review of each patient's medical history, clinical laboratory data, ultrasonography, and CT examinations.

Eligibility criteria included: (1) documented hypersplenism caused by cirrhosis established by a review of the patient's history, CT results or ultrasound; and (2) hepatitis B virus/hepatitis C virus-related active cirrhosis, with neutropenia (neutrophil count $\leqslant 2.0 \times 10^9$/L), thrombocytopenia (PLT count $\leqslant 50 \times 10^9$/L), or both. Patients who had severe jaundice (total serum bilirubin level $\geqslant 81.4$ μmol/L) or spontaneous bacterial peritonitis were excluded.

Total embolization of the main splenic artery

All embolization procedures were performed by two experienced interventional radiologists. Prior to embolization, selective angiography of the celiac trunk, the splenic artery and the superior mesenteric artery was performed in all patients *via* the right femoral artery using a 5-Fr diagnostic catheter. To avoid total splenic infarction, we confirmed patency of the collateral arteries, which were confirmed as connected to the hilar splenic artery from

Figure 1 Hypersplenism in a 76 year old man with hepatitis B virus-related cirrhosis. A: Celiac digital subtraction arteriogram. The splenic artery is markedly dilated and tortuous. The spleen is enlarged. The right gastroepiploic artery arises from the celiac axis; B: Celiac arteriogram after coil embolization of the distal splenic artery and its primary branches. The hilar splenic arterial branches are faintly filled by collateral circulation.

the left gastric artery or from the gastroepiploic artery on a celiac arteriogram (Figure 1).

Once these connections were confirmed, TSAE was performed, using details of the coiling procedure as has been previously described[11]. Coils and gelfoam were used as embolization material, either alone or in combination. In general, the embolization coils used in this series were standard 0.089 cm fibered coils, microcoils (Tornado; Cook Inc., Bloomington, IN, United States). Post-embolization check angiograms were performed using selective splenic artery, celiac and superior mesenteric artery angiograms to confirm occlusion of the main splenic artery and patency of the collateral arteries (Figure 1). Preoperative antibiotic prophylaxis was used routinely for 3 d. Following embolization, patients were monitored clinically, and antibiotics were administered after the procedure for several days in order to avoid infection.

PSE

The embolic agents most commonly used for splenic arterial embolization are gelatin sponge pledgets (Gelfoam cube, Upjohn, Kalamazoo, United States) and polyvinyl alcohol particles (PVP, lvalon, Ingenor, Paris; and Contour, Interventional Therapeutics Corp., South San Francisco, CA, United States). Details of PSE with gelatin sponge pledgets[12] or with PVP[13] have been described previously, and therefore we do not repeat the methodology here.

Figure 2 Computed tomographic angiography, arterial phases, before and after splenic artery embolization in the same patient as Figure 1. A: Before embolization the spleen is enlarged; B: One month after embolization the major portion of the spleen is avascular with minute areas of low attenuation representing residual gas collection. A small part of the spleen was perfused with contrast medium; C: Six months after embolization the low attenuation area has decreased in size; D: One year later the low attenuation area has further decreased in size; E: Two years later the necrotic area has further decreased in size; F: Four years later no significant change is seen.

Follow-up protocol and postoperative outcome evaluation

All patients were followed up at our outpatient clinic. Peripheral blood cell parameters, including white blood cell (WBC), PLT and red blood cell (RBC) counts, were monitored at different time-points prior to the procedure, and at day 3, 14 and 30, and subsequently at 6-monthly intervals for a total of 5 years. To determine any possible effects on liver function, serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), albumin (Alb), as well as the prothrombin time (PT), were measured before and after the procedures at the same time-points. The frequency and type of complications associated with the procedures were recorded.

Abdominal CT scans were routinely performed before and two weeks after the procedure, and subsequently every 6 mo during follow-up (Figure 2). Based on enhanced CT images, we measured and compared the pretreatment splenic volume and the post-embolization residual splenic volume on a 3.1 workstation (GE Medical Systems, Milwaukee, WI, United States) using volumetric analysis software. The infarcted splenic volume (mL) was calculated by subtracting the noninfarcted splenic volume from the pretreatment volume. The splenic infarction rate was calculated by dividing the infarcted splenic volume by the pretreatment volume ($\times 100\%$).

Complications related to the procedures were categorized as either major or minor. A major complication was defined as a complicated disease requiring surgical or interventional treatment or a prolonged postoperative hospital stay of more than 30 d, and included splenic abscess, splenic rupture, pneumonia, refractory ascites, pleural effusions, gastrointestinal bleeding, variceal rupture and liver failure. A minor complication was defined as a complication that had minimal consequences and could be tolerated by patients or treated using conservative methods, including post-embolization syndrome, abdominal fullness, and loss of appetite.

Statistical analysis

We predicted that rates for a WBC count $> 4 \times 10^9/\mathrm{L}$ at the 2-year follow-up assessment would be approximately 95% in group A and 60% in group B. We estimated that at least 25 patients with hypersplenism would need to be enrolled into each group for the study to have a statistical power of 80% with a two-sided alpha level of 0.05. This power was based on the ability to detect an absolute difference of 30% in patients with WBC counts $> 4 \times 10^9/\mathrm{L}$ at the 2-year follow-up assessment.

All data are expressed as mean ± SD. Comparisons of variables between the two groups were performed by applying the Mann-Whitney test, χ^2-test or the Fisher's exact test as appropriate. All statistical analyses were performed using the SPSS package, version 13.0 (SPSS, Chicago, Illinois, United States).

RESULTS

A total of 109 patients with hypersplenism were enrolled. Before each procedure, patients or family members had the right to choose either TSAE or PSE. Initially, 51 and

Table 1 Demographic and clinical characteristics of patients with hypersplenism (mean ± SD)

	Group A (n = 27)	Group B (n = 34)	P value
Age (yr)	48.48 ± 8.87	47.945 ± 7.32	0.445
Female/male	11/15	14/20	0.930
Child-Pugh classification, *n* (%)			0.441
A	19 (70.4)	23 (676)	
B	6 (22.2)	9 (26.5)	
C	2 (7.4)	2 (5.8)	
Virus, *n* (%)			0.463
B	22 (81.5)	25 (73.5)	
C	5 (19.5)	9 (26.5)	
WBC counts × 10^9	1.50 ± 0.32	1.54 ± 0.30	0.453
PLT counts × 10^9	40.33 ± 6.16	39.74 ± 4.63	0.574
Splenic volume (cm^3)	769.93 ± 61.40	745.73 ± 50.09	0.201

WBC: White blood cell; PLT: Platelet.

Table 2 Post-procedural computed tomography results, clinical outcomes, and complications (mean ± SD)

	Group A (n = 27)	Group B (n = 34)	P value
Technical success, *n* (%)	27 (100)	34 (100)	0.999
Hospital stay (d)	8.52 ± 1.91	15.88 ± 6.36	< 0.001
Complications, *n* (%)			0.007
Minor complications			0.005
Post-embolization syndrome	21 (77.8)	34 (100)	
Major complications	0 (0)	7 (20.6)	0.014
Splenic abscess	0	2	
Pleural effusion	0	3	
Ascites	0	2	
CT follow-up (mo)	36.44 ± 12.67	36.00 ± 11.82	0.926
Clinical follow-up (mo)	40.63 ± 12.60	40.14 ± 11.58	0.965
WBC count > 4 × 10^9/L at 2-year follow-up, *n* (%)	27 (100)	21 (62)	< 0.001

WBC: White blood cell; CT: Computed tomography.

58 patients were assigned to group A and group B, respectively. Of these, 48 patients did not meet the inclusion criteria, with a total follow-up of < 2 years in 43 patients, and five patients were lost to follow-up (two in group A and three in group B). The remaining 61 patients were enrolled. Subjects included 36 males and 25 females, with a mean age of 47.92 ± 8.00 years (range: 28-60 years). Demographic features and clinical presentation of included patients are summarized in Table 1.

Primary procedural results

The technical and initial clinical outcomes of the two groups are shown in Table 2. Both TSAE and PSE were technically successful in all patients, with no procedure-related complications. The thirty-day mortality rate was zero.

Post-embolization syndrome, which included abdominal pain, fever and vomiting, occurred in 21 patients (77.8%) in group A and 34 in group B (100%) (*P* = 0.014). Abdominal pain was severe enough to require morphine administration in 15 patients, including two in group

A (7.4%) and 13 in group B (38.2%) (*P* < 0.01). Severe complications, which occurred in seven patients (20.6%) in group B and no patient (0%) in group A, are listed in Table 2. Three patients had pleural effusions, which resolved with thoracocentesis. Two patients with ascites were treated with abdominal paracentesis. Two patients suffered from a persistent high fever and were found to have a splenic abscess on abdominal CT scan. The abscess was drained with a catheter and had resorbed within 2 mo. The length of hospital stay was significantly less in group A, 5-13 d (mean, 8.52 ± 1.91 d), than in group B, 9-37 d (mean, 15.88 ± 6.36 d) (*P* < 0.001).

Changes in peripheral blood cell counts

At the time of writing, all patients in group A had been followed up and assessed at the 2-wk, 6-mo, 1- and 2-year time-points; 35 had been assessed for 3 years and 23 had been assessed for ≥ 4 years, prospectively, with a mean follow-up of 40.36 ± 11.94 mo (range: 24-64 mo). There were no significant differences in the number of patients in each follow-up period between the two groups.

Changes in WBC and PLT counts before the procedures and between 2 wk and 4 years after the procedures are shown in Figure 3A and B. After the procedures, in both groups, WBC and PLT counts increased significantly, peaked at 2 wk, and then gradually fell during the 4-year follow-up period (Figure 3A and B). There were no significant changes in RBC counts either after PSE or during the long-term follow-up period.

Before PSE, there were no significant differences between the two groups with respect to sex, age, splenic volume, Child-Pugh class, and peripheral blood cell counts (Table 1). In both group A and group B, WBC and PLT counts increased significantly and remained higher than the pre-procedure counts from the second week to the fourth year after the procedures (*P* < 0.05, Figure 3A and B). Although there were no significant differences in WBC and PLT counts between the two groups 2 wk after the procedures, both WBC and PLT counts in group A were significantly higher than those observed in group B at 6 mo, 1, 2, 3 and 4 years after the procedures (*P* < 0.05, Figure 3A and B). A WBC count > 4 × 10^9/L at the 2-year follow-up assessment was observed in all 27 (100%) patients in group A and in 21 (62%) patients in group B (*P* < 0.001).

Changes in liver function parameters

In comparison with pre-procedure levels of liver function parameters values, including AST, ALT, TB, Alb and PT, in each of the two groups, there were no significant short- and long-term changes after the procedures.

Changes in splenic volume after embolization

Splenic volume changes before the procedures and between one month and 4 years after the procedures are shown in Figure 3C. All patients in group A were followed up and assessed at 2 wk, 6 mo, 1 and 2 years; 35 were assessed for 3 years and 23 were assessed for ≥ 4 years, prospectively, with a mean follow-up of 40.36 ±

A

B

C

Figure 3 Curves of the white blood cell counts, platelet counts, and splenic volume in cirrhotic patients of group A (total splenic artery embolization) and group B (partial splenic embolization) before embolization and at the different follow-up time intervals. A: Curves of long-term follow-up of white blood cell counts between the two groups; B: Curves of long-term follow-up of platelet counts between the two groups; C: Curves of long-term follow-up of splenic volume between the two groups. All values are expressed as mean ± SD. [a]$P < 0.05$ *vs* pre-embolization within each group; [c]$P < 0.05$ group A *vs* group B at each time point determined with the Mann-Whitney test. WBC: White blood cell; PLT: Platelet.

11.94 mo (range: 24-64 mo). After the procedures, splenic volume decreased significantly, peaked one month after the procedures, and then gradually increased during the 4-year follow-up period (Figure 3C). In groups A and B, splenic volume gradually increased and remained lower than the pre-procedure volume from 1 mo to four years after the procedures ($P < 0.05$, Figure 3C). There were no significant differences in splenic volume between the two groups before embolization, and at 1 and 6 mo post-procedure, but the residual splenic volume in group A was significantly smaller than that observed in group B 1, 2, 3 and 4 years after the procedures ($P < 0.05$, Figure 3C).

DISCUSSION

This nonrandomized trial was designed to compare the clinical outcomes of two endovascular regimens in patients with hypersplenism. The results demonstrate a sustained and long-term increase in WBC and PLT counts in group A, with no major complications, compared with group B. The significant increase in WBC and PLT counts in group A during follow-up appears to be predominantly attributable to the complete occlusion of the main splenic artery.

Hypersplenism is a well-known complication of portal hypertension in cirrhosis, which can result in thrombocytopenia and/or leukocytopenia. There are several possible approaches to management, for example splenectomy[14-16], PSE[1-6], ligation and banding of the splenic artery[17] and percutaneous placement of a narrowed stent into the splenic artery[18]. Surgical splenectomy can eliminate hypersplenism-induced blood cell destruction, but the morbidity of severe complications after splenectomy, including laparoscopic and open splenectomy, still ranges from 9.6% to 26.6%[14,15]. In addition, splenectomy is often associated with an increased long-term risk of septic events[14,15]. Ligation and banding of the splenic artery are also used to treat hypersplenism, but complications such as portal vein thrombosis, sepsis and multi-organ failure may occur with these methods[17]. Although percutaneous placement of a narrowed stent into the splenic artery is a promising new technique for treating hypersplenism[18], it is difficult to ensure a sustained and long-term increase in WBC and PLT counts as the splenic artery is not completely occluded, which cannot provide a sustained splenic infarction rate (> 50%).

Today, PSE is the most commonly used alternative to splenectomy for patients with hypersplenism, with the aim of improving the peripheral blood cell count[2-4], but it is often associated with minor and major complications, such as post-embolization syndrome, splenic abscess, splenic rupture, pneumonia, refractory ascites, pleural effusions and gastrointestinal bleeding[5,6], as reported in our study and in many other series[2-6]. Post-embolization syndrome is the most frequent minor complication, which is usually well-tolerated by patients and controlled with conservative therapy[3]. Major complications, on the other hand, such as splenic abscess, splenic rupture and pleural effusions, require surgical or other interventional treatment. Many reports have demonstrated a close association between the incidence of complications and the splenic infarction rate, and it appears that complications occur more frequently in patients with a splenic embolization > 50%[6,12,19,20]. In this study, the embolized splenic volume rate was the same for both groups, but we observed that both minor and major complications were sig-

nificantly less in group A than in group B post-procedure ($P < 0.05$). Moreover, no major complications occurred. Therefore, we do not believe that there is a close relationship between the rate of complications and splenic infarction rates in patients with hypersplenism treated with TSAE. This may be explained by the collateral arteries, which connect to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery, and provide a small amount of blood to the spleen, thereby avoiding complete splenic infarction.

In this study, both WBC and PLT counts in group A were significantly higher than those observed in group B at 6 mo, 1, 2, 3 and 4 years post-procedures. Although WBC and PLT counts in both groups decreased with time, the counts in group A revealed a sustained and long-term increase above normal levels and decreased very slowly, whereas those in group B decreased more quickly. The relatively high WBC and PLT counts in group A were mainly attributable to differences in treatment behavior, which may provide one explanation. As PSE embolizes the peripheral artery in the spleen, leaving the main splenic artery open, it is relatively easy for relapse to occur with resumption of blood flow, which leads to the residual splenic volume increasing relatively quickly. On the contrary, when the main splenic artery is completely embolized, the blood flow supply to the spleen from the splenic artery is stopped, with only a small amount of blood supplied from collateral arteries. This ensures that the residual splenic volume increases very slowly. Our study demonstrated that the residual splenic volume in group A increased very slowly and was significantly smaller than that observed in group B ($P < 0.05$), whereas the residual splenic volume in group B increased much more quickly.

The study has the following limitations. Firstly, a major weakness remains the lack of randomization, and the patient population was relatively small, which restricted our ability to detect possible differences between the two groups. As it was a preliminary prospective clinical study, and many patients were unwilling to choose TSAE at the outset, it was difficult to randomly select patients. Moreover, the two approaches varied with regard to different pathologic changes. The pathologic changes developed in distinctive patterns during follow-ups and were not affected by individual or group. Furthermore, our results revealed a sustained and long-term increase in WBC and PLT counts in group A compared with group B, for all follow-up time-points. Therefore, we believe that the non-randomized allocation of patients into two groups did not affect the results. Secondly, severe complications may occur when collateral arteries are not present or patent. Confirmation of the patency of the collateral arteries, which were connected to the hilar splenic artery from the left gastric artery or from the gastroepiploic artery, was a necessary step prior to TSAE. Lastly, the clinical indications for splenic embolization can be bleeding from varices or hypersplenism, and therefore great attention needs to be directed at these patients after embolization in order to prevent bleeding.

In conclusion, the results of this nonrandomized, prospective trial indicate that TSAE for patients with hypersplenism not only delivers better long-term outcomes but is also associated with lower rates of complications and a shorter hospital stay than PSE. Further clinical trials and longer-term follow-up studies are needed.

COMMENTS

Background
Partial splenic embolization (PSE) has been proposed as an effective alternative to splenectomy to improve peripheral blood cell counts. However, PSE is often associated with complications such as intermittent fever, abdominal pain, nausea and vomiting.

Research frontiers
Total splenic artery embolization (TSAE) has been widely used for the treatment of splenic artery aneurysms, but treatment of patients with hypersplenism with TSAE has rarely been reported. The authors present the comparative clinical outcomes of TSAE and PSE in patients with hypersplenism.

Innovations and breakthroughs
The use of TSAE for the treatment of hypersplenism was devised for the management of patients with thrombocytopenia or leukocytopenia associated with cirrhosis of the liver. All procedures were performed under fluoroscopic control. This is the first study comparing TSAE and PSE in patients with hypersplenism.

Applications
TSAE is a safe and feasible procedure and may serve as supplemental treatment for hypersplenism with thrombocytopenia or leukocytopenia accompanying cirrhosis of the liver, as a result of low complication rates and good medium-term clinical efficacy.

Peer review
The authors present a nonrandomized prospective trial of the endovascular treatment of hypersplenism with TSAE or PSE, with the aim of comparing the clinical outcomes of these two treatment approaches. The results reveal that treatment with TSAE ensured a sustained and long-term increase in white blood cell and platelet counts, as well as lower complication rates and a shorter hospital stay compared to PSE. In addition, post-procedure residual splenic volume in patients treated with TSAE was significantly smaller than that of PSE during follow-up. These results suggest an alternative approach for the management of patients with hypersplenism.

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BRIEF ARTICLE

Gastric mucosal damage in water immersion stress: Mechanism and prevention with GHRP-6

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Abstract

AIM: To investigate the mechanism of gastric mucosal demage induced by water immersion restraint stress (WRS) and its prevention by growth hormone releasing peptide-6 (GHRP-6).

METHODS: Male Wistar rats were subjected to conscious or unconscious (anesthetized) WRS, simple restraint (SR), free swimming (FS), non-water fluid immersion, immersion without water contact, or rats were placed in a cage surrounded by sand. To explore the sensitivity structures that influence the stress reaction besides skin stimuli, a group the rats had their eyes occluded. Cervical bilateral trunk vagotomy or atropine injection was performed in some rats to assess the parasympathetic role in mucosal damage. Gastric mucosal lesions, acid output and heart rate variability were measured. Plasma renin, en-

dothelin-1 and thromboxane B2 and gastric heat shock protein 70 were also assayed. GHRP-6 was injected [intraperitoneal (IP) or intracerebroventricular (ICV)] 2 h before the onset of stress to observe its potential prevention of the mucosal lesion.

RESULTS: WRS for 6 h induced serious gastric mucosal lesion [lesion area, WRS 81.8 ± 6.4 mm^2 *vs* normal control 0.0 ± 0.0 mm^2, $P < 0.01$], decreased the heart rate, and increased the heart rate variability and gastric acid secretion, suggesting an increase in vagal nerve-carrying stimuli. The mucosal injury was inversely correlated with water temperature (lesion area, WRS at 35 ℃ 56.4 ± 5.2 mm^2 *vs* WRS at 23 ℃ 81.8 ± 6.4 mm^2, $P < 0.01$) and was consciousness-dependent. The injury could not be prevented by eye occlusion, but could be prevented by avoiding contact of the rat body with the water by dressing it in an impermeable plastic suit. When water was replaced by vegetable oil or liquid paraffin, there were gastric lesions in the same grade of water immersion. When rat were placed in a cage surrounded by sand, there were no gastric lesions. All these data point to a remarkable importance of cutenuous information transmitted to the high neural center that by vagal nerves reaching the gastric mucosa. FS alone also induced serious gastric injury, but SR could not induce gastric injury. Bilateral vagotomy or atropine prevented the WRS-induced mucosal lesion, indicating that increased outflow from the vagal center is a decisive factor in WRS-induced gastric injury. The mucosal lesions were prevented by prior injection of GHRP-6 *via* IP did, but not *via* ICV, suggesting that the protection is peripheral, although a sudden injection is not equivalent to a physiological release and uptake, which eventually may affect the vagal center.

CONCLUSION: From the central nervous system, vagal nerves carry the cutaneous stimuli brought about by the immersion restraint, an experimental model for inducing acute gastric erosions. GHRP-6 prevents the occurrence of these lesions.

INTRODUCTION

Stress is an adaptive physiological response to disruption of homeostasis. Serious stress can induce organ injury or contribute to diseases, such as gastric ulcers, hypertension, diabetes, and cancer. The stomach is one of the main targets of stress. Stress-induced gastric ulceration is a typical example of stress-associated organ injuries[1]. Water immersion restraint stress (WRS) mimics the clinical acute gastric ulcerations caused by trauma, surgery, or sepsis[2] and has been widely accepted for studying stress ulceration[3]. It is theoretically and clinically significant to demonstrate the mechanism of stress-induced gastric injury and develop respective therapeutic drugs.

Both psychological and physiological responses occur during stress and these are involved in the pathogenesis of gastric ulceration. The psychological responses include anxiety, depression, feeling of helplessness, fear, threat of drowning, *etc*. The physiological responses include neurohormonal and immunological activations, including the involvement of corticotropin-releasing factor. These two systems may interact during stressful challenges[4,5], known as psychosomatic reactions. Nonetheless, the mechanisms of gastric stress ulceration remain unclear.

Developing protective drugs against gastric stress ulceration is an important clinical issue. Based on previous studies, agonizing the growth hormone secretagog receptor (GHSR) might be a strategy. Growth hormone releasing peptides (GHRP) are peptidyl growth hormone secretagogs (GHS) and are the synthetic ligands for the GHSR. The family members of GHRP include GHRP-1, GHRP-2, GHRP-6 and hexarelin[6]. GHSR, and its natural ligand ghrelin, are widespread in many tissues, including the gastrointestinal tract[7] and cardiovascular system[8]. Although the GH-releasing actions of both the natural and synthetic GHS have been demonstrated in different species, the function of GHS on alternative physiological systems has not been clearly elucidated. Studies over the past two decades have demonstrated that GHS exerts its physiological or pharmacological actions *via* GH-independent pathways, except for its GH-dependent action[9]. In the cardiovascular system, GHRP and ghrelin exert protective effects, especially on myocardial infarction[10] and heart failure[8,11,12]. Ghrelin and GHSR are expressed in the rat and human stomach and may have significant physiological/pharmacological effects on gastric function and diseases[13,14]. Ghrelin exerts a potent protective action on the stomach of rats exposed to WRS[15]. However, whether or not GHRP also protects against stress-induced gastric injury is unknown. GHRP are much smaller in molecular weight, effective when administered orally, more stable and economically cheaper than ghrelin, and with minimal toxicity, they are better prospects for developing drugs for gastric protection. The purpose of the study was to further investigate the mechanism of gastric stress ulceration using the WRS rat as a model and observe the potential protective effect of GHRP-6 on this gastric injury.

MATERIALS AND METHODS

Stress procedures and animal grouping

A 78 4-mo old male Wistar rats of 310 ± 10 g, were involved in the study. Before the experiment, each animal was housed in a single cage that had wire-net bottoms to avoid coprophagy and had free access to tap water and regular chow for at least 7 d. All animals were starved for 24 h before the onset of stress, but had free access to tap water. Animals were conscious during the stress procedures except those in the "WRS + anesthesia" group (described below), in which rats were anesthetized with 50 mg/kg of sodium pentothal intraperitoneal (IP) during the whole 6-h stress procedures. The water temperature was set to 23 ± 0.5 ℃, except in the WRS group, in which three water temperatures were tested (see below).

The animals were randomly divided into 11 groups (n = 6 in each group/treatment): (1) WRS: rats were lightly anesthetized by ether inhalation and four limbs of each rat were restrained on a wooden plate (25 cm × 19 cm), with the upper limbs anchored at a horizontal position and the lower limbs extended downward. After awakening (usually 10-15 min after ether anesthesia), rats anchored on the wooden plates were immersed vertically (head up) in water to the level of xiphoid process in a water bath thermostatically controlled at 23 ± 0.5 ℃, 19 ± 0.5 ℃ or 35 ± 0.5 ℃, with or without constant pentothal anesthesia, respectively (each n = 6). Anesthesia was achieved with 50 mg/kg of sodium pentothal IP over the whole 6-h stress procedure; (2) simple restraint (SR): the procedure was the same as in the WRS group except that the water bath was empty; (3) free swimming (FS): rats were put into water (water depth 7 cm to avoid drowning) and allowed free movement in the water for 6 h; (4) shallow water touch: rats were put into a water both (water

depth 1 cm) and kept for free moving in the water for 6 h; (5) WRS + eye occlusion: animals were eye-occluded with adhesive plasters and then underwent the WRS procedures, in an attempt to determine whether vision plays a role in the development of stress ulcers; (6) immersion without water contact (NWCI): water immersion with the rat body into a plastic bag to avoid water contact but the rat could see the surrounding water; (7) non-water fluid immersion: the procedure was the same as WRS except that water was replaced by salad oil or liquid paraffin (each $n = 6$), in an attempt to elucidate if skin sensation can differentiate different liquids and induce different gastric responses; (8) "burial" in sand: the restrained rat was placed in a box, the space between the box wall and the rat body was filled with fine sand, the level of filling sand was also to the xiphoid process. To avoid compression of the body, pieces of spongeous material were introduced into the sand; (9) WRS + vagotomy and WRS + atropine: rats underwent bilateral vagal nerve trunk cutting and then underwent WRS. Additional 6 WRS rats (without vagotomy) received atropine (1 mg/kg) IP injection 10 min before the onset of WRS; (10) WRS + GHRP-6: the rat received GHRP-6 (100 μg/kg) (ProSpec-Tany, Israel) IP or intracerebroventricular (ICV) injection 2 h before the WRS procedure. For IP injection, GHRP-6 was dissolved in saline, with a total volume of 0.25 mL per injection; for ICV injection, GHRP was diluted in artificial cerebrospinal fluid, the volume and dosage of GHRP per injection were 5 μL and 20 μg/kg; and (11) normal control: rats were not submitted to any stress procedure. Animals without GHRP-6 IP injection received same volume (0.25 mL) of saline injection (placebo).

The animal use protocol was approved by the Life Ethics Committee of Peking Union Medical College and was conducted in compliance with the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85-23).

Evaluation of gastric mucosal lesion

After the stress procedures, animals were released from the plate and were decapitated under pentobarbital anesthesia. The stomachs were then harvested and opened along the lesser curvature. The severity of mucosal lesions was grossly inspected and digitally photographed. Gastric tissues were then fixed in 10% formalin, dehydrated and imbedded in paraffin wax. Paraffin sections of 5 μm were cut and stained with hematoxylin and eosin. Histological changes were checked under a microscope. The length and width of each lesion, including epithelial cell damage, glandular disruption, vasocongestion, hemorrhage and deep necrosis, were measured by stereoscopy and the total area of the lesions in one stomach was assessed by planimetry[16]. The measurement of ulcer index was determined by protocol-blinded researcher. The number of animals showing these histopathological lesions in each group was compared with that of other groups.

Surface electrocardiograms recording and heart rate variability analysis

Surface electrocardiograms (ECG) were recorded with a computer assisted BL-420S system (Chengdu Technology and Market Co. Ltd., Chengdu, China) with a sample rate of 1000 HZ. To avoid the influence of circadian variation of gastric susceptibility, we restricted the ECG recording time to 09:00-15:00 for all subjected animals. R waves were detected off-line with wavelet transform algorithm and then by manual artifact removal. Linear parameters of heart rate variability (HRV), including mean R-R intervals, standard deviation of the normal-to-normal R-R intervals (SDNN) and root mean square of successive difference (RMSSD) of R-R intervals, and non-linear parameter (Poincaré plot) were analyzed as we previously described[17].

Vagotomy

Under light ether inhalation anesthesia, bilateral cervical vagal nerve trunks were exposed and cut off. After closure of the incision, rats were allowed 3 h to recover from the surgery and anesthesia before the WRS procedure.

Measurement of gastric acid secretion

To avoid interrupting the development and observation of gastric erosion, an additional 24 rats were used to measure gastric acid secretion during the stress. These rats were randomly assigned to four groups: WRS, WRS + GHRP-6, RS and Normal + GHRP-6 groups, respectively (each $n = 6$). Gastric acid outputs were measured according to the reported protocols[18,19] with minor modifications. After a 24-h fast, animals were anesthetized by light ether inhalation. For each animal, a transverse incision was made in the abdomen. Both cardia and pylorus were intubated *via* incisions with open polyethylene cannulae and then ligated. The incisions were closed with thread adhesive to avoid water invasion, and ether was discontinued. To remove any solid contents, the stomach was gently rinsed with 2 mL of saline at 37 ℃ three times before the drainage of the gastric juice. Two milliliters of saline warmed to 37 ℃ were then injected into the stomach, left for 30 min and then aspirated and replaced by a fresh saline solution. The process was repeated twice to obtain acid secretion before stress and once every 30 min after the beginning of stress, for 3 h. The aspirated fluids were titrated to pH 7.0 with 0.01 mol/L (normality) NaOH using a pH meter, and acid output was calculated as μEq/30 min.

Intracerebroventricular cannulation

At least one week before the ICV injection, a brain cannula made of polyethylene tubing (PE-10; Clay Adams, Parsippany, NJ) was inserted into the left lateral cerebral ventricle (A-P, 1.5 mm caudal to the bregma; L, 2.0 mm lateral to the midline; V, 3.0 mm below the skull surface) under pentobarbital anesthesia (35 mg/kg, IP), as recently reported[20]. The cannula implanted into the brain

was securely fixed by dental cement and synthetic resin. When injections were given to the rats, a microsyringe for injection was directly connected to the cannula. ICV injections were performed only in conscious rats.

Measurement of plasma renin activity, endothelin-1 and thromboxane B2

Blood was sampled from the ventroartery and prepared for the measurements of stress-related vasoconstrictive factors. Kits for assaying the factors were purchased from the People's Liberation Army General Hospital, Beijing, China. Plasma renin activity (PRA) was indicated by the production of angiotensin I (Ang I) in a reaction system including rat plasma (containing renin and angiotensinogen), rabbit anti-human Ang I antiserum, Ang I standards, and ^{125}I-Ang I. Ang I was measured by the respective radioimmunoassay (RIA) kit. The direct reaction between sample plasma and Ang I antiserum served as a control. The radiation intensity (counts/min) in each tube was converted to nanograms per milliliter (ng/mL), with reference to the Ang I standard curve. PRA was calculated by the equation: PRA (ng/mL per hour) = (Ang I concentration in test tube-Ang I concentration in control tube)/incubation time (h). All assays were performed in duplicate.

Plasma endothelin-1 (ET-1) was measured with a RIA kit according to the manufacturer's protocols. The primary antibodies were rabbit anti-human ET-1 which showed interactions with rat ET-1. We also used standards of these hormones and blank controls to guarantee the quality of the measurement. The measuring sensitivities were < 5 pg/mL for ET-1. The intra- and interassay variabilities were < 10% and < 15% for ET-1.

Plasma thromboxane B2 (TXB2) was measured with an RIA kit (People's Liberation Army General Hospital, Beijing), according to the manufacturer's protocols. Thromboxane A2 (TXA2) is unstable (half-life 30 min) and is rapidly metabolized to the relatively stable TXB2; therefore, we used TXB2 as an indicator of TXA2 level. One milliliter of blood was drawn from the abdominal aorta into a test tube containing 0.06 mL of indomethacin-EDTA solution and then mixed. The blood was centrifuged at 3500 r/min for 15 min and the plasma was separated and stored at -20 ℃. At the beginning of the measurement, the plasma was defrosted and centrifuged again at 3500 r/min for 10 min. The supernatant was used to measure the TXA2 level using the RIA kit.

Western blotting

The gastric mucosal tissues were harvested immediately after decapitation, and 100 mg of mucosal tissues for each animal were used for the following procedures. Total protein extracts were prepared by homogenizing mucosal tissues in lysis buffer. Protein (80 μg per sample) electrophoresis were subjected to sodium dodecylsulfate-polyacrylamide gel and then transferred onto a nitrocellulose membrane. Primary antibodies [goat anti-rat heat shock proteins 70 (HSP70), polyclonal] (Santa Cruz Biotechnol-

Table 1 The lesion areas of gastric mucosa in different groups	
Group	**Lesion area (mm²)**
WRS (23 ℃)	81.8 ± 6.4[b]
WRS (19 ℃)	97.5 ± 8.7[b]
WRS (35 ℃)	56.4 ± 5.2[d]
WRS + GHRP-6	12.0 ± 2.8[d]
SR	0.0 ± 0.0[d]
FS (23 ℃)	99.5 ± 6.9[b]
Shallow water touch	0.0 ± 0.0[d]
WRS + eye occlusion	91.2 ± 8.4[b]
R + NWCI	0.0 ± 0.0[b]
R + SO immersion	80.6 ± 6.9[b]
R + LP immersion	82.3 ± 7.1[b]
R + sand immersion	0.0 ± 0.0[d]
WRS + anesthesia	0.0 ± 0.0[d]
WRS + vagotomy	0.0 ± 0.0[d]
WRS + atropine	0.0 ± 0.0[d]
Normal control	0.0 ± 0.0

[b]$P < 0.01$ *vs* simple restraint (SR) or normal control; [d]$P < 0.01$ *vs* water immersion restraint stress (WRS) group. FS: Free swimming; NWCI: Non-water contact immersion; SO: Salad oil; LP: Liquid paraffin; R: Restraint.

ogy, Inc., dilution 1:500) were added onto the membrane to react overnight at 4 ℃ and then incubated with rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Inc., dilution 1:2500) for 1 h. The immunoreactive bands were visualized using Western blotting luminal reagents and were scanned with Image Analysis software (Alpha Innotech, United States).

Statistical analysis

Data are presented as mean ± SD. Student's *t*-test was used for two group comparison and analysis of variance followed by Newman-Keuls multiple comparisons were used in case for multiple comparisons. Differences with *P* value < 0.05 were considered significant.

RESULTS

The water immersion restraint stress-induced gastric mucosal lesion is skin-sensing dependent but is vision or restraint independent

Visual inspection showed that WRS for 6 h (water temperature 23 ℃) induced serious gastric bleeding erosions, as indicated by the mucosal hemorrhage and mucosal erosive lesion (Figure 1) and the calculated area of the lesions (Table 1). The hemorrhage was observed mainly in the gastric body and antrum, but not in the fundus and duodenum (Figure 1). Under the microscope, the mucosa in the WRS rat was disrupted and covered with coagulated blood and inflammatory cell infiltration (Figure 1). Rats with SR did not show gastric mucosal lesions (Figure 1, Table 1). Compared with the serious gastric mucosal damage in all the WRS rats (Figures 1 and 2), eye occlusion of WRS rats did not provide any protection from the mucosal lesions (Figure 2, Table 1), suggesting that vision does not play an important role in the pathogenesis of gastric damage. Rats with NWCI showed an intact mucosa (Figure 2, Table 1), again supporting that

Figure 1 Gross anatomy of rat gastric mucosa in different groups. Left three columns: The gross inspections of the gastric mucosa in water immersion restraint stress (WRS), WRS + growth hormone releasing peptide-6 (GHRP-6) and simple restraint (SR) groups, respectively. Note that there were serious mucosal hemorrhages (black color) in the WRS group, while the hemorrhage was minimal in the WRS + GHRP-6 group. No mucosal hemorrhage was observed in the SR group; the forth column: Hematoxylin-eosin staining of the mucosa, mucosal structure disruption and hemorrhage (brown color) were observed in the WRS group, but not in the WRS + GHRP-6 and SR groups.

the view that vision is not important in inducing gastric mucosal lesion. FS for 6 h also induced serious mucosal bleeding erosions (Figure 2, Table 1), indicating that water immersion without restraint is also sufficient for inducing gastric mucosal lesions.

We also observed the influence of water temperature on WRS-induced gastric mucosal lesions. WRS with cool water (23 ℃) (Figure 1) or cold water (19 ℃) (Figure 2) both induced serious mucosal lesions, but the extent of the lesions was smaller when warm water (35 ℃) was used (Figure 2, Table 1).

The liquid immersion-induced gastric mucosal lesion depends on the deepening of immersion but not depends on the nature of a liquid

Water immersion (WI) to the level of the xiphoid induced serious gastric mucosal lesions (Figure 1), but partial WI (shallow water tough) did not induce mucosal lesions (Figure 2, Table 1), suggesting that the depth of the immersion determines the occurrence of mucosal lesions.

In an attempt to determine if different liquids would lead to different response patterns in the gastric mucosa, we observed the effects of immersion with two other liquids (salad oil and liquid paraffin, which are not obviously skin-hazardous) on gastric mucosa. Immersion to the level of xiphoid process with either of the two liquids induced similar gastric mucosal lesion (Figure 2, Table 1) as WRS did (Figure 2). "Burying" the body into sand (with the head exposed) did not induce mucosal lesions (Figure 2, Table 1). These results suggest that it is the liquid, but not the chemical nature of the liquid, that determines whether the mucosal lesions would occur, and "burying" the body in solid materials does not induce gastric mucosal lesions.

The WRS-induced gastric mucosal lesion depends on the functional neural integrity and increased vagal outflow to the stomach

WRS without anesthesia (i.e., conscious rats) induced serious gastric mucosal lesions (Figure 1, Table 1), but

Figure 2 Representative pictures of the gastric mucosa in different groups. WRS: Water immersion restraint stress; R: Restraint; NWCI: Immersion without water contact; LP: Liquid paraffin; SO: Salad oil; Anes.: Anesthesia; ICV: Intracerebroventricular injection; IP: Intraperitoneal injection.

Figure 3 Heart rate variability analyses showing the differences in mean R-R intervals, standard deviation of the normal-to-normal R-R intervals and root mean square of successive difference of R-R intervals in different groups. Note that the mean R-R intervals gradually prolonged along with the progress of water immersion restraint stress (WRS), whereas this prolongation was much less in the WRS + growth hormone releasing peptide-6 (GHRP-6) group. The mean R-R intervals were shortened in the simple restraint (SR) group. The changes in standard deviation of the normal-to-normal (SDNN) and root mean square of successive difference (RMSSD) was more significant in WRS group than the other two groups. [a]$P < 0.05$, [b]$P < 0.01$ *vs* WRS+GHRP-6 group.

WRS with anesthesia (unconscious rats) did not, no matter what a water temperature was used (Figure 2, Table 1). The HRV analyses (Figures 3 and 4) showed that the R-R intervals of the ECG in WRS rats gradually became longer, in other words, the HR gradually decreased; the SDNN and RMSSD of the R-R intervals increased, suggesting an increase of HRV, underlying an increase of the vagal outflow. Simple restraint induced a gradual shorting in R-R intervals and decreases in SDNN and RMSSD (Figure 3), suggesting an increase in sympathetic outflow to the heart. The Poincaré plot of R-R intervals (Figure 4) also supported the above observations. Previous injec-

tion of atropine also abolished the WRS-induced gastric mucosal lesion (Figure 2), further supporting the vagal hypothesis of this injury.

WRS stimulated gastric acid secretion (Figure 5), also indicative of increased vagal efferent activity. Simple restraint did not affect gastric acid output (Figure 5), indicating that restraint alone did not stimulate parasympathetic activity. Bilateral vagotomy totally prevented the development of WRS-induced mucosal lesions (Figure 2), also supporting the hypothesis that increased vagal outflow to the stomach plays a leading role in the development of WRS-induced mucosal lesions.

Figure 4 Pointcaré plots of the R-R intervals in different groups. A: The plots indicate that heart rate decreased gradually with time, and heart rate variability (HRV) was increased as shown by the scattered pattern of the plots in the water immersion restraint stress (WRS) group, suggesting vagal overactivity in this group; B: Changes of the heart rate and HRV in the WRS + growth hormone releasing peptide-6 (GHRP-6) group were much less than the WRS group; C: In the simple restraint (SR) group, the heart rate increased and the HRV was decreased as shown by the condensed geometry of the plots, suggesting increased sympathetic activity in this group.

Figure 5 Gastric acid output in different groups. Note that water immersion restraint stress (WRS) induced significant increase in gastric acid output and growth hormone releasing peptide-6 (GHRP-6) (intraperitoneal) significantly suppressed this increase. Simple restraint (SR) and GHRP-6 alone did not change the gastric acid output. [a]$P < 0.05$, [b]$P < 0.01$ *vs* WRS group.

Figure 6 Western blotting showing gastric mucosal protein levels of heat shock proteins 70 in different groups. Note that there was substantial expression in the water immersion restraint stress (WRS) and simple restraint (SR) groups, whereas the expression was minimal in the WRS + growth hormone releasing peptide-6 (GHRP-6) group. HSP70: Heat shock proteins 70.

GHRP-6 prevents WRS-induced gastric mucosal lesion mainly by suppressing the vagal effect on the stomach

GHRP-6 pre-injection *via* IP 2 h before the start of WRS dramatically prevented the WRS-induced mucosal bleeding erosion; only very slight or no hemorrhaging was observed in the WRS + GHRP-6 group (Figure 1). The HE stains of the gastric tissues (Figure 1) also confirmed that the mucosal injury/hemorrhage was minimal or not observed in WRS rats pretreated with GHRP-6. Planimetry analyses (Table 1) showed that the lesion area was large in the WRS group; but was minimal in the WRS + GHRP-6

group; lesion area was zero in the SR group. GHRP-6 did not have a protective effect on the mucosa of WRS rats if administrated centrally *via* ICV (Figure 2), suggesting that the protective effect of GHRP-6 is mainly peripheral.

GHRP-6 alleviated the changes of HRV parameters induced by WRS (Figures 3 and 4), and decreased the gastric acid output during WRS (Figure 5), suggesting that GHRP-6 protects the mucosa, at least in part, by suppressing the vagal efferent effect on the stomach.

GHRP-6 could alleviate the intensity of gastric stress response, which is reflected by the level of expression of HSP70 in the mucosa. Western blotting results showed that both the WRS and SR induced a high expression of HSP70 in the gastric mucosal tissues (Figure 6), indicating a nonspecific response of HSP70 expression to stress. GHRP-6 pretreatment significantly decreased the protein level of HSP70 in the WRS rats (Figure 6), suggesting a

Table 2 The effect of growth hormone releasing peptide-6 on the plasma levels of stress-related factors in rats

	ET-1 (pg/mL)	Renin activity (pg/mL)	TXB2 (ng/mL)
WRS	152.23 ± 10.70[d]	1.71 ± 0.59[d]	311.31 ± 50.54[d]
WRS + GHRP-6	115.11 ± 4.08[b]	0.65 ± 0.29[a]	125.84 ± 8.36[b]
SR	97.11 ± 4.71	0.11 ± 0.03	69.93 ± 22.13

[a]$P < 0.05$, [b]$P < 0.01$ *vs* WRS group; [d]$P < 0.01$ *vs* SR group. WRS: Water immersion restraint stress; SR: Simple restraint; GHRP-6: Growth hormone releasing peptide-6; ET-1: Endothelin-1; TXB2: Thromboxane B2.

decrease in the stress intensity.

WRS significantly increased the plasma levels of ET-1, renin activity and TXB2 compared with that of the SR group, while GHRP-6 pretreatment significantly attenuated the increases in these vasoconstrictive hormones (Table 2).

DISCUSSION

The mechanism of WRS-induced gastric mucosal lesion is complicated and not yet fully understood. The pathogenesis of the injury may be recognized at different levels, for example, at psychological, physiological, psychosomatic[21], integrative, organic, cellular and molecular levels. This study focuses on the psychosomatic mechanism of WRS-induced gastric mucosal lesions *in vivo*.

We first differentiated the relative importance of the pathways by which the stress stimulus signals were sensed and transferred to the central nervous system (CNS). When a conscious rat was immersed in water, it saw (intact vision) that its body was almost drowning, which may have induced fear. At the same time, the rat's skin also sensed liquid immersion and generated a physiological response and subsequent psychological stress response. By eye occlusion or NWCI manipulations, we determined that vision alone is not sufficient to induce gastric mucosal lesion, while WI alone is sufficient for the induction of the lesion, because free swimming rats showed serious mucosal injury. These results also showed that immersion depth significantly affects the severity of mucosal lesions, as partial immersion in shallow water could not induce mucosal lesions. These results indicate that skin sensation is the leading input pathway for WRS-induced gastric bleeding erosion. The cutaneous stimuli may reach the integrative structures of the upper central nervous system and, by the vagal pathway, produce gastric mucosa lesions. Rat is an animal of nocturnal habit; therefore vision is not a fundamental sense for its defense. In all environments, rat performs a cognitive map to run away from an eventual predator. In an unstable environment, such as fluid, they may aware of the difficulty of running away.

Skin receptors can sense temperature, touch and noxious stimulation. Whether or not skin sensors can also distinguish different liquids is unknown. To determine

this point, we examined gastric mucosal responses to immersion in different liquids or solid materials. Immersion in either salad oil or liquid paraffin induced serious mucosal hemorrhage similarly to WI, indicating that liquid immersion-induced gastric mucosal injury does not depend on the chemical nature of a liquid, but depends on liquid itself. "Burial" in sand did not induce gastric lesions, even when combined with restraint, suggesting that skin sensors can differentiate whether a material is fluid or dry matter, and immersion in a liquid or "burial" in dry material would lead to different gastric mucosal responses. Another possibility is that liquid immersion leads to lower body temperature compared with surrounding the body with solid materials.

The present data also indicated that the functional integrity of neural regulation is essential for the induction of gastric mucosal injury by WRS. In conscious animals, WRS induced injury that was inversely related to water temperature. However, in unconscious rats exposed to even the most severe condition (19 ℃), no ulceration occurred, which agreed with the result of Murison *et al*[21]. Pentobarbital does not block vagal output to the stomach[22], but even enhances vagal output[21] ; therefore, the lack of gastric erosion by WRS in unconscious rats may be caused by certain selective interruptions of CNS-stomach communications by the anesthesia, potentially including sensation of body temperature and mobilization of vasoconstrictive hormones, such as renin-angiotensin system[23], ET-1[24] and TXB2[25]. These vasoconstrictive factors may reduce gastric blood flow and lead to changes in the ratio of gastric blood flow/acid output, which favors the formation of gastric ulcers[26].

Gastric acid secretion is controlled by sympathetic and parasympathetic nerves, and by certain hormones, such as gastrin. Generally, sympathetic activity inhibits, and parasympathetic activity stimulates, gastric acid secretion. Occasionally, sympathetic stimulation may also increase gastric acid secretion, because adrenalin releases gastrin; and vagal nerves may exert some sympathetic-like effect as they have adrenergic fibers. Our results indicate that increased vagal efferent activity is the leading cause of WRS-induced gastric mucosal injury, because HRV analyses showed heart rate slowing and increasing of some HRV parameters, and furthermore, bilateral vagotomy or atropine totally prevented the injury induced by WRS. This result is consistent with our previous study[17]. Our HRV analyses also showed that restraint alone induced moderate sympathetic hyperactivity, while sympathetic hyperactivity in the stomach prevents WRS-induced gastric injury formation mainly *via* the inhibition of gastric acid secretion, as observed in stroke prone spontaneously hypertensive rats[27].

Heart rate slowing is a universal response in all air-breathing vertebrates when immersed in water (drowning or diving), this is called diving bradycardia[28]. Diving bradycardia is triggered by apnea and accentuated by immersion of the face or whole body in cold water[28]. The

diving response is mainly characterized by bradycardia, decreased cardiac output, peripheral vasoconstriction and increased arterial blood pressure[29,30]. The physiological significance of this response is to conserve oxygen, a mechanism of defense against hypoxic damage[28,31]. Our previous[17] and present observations in the rat model indicate that bradycardia appears even when the immersion is partial and the face is not immersed (for example, immersed to the xiphoid process in the present study), suggesting that heart rate slowing during water immersion does not necessarily depend on face immersion. This reaction pattern may be formed in development, and is a heritable trait[32]. However, when humans are diving or swimming for longer times (for example, 8-h swimming), they usually do not develop gastric mucosal injury, while rats do. One potential mechanism for these differential gastric responses may be the psychological component: a man who is diving or swimming knows that he is just at work or recreation and will not drown; therefore, he has no severe psychological reactions. By contrast, a rat would not think so, it would feel it was about to drown and die, and therefore severe psychological responses would be triggered, which may partially contribute to gastric injury.

Ghrelin, a peptide hormone originally isolated mainly from the stomach, is the endogenous ligand for the GHSR. In the gastrointestinal tract, ghrelin regulates the motility of the stomach and gut[33], gastric acid secretion[34] and gastric mucosal defense[35,36]. Intravenous administration of rat ghrelin dose-dependently increases both gastric acid secretion and gastric motility, actions that are blocked by pretreatment with either atropine or bilateral cervical vagotomy, but not by the histamine H_2-receptor antagonist famotidine, suggesting ghrelin might have a physiological role in the vagal control of gastric function in rats[37]. Another study indicated that ghrelin inhibits gastric acid secretion[38]. This controversy deserves further investigation. GHRP, the mimetic of ghrelin, has been shown to have gastric motor effects[39]. However, the effect of GHRP on gastric acid secretion is unknown. We show here that WRS significantly increased the gastric acid output, but restraint did not; GHRP-6 significantly suppressed WRS-stimulated gastric acid secretion, although GHRP-6 did not significantly affect the basal gastric acid output in normal rats. These results, combined with the HRV data, suggest that the protective effect of GHRP-6 on WRS-induced gastric mucosal injury is affected, at least in part, by suppressing vagal efferent effect on the stomach, including gastric acid secretion, as gastric acid play an important role in the development of WRS-induced gastric ulcers[40]. Our results also indicate that the protective effect of GHRP-6 is likely peripheral, potentially by affecting the function of vagal efferent terminals and/or cell protection. However, we cannot exclude the possibility that GHRP-6 might also affect the vago-vagal or vago-sympathetic reflexes. One possibility is that GHRP-6 injected *via* ICV may not effectively reach its target CNS site (for example, the dorsal vagal complex); the other possibility is that GHRP-6 may also affect the vagal afferent nerves, which in turn affects the neuronal reflex.

The protection of GHRP-6 on WRS-induced gastric injury could also be reflected by the level of expression of HSP70 in the gastric mucosal tissue. HSP are crucial for cell survival during and after various cellular stresses. WRS rapidly induces HSP70 expression and accumulation; the HSP70 level is inversely correlated with the severity of mucosal lesions[41]. GHRP-6 significantly decreased the HSP70 protein level in the gastric mucosa of WRS rats compared with WRS alone, indicating that the stress intensity is low in the GHRP-6 treated animals. This result also suggests that GHRP-6 can exert a cell protective effect.

Interestingly, we found that gastric mucosal injury never occurred in the gastric fundus, while ghrelin is secreted predominantly by enteroendocrine cells in the gastric fundus, although ghrelin gene transcripts and ghrelin-producing cells are found throughout the gastrointestinal tract[13]. Whether the ghrelin-secreting fundus is ulcer-resistant or only the acid-secreting areas (gastric body and antrum) are vulnerable to stress, deserves further investigation. It is possible that locally released ghrelin may have a protective action on the fundic gastric mucosa.

In conclusion, this study demonstrates that vision-triggered psychosomatic responses do not play an important role in WRS-induced gastric mucosal lesions; however, skin sensation-induced increase of vagal outflow and subsequent increase of gastric acid secretion do play a leading role. Skin receptors cannot differentiate different liquids, and immersion with different liquids induced the same gastric injury as WI does. GHRP-6 protects against WRS-induced gastric lesions mainly by suppressing the vagal effect on gastric mucosa, and this protection is likely peripheral. The protective effect of GHRP-6 on gastric stress ulceration suggests a clinical application in treating stress-related gastric injury.

COMMENTS

Background
Gastric ulcers are among the most frequently occurring stomach diseases across the world and stress is an important inducer of this disease. Therefore, an understanding of the key mechanism of gastric stress ulceration and the development of preventive/therapeutic drugs are important in treating this disease.

Research frontiers
How stress induces gastric ulcers is an old question that needs a new answer. Most previous studies only looked at restricted areas, especially at the physiological and molecular levels. Exploring the key mechanism and developing therapeutic drugs for gastric stress ulcer are urgently required.

Innovations and breakthroughs
In contrast to other mechanistic studies on gastric stress ulceration, this investigation focuses on the psychosomatic mechanisms of water immersion and restraint stress (WRS)-induced gastric bleeding erosions, and found that increased outflow from the vagal center is the leading cause of WRS-induced gastric injury. Skin sensation, but not vision, triggers the stress reaction *via* vago-vagal reflex. The study also found that growth hormone releasing peptide-6 (GHRP-6), a synthetic agonist for growth hormone secretagogues receptor, prevents the occurrence of gastric mucosal lesions, mainly by suppressing the vagal effect on the stomach.

Applications

The study demonstrates the key signaling pathway by which water immersion induces gastric mucosal damage in the rat, and provides the first evidence that GHRP-6 can prevent this damage. The study suggests a clinical application of GHRP in treating gastric stress ulceration.

Peer review

Discovering the mechanism of gastric stress ulceration is a prerequisite for the prevention and treatment of this disease. This study shows that skin sensation and the subsequent vago-vagal reflex play a key role in the development of water immersion-induced gastric mucosal damage in the rat. GHRP-6 prevents this damage, probably by suppressing the vagal effect on the stomach. The study is innovative and with potential therapeutic interest.

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BRIEF ARTICLE

Two-stage *vs* single-stage management for concomitant gallstones and common bile duct stones

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Abstract

AIM: To evaluate the safety and effectiveness of two-stage *vs* single-stage management for concomitant gallstones and common bile duct stones.

METHODS: Four databases, including PubMed, Embase, the Cochrane Central Register of Controlled Trials and the Science Citation Index up to September 2011, were searched to identify all randomized controlled trials (RCTs). Data were extracted from the studies by two independent reviewers. The primary outcomes were stone clearance from the common bile duct, postoperative morbidity and mortality. The secondary outcomes were conversion to other procedures, number of procedures per patient, length of hospital stay, total operative time, hospitalization charges, patient acceptance and quality of life scores.

RESULTS: Seven eligible RCTs [five trials (*n* = 621) comparing preoperative endoscopic retrograde cholangiopancreatography (ERCP)/endoscopic sphincterotomy (EST) + laparoscopic cholecystectomy (LC) with LC + laparoscopic common bile duct exploration (LCBDE);

two trials (*n* = 166) comparing postoperative ERCP/EST + LC with LC + LCBDE], composed of 787 patients in total, were included in the final analysis. The meta-analysis detected no statistically significant difference between the two groups in stone clearance from the common bile duct [risk ratios (RR) = -0.10, 95% confidence intervals (CI): -0.24 to 0.04, *P* = 0.17], postoperative morbidity (RR = 0.79, 95% CI: 0.58 to 1.10, *P* = 0.16), mortality (RR = 2.19, 95% CI: 0.33 to 14.67, *P* = 0.42), conversion to other procedures (RR = 1.21, 95% CI: 0.54 to 2.70, *P* = 0.39), length of hospital stay (MD = 0.99, 95% CI: -1.59 to 3.57, *P* = 0.45), total operative time (MD = 12.14, 95% CI: -1.83 to 26.10, *P* = 0.09). Two-stage (LC + ERCP/EST) management clearly required more procedures per patient than single-stage (LC + LCBDE) management.

CONCLUSION: Single-stage management is equivalent to two-stage management but requires fewer procedures. However, patient's condition, operator's expertise and local resources should be taken into account in making treatment decisions.

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INTRODUCTION

Choledocholithiasis is concomitant with gallstones in approximately 3%-10% of the patients[1-4]. In the pre-endoscopy and pre-laparoscope era, the standard treatment for patients suffering from gallstones accompanied with common bile duct stones (CBDS) was open cholecystectomy and common bile duct exploration[5]. With the advent of laparoscopic and endoscopic techniques, several alternative treatments, such as laparoscopic cholecystectomy (LC), preoperative or postoperative endoscopic retrograde cholangiopancreatography and endoscopic sphincterotomy (ERCP + EST) and laparoscopic common bile duct exploration (LCBDE), have been developed to treat cholelithiasis. In the past two decades, LC has become gradually accepted as the first choice for the treatment of cholecystolithiasis. Consequently, confirmed or suspected cases of CBDS have been routinely removed *via* a two-stage management using preoperative ERCP/EST followed by LC. However, even with the strictest selection criteria, over 10% of the preoperative ERCP are normal[6,7], and only 10%-60% of patients will have stones at the time of ERCP[8-11]. As a result, a number of unnecessary ERCP procedures have been performed. To avoid these unnecessary procedures, laparoscopic intraoperative cholangiography combined with selective postoperative ERCP has been proposed[12]. Nevertheless, preoperative ERCP/EST and postoperative ERCP/EST can both result in unpredictable severe complications, even death[13]. Although ERCP/EST has been proven to be a safe and effective option for extracting CBDS in most cases, it also has some adverse effects. This procedure can not only induce several postoperative complications, including bleeding, perforation and pancreatitis[14-17], but also lead to the disruption of the intact sphincter of Oddi[18,19]. Currently, as the laparoscopic technique matures, more and more centers prefer conducting LCBDE through either the transcystic duct or *via* the choledochotomy to remove CBDS, thus preventing unnecessary preoperative ERCP[20-22]. Above all, LCBDE has the advantage of reducing the two-stage approach to a single-stage approach by minimally invasive surgery. Previously published trials are unclear as to whether two-stage (LC + ERCP/EST) management is better or worse than single-stage (LC + LCBDE) management for choledocholithiasis complicated with cholecystolithiasis. Therefore, we conducted a systematic review and meta-analysis of all randomized controlled trials (RCTs) to evaluate the clinical safety and effectiveness of the two-stage (LC + ERCP/EST) management *vs* single-stage (LC + LCBDE) management in patients with concomitant gallstones and CBDS.

MATERIALS AND METHODS

Searching strategy

We searched databases, including PubMed, Embase, the Cochrane Central Register of Controlled Trials and the Science Citation Index updated to September 2011, to identify all related published RCTs. The keywords used in the search were as follows: LC, LCBDE, ERCP, EST, gallstones and CBDS. The language of all publications was restricted to English only. The citations within the reference lists of the articles were searched manually to identify additional eligible studies.

Inclusion and exclusion criteria

All studies, published up to and including September 2011, that compared two-stage (LC + ERCP/EST) with single-stage (LC + LCBDE) management in patients with concomitant gallstones and CBDS were eligible for inclusion. The inclusion criteria were: (1) study design, RCT; (2) types of participants, those with proven or suspected CBDS before LC or those with gallstones that were found to have CBDS at LC by intraoperative cholangiography; (3) intervention, preoperative ERCP/EST + LC *vs* LC + LCBDE or (4) postoperative ERCP/EST + LC *vs* LC + LCBDE. Non-randomized trials, retrospective analyses and reviews were not included, and studies were excluded if there were no postoperative major outcomes. In addition, those studies comparing intraoperative ERCP/EST + LC with LC + LCBDE were also excluded because both managements were single-stage.

Data extraction and validity assessment

Two authors (Lu J and Cheng Y) independently extracted the data, evaluated the study quality by applying a predesigned standardized form, and then cross-checked. Any disagreement in the two reviewers' data collection and quality assessment was discussed until a consensus was reached; otherwise, a third reviewer (Xiong XZ) would take part in the discussion as the referee. The general information extracted from the studies included the authors, publication year, study period, country, characteristics of patients, sample size, interventions and outcomes. The risk of bias in the included studies was assessed using the Cochrane collaboration' tool. The assessment contained six dimensions: (1) random sequence generation; (2) allocation concealment; (3) blinding; (4) incomplete outcome data addressed; (5) selective reporting; and (6) other bias.

Outcomes of interest and definitions

The primary outcomes were stone clearance from the common bile duct (CBD), postoperative morbidity and mortality, while secondary outcomes were conversion to other procedures, length of hospital stay, number of procedures used per patient, total operative time, hospitalization charges, patient acceptance and quality of life scores. Stone clearance from the CBD was determined by ERCP or intraoperative cholangiography, and it was defined as successful stones extracted from the CBD *via* the planned procedure only once. The overall postoperative morbidity consisted of surgical and nonsurgical complications. The surgical complications included hemorrhage, bile leak, acute pancreatitis, cholangitis, perforation, wound infection, abdominal and wall hematoma that were directly

Table 1 Characteristics of 7 included randomized controlled trials

Included studies	Country	Study period	Sample size	Comparison	Measured outcomes
Bansal *et al*[5], 2010	India	2007-2008	30	Preoperative ERCP/EST + LC (*n* = 15) *vs* LC + LCBDE (*n* = 15)	Successful removal of gallbladder and CBD clearance, complications
Rogers *et al*[24], 2010	United States	1997-2003	122	Preoperative ERCP/EST + LC (*n* = 61) *vs* LC + LCBDE (*n* = 61)	Stone clearance from CBD, length of hospital stay, cost of index hospitalization, hospital charges, professional fees, patient acceptance, morbidity, mortality, quality of life scores
Rhodes *et al*[25], 1998	United Kingdom	1995-1997	80	Postoperative ERCP/EST + LC (*n* = 40) *vs* LC + LCBDE (*n* = 40)	Duct-clearance rates, morbidity, operating time and hospital stay
Cuschieri *et al*[26], 1999	Scotland	1994-1997	300	Preoperative ERCP/EST + LC (*n* = 150) *vs* LC + LCBDE (*n* = 150)	Hospital stay, success rates, conversion rates, morbidity and mortality
Nathanson *et al*[27], 2005	Australia	1998-2003	86	Postoperative ERCP/EST + LC (*n* = 45) *vs* LC + LCBDE (*n* = 41)	Operative time, morbidity, retained stone rate, reoperation rate and hospital stay
Sgourakis *et al*[28], 2002	Greece	1997-2000	78	Preoperative ERCP/EST + LC (*n* = 42) *vs* LC + LCBDE (*n* = 36)	Stone clearance, morbidity, mortality, conversion, hospital stay, complications
Noble *et al*[29], 2009	United Kingdom	2000-2006	91	Preoperative ERCP/EST + LC (*n* = 47) *vs* LC + LCBDE (*n* = 44)	Duct clearance, complications, number of procedures per patient, conversion and hospital stay

RCTs: Randomized controlled trial; CBD: Common bile duct; LC: Laparoscopic cholecystectomy; ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; LCBDE: Laparoscopic common bile duct exploration.

Figure 1 Flow diagram of literature screening. ERCP: Endoscopic retrograde cholangiopancreatography; LC: Laparoscopic cholecystectomy; LCBDE: Laparoscopic common bile duct exploration.

related with the operation, and the nonsurgical complications included myocardial infarction and pulmonary embolism, which had nothing to do with the operation. Mortality was defined as postoperative death before discharge or within 30 postoperative days. Conversion to other procedures was defined as any case in which stones from the CBD were not successfully extracted or other scenarios, such as dense gallbladder adhesions and fibrosis, which resulted in converting the planned procedure into another procedure.

Statistical analysis

All statistical analyses of the extracted data was performed with Review Manager (Review Manager version 5.1, Copenhagen, the Nordic Cochrane Centre, the Cochrane Collaboration, 2008). The results of the meta-analysis were expressed as the risk ratios (RRs) and mean difference (MD) for dichotomous data and continuous data, respectively, with 95% confidence intervals (CIs) for both. The Mantel-Haenzsel method was used for dichotomous variables, while the inverse variance method was used for continuous variables. *P* values were computed with the *Z* test, and $P < 0.05$ was regarded as statistically significant. The heterogeneity among the studies was evaluated using the χ^2 test, with its significance set at $P < 0.1$, and the extent of inconsistency was assessed by the I^2 statistic[23]. If significant heterogeneity existed, a random-effect model was used to attempt to explain it. In the absence of significant heterogeneity, a fixed-effect model was adopted. Generally, the estimates of the mean and SD were required to calculate the CIs for continuous data. However, a few published clinical trials reported a median and a range instead of a mean and SD. To adjust for this difference, we assumed that the median was equal to the mean, and we estimated the SD as a quarter of the reported range. Potential publication bias was appraised visually by funnel plots.

RESULTS

Literature search and selection

The literature search identified 792 potentially relevant studies according to our predefined search strategy (Figure 1). Ninety-three studies were removed by the Endnote X4 software, and 667 studies were excluded through scanning titles and abstracts. Full-text papers were retrieved for the remaining 32 eligible studies. Of the remaining 32 studies, 20 studies were excluded because they were non-randomized trials. Three studies were excluded because they were duplicated trials, and two were not included because they compared intraoperative ERCP/EST + LC with LC + LCBDE. Eventually, seven RCTs[5,24-29] were considered to be suitable for the final meta-analysis. There were five

Table 2 Outcomes of 7 included randomized controlled trials (endoscopic retrograde cholangiopancreatography/endoscopic sphincterotomy + laparoscopic cholecystectomy) *vs* (laparoscopic cholecystectomy + laparoscopic common bile duct exploration)

Included studies	Stone clearance from the CBD (%)	Postoperative morbidity (%)	Mortality (%)	Conversion to other procedures (%)	Number of procedures per patient	Length of hospital stay (d)	Total operating time (min) (SD or range)	Hospitalization charges ($) (SD or range)
Bansal *et al*[5], 2010	86.7 *vs* 93.3	Not mentioned	0 *vs* 0	15.4 *vs* 6.7	Not mentioned	4 (2-11) *vs* 4.2 (3-9)	153 (120-240) *vs* ?	Not mentioned
Rogers *et al*[24], 2010	96.8 *vs* 88.2	9.1 *vs* 10.5	0 *vs* 0	1.8 *vs* 3.5	2.0 *vs* 1.0	4.1 (3.5) *vs* 2.3 (1.9)	183 (39) *vs* 174 (67)	30 617 (16 384) *vs* 27 675 (11 256)
Rhodes *et al*[25], 1998	75 *vs* 75	15 *vs* 17.5	Not mentioned	0 *vs* 25	2.4 *vs* 1.3	3.5 (1-11) *vs* 1 (1-26)	105 (60-255) *vs* 90 (25-310)	Not mentioned
Cuschieri *et al*[26], 1999	83.7 *vs* 82.6	12.5 *vs* 15.8	1.5 *vs* 0.8	14.7 *vs* 15	2.0 *vs* 1.2	9 (5.5-14) *vs* 6 (4.2-12)	Not mentioned	Not mentioned
Nathanson *et al*[27], 2005	71.1 *vs* 97.6	13.3 *vs* 17.1	0 *vs* 0	6.7 *vs* 4.9	2.3 *vs* 1.2	7.7 *vs* 6.4	147.9 *vs* 158.8	Not mentioned
Sgourakis *et al*[28], 2002	84.3 *vs* 85.7	18.8 *vs* 17.9	3.1 *vs* 0	15.6 *vs* 14.3	2.1 *vs* 1.1	9 *vs* 7.4	105 (60-255) *vs* 90 (70-310)	Not mentioned
Noble *et al*[29], 2009	55.6 *vs* 100	29.8 *vs* 43.2	Not mentioned	42.6 *vs* 9.1	2.3 *vs* 1.0	3 (2-7) *vs* 5 (2-7)	Not mentioned	Not mentioned

CBD: Common bile duct.

Figure 2 Funnel plot of trials of stone clearance from the common bile duct. ERCP: Endoscopic retrograde cholangiopancreatography; LC: Laparoscopic cholecystectomy; LCBDE: Laparoscopic common bile duct exploration. SE: Standard error; RD: Risk difference.

trials[4,24,26,28,29] (n = 621) comparing preoperative ERCP/EST + LC with LC + LCBDE and two trials[25,27] (n = 166) comparing postoperative ERCP/EST + LC with LC + LCBDE. The characteristics, outcomes and risk of bias for the included studies are summarized in Tables 1-3. A manual search and examination of the bibliographies in these reference lists were also performed, and no additional eligible studies were found.

Description of various RCTs including inclusion and exclusion criteria

All of the patients were proven or suspected of having gallstones and CBDS on the basis of clinical presentation (jaundice, biliary pancreatitis and cholangitis) or liver function tests or imaging (ultrasound, magnetic resonance cholangiopancreatography (MRCP) and intraoperative cholangiography) before being enrolled in the trial. Three

trials[24,26,28] restricted the participants to the ASA risk grade at the level of Ⅰ and Ⅱ. In addition, one trial[29] was specially designed to compare the two-stage (ERCP/EST + LC) approach with the single-stage (LC + LCBDE) approach in higher risk patients, who were defined as those over 60 years of age with comorbidity, those over 70 years of age, or those over 50 years of age with a body mass index exceeding 40. Three trials[5,26,28] did not mention their exclusion criteria. Additionally, three studies[25,27,29] excluded patients who had accepted previous ERCP/EST prior to recruitment into the trials. Patients with severe pancreatitis and cholangitis, which required emergency ERCP/EST, were also excluded from two trials[27,29].

Publication bias

A funnel plot analysis was performed by adopting the occurrence of stone clearance from the CBD as the index, and it appeared to be asymmetrical, which suggested the presence of publication bias (Figure 2).

Meta-analysis results

Stone clearance from the CBD: Stone clearance from the CBD was achieved in 78.8% (234 of 297) of patients in the two-stage (ERCP/EST + LC) group and in 87.2% (251 of 288) of patients in the single-stage (LC + LCBDE) group. The meta-analysis revealed that the difference between the two groups was not statistically significant (RR = -0.10, 95% CI: -0.24 to 0.04, P = 0.17), and there was statistically significant heterogeneity among the studies (χ^2 = 33.55, P < 0.00 001, I^2 = 82%) (Figure 3A).

Postoperative morbidity: Postoperative morbidity was reported in six trials[24-29]. Overall, postoperative morbidity occurred in 15.2% (54 of 355) of patients in the two-stage (ERCP/EST + LC) group *vs* 19.0% (65 of 343) of patients in the single-stage (LC + LCBDE) group. The meta-analysis demonstrated that there was no statistically

Table 3 Cochrane risk of bias summary

Included studies	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias
Bansal *et al*[5], 2010	Low risk	Low risk	High risk	Unclear risk	High risk	High risk	Unclear risk
Rogers *et al*[24], 2010	Low risk	Low risk	High risk	Unclear risk	High risk	Low risk	Low risk
Rhodes *et al*[25], 1998	Unclear risk	Unclear risk	High risk	Unclear risk	Low risk	High risk	Unclear risk
Cuschieri *et al*[26], 1999	Low risk	Unclear risk	High risk	Unclear risk	Low risk	High risk	Unclear risk
Nathanson *et al*[27], 2005	Low risk	Low risk	High risk	Unclear risk	Low risk	High risk	Unclear risk
Sgourakis *et al*[28], 2002	High risk	Unclear risk	High risk	Unclear risk	High risk	High risk	Low risk
Noble *et al*[29], 2009	Low risk	Unclear risk	High risk	Unclear risk	Low risk	High risk	Low risk

significant difference between the two groups (RR = 0.79, 95% CI: 0.58 to 1.10, P = 0.16). As evidenced by the values of the χ^2 and I^2 indices (χ^2 = 0.55, P = 0.99, I^2 = 0%), no significant heterogeneity was found from the trials (Figure 3B).

Mortality: Five trials[5,24,26-28] reported mortality; however, only two of these trials[26,28] reported postoperative deaths. The results of the pooled analysis revealed no statistically significant difference between the two groups (RR = 2.19, 95% CI: 0.33 to 14.67, P = 0.42); and there was no significant heterogeneity between the trials (χ^2 = 0.02, P = 0.88, I^2 = 0%) (Figure 3C).

Conversion to other procedures: We identified all the trials existing in the data, and the occurrence of conversion was 13.9% (51 of 368) and 12.0% (43 of 358) in the two-stage (ERCP/EST + LC) group and the single-stage (LC + LCBDE) group, respectively. Significant heterogeneity was present in the trials (χ^2 = 13.83, P = 0.03, I^2 = 57%), and there was no statistically significant difference between the two groups (RR = 1.21, 95% CI: 0.54 to 2.70, P = 0.39) (Figure 3D).

Length of hospital stay: The length of hospital stay was evaluated in all the studies, but only one study[24] reported this data in the form of the mean and the SD. There were two studies[27,28] that provided the mean without the SD, and the rest of studies[5,25,26,29] provided the median and the range. Consequently, according to our predefined plan, we presumed that the median was equal to the mean, and we equated the SD with a quarter of the reported range. Significant heterogeneity was found among the trials (χ^2 = 209.70, P < 0.001, I^2 = 98%), and the meta-analysis indicated no statistically significant difference between the two groups (MD = 0.99, 95% CI: -1.59 to 3.57, P = 0.45) (Figure 3E).

Total operative time: There were five trials[5,24,25,27,28] that included information about the total operative time; however, only one trial[24] reported the mean and the SD. Two trials[25,28] offered the median and the range instead of the mean and the SD, while one trial[27] offered the mean without the SD. Furthermore, there was one trial[5] that reported the total operative time of the two-stage (ERCP/EST + LC) group, but not the single-stage (LC + LCBDE) group. There was no statistically significant dif-

ference between the two groups (MD = 12.14, 95% CI: -1.83 to 26.10, P = 0.09), and no significant heterogeneity was found among the trials (χ^2 = 0.18, P = 0.92, I^2 = 0%) (Figure 3F).

Hospitalization charges: The hospitalization charges were recorded in only one trial. Rogers *et al*[24] stated that there was no statistically significant difference in total hospitalization charges between the two groups.

Patient acceptance and quality of life scores: Only one trial[24] reported the patient acceptance and quality of life scores. This article mentioned that the patient acceptance and quality of life scores were the same in both groups using standardized scoring system. However, the study did not provide specific data.

Number of procedures used per patient: It was obvious that the two-stage (LC + ERCP/EST) approach required more procedures per patient than the single-stage (LC + LCBDE) approach (Table 2).

Subgroup analysis: Because there were two options (preoperative ERCP/EST + LC and postoperative ERCP/EST + LC) for the two-stage (ERCP/EST + LC) management technique, which may have influenced the eventual conclusion, we performed a subgroup analysis for three outcomes (stone clearance from the CBD, postoperative morbidity and conversion to other procedures). In the subgroup analysis, the outcomes were also equivalent, and no statistically significant difference was found among the subgroups.

DISCUSSION

In summary, our meta-analysis revealed that both single-stage (LC + LCBDE) and two-stage (LC + ERCP/EST) management achieved equivalent stone clearance from the CBD, but the former procedure was associated with fewer procedures per patient. In addition, there was no statistically difference between the two approaches in terms of postoperative morbidity, mortality, conversion to other procedures, length of hospital stay, total operative time and hospitalization charges.

Currently, the optimal treatment for concomitant gallstones and CBDS is still in dispute[24,30,31]. In the laparoscopic era, the vast majority of patients who suffered

A

Study or subgroup	Experimental Events	Total	Control Events	Total	Weight	Risk difference M-H, random, 95% CI	Year	Risk difference M-H, random, 95% CI
1.1.1 Preoperative ERCP + LC *vs* LC + LCBDE								
Cuschieri *et al*[26]	82	98	90	109	16.3%	0.01 [-0.09, 0.11]	1999	
Sgourakis *et al*[28]	27	32	24	28	13.8%	-0.01 [-0.19, 0.17]	2002	
Noble *et al*[29]	20	36	38	38	14.4%	-0.44 [-0.61, -0.28]	2009	
Bansal *et al*[5]	13	15	14	15	12.7%	0.07 [-0.28, 0.15]	2010	
Rogers *et al*[24]	30	31	15	17	14.3%	0.09 [-0.08, 0.25]	2010	
Subtotal (95% CI)		212		207	71.4%	-0.08 [-0.27, 0.10]		
Total events	172		181					

Heterogeneity: Tau^2 = 0.04, χ^2 = 26.23, df = 4 (*P* < 0.0001); *I*^{2} = 85%
Test for overall effect: *Z* = 0.91 (*P* = 0.36)

1.1.2 LC + postperative ERCP *vs* LC + LCBDE								
Rhodes *et al*[25]	30	40	30	40	13.5%	0.00 [-0.19, 0.19]	1998	
Nathanson *et al*[27]	32	45	40	41	15.1%	-0.26 [-0.41, -0.12]	2005	
Subtotal (95% CI)		85		81	28.6%	-0.14 [-0.41, 0.13]		
Total events	62		70					

Heterogeneity: Tau^2 = 0.03, χ^2 = 5.16, df = 1 (*P* = 0.002); *I*^{2} = 81%
Test for overall effect: *Z* = 1.02 (*P* = 0.31)

Total (95% CI)		297		288	100.0%	-0.10 [-0.24, 0.04]		
Total events	234		251					

Heterogeneity: Tau^2 = 0.03, χ^2 = 33.55, df = 6 (*P* < 0.00 001); *I*^{2} = 82%
Test for overall effect: *Z* = 1.39 (*P* = 0.17)
Test for subgroup differences : χ^2 =0.11, df = 1 (*P* = 0.74); *I*^{2} = 0%

-2 -1 0 1 2
Favours LC + ERCP/EST   Favours LC + LCBDE

B

Study or subgroup	Experimental Events	Total	Control Events	Total	Weight	Risk ratio M-H, random, 95% CI	Year	Risk ratio M-H, fixed, 95% CI
1.2.1 Preoperative ERCP/EST + LC *vs* LC + LCBDE								
Cuschieri *et al*[26]	17	136	21	133	32.0%	0.79 [0.44, 1.43]	1999	
Sgourakis *et al*[28]	6	32	5	28	8.0%	1.05 [0.36, 3.07]	2002	
Noble *et al*[29]	14	47	19	44	29.6%	0.69 [0.40, 1.20]	2009	
Rogers *et al*[24]	5	55	6	57	8.9%	0.86 [0.28, 2.67]	2010	
Subtotal (95% CI)		270		262	78.4%	0.79 [0.55, 1.13]		
Total events	42		51					

Heterogeneity: χ^2 = 0.52, df = 3 (*P* = 0.91); *I*^{2} = 0%
Test for overall effect: *Z* = 1.29 (*P* = 0.20)

1.1.2 LC + postperative ERCP/EST *vs* LC + LCBDE								
Rhodes *et al*[25]	6	40	7	40	10.5%	0.86 [0.32, 2.33]	1998	
Nathanson *et al*[27]	6	45	7	41	11.0%	0.78 [0.29, 2.13]	2005	
Subtotal (95% CI)		85		81	21.6%	0.82 [0.40, 1.66]		
Total events	12		14					

Heterogeneity: χ^2 = 0.02, df = 1 (*P* = 0.90); *I*^{2} = 0%
Test for overall effect: *Z* = 0.56 (*P* = 0.58)

Total (95% CI)		355		343	100.0%	0.79 [0.58, 1.10]		
Total events	54		65					

Heterogeneity: χ^2 = 0.55, df = 5 (*P* = 0.99); *I*^{2} = 0%
Test for overall effect: *Z* = 1.40 (*P* = 0.16)
Test for subgroup differences : χ^2 =0.01, df = 1 (*P* = 0.93); *I*^{2} = 0%

0.005 0.1 0 10 200
Favours LC + ERCP/EST   Favours LC + LCBDE

C

Study or subgroup	Experimental Events	Total	Control Events	Total	Weight	Risk ratio M-H, random, 95% CI	Year	Risk ratio M-H, fixed, 95% CI
Sgourakis *et al*[28]	1	32	0	28	34.5%	2.64 [0.11, 62.23]	2002	
Cuschieri *et al*[26]	2	136	1	133	65.5%	1.96 [0.18, 21.31]	1999	
Total (95% CI)		168		161	100.0%	2.19 [0.33, 14.67]		
Total events	3		1					

Heterogeneity: χ^2 = 0.02, df = 1 (*P* = 0.88); *I*^{2} = 0%
Test for overall effect: *Z* = 0.81 (*P* = 0.42)

0.001 0.1 0 10 1000
Favours LC + ERCP/EST   Favours LC + LCBDE

D

	Experimental		Control			Risk ratio		Risk ratio
Study or subgroup	Events	Total	Events	Total	Weight	M-H, random, 95% CI	Year	M-H, random, 95% CI
1.4.1 Preoperative ERCP/EST + LC vs LC + LCBDE								
Cuschieri *et al*[26]	20	136	20	133	25.9%	0.98 [0.55, 1.73]	1999	
Sgourakis *et al*[28]	5	32	4	28	17.7%	1.09 [0.33, 3.68]	2002	
Noble *et al*[29]	20	47	4	44	20.5%	4.68 [1.74, 12.62]	2009	
Bansal *et al*[5]	2	13	1	15	8.8%	2.31 [0.24, 22.62]	2010	
Rogers *et al*[24]	1	55	2	57	8.3%	0.52 [0.05, 5.55]	2010	
Subtotal (95% CI)		283		277	81.1%	1.53 [0.70, 3.37]		
Total events	48		31					

Heterogeneity: Tau^2 = 0.38, χ^2 = 8.35, df = 4 (P = 0.08); I^2 = 52%
Test for overall effect: Z = 1.06 (P = 0.29)

1.4.2 LC + postperative ERCP/EST vs LC + LCBDE								
Rhodes *et al*[25]	0	40	10	40	6.4%	0.05 [0.00, 0.79]	1998	
Nathanson *et al*[27]	3	45	2	41	12.4%	1.37 [0.24, 7.77]	2005	
Subtotal (95% CI)		85		81	18.9%	0.30 [0.01, 11.05]		
Total events	3		12					

Heterogeneity: Tau^2 = 5.44, χ^2 = 4.84, df = 1 (P = 0.003); I^2 = 79%
Test for overall effect: Z = 0.66 (P = 0.51)

Total (95% CI)		368		358	100.0%	1.21 [0.54, 2.70]		
Total events	51		43					

Heterogeneity: Tau^2 = 0.57, χ^2 = 13.81, df = 6 (P = 0.03); I^2 = 57%
Test for overall effect: Z = 0.46 (P = 0.65)
Test for subgroup differences : χ^2 = 0.75, df = 1 (P = 0.39); I^2 = 0%

0.001 0.1 1 10 1000
Favours LC + ERCP/EST   Favours LC + LCBDE

E

	Experimental			Control				Mean difference		Mean difference
Study or subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, random, 95% CI	Year	IV, random, 95% CI
Rhodes *et al*[25]	3.5	2.5	40	1	6.25	40	18.4%	2.50 [0.41, 4.59]	1998	
Cuschieri *et al*[26]	9	2.125	150	6	1.95	150	20.8%	3.00 [2.54, 3.46]	1999	
Noble *et al*[29]	3	1.25	47	5	1.25	44	20.8%	-2.00 [-2.51, -1.49]	2009	
Rogers *et al*[24]	4.1	3.5	55	2.3	1.9	57	20.2%	1.80 [0.75, 2.85]	2010	
Bansal *et al*[5]	4	2.25	15	4.2	1.5	15	19.8%	-0.20 [-1.57, 1.17]	2010	
Total (95% CI)			307			306	100.0%	0.99 [-1.59, 3.57]		

Heterogeneity: Tau^2 = 8.28; χ^2 = 209.31, df = 4 (P < 0.00 001); I^2 = 98%
Test for overall effect: Z = 0.75 (P = 0.45)

-20 -10 0 10 20
Favours LC + ERCP/EST   Favours LC + LCBDE

F

	Experimental			Control				Mean difference		Mean difference
Study or subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, fixed, 95% CI	Year	IV, random, 95% CI
Rhodes *et al*[25]	105	48.75	40	90	71.25	40	27.3%	15.00 [-11.75, 41.75]	1998	
Sgourakis *et al*[28]	105	48.75	32	90	60	28	25.2%	15.00 [-12.91, 42.91]	2002	
Rogers *et al*[24]	183	39	55	174	67	57	47.7%	9.00 [-11.22, 29.22]	2010	
Total (95% CI)			127			125	100.0%	12.14 [-1.83, 26.10]		

Heterogeneity: χ^2 = 0.18, df = 2 (P = 0.92); I^2 = 0%
Test for overall effect: Z = 1.70 (P = 0.09)

-100 -50 0 50 100
Favours LC + ERCP/EST   Favours LC + LCBDE

Figure 3 Forest plot of meta-analysis. A: Two-stage [endoscopic retrograde cholangiopancreatography (ERCP)/endoscopic sphincterotomy (EST) + laparoscopic cholecystectomy (LC)] *vs* single-stage [LC + laparoscopic common bile duct exploration (LCBDE)] in stone clearance from the common bile duct; B: Two-stage (ERCP/EST + LC) *vs* single-stage (LC + LCBDE) in postoperative morbidity; C: Two-stage (ERCP/EST + LC) *vs* single-stage (LC + LCBDE) in mortality; D: Two-stage (ERCP/EST + LC) *vs* single-stage (LC + LCBDE) in conversion to other procedures; E: Two-stage (ERCP/EST + LC) *vs* single-stage (LC + LCBDE) in length of hospital stay; F: Two-stage (ERCP/EST + LC) *vs* single-stage (LC + LCBDE) in total operating time. CI: Confidence interval.

from concomitant gallstones and CBDS were routinely managed by ERCP/EST, either preoperatively or postoperatively, prior to LC[25,26]. Although this approach is effective and safe for removing the CBDS, it also has several drawbacks. First, it requires two periods of anesthesia and occasionally two hospital admissions, which may increase the length of hospital stay and hospitalization expenses. Furthermore, if patients still have CBDS detected by intraoperative cholangiography in LC after successful ERCP/EST, surgeons will face the dilemma of depending on LCBDE, postoperative ERCP/EST or traditional open choledochotomy. Most importantly, even in those patients with clinical, biochemical and imaging risk factors for CBDS, preoperative ERCP/EST can produce false-

negative results, leading to the possibility of morbidity and mortality[26,32]. Despite postoperative ERCP/EST can indeed avoid the risk inherent in preoperative ERCP/EST to patients without CBDS, it necessitates another surgical procedure when it fails to remove the CBDS[27]. Both preoperative and postoperative ERCP/EST are likely to lead to some short-term and long-term complications. For instance, they may result in postoperative complications, including bleeding, perforation, pancreatitis and even death[14-17]. Moreover, it is notable that the intact sphincter of Oddi is destroyed after EST so that biliary sphincter function is permanently lost, which damages the barrier of the sphincter that prevents duodenobiliary reflux[33]. Reflux from the duodenum into the bile duct is responsible for the high rate of bacterobilia occurring after EST[34], and chronic bacterobilia may even cause neoplastic changes in the biliary epithelium[35]. Therefore, ERCP/EST should be adopted on a selective basis, i.e., in patients with acute obstructive suppurative cholangitis, severe biliary pancreatitis, ampullary stone impaction or severe comorbidity. If possible, MRCP should be used for preoperative diagnosis. According to a meta-analysis, MRCP achieved a high overall sensitivity of 95% and a specificity of 97% for detecting CBDS[36].

With the improvement in laparoscopic equipment and skills, LCBDE has been increasingly used to remove the CBDS. It is considered to be a safe, efficient and cost-effective treatment for choledocholithiasis; it is associated with a high stone clearance rate ranging from 84%-97%, a postoperative morbidity rate of 4%-16%, and a mortality rate of approximately 0%-0.8%[26,37-39]. However, to decompress the bile duct and decrease biliary complications, T-tube drainage has been routinely employed after choledochotomy[40,41], which is inevitable with complications including bile leakage, bile infection and wound infection[41]. Furthermore, the patients have to keep the bile drainage tube in place for several weeks before removal, causing great discomfort and delaying their return to work[42].

Nevertheless, according to a recent meta-analysis[43], primary closure might be as effective as T-tube drainage in the prevention of postoperative complications after choledochotomy. Consequently, it seems that LCBDE is a commendable alternative to the use of ERCP/EST.

Previously published trials have demonstrated that single-stage (LC + LCBDE) and two-stage (LC + ERCP/EST) management are equivalent with respect to stone clearance from the CBD, morbidity and mortality[5,24-26]. However, most of the trials were limited by their small sample size. In 2006, Clayton *et al*[44] reported a meta-analysis concerning endoscopy and surgery *vs* surgery alone for CBDS with the gallbladder *in situ*. In the subgroup analysis, they concluded that the endoscopic and laparoscopic surgery groups had similar outcomes; however, treatment should depend on local resources and expertise. Furthermore, the number of patients included was insufficient, and up-to-date trials were not included. Unlike previous studies, this meta-analysis took patients'

characteristics into consideration and gave suggestions for the optimum management of different patients.

Concerning stone clearance from the CBD, this meta-analysis demonstrated that single-stage (LC + LCBDE) management was as effective as two-stage (LC + ERCP/EST) management (P = 0.17), but one trial[29] was more strongly in favor of the single-stage (LC + LCBDE) management than any other included studies. One possible reason was that they abandoned ERCP/EST at an earlier stage when they detected multiple and large stones in the CBD, and they favored a transductal approach if the bile duct diameter was large or if the stones were large and multiple. Another reason might be the use of intention-to-treat analysis. Our meta-analysis showed that the difference in the length of hospital stay between the two groups was not statistically significant (P = 0.45). Two of the included trials reported that the length of hospital stay was shorter for the single-stage (LC + LCBDE) approach with a statistically significant difference compared with the two-stage (LC + ERCP/EST) management[24,25]. Other studies showed that there was no significant difference between the two groups, but a recent review suggested that single-stage management had the potential merit of a shorter hospital stay[45]. One probable reason was that the definitions of hospital stay varied, which had an impact on the validity of the data. Some trials defined it as the duration from the last finished procedure to discharge, while other trials defined it as the entire duration from hospital admission to discharge. Another explanation for the discrepancy in the studies might be the use of data conversions to produce estimates. In our meta-analysis, only one trial reported hospitalization charges, and there was no significant difference in total hospitalization charges and hospital service charges between the two groups. However, the charges for single-stage (LC + LCBDE) management were lower than those for two-stage (LC + ERCP/EST) management[24]. Other studies[46,47] showed that single-stage management is a cost-effective method compared to two-stage management.

The postoperative morbidity, mortality and total operating time were similar between the two-stage (LC + ERCP/EST) and single-stage (LC + LCBDE) management with no statistically significant difference in this meta-analysis (P = 0.16, P = 0.42 and P = 0.09, respectively). When considering preoperative ERCP/EST + LC *vs* LC + LCBDE and postoperative ERCP/EST + LC *vs* LC + LCBDE separately in the subgroup analysis, the outcomes, as stated, remained consistent.

This meta-analysis was subject to some limitations. First, the funnel plot analysis detected publication bias, which results in over-representation of significant or positive studies. However, the random effects model was utilized for this analysis, and this model is known to enlarge the presence of publication bias by attributing heavier weighting to smaller trials compared to the fixed effects models. Second, the different methodological quality and the heterogeneity of the effect after intervention could also account for the asymmetry in the funnel plot. A

second potential limitation was the presence of significant heterogeneity for three outcomes, including stone clearance from the CBD, conversion to other procedures and length of hospital stay, which might influence the reliability and validity of the conclusions to some extent. Finally, the restriction of only including studies published in English was another possible limitation.

For future research studies on this topic, the following suggestions may be helpful (1) hospitalization expenses data were available in only one included trial, which was far from sufficient, and future studies should evaluate this significant endpoint; (2) follow-up was poorly reported in most of the included trials, and long-term outcomes, which largely rely on the follow-up, are as yet unknown. Future trials should strengthen the work of follow-up; (3) future studies need to assess outcomes such as pain scores, health economics, patients satisfaction and quality of life scores because it is more practical for patients to choose their optimal treatment; (4) blinded outcome assessment should be employed in future trials to better reduce bias; and (5) as a result of the different data types provided by the included trials, the outcomes, including the length of hospital stay and total operative time were weakened. Thus, future researchers should use unified data types.

In conclusion, single-stage (LC + LCBDE) management is not only as effective as two-stage (LC + ERCP/EST) management and equivalent in terms of postoperative morbidity, mortality and conversion, but it also reduces the number of procedures used per patient and potentially shortens the length of hospital stay. In addition, single-stage (LC + LCBDE) management also eliminates the underlying risk of ERCP/EST and keeps the sphincter of Oddi intact. It is likely that as laparoscopic expertise and operators' experience improve, the need for two-stage (LC + ERCP/EST) management will decrease, and single-stage (LC + LCBDE) management should be ultimately available for most patients. However, the optimal management of patients with CBDS should depend on the condition of patients, the expertise of operators and local resources.

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COMMENTS

Background

Patients with concomitant gallstones and common bile duct stones (CBDS) are very common, but surgeons and patients are often faced with difficulties in making treatment decisions when choosing the optimal treatment. Previously published trials were inconclusive as to whether two-stage [laparoscopic cholecystectomy (LC) + preoperative endoscopic retrograde cholangiopancreatography (ERCP)/endoscopic sphincterotomy (EST)] management is better or worse than single-stage [LC + laparoscopic common bile duct exploration (LCBDE)] management. The authors in this study conducted a systematic review and meta-analysis to provide the current best evidence for the management of patients with concomitant gallstones and CBDS.

Research frontiers

Both two-stage (LC + ERCP/EST) and single-stage (LC + LCBDE) management are used for the patients with concomitant gallstones and CBDS. Two-stage (LC + ERCP/EST) management has become very popular in recent years, because it is considered to be safe and effective that can reduce the number of procedures used per patient.

Innovations and breakthroughs

This systematic review and meta-analysis summarized all available randomized controlled trials (RCTs) comparing the two techniques. The authors found that single-stage (LC + LCBDE) management was as effective as two-stage (LC + ERCP/EST) management and required fewer procedures used per patient.

Applications

Single-stage (LC + LCBDE) management is proven to be a safe and effective treatment compared with two-stage (LC + ERCP/EST) management, and it should be recommended for patients with concomitant gallstones and CBDS.

Terminology

ERCP: a technique that combines the use of endoscopy and fluoroscopy to diagnose and treat certain problems of the biliary or pancreatic ductal systems; EST: a minimally invasive surgery that was developed on the basis of ERCP to treat biliary or pancreatic ductal disease; LC: a technique that removes the gallbladder through small punctures in the abdomen to permit the insertion of a laparoscope and surgical instruments; LCBDE: a technique that combines the use of laparoscopy and choledochoscopy to treat biliary tract disease, especially CBDS.

Peer review

This is a good descriptive study in which authors evaluate the safety and effectiveness of two-stage *vs* single-stage management for concomitant gallstones and CBDS. The results are interesting and suggest that two-stage (LC + ERCP/EST) management clearly required more procedures per patient than single-stage (LC + LCBDE) management.

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BRIEF ARTICLE

Poor awareness of preventing aspirin-induced gastrointestinal injury with combined protective medications

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Abstract

AIM: To investigate prescribing pattern in low-dose aspirin users and physician awareness of preventing aspirin-induced gastrointestinal (GI) injury with combined protective medications.

METHODS: A retrospective drug utilization study was conducted in the 2nd Affiliated Hospital, School of Medicine, Zhejiang University. The hospital has 2300 beds and 2.5 million outpatient visits annually. Data mining was performed on all aspirin prescriptions for outpatients and emergency patients admitted in 2011. Concomitant use of proton-pump inhibitors (PPIs), histamine 2-receptor antagonists (H2RA) and muco-protective drugs (MPs) were analyzed. A defined daily dose (DDD) methodology was applied to each MP. A further investigation was performed in aspirin users on combination use of GI injurious medicines [non-steroid anti-inflammatory drugs (NSAIDs), corticosteroids and clopidogrel and warfarin] or intestinal protective drugs (misoprostol, rebamipide, teprenone and gefarnate). Data of major bleeding episodes were derived from medical records and adverse drug reaction monitoring records. The annual incidence of major GI bleeding due to low-dose aspirin was estimated for outpatients.

RESULTS: Prescriptions for aspirin users receiving PPIs, H2RA and MPs (n = 1039) accounted for only 3.46% of total aspirin prescriptions (n = 30 015). The ratios of coadministration of aspirin/PPI, aspirin/H2RA, aspirin/MP and aspirin/PPI/MP to the total aspirin prescriptions were 2.82%, 0.12%, 0.40% and 0.12%, respectively. No statistically significant difference was observed in age between patients not receiving any GI protective medications and patients receiving PPIs, H2RA or MPs. The combined medication of aspirin and PPI was used more frequently than that of aspirin and MPs (2.82% *vs* 0.40%, P < 0.05) and aspirin/H2RA (2.82% *vs* 0.12%, P < 0.05). The values of DDDs of MPs in descending order were as follows: gefarnate, hydrotalcite > teprenone > sucralfate oral suspension > L-glutamine and sodium gualenate granules > rebamipide > sucralfate chewable tablets. The ratio of MP plus aspirin prescriptions to the total MP prescriptions was as follows: rebamipide (0.47%), teprenone (0.91%), L-glutamine and sodium gualenate granules (0.92%), gefarnate (0.31%), hydrotalcite (1.00%) and sucralfate oral suspension (0.13%). Percentages of prescriptions containing aspirin and intestinal protective drugs among the total aspirin prescriptions were: rebamipide (0.010%), PPI/rebamipide (0.027%), teprenone (0.11%), PPI/teprenone (0.037%), gefarnate (0.017%), and PPI/gefarnate (0.013%). No prescriptions were found containing coadministration of aspirin and other NSAIDs. Among the 3196 prescriptions containing aspirin/clopidogrel,

3088 (96.6%) prescriptions did not contain any GI protective medicines. Of the 389 prescriptions containing aspirin/corticosteroids, 236 (60.7%) contained no GI protective medicines. None of the prescriptions using aspirin/warfarin (*n* = 22) contained GI protective medicines. Thirty-five patients were admitted to this hospital in 2011 because of acute hemorrhage of upper digestive tract induced by low-dose aspirin. The annual incidence rates of major GI bleeding were estimated at 0.25% for outpatients taking aspirin and 0.5% for outpatients taking aspirin/warfarin, respectively.

CONCLUSION: The prescribing pattern of low-dose aspirin revealed a poor awareness of preventing GI injury with combined protective medications. Actions should be taken to address this issue.

INTRODUCTION

We read with great interest in the study by Mizukami *et al*[1], who evaluated the influence of taking low-dose aspirin for 4 wk on small intestinal injury and examined the preventive effect of rebamipide. The results showed that long-term aspirin induced small bowl damage (7 cases with a mucosal break at 4 wk on the ileum and 1 on the jejunum at 4 wk among 11 healthy subjects) and rebamipide significantly prevented this damage, and it may be a candidate drug for treating aspirin-induced small bowel complications.

Long-term low-dose aspirin (75-325 mg) has been increasingly prescribed to elderly patients for primary and secondary prevention of cardiovascular and cerebral diseases. Nonetheless, aspirin's efficacy in such disease prevention is limited by the risk of gastrointestinal (GI) injury. Aspirin treatment can increase the GI risk by 2-fold in middle-aged patients without a prior history of peptic ulcer and without using concomitant drugs[2]. Compared

with low-dose aspirin monotherapy, the risk of upper GI injury increased when low-dose aspirin was used in combination with clopidogrel [relative risk (RR), 2.08], oral anticoagulants (RR, 2.00), nonsteroidal antiinflammatory drugs (NSAIDs) (RR, 2.63), or high-dose oral corticosteroids (RR, 4.43)[3]. While the aspect on preventing upper GI complications induced by aspirin[4-7] was emphasized, the study by Mizukami *et al*[1] has reminded us of attaching equal importance to prevention of small bowel injury. We have investigated the status of prescribing pattern of low-dose aspirin in an attempt to recommend the use of combined therapy of proton-pump inhibitors (PPIs), histamine 2-receptor antagonists (H2RA) or mucoprotective drugs (MPs) to prevent aspirin-induced GI injuries. We would discuss and share our perspectives below.

MATERIALS AND METHODS

The prescribing pattern of low-dose aspirin was investigated from the perspective of concomitant use of PPIs, H2RA or MPs in the 2nd Affiliated Hospital, School of Medicine, Zhejiang University. The hospital has 2300 beds and 2.5 million outpatient visits annually. Prescription data was obtained from the hospital information system and processed with Visual FoxPro 9.0. Statistical analysis was performed on the number of prescriptions with aspirin, aspirin/PPI, aspirin/H2RA, aspirin/MP, aspirin/PPI/MP and aspirin/H2RA/MP for outpatients and emergency patients admitted in 2011.

Oral PPIs included omeprazole (or magnesium salt), pantoprazole (or sodium salt), rabeprazole, lansoprazole and esomeprazole magnesium. Intravenous PPIs included omeprazole sodium, pantoprazole sodium and esomeprazole sodium. MPs included teprenone, L-glutamine and sodium gualenate granules (Marzulene-S®), misoprostol, rebamipide, gefarnate, sucralfate oral suspension, hydrotalcite and sucralfate chewable tablets.

Annual amount of each MP consumed was calculated. A defined daily dose (DDD) methodology was applied[8]. The DDD value of each MP was derived from its package insert. DDDs and daily expenditure were estimated using the following equations:

DDDs = Total dosage (amount of drug consumed)/DDD

Daily expenditure = Overall expenditure/DDDs

A further investigation was performed in aspirin users taking GI injurious medicines (NSAIDs, corticosteroids, clopidogrel and warfarin) or intestinal protective drugs (misoprostol, rebamipide, teprenone and gefarnate) which prevented small bowel injury induced by low-dose aspirin or other NSAIDs in human[1,9-12] or rats[13,14]. Data of major bleeding episodes were derived from the medical records and adverse drug reaction monitoring records. The annual incidence of major GI bleeding due to low-dose aspirin was estimated for outpatients.

Age differences between patient groups were tested using Student's *t* test. χ^2 test was used for comparisons of ratios of prescriptions with combined aspirin and other drugs to the total aspirin prescriptions. Difference

was considered statistically significant at $P < 0.05$ for all analyses.

RESULTS

Prescriptions for aspirin users receiving PPIs, H2RA and MPs (n = 1039) only accounted for 3.46% of total aspirin prescriptions (n = 30 015). Among 1039 patients, there were 924 (88.5%) patients on 100 mg aspirin, 108 (10.8%) patients on 200 mg aspirin and 7 (0.7%) patients on 300 mg aspirin. No statistically significant difference in age was observed between patients not receiving any GI protective medications (n = 28 976, aged 63.3 ± 12.4 years) and patients receiving PPIs, H2RA or MPs (n = 1039, 61.8 ± 17.9 year) ($P > 0.05$).

Coadministration of aspirin/PPI, aspirin/H2RA, aspirin/MP and aspirin/PPI/MP accounted for 2.82%, 0.12%, 0.40% and 0.12%, respectively of the total aspirin prescriptions (Table 1). Combined use of aspirin/PPI was more frequent than that of aspirin/MP (2.82% *vs* 0.40%, $P < 0.05$) and aspirin/H2RA (2.82% *vs* 0.12%, $P < 0.05$). Combined therapy of aspirin and two MPs was not found among the prescriptions.

Prescriptions with combination use of pantoprazole, esomeprazole and rabeprazole accounted for 82.6% of all prescriptions containing aspirin/oral PPIs. Omeprazole only accounted for 17.1%.

Pharmacoeconomic indices of MPs for outpatients are listed in Table 2. The values of DDDs of MPs in descending order were as follows: gefarnate, hydrotalcite > teprenone > sucralfate oral suspension > L-glutamine and sodium gualenate granules > rebamipide > sucralfate chewable tablets.

The total dosage (amount of drug consumed) of misoprostol in 2011 was 564.6 mg. However, misoprostol was prescribed for termination of early pregnancy in combination with mifepristone instead of indication for reducing the risk of NSAIDs-induced GI injury. No combined use of misoprostol and aspirin was found among the prescriptions.

The ratio of amount of MP comedicated with aspirin to total amount of MP consumed in 2011 was 0.47% (rebamipide), 0.91% (teprenone), 0.92% (marzulene-S), 0.31% (gefarnate), 1.00% (hydrotalcite) and 0.13% (sucralfate oral suspension), respectively.

Percentages of precriptions containing aspirin and intestinal protective drugs to the total aspirin prescriptions were: aspirin/rebamipide (n = 3, 0.010%), aspirin/PPI/rebamipide (n = 5, 0.027%); aspirin/teprenone (n = 33, 0.11%), aspirin/PPI/teprenone (n = 11, 0.037%), aspirin/gefarnate (n = 5, 0.017%), and aspirin/PPI/gefarnate (n = 4, 0.013%).

No prescriptions were found containing coadministration of low-dose aspirin and other NSAIDs. There were 3196 prescriptions with concomitant use of aspirin/clopidogrel in 2011. However, only 108 (3.4%) prescriptions contained aspirin/clopidogrel/PPI (n = 101), aspirin/clopidogrel/MP (n = 4) and aspirin/ clopidogrel/PPI/MP (n = 3). None of 3088 (96.6%) prescriptions

Table 1 Concomitant use of proton-pump inhibitors, H2-receptor antagonists or mucoprotective drugs in patients taking low-dose aspirin

Drug name	Number of prescriptions	Percentage of prescriptions with combination therapy to total aspirin prescriptions (%)
H2RA	36	0.12[a]
Oral PPIs	708	2.36
Pantoprazole	342	1.14
Esomeprazole	157	0.52
Omeprazole	121	0.40
Rabeprazole	85	0.28
Lansoprazole	3	0.01
i.v. PPIs	139	0.46
Pantoprazole sodium	132	0.44
Omeprazole sodium	7	0.02
MPs	120	0.40[c]
Hydrotalcite	43	0.14
Teprenone	33	0.11
Marzulene-S	25	0.083
Sucralfate	11	0.037
Gefarnate	5	0.017
Rebamipide	3	0.010
Misoprostol	0	0
PPI/MPs	36	0.12
Teprenone	11	0.037
Hydrotalcite	9	0.030
Rebamipide	5	0.017
Marzulene-S	4	0.013
Gefarnate	4	0.013
Sucralfate	3	0.010
Misoprostol	0	0

[a]$P < 0.05$ *vs* oral proton-pump inhibitors (PPIs) plus i.v. PPIs; [c]$P < 0.05$ *vs* oral PPIs plus i.v. PPIs. MPs: Mucoprotective drugs; H2RA: H2-receptor antagonists.

Table 2 Pharmacoeconomic indices of mucoprotective drugs for outpatients in 2011

Drug name	Total dose (g)	DDD (g)	DDDs	Overall expenditure (CNY)	Daily expenditure (CNY)
Gefarnate	14 252	0.15	95 013	312 000	3.3
Hydrotalcite chewable tablet	282 842	3	94 280	557 000	7.4
Teprenone	11 345	0.15	75 633	380 000	5.0
Sucralfate oral suspension	459 648	8	57 456	444 000	7.7
Marzulene-S	89 880	2	44 940	250 000	5.6
Rebamipide	11 195	0.3	37 316	214 000	5.8
Sucralfate chewable tablet	13 950	4	3488	3600	1.0

MPs: Mucoprotective drugs; DDD: Defined daily dose; CNY: China Yuan.

contained any GI protective medications. PPIs coadministered with aspirin and clopidogrel included: oral pantoprazole (n = 58), esomeprazole (n = 32), i.v. pantoprazole (n = 9) and rabeprazole (n = 2). No prescriptions were found containing aspirin/clopidogrel/ omeprazole.

There were 389 prescriptions with concomitant use of aspirin/corticosteroids (prednisone, methylpredisolone and dexamethasone) in 2011. However, only 153 (39.3%) prescriptions contained aspirin/corticosteroid/

PPI (*n* = 148), aspirin/ corticosteroid/MP (*n* = 4) and aspirin/corticosteroid/PPI/MP (*n* = 1). Two hundred and thirty-six prescriptions (60.7%) did not contain any GI protective medications.

There were 22 prescriptions with concomitant use of aspirin/warfarin in 2011. Again, none of these prescriptions contained GI protective medications.

There were 35 patients admitted to this hospital because of acute hemorrhage of upper digestive tract induced by low-dose aspirin in 2011. The annual incidence rates of major GI bleeding were estimated at 0.25% for outpatients on aspirin and 0.5% for outpatients on aspirin/warfarin, respectively. For example, an 81-year-old male patient with coronary artery disease treated by percutaneous coronary interventions and arterial embolism of lower limb, received aspirin (100 mg, q.d.), warfarin (3 mg, q.d.), clopidogrel (75 mg, q.d.) and beraprost sodium (20 μg, t.i.d.) but without concomitant use of any PPIs or MPs. Two weeks later he was sent to the Emergency Center because of GI hemorrhage. Aspirin therapy was stopped and the patient was treated with i.v. pantoprazole. Five days later, he was discharged and continued to take oral pantoprazole (40 mg, q.d.) and rebamipide (0.1 g, t.i.d.) plus warfarin and clopidogrel. No adverse GI event was observed.

DISCUSSION

Yamamoto *et al*[5] examined the effects of gastroprotective drugs on aspirin-related gastroduodenal toxicity in 530 patients who had taken low-dose aspirin for 1 mo or more. Use of a PPI alone was significantly more protective against bleeding (9.3% *vs* 2.1%, *P* < 0.01) and mucosal injury (49.1% *vs* 18.6%, *P* < 0.01) than non-use of any gastroprotective medicine. Among the background characteristics, such as *Helicobacter pylori* infection, concomitant use of anticoagulants, anti-platelet agents, NSAIDs and PPI, a bleeding history, age and gender, only the co-administration of a PPI was found significantly associated with reduced bleeding events. Patients taking any medicine PPI, H2RA, MP, PPI (or H2RA) plus MP showed significantly better outcomes with respect to mucosal injury as compared with the patients not receiving any gastroprotective medication.

Our study indicated that only 3.46% of the patients taking low-dose aspirin received concurrent therapy of PPI, H2RA and MPs. Thus it is imperative to enhance the awareness of preventing GI injury induced by low-dose aspirin among both physicians and patients.

The combined therapy of aspirin and PPI was more frequently used than that of aspirin and MPs (2.82% *vs* 0.40%, *P* < 0.05) and aspirin/H2RA (2.82% *vs* 0.12%, *P* < 0.05) in this hospital and it may be due to the more potent effects of PPIs in prevention of NSAIDS-related GI injuries[15]. However, Nema *et al*[7] observed that the healing rate of gastroduodenal ulcers during continuous use of low-dose aspirin was higher than 80% in both the PPI group and the H2RA group, with no significant difference between the two groups. Nakashima *et al*[16] con-

cluded that H2RA may be the most beneficial drug for both the prevention and treatment of low-dose aspirin-induced peptic ulcers, in which it has the similar anti-ulcer effects to PPIs, but with lower cost and fewer adverse effects as compared with PPIs and prostaglandins.

Concomitant use of NSAIDs, corticosteroids, clopidogrel or anticoagulants increases GI risk further in patients on low-dose aspirin[2-4]. A meta-analysis by Lanas *et al*[4] showed that the risk for GI bleeding in aspirin users increased with concomitant use of clopidogrel and anticoagulant therapies, but decreased in patients who took PPIs. Astoundingly, our investigation showed that 96.6% of patients on aspirin plus clopidogrel, 60.7% of patients on aspirin plus oral corticosteroids and 100% of patients on aspirin plus warfarin did not receive any GI protective medications.

The anti-platelet effect of clopidogrel was activated by biotransformation *via* CYP2C19 and CYP3A4. Different PPIs have different effects on CYP2C19. It has been generally acknowledged that drug interaction between omeprazole and clopidogrel was of clinically significance and can reduce the efficacy of clopidogrel[17]. Pantoprazole, esomeprazole and rabeprazole were alternatives[18-20]. Coadministration of aspirin, clopidogrel and omeprazole was not observed in this study, indicating that this hospital is good at clopidogrel therapy management.

DDDs values of seven mucoprotective drugs were compared. Gefarnate's DDDs ranked first and this result may be associated with its relatively low price. Teprenone's DDDs ranked second. Murakami *et al*[21] concluded that the effects of teprenone on aspirin-induced gastric ulcers in rats were more potent and more definite than those of gefarnate. Fang *et al*[14] reported that teprenone (15.63 mg/kg daily) and gefarnate (31.25 mg/kg daily) can exert protective effects against the intestinal injury induced by NSAIDs in rats. Niwa *et al*[12] found that teprenone (300 mg/d) reduced diclofenac-induced gastric and small intestinal injuries in 10 healthy volunteers (*P* < 0.05). Shiotani *et al*[22] reported that 1 wk administration of low-dose aspirin to 20 healthy volunteers was associated with visible small bowel damage in the majority of users whereas teprenone (150 mg/d) could not prevent aspirin-induced small bowel injury. The inconsistent findings about the preventive effects of teprenone against small intestinal injury may be associated with the dosage of teprenone, sample size of clinical trials and species differences. Although gefarnate used 50 mg twice daily was inferior to lansoprazole at 15 mg daily in reducing the risk of gastric or duodenal ulcer recurrence in patients with a definite history of gastric or duodenal ulcers who required long-term low-dose aspirin therapy[23], the proven effects of gefarnate in prevention of small intestinal injury induced by NSAIDs in rats provoked further investigations on whether gefarnate could prevent small intestinal injury in aspirin users.

Sucralfate is proved to have protective effects against NSAID-associated ulcer due to the enhanced prostaglandin synthesis, increased mucus secretion, suppression of pro-inflammatory cytokines such as tumor necrosis

factor-α (TNF-α), and induction of constitutive nitric oxide synthase as well as its antioxidant capability[24]. Although it had the lowest daily expenditure, sucralfate chewable tablets had the lowest DDDs value. The DDDs value of sucralfate oral suspension was 16 times more than that of sucralfate chewable tablets, although oral suspension had a higher daily expenditure than chewable tablets. In this investigation, we observed that some patients swallowed the chewable tablets directly without chewing, and this implies that sucralfate suspension has a better medication compliance than chewable tablets.

Misoprostol exerts a protective effect on the gastrointestinal mucosa by increasing mucus and bicarbonate ion secretion as well as mucosal blood flow. In addition, it inhibits acid secretion. Watanabe *et al*[9] reported that misoprostol 200 μg 4 times a day could effectively prevent aspirin-induced small intestinal injuries. However, drug compliance of misoprostol was not good due to its side effects and dosing frequency. In clinical trials, misoprostol-induced diarrhoea occurred in approximately one-tenth of the patients despite that it was usually mild and self-limiting. Donnelly *et al*[25] conducted a double-blind placebo-controlled parallel group endoscopic study in 32 healthy volunteers over 28 d and concluded that low-dose misoprostol 100 μg daily can prevent the gastric mucosal injury induced by aspirin 300 mg daily without causing identifiable adverse effects. However, many physicians seem unaware of misoprostol use at such a low-dose. Off-label use of misoprostol (Cytotec®, Piramal Healthcare Ltd., United Kingdom) is very common in China. In this investigation, all of Cytotec® was prescribed for termination of early pregnancy in combination with mifepristone. Thus misoprostol was eliminated by the Drug and Therapeutics Committee of this hospital in September 2011.

Rebamipide provides mucoprotective effect by inducing the production of intracellular prostaglandins and epidermal growth factor, improving blood flow, suppressing increases in permeability, scavenging free radicals and exerting anti-infammatory effect[1]. Yamamoto *et al*[5] reported that in the patients taking rebamipide concomitantly with PPIs, aspirin-induced gastroduodenal mucosal injuries occurred less frequently than in those taking PPIs plus MPs other than rebamipide (14.8% *vs* 38.1%, *P* < 0.01). From this viewpoint, rebamipide had an obvious advantage over other MPs. Mizukami *et al*[1] proved that rebamipide could prevent effectively aspirin-induced small bowel injury. If the exciting finding of this research can be applied to routine clinical practices, good clinical outcomes would be anticipated in patients taking low-dose aspirin.

Our investigation indicated a 0.25% incidence of upper GI bleeding in the low-dose aspirin users, a value nearly four times that of the documented baseline rate of 0.06% noted for the general population without medications or conditions predisposing to bleeding[26], suggesting that low-dose aspirin users did have a relative higher risk of GI injury.

Physician education and computer alert were proved to improve targeted use of gastroprotection among NSAID users[27]. The poor awareness of preventing aspirin-induced GI injury with combined protective medications has attracted the attention of the Drug and Therapeutics Committee of this hospital. Actions to address this issue include academic lectures and computer alert to encourage prescriptions of GI protecting agents, a multidisciplinary team building and initiation of risk-benefit long-term study on the association between increase in expenditure of GI protecting agents and outcomes such as significant reduction in hospital admissions/stays related to GI bleeding.

In conclusion, the study of Mizukami *et al*[1] inspired us to perform this retrospective drug utilization study on the prescribing pattern of low-dose aspirin from the perspective of combined therapy in a large university teaching hospital in China. The results of our survey indicated the poor awareness of preventing gastric and small intestinal injury in patients taking low-dose aspirin with combined protective medications. Good clinical outcomes would be anticipated in aspirin users, especially in patients with a high risk of GI injury, given that the importance of administration of gastroduodenal protective PPIs and intestinal protective rebamipide as well as other GI mucoprotectives is being recognized.

COMMENTS

Background
Low-dose aspirin is currently recommended for the secondary prevention of cardiovascular and cerebral diseases. However, gastroduodenal mucosal injury may be induced by aspirin and concomitant use of non-steoid anti-inflammatory drugs, corticosteroids, clopidogrel or anticoagulants further increases gastrointestinal (GI) risk in patients taking low-dose aspirin. Concomitant use of proton-pump inhibitors (PPIs), histamine 2-receptor antagonists (H2RA) and mucoprotective drugs (MPs) with aspirin can help prevent GI injuries. Recently, aspirin-induced small bowel injuries have attracted clinical attention and preventive effects of some MPs have been observed. This may enhance the awareness of preventing aspirin-induced GI injury with combined protective medication in clinical practice.

Research frontiers
A retrospective drug utilization study on prescribing pattern of low-dose aspirin from the perspective of combination therapy was conducted in a large university teaching hospital of China. Clinical awareness of preventing aspirin-induced GI injury with combined protective medications was evaluated systematically.

Innovations and breakthroughs
The ratio of prescriptions for aspirin users receiving PPIs, H2RA and MPs to the total aspirin prescriptions was revealed for the first time. The survey indicated a poor awareness of preventing gastric and small intestinal injury in patients taking low-dose aspirin with combined protective medications. Combined use of GI injurious medicines or intestinal protective drugs in aspirin users was investigated. The annual incidence of major GI bleeding due to low-dose aspirin was also estimated in the outpatients admitted to the the hospital in 2011. Additionally, a defined daily dose (DDD) methodology was applied to study the prescription pattern.

Applications
The findings of the study will enhance the awareness of preventing aspirin-induced GI injury with combined protective medications from physicians, pharmacists and nurses. Good clinical outcomes would be anticipated in aspirin users, especially in patients with a high risk of GI injury, as the importance of gastroduodenal protective PPIs and intestinal protective rebamipide as well as other MPs is being recognized. Actions should be taken such as encouraging prescription of GI protective agents, building a multi-disciplinary team and initiating a risk-benefit long-term study on the association between increase in

expenditure of GI protective agents and outcomes such as significant reduction in hospital admissions/stays related to GI bleeding.

Terminology

The DDD, a statistical measure of drug consumption, is the assumed average maintenance dose per day for a drug used for its main indication in adults. DDDs value is determined by the formula: DDDs = annual consumption of a drug/drug DDD value. Higher DDDs value means that the drug is prescribed more frequently. Drug utilization studies aim to evaluate the factors related to the prescription, dispensation, administration and intake of medicines and its associated events (either beneficial or adverse).

Peer review

This is a very relevant piece of work summarizing the prescription pattern of low-dose aspirin. Combination use of GI injurious medicines or intestinal protective drugs in aspirin users was also investigated. Based on retrospective data obtained from large number of patients in China, the authors conclude that there is a need for increasing the awareness of preventing aspirin-induced GI injury by concomitant use of gastroduodenal or small intestinal protective agents. The manuscript is very well written, very interesting for the readers.

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CASE REPORT

An unusual case of fatty liver in a patient with desmoid tumor

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Abstract

A desmoid tumor, also known as aggressive fibromatosis, is a rare benign neoplasm that arises from fascial or musculoaponeurotic tissues. It can occur in any anatomical location, most commonly the abdominal wall, shoulder girdle and retroperitoneum. The typical clinical presentation is a painless mass with a slow and progressive invasion of contiguous structures. It is associated with a high local recurrence rate after resection. Many issues regarding the optimal treatment of desmoid tumors remain controversial. Aggressive surgical resection with a wide margin (2-3 cm) remains the gold standard treatment with regard to preserving quality of life. Radiotherapy alone has been shown to be effective for the control of unresectable or recurrent lesions. Desmoid tumors tend to be locally infiltrative, therefore, the fields must be generous to prevent marginal recurrence. The radiation dose appropriate for treating desmoid tumors remains controversial. We present a 25-year-old Caucasian man with local recurrence of a desmoid tumor after repeated surgical resection, treated with radiotherapy. The patient achieved complete tumor regression at 4 mo after radiotherapy, and he is clinically free of disease at 12 mo after the end of treatment, with an acceptable quality of life. The patient developed short bowel syndrome as a complication of second surgical resection. Consequently, radiotherapy might have worsened an already present malabsorption and so led to steatohepatitis.

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Key words: Desmoid tumor; Aggressive fibromatosis; Fatty liver

INTRODUCTION

A desmoid tumor, also known as aggressive fibromatosis, is a rare benign neoplasm that arises from fascial or musculoaponeurotic tissues. It is usually associated with preneoplastic conditions, such as familial adenomatous polyposis and Gardner syndrome. The sporadic form is uncommon, presenting with a mass, sometimes associated with pain and weight loss. It can occur in any anatomical location, but most commonly in the abdominal wall, shoulder girdle and retroperitoneum. Histological

analysis of this type of tumor reveals mesenchymal characteristics, a lack of mitotic activity, and cellular pleomorphism[1-4]. Although desmoid tumors are considered benign, without the ability to metastasize, they do have significant potential for local invasiveness and recurrence[5,6].

We present a case of local recurrence after aggressive surgical resection in a 25-year-old man.

CASE REPORT

A 25-year-old Caucasian man presented to our department with a histologically confirmed diagnosis of local recurrence of sporadic desmoid tumor. The patient's disease dated back 2 years, when the patient was admitted to another hospital because of pain in the right anterolateral abdomen. Abdominal computed tomography (CT) revealed a large solid mass (22 cm × 10 cm × 15 cm) with mild enhancement and density equal to that of soft tissue. The mass appeared to arise from the retroperitoneum and caused superior displacement of the stomach and adjacent bowel loops. There was no pathological lymphadenopathy (Figure 1A).

There was no known history of familial polyposis and no medical history of any abdominal trauma or surgery. His blood parameters were normal, and laboratory studies showed normal renal function and electrolytes.

Radical surgical resection was performed, including 30 cm of small intestine and right hemicolectomy, which was necessary to obtain a wide margin. The histological diagnosis was desmoid tumor. There were no complications in the postoperative phase.

After 10 mo, the patient came to our hospital because of persistent diarrhea. A CT scan was performed, which demonstrated a perianastomotic solid mass (13 cm × 11 cm × 10 cm) arising from the mesentery. The mass caused displacement of the common iliac artery and spread downward to the psoas muscle, without infiltrating it (Figure 1B). A second surgical resection was performed, without complications, including anastomosis and part of the intestinum tenue. The histological diagnosis was aggressive fibromatosis, with uninvolved surgical margins. The patient tolerated the surgical excision of the mass, but he had a nutritional examination because of short bowel syndrome acquired due to the surgery (leaving a residual 75 cm of small intestine). The patient received an optimized dietary and medication plan.

One month later, the patient underwent an abdominal CT scan that revealed an oval mass with a diameter of 18 mm in the mesenteric root, close to the metallic clips of the second surgical excision. This mass was open to a different interpretation. Therefore, the patient underwent an oncological examination, and as we suspected, it was found to be a recurrent lesion. We also recommended a biopsy. The test reported a histological diagnosis of desmoid tumor, and the patient was treated with curative radiotherapy. He received 3D-conformal external beam irradiation using megavoltage photon and conventional fractionation: 2 Gy daily for 5 d/wk, for a total dose of 50 Gy. The planned target volume included the previous tumor bed and actual relapse. Acute toxicity was observed, primarily as a function of the volume of the radiation field. After 2 wk of radiotherapy, the treatment was interrupted for 13 d because of grade 3 gastrointestinal toxicity, according to the Common Terminology Criteria for Adverse Events v3.0. The patient had progressive weight loss, fatigue and severe radiation enterocolitis, resulting in urgency, increased frequency (> 7/d) and decreased consistency of bowel movements. The radiotherapy was temporarily interrupted, and the patient received parenteral nutrition for 1 wk.

We radiologically assessed the response to radiotherapy after 4, 8 and 12 mo, and the outcome was complete remission. It is important to note that abdominal CT carried out 8 mo after the end of radiotherapy revealed liver alterations with no distinguishing marks (Figure 2). Such alteration had not been observed in previous examinations; therefore, the patient underwent magnetic resonance imaging and then liver biopsies, because the first was not sufficient to clarify the situation (Figure 2). The histological diagnosis was macrovesicular and microvesicular steatosis. Radiotherapy increases the risk of hepatobiliary complications in short bowel syndrome, therefore, a wait-and-see approach, consisting of radiological and clinical evaluations performed every 3 mo, was chosen. The patient achieved complete tumor regression at 4 mo after radiotherapy, and he is clinically free of disease at 12 mo after the end of treatment, with an acceptable quality of life.

DISCUSSION

The definition of a rare tumor is based on an incidence of ≤ 3/100 000/year[7]. Desmoid tumors fall under this category because they have an annual incidence of 0.2-0.4/100 000 population. For that reason, desmoids are not extensively discussed in the literature: most reports are case reports[1,3,8-11], and some are retrospective analyses[5,6,12,13], but randomized series do not exist.

The etiopathogenesis is poorly defined; trauma, endocrine factors and genetic predisposition all seem to play important roles in the development of the disease. It is significantly associated with familial adenomatous polyposis and has a more pronounced association with the subgroup of Gardner syndrome. Desmoid tumors are a fibroblastic proliferations that arise mainly from fascial or musculoaponeurotic structures. The typical clinical presentation is a painless mass with a slow and progressive invasion of contiguous structures, and it is associated with a high local recurrence rate after resection. Factors predictive of relapse include an age between 18 and 30 years, a tumor size > 8 cm, incomplete resection, or close positive margins, and no adjuvant radiotherapy for gross residual disease[5,6,12].

The rarity of these tumors, coupled with the variability in their clinical course, has prevented the demonstration

Figure 1 Abdominal computed tomography of desmoid tumour. A: A solid large mass (22 cm × 10 cm × 15 cm) with density equal to that of soft tissue; B: A peri-anastomotic solid mass (13 cm × 11 cm × 10 cm) arising from the mesentery.
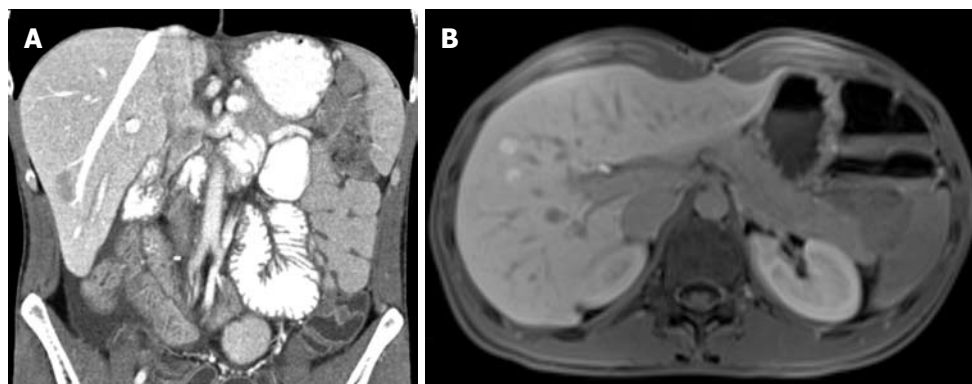

Figure 2 Fatty liver. Abdominal computed tomography (A) and magnetic resonance imaging (B) demonstrated liver alterations with no distinguishing marks.

of the efficacy of any specific intervention[13]. Many issues regarding the optimal treatment of desmoid tumors remain controversial. However, true R0 margins are difficult to achieve because of the indistinct borders in this type of tumor[14,15]. Aggressive surgical resection with a wide margin (2-3 cm) remains the gold standard treatment with regard to the preservation of quality of life[16].

Radiotherapy alone has been shown to be effective for the control of unresectable or recurrent lesions. In 1928, Ewing *et al* proposed radiotherapy as a treatment modality for the management of these lesions and reported that desmoid tumors respond slowly but satisfactorily to radiation[16]. In subsequent years, several studies[17-20] have confirmed that radiotherapy can result in lasting local control in inoperable or incompletely resected tumors. A comparative review of 22 articles[12] has shown that radiotherapy, delivered as a single modality or as an adjuvant treatment, resulted in significantly better local control than surgery alone (78%, 75% and 61%,

respectively). However, the role of radiotherapy in the management of desmoid tumors is still not defined.

Strong indications for radiotherapy include unresectable lesions, macroscopic or microscopic residual disease after resection and recurrent tumors.

The radiation dose appropriate for treating desmoid tumors remains controversial. Ballo *et al*[14] have demonstrated that doses of 56 Gy at 2 Gy per fraction (equivalent to approximately 60 Gy at 1.8 Gy per fraction) produce significantly higher control rates than doses ≤ 50 Gy. In other published studies[17,19-22], patients were treated with doses of 50-60 Gy. To date, no improvement of results with total doses higher than 60 Gy has been observed, while an increased incidence of radiation-related toxicity has been documented[12-15,17,20]. As desmoid tumors tend to be locally infiltrative, the fields must be generous to prevent marginal recurrence. Of course, normal tissue toxicity and potential late radiation effects are important to consider in the treatment. Radiotherapy for gross dis-

ease is associated with a relatively high rate of complications, which are dose-dependent. Complications have included edema, fibrosis, ulcers, paraesthesia and secondary malignancy[8]. Radiation enteritis and radiation-induced liver disease are also potential complications of radiotherapy. In the study of Boland *et al*[23], a total of 48 patients treated with radiotherapy for intra-abdominal malignancy were evaluated: 16 (33%) patients developed short bowel syndrome within 12 mo of treatment. Thompson *et al*[24] found that patients with short bowel syndrome and a history of radiotherapy were more likely to develop cirrhosis and portal hypertension than short bowel syndrome patients not subject to radiotherapy. Although diabetes mellitus and obesity are the conditions most frequently associated with nonalcoholic fatty liver disease, which is histologically defined by the presence of macrovesicular steatosis, intestinal resection has also been recorded as one of the predisposing factors[25]. Our patient, who did not conform to the usual profile of an obese and diabetic man, developed short bowel syndrome as a complication of the second surgical resection. Consequently, radiotherapy might have worsened an already present malabsorption and so led to steatohepatitis. Immediate adjuvant radiotherapy after the first radical surgical approach would likely have produced a better result in terms of toxicity and quality of life, reducing the likelihood of malabsorption due to repeated surgical resections.

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CASE REPORT

Rectal perforations and fistulae secondary to a glycerin enema: Closure by over-the-scope-clip

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Abstract

Rectal perforations due to glycerin enemas (GE) typically occur when the patient is in a seated or lordotic standing position. Once the perforation occurs and peritonitis results, death is usually inevitable. We describe two cases of rectal perforation and fistula caused by a GE. An 88-year-old woman presented with a large rectal perforation and a fistula just after receiving a GE. Her case was further complicated by an abscess in the right rectal wall. The second patient was a 78-year-old woman who suffered from a rectovesical fistula after a GE. In both cases, we performed direct endoscopic abscess lavage with a saline solution and closed the fistula using an over-the-scope-clip (OTSC) procedure. These procedures resulted in dramatic improvement in both patients. Direct endoscopic lavage and OTSC closure are very useful for pararectal abscess lavage and fistula closure, respectively, in elderly patients who are in poor general condition. Our two cases are the first reports of the successful endoscopic closure of fistulae using double OTSCs after endoscopic lavage of the debris and an abscess of the rectum secondary to a GE.

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Key words: Rectal perforation; Glycerin enema; Abscess lavage; Fistula closure; Over-the-scope-clip

INTRODUCTION

Glycerin enemas (GEs) are widely performed, but rectal perforations secondary to GEs are only occasionally reported[1]. These perforations usually occur when patients are in a seated or lordotic standing position. Once they lead to peritonitis, death is usually inevitable[2]. We successfully performed direct endoscopic abscess lavage and fistula closure using a double over-the-scope-clip (OTSC) technique (Ovesco Endoscopy GmbH, Tuebingen, Germany)[3,4] in two patients with rectal perforation and fistula caused by a GE. These procedures resulted in a dramatic improvement in each patient's condition. Direct endoscopic lavage with a saline solution and double OTSC

closure are very useful for pararectal abscesses and large fistulae closure (greater than 20 mm), respectively, in elderly patients in poor general condition who cannot undergo surgery[5-9].

CASE REPORT

An 88-year-old bedridden woman experienced an acute onset of abdominal pain, high fever and melena. The laboratory examination revealed severe inflammation and anemia [white blood cell (WBC) 23 000/μL, hemoglobin (Hb) 6.6 g/dL, C-reactive protein (CRP) 23 mg/dL]. Three days later, a colonoscopy was performed, revealing a large fistula (20 mm) in the right rectal wall (Figure 1A). Computed tomography also revealed a large abscess in the right rectal wall (6 cm × 3 cm × 3 cm) (Figure 1B). Surgical closure was indicated but was impossible to perform given the patient's poor overall condition. We informed her family that direct endoscopic abscess lavage and fistula closure with an OTSC would thus be required, and written informed consent was obtained from the family. The rectum was thoroughly washed with saline, and an endoscope with a water jet function was inserted into the abscess cavity that resulted from the fistula to wash out and lavage the pus and debris associated with the abscess. The abscess cavity was undercoated with pararectal fat tissue, which bled readily (Figure 2). The fistula was lavaged with 2 L of saline and then closed completely using the OTSC technique (Figure 1C). Five days later, the patient's laboratory parameters had significantly improved (WBC 7800/μL, Hb 8.3 g/dL, CRP 7 mg/dL), and computed tomography revealed the disappearance of the abscess (Figure 1D). The overall time was 10 min, and there were no procedure-related complications. We administered an intravenous drip infusion of cefazolin (2 g/d) for two days following the closure of the fistula.

The second patient was a 78-year-old woman suffering from constipation. After receiving a GE, she experienced sudden, severe and sharp lower abdominal pain, a high fever and melena. Immediately after the onset of this sudden abdominal pain, an emergency colonoscopy revealed a large fistula (25 mm) in the ventral wall of the rectum (Figure 3A). After contrast radiography was used in the colonoscopy, computed tomography revealed the pooling of the radiocontrast agent in the urinary bladder and fistula (Figure 3B). We completely closed the fistula with a series of OTSCs (double OTSCs) (Figure 3C).

Computed tomography revealed the pooling of the radiocontrast agent in the urinary bladder and the complete closure of the fistula with double OTSCs (Figure 3D). The lateral view of the contrast radiography with the colonoscopy revealed a fistula leading to the urinary bladder, with inflowing and pooling of the radiocontrast agent in the urinary bladder (Figure 4A). Given the onset of the patient's symptoms and these findings, we diagnosed a rectovesical fistula secondary to the tip of the GE. Because the fistula was a little larger than the OTSC, the single OTSC closure resulted in 1/3 of the fistula remaining (Figure 4B). We decided to close the fistula us-

Figure 1 Colonoscopy and computed tomography of case 1. A: Colonoscopy revealed a large fistula in the right side wall of the rectum; B: Computed tomography revealed a large abscess in the right rectal wall (6 cm × 3 cm × 3 cm in diameter) (arrowheads) and a fistula (arrow); C: After the lavage with 2 liters of saline, a colonoscopy was performed, and the fistula was completely closed using the over-the-scope-clip (OTSC) technique (arrow); D: Computed tomography revealed a remarkable disappearance of the abscess (arrowheads) and showed the OTSC (arrow).

ing double OTSCs. We succeeded in immediately closing the fistula with two OTSCs (Figure 4C). The overall pro-

Figure 2 The abscess cavity lumen of case 1. The abscess cavity was undercoated by pararectal fat tissue, which bled easily.

Figure 4 The contrast radiography of case 2. A: The lateral view of the contrast radiography and the colonoscopy revealed a fistula communicating with the urinary bladder and the inflowing and pooling of the radiocontrast agent in the urinary bladder (arrow); B: The fistula was slightly larger than the single over-the-scope-clip (OTSC). Single OTSC closure (arrow) resulted in 1/3 of the fistula remaining. Again, the contrast radiography revealed a residual fistula leading to the urinary bladder (arrowheads); C: The remaining fistula was completely closed with double OTSCs. The contrast radiography revealed no fistula leading to the urinary bladder (arrowheads). Tests revealed the successful closure of the fistula without any inflowing of the radiocontrast agent (arrow).

cedure time was 20 min, and there were no procedure-related complications. After this series of OTSC closures, the patient's laboratory data improved significantly, and computed tomography revealed the disappearance of the fistula.

DISCUSSION

In the first case, rectal perforation led to a pararectal abscess. In the second case, the rectal fistula was caused by rectal perforation. Rectal perforation has been reported after GE when patients are in a seated or lordotic standing position[1,2]. We diagnosed the rectal perforation secondary to a GE partly based on the presentation of sudden abdominal pain, melena and a high fever. The perforation rate after a double-contrast barium enema is between 0.02% and 0.24%. Perforation remains an infrequent and almost certainly under-reported complication[3-5]. An iatrogenic perforation caused by a barium or GE as in our cases leads to serious conditions and adverse outcomes in elderly patients who are already in poor general condition.
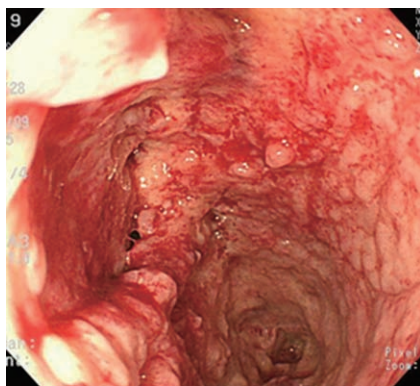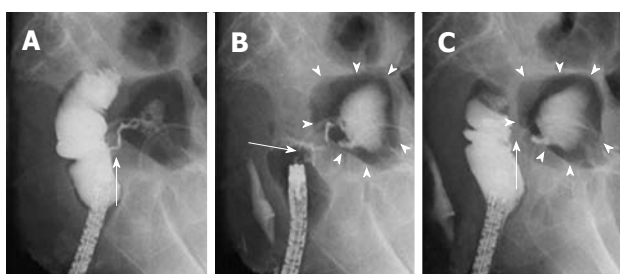

Figure 3 Colonoscopy and computed tomography of case 2. A: Colonoscopy revealed a large fistula (25 mm) (arrow) in the ventral wall of the rectum; B: Following contrast radiography with a colonoscopy, computed tomography revealed the pooling of the radiocontrast agent in the urinary bladder and fistula (arrow); C: Complete closure of the fistula with a series of over-the-scope-clips (OTSCs) (double OTSCs) was performed (arrow); D: Computed tomography revealed the pooling of the radiocontrast agent in the urinary bladder and the complete closure of the fistula with double OTSCs (arrow).

Although perforation is a severe endoscopic complication, the OTSC procedure is reportedly very useful for the closure of the perforation site[6,7]. The OTSC is an endoscopic tool that allows for the application of a large claw-like clip for the endoscopic closure of full thickness wall defects within the gastrointestinal tract. It is a novel tool that can be safely and successfully employed to en-

doscopically close a fistula of the intestinal tract[8,9]. Studies report that the best indications for the use of OTSC are related to post-surgical fistulae and perforations due to colonoscopy. Defects ranging from 5 to 20 mm in the stomach and from 10 to 30 mm in the colon can be closed with a single OTSC, but tissue defects larger than 20-30 mm may require more than one OTSC to achieve adequate closure[10-15]. We successfully closed a perforation larger than 20 mm using a single OTSC and assessed the healing of the fistula by endoscopic or radiological means. Direct endoscopic lavage with a saline solution and double OTSC closures are very useful for the management of pararectal abscesses. In case 2, we closed a fistula larger than 25 mm with a series of OTSCs (double OTSCs) in an elderly patient in poor overall condition who could not undergo surgery. We did not experience any OTSC-related complications. The endoscopic closure of perforations and fistulae with OTSC is a simple and minimally invasive technique. Given the complete closure and healing of large fistulae with OTSC in our two cases, this approach may be less expensive and more advantageous than a surgical closure.

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LETTERS TO THE EDITOR

More attention should be paid on the interpretation of gene expression data

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Abstract

Molecular profiling of gene expression is important for determining signatures in cancer progression and diagnosis. For this purpose, polymerase chain reaction-based techniques are preferentially used as a feasible and sensitive approach. Nevertheless, when relative quantitative analyses are performed on gene expression, the interpretation of mathematical equations must be carefully done. This letter to the editor is focused on recently published gene expression data in *World Journal of Gastroenterology* by Ozmen *et al* demonstrating increased levels of *LYVE-1*, *VEGFR-3* and *CD44* genes in gastric cancer samples compared to non-neoplastic gastric tissues. However, there are major concerns about misinterpretation of the gene expression data obtained with the $2^{-\Delta\Delta Ct}$ relative quantitative method. In the study, $2^{-\Delta\Delta Ct}$ values calculated for many samples were smaller than 1 ($2^{-\Delta\Delta Ct} < 1$) which indicate decreased levels of *LYVE-1*, *VEGFR-3* and *CD44* gene expression in the gastric cancer tissues. This unfortunate mistake is an important example showing how a simple error in the interpretation of relative-quantitative gene expression data may result in misleading scientific conclusions. In this letter, a brief explanation of the $2^{-\Delta\Delta Ct}$ method is given. In addition, the importance of technical quality and interpretation in gene expression studies is discussed.

LETTER TO THE EDITOR

I read with great interest the article "relationship between *LYVE-1*, *VEGFR-3* and *CD44* gene expressions and lymphatic metastasis in gastric cancer" by Ozmen *et al*[1] in *World Journal of Gastroenterology* published in July 21, 2011. The expression of three genes playing important roles in lymphangiogenesis was studied in a large group of gastric cancer patients. In their study, a relative quantitative method was applied to assess the levels of gene expression. In addition, it was a good approach to compare the gene expression of the gastric tumors' data with that of the surgically-resected non-neoplastic (so-called "normal tissues" by the authors) samples. However, I am concerned about the misinterpretation of the gene expression data calculated by using "$2^{-\Delta\Delta Ct}$ method". Rather than being increased as presented in the manuscript, the gene expression values calculated with $2^{-\Delta\Delta Ct}$ indicate decrement ($2^{-\Delta\Delta Ct} < 1$) for many samples.

According to $2^{-\Delta\Delta Ct}$ method, the polymerase chain reaction (PCR) threshold cycle (Ct) values of a specific gene and of a house keeping gene are obtained both from the case and control groups (tumor and non-neoplastic tissue samples, respectively in the manuscript by Ozmen *et al*[1])[2,3]. The difference between the Ct values of the specific gene and the house keeping gene is calculated (which is ΔCt) for individual cases and controls[2,3].

This provides an internal normalization for each sample. Then, ΔCt value of the control sample is subtracted from the ΔCt of the case sample (which is ΔΔCt, giving raw information about the difference in gene expression levels). Since DNA is amplified by the power of two for each PCR cycle, ΔΔCt is also presented as power of "2"[2,3]. Simply, if there is no difference between the case and control samples, ΔΔCt value will be "0" and two to the zero power is "1". Therefore, in the $2^{-\Delta\Delta Ct}$ method, if the gene expression level of the case is higher than that of the control sample, the $2^{-\Delta\Delta Ct}$ value is "> 1"; but, if it is lower than the control sample $2^{-\Delta\Delta Ct}$ value is between "0" and "1"[2,3].

In the manuscript of Ozmen et al[1], although $2^{-\Delta\Delta Ct}$ values are calculated correctly, they presumed that the values higher than zero give an indication of increment in the expression levels of the genes studied. When their data is distributed according to the $2^{-\Delta\Delta Ct}$ with an appropriate interpretation, two groups appear with high and low gene expression patterns, especially for LYVE-1 and VEGFR-3 genes (Figure 2 in the manuscript by Ozmen et al[1]). Additionally, to obtain a true Ct value, real-time PCR con-

ditions should be finely optimized and final product should be devoid of non-specific products (extra bands) or primer dimers. However, there are several non-specific amplicons in the PCR products of VEGFR-3 gene (Figure 1 in the manuscript by Ozmen et al[1]). This basic technical problem hampers the reliability of quantitative gene expression results. I believe the correction of this misinterpretation would provide additional value to their study.

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MEETINGS

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori*
Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

March 1-3, 2012
International Conference on
Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal
and Endoscopic Surgeons Annual
Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on
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Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and
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Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
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March 31-April 1, 2012
27th Annual New Treatments in
Chronic Liver Disease
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April 8-10, 2012
9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
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April 15-17, 2012
European Multidisciplinary
Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress
2012
Barcelona, Spain

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Internal Medicine 2012
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United States

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Diffuse Small Bowel and Liver
Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual

Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian
Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-
American Society of Colon and
Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology
Nurses and Associates Annual
Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and
Rectal Surgeons Annual Meeting
San Antonio, TX 78249,
United States

June 18-21, 2012
Pancreatic Cancer: Progress and
Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International

Neurogastroenterology and Motility
Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory
Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society
2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of
Gastroenterology and Abdominal
Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies
in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of
Gastroenterology 77th Annual
Scientific Meeting and Postgraduate
Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in
Diagnosis and Treatment of
Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101,
United States

November 9-13, 2012
American Association for the Study
of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel
Diseases
Hollywood, FL 33028, United States

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1352 experts in gastroenterology and hepatology from 64 countries.

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Name of journal

World Journal of Gastroenterology

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Key words
Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Acknowledgments
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Chinese journal article (list all authors and include the PMID where applicable)
2   **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press
3   **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author
4   **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462   PMCID:2516377   DOI:10.1161/01.HYP.00000 35706.28494.09]

Both personal authors and an organization as author
5   **Vallancien G**, Emberton M, Harving N, van Moorse-laar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given
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]

Volume with supplement
7   **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatrip-tan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume
8   **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue
9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books
Personal author(s)
10   **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)
11   **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)
12   **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings
13   **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper
14   **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)
15   Morse SS. Factors in the emergence of infectious dis-eases. 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

Statistical data

Write as mean $\pm$ SD or mean $\pm$ SE.

Statistical expression

Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as υ (in Greek), sample number as n (in italics), and probability as P (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 $\pm$ 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.
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