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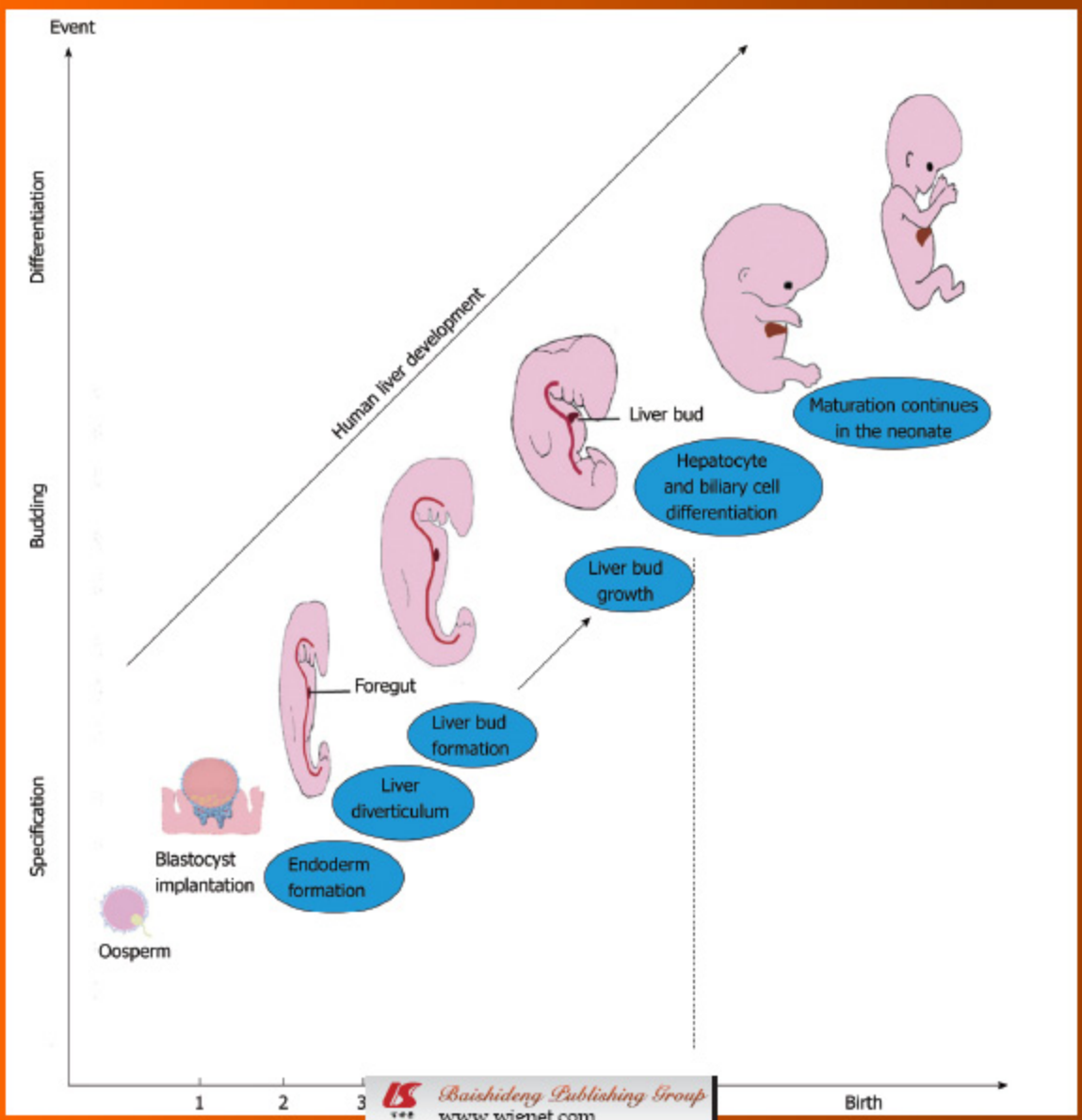

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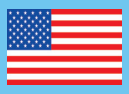

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EDITING
Editorial Board of *World Journal of Gastroenterology*
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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EDITORIAL OFFICE
Jian-Xia Cheng, Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
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EDITORIAL

Idiopathic sclerosing encapsulating peritonitis: Abdominal cocoon

Jenny N Tannoury, Bassam N Abboud

Jenny N Tannoury, Bassam N Abboud, Department of General Surgery, Faculty of Medicine, Hotel Dieu de France Hospital, Saint-Joseph University, Beirut 16-6830, Lebanon
Author contributions: Abboud BN designed the research; Tannoury JN and Abboud BN performed the research; Tannoury JN and Abboud BN analyzed the data; Tannoury JN and Abboud BN wrote the paper.
Correspondence to: Bassam N Abboud, MD, Professor, Department of General Surgery, Faculty of Medicine, Hotel Dieu de France Hospital, Saint-Joseph University, Alfred Naccache Street, Beirut 16-6830, Lebanon. dbabboud@yahoo.fr
Telephone: +961-1-615300 Fax: +961-1-615295
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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

Peer reviewers: A Ibrahim Amin, MD, Department of Surgery, Queen Margaret Hospital, Dunfermline, Fife KY12 0SU, United Kingdom; Vincenzo Stanghellini, Professor, Internal Medicine and Gastroenterology, University of Bologna, VIA MASSARENTI 9, Ⅰ-40138 Bologna, Italy

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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 etiologies. 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

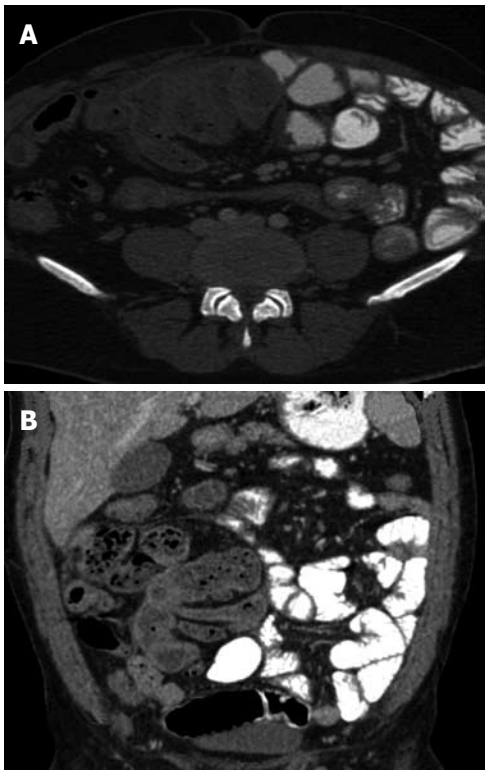

Figure 1 Computed tomography scan. A: Axial contrast enhanced computed tomography (CT) scan of the abdomen showing bowel loop mass encased in a membrane; B: Anterio-posterior CT scan of the abdomen showing thin membrane around bowel loops.

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-

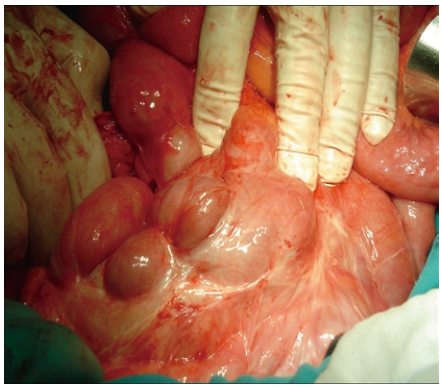

Figure 2 Laparotomy showing an encapsulating thick, white adherent membrane encasing the small bowel.

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 associated 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

Toru Ishikawa

Toru Ishikawa, Department of Gastroenterology and Hepatology, Saiseikai Niigata Daini Hospital, 280-7 Teraji, Niigata 950-1104, Japan
Author contributions: Ishikawa T contributed solely to this manuscript.
Correspondence to: Toru Ishikawa, MD, Department of Gastroenterology and Hepatology, Saiseikai Niigata Daini Hospital, 280-7 Teraji, Niigata 950-1104, Japan. toruishi@ngt.saiseikai.or.jp
Telephone: +81-25-2336161 Fax: +81-25-2338880
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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.

Key words: Branched-chain amino acids to tyrosine ratio; Fischer's ratio; Prognostic factor; Hepatocellular carcinoma

Peer reviewer: Gabriele Grassi, Associate Professor, Department of Medical, Technological and Translational Sciences, University Hospital of Cattinara, Strada di Fiume 447, 34100 Trieste, Italy

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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

Varut Lohsiriwat

Varut Lohsiriwat, Division of Colon and Rectal Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Author contributions: Lohsiriwat V was the sole contributor to literature review, acquisition, analysis of data and manuscript preparation.
Supported by Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
Correspondence to: Varut Lohsiriwat, MD, PhD, Division of Colon and Rectal Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Prannok Road, Bangkok 10700, Thailand. bolloon@hotmail.com
Telephone: +66-0-24198077 Fax: +66-0-24115009
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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.

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

Peer reviewer: Rasmus Goll, MD, Department of Gastroenterology, University Hospital of North Norway, 9038 Tromso, Norway

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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)

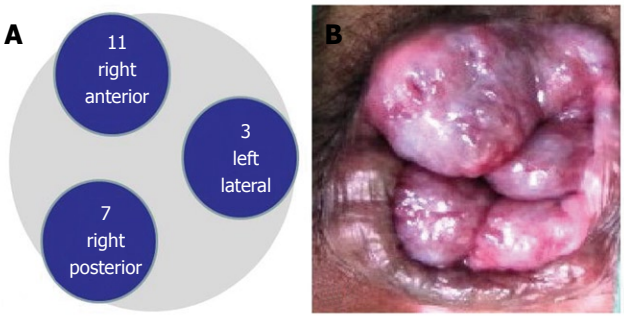

Figure 1 Diagram of common sites of major anal and internal hemorrhoids. A: Diagram of common sites of major anal cushions; B: Common sites of internal hemorrhoids.

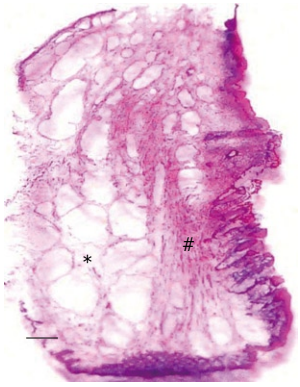

Figure 2 Pathological changes in hemorrhoids. *: Marked dilatation of hemorrhoidal venous plexus; #: Fragmented anal subepithelial muscle (the Treitz's muscle or mucosal suspensory ligament) (Scale bar = 1 mm).

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, dysregulation 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 dietary 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 $\geqslant$ 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

Wen-Li Zhou, Claire N Medine, Liang Zhu, David C Hay

Wen-Li Zhou, Liang Zhu, Department of Gastroenterology, Changzheng Hospital, Second Military Medical University of China, Shanghai 200003, China
Wen-Li Zhou, Claire N Medine, David C Hay, Medical Research Council Centre for Regenerative Medicine, University of Edinburgh, Edinburgh EH16 4UU, Scotland, United Kingdom
Author contributions: Zhou WL and Medine CN contributed towards the conception and design of the review; Zhu L contributed to supervision; Hay DC provided supervision and major input into manuscript preparation.
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Correspondence to: David C Hay, PhD, Medical Research Council Centre for Regenerative Medicine, University of Edinburgh, 5 Little France Drive, Edinburgh EH16 4UU, Scotland, United Kingdom. davehay@talktalk.net
Telephone: +44-131-6519549 Fax: +44-131-6519501
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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.

Key words: Differentiation; Pluripotent stem cells; Hepatocyte-like cells; Liver development; Polymer chemistry; Regenerative medicine; Transplantation; Bio-artificial liver

Peer reviewer: Dr. Run Yu, Cedars-Sinai Medical Center, 8700 Beverly Blvd, B-131, Los Angeles, CA 90048, United States

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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 demonstrate 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.

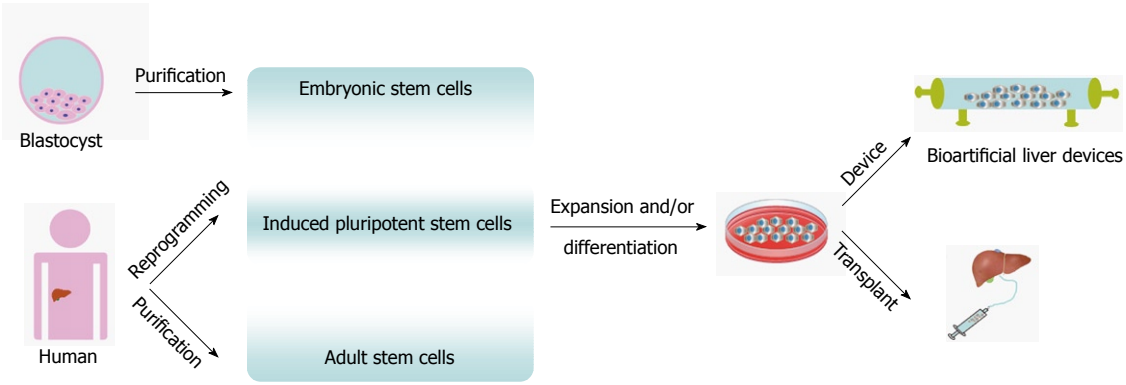

Figure 1 Potential application of human stem cells in cell based therapy for liver disease. Pluripotent and multipotent stem cells can be reprogrammed or purified from human material that was been ethically sourced. Following expansion and differentiation the derivative hepatocyte like cells can be used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

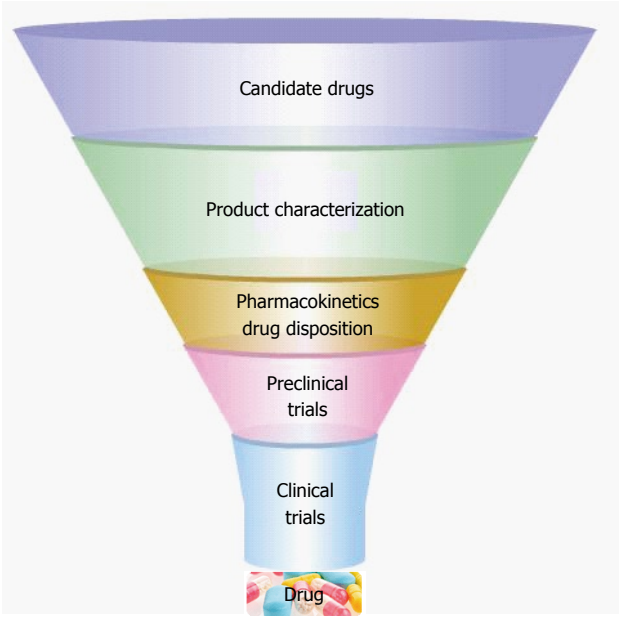

Figure 2 Human liver cells in drug discovery[7,53,66,67]. Drug development process is a lengthy and expensive process. The derivation of hepatocyte-like cells from different human genotypes may provide novel *in-vitro* models for the screening of new compounds in the drug discovery process.

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

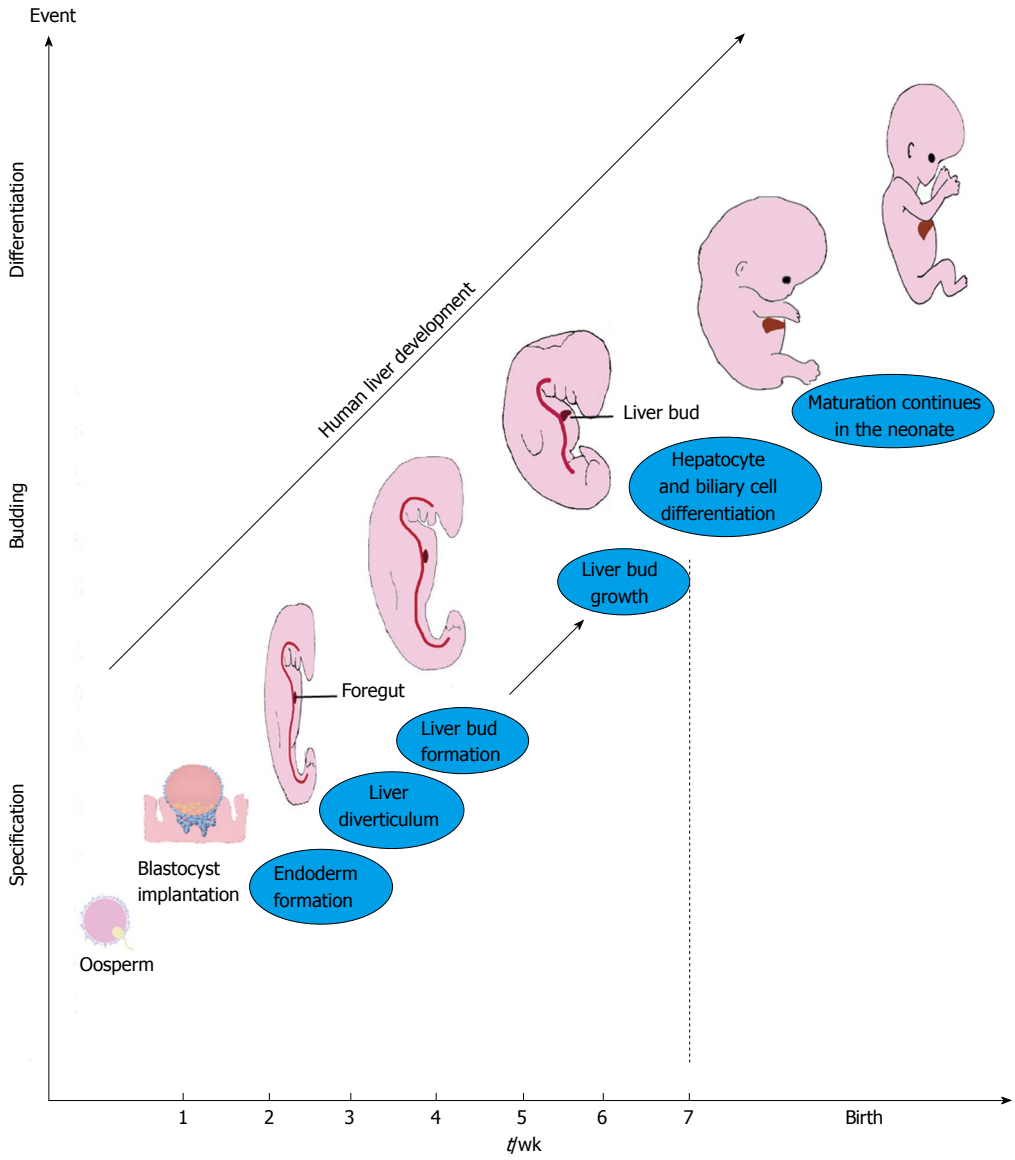

Figure 3 Human fetal liver development[31,74]. The key stages of human liver development are shown in pink and blue. Endoderm formation occurs in the 2nd-3rd wk of fetal development. The liver bud forms between week 3-4 and expands rapidly. Hepatocytes and biliary epithelia differentiate and mature from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

Sahar K Hegazy, Mohamed El-Bedewy, Akira Yagi

Sahar K Hegazy, Department of Clinical Pharmacy, Faculty of Pharmacy, Tanta University, Tanta 8130, Egypt
Mohamed El-Bedewy, Department of Internal Medicine, Faculty of Medicine, Tanta University, Tanta 8130, Egypt
Akira Yagi, Placenta/Aloe Research Institute, Japan Bio-Products Co., Ltd. 1488-4, Aikawa-machi, Kurume-shi, Fukuoka Bio Factory 204, Fukuoka 839-0861, Japan
Author contributions: Hegazy SK designed the study, analyzed the data, and wrote the paper; El-Bedewy M followed up the patients, obtained the samples for analysis and revised the paper; Yagi A obtained the high molecular weight fractions of aloe vera, and made the extraction and preparation of aloe vera.
Correspondence to: Sahar K Hegazy, Assistant Professor, Department of Clinical Pharmacy, Faculty of Pharmacy, Tanta University, Tanta 8130, Egypt. s_hgz@yahoo.com
Telephone: +20-40-2243391 Fax: +20-40-2233622
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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.

Key words: Hepatic fine periportal fibrosis; Aloe vera; α-smooth muscle actin; Transforming growth factor-β; Hyaluronic acid

Peer reviewer: Ali Reza Mani, Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Jalal-Ale-Ahmad Highway, Tehran 14115, Iran

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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 determined 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 $P < 0.05$.

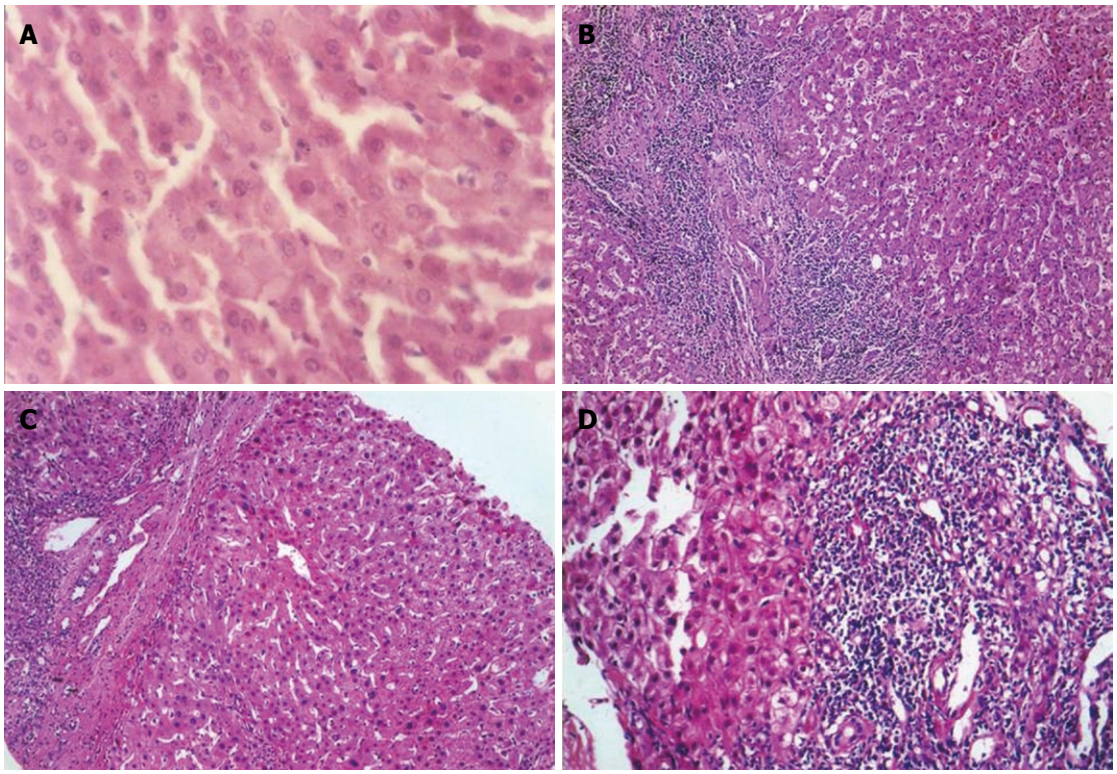

Figure 1 Hematoxylin and eosin staining of liver tissues (HE, × 200). A: Control group showed normal lobular architecture and cell structure; B: Patients before treatment showed fibro-proliferated bile ductules, thick fibrous septa and dense inflammatory cellular infiltration; C: The conventional group 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.

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.

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, 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, antioxidative 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.

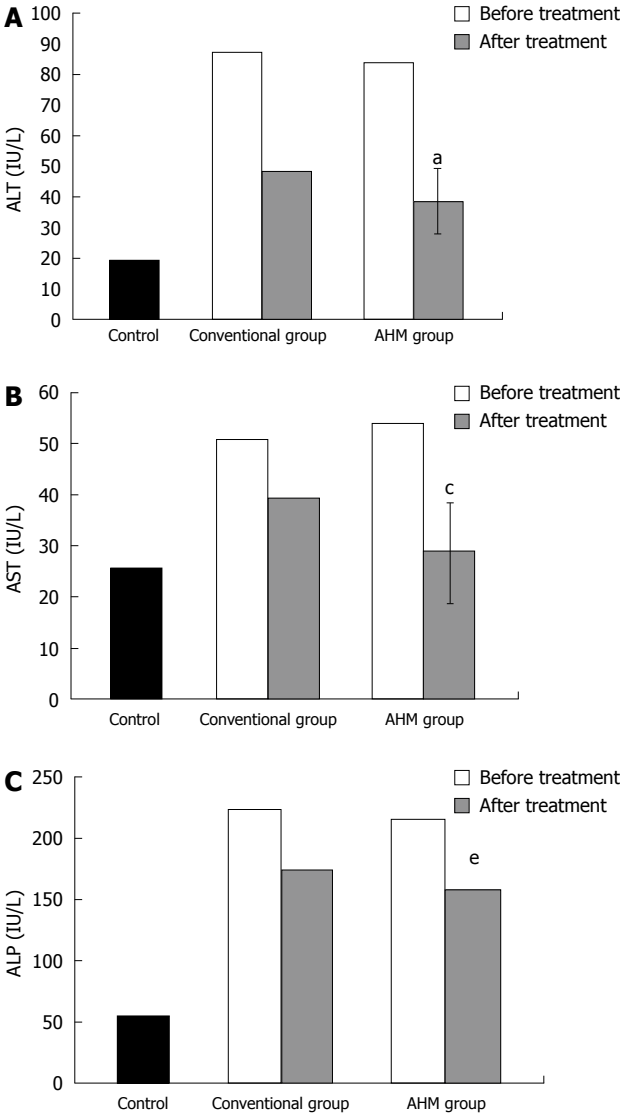

Figure 2 Serum activity of alanine aminotransferase, alkaline phosphatase and aspartate aminotransferase of hepatic fibrosis patients and controls. A: Serum activity of alanine aminotransferase (IU/L) of hepatic fibrosis patients and controls; B: Serum activity of aspartate aminotransferase (IU/L) of 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-

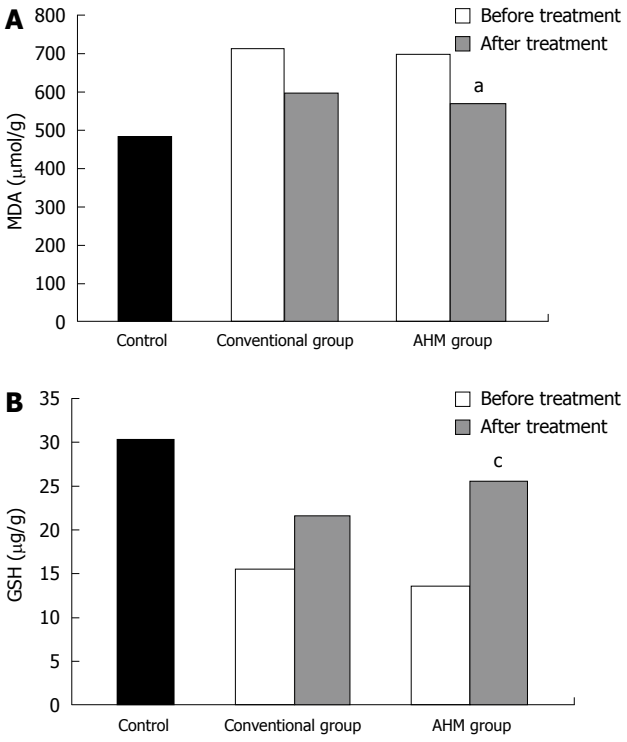

Figure 3 Hepatic malondialdhyde and glutathione of hepatic fibrosis patients and controls. A: Hepatic malondialdhyde (μmol/g) of hepatic fibrosis patients and controls; B: Hepatic reduced glutathione (μg/g) of hepatic fibrosis patients and controls. $^{a}P < 0.05$, $^{c}P < 0.05$ *vs* before treatment 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

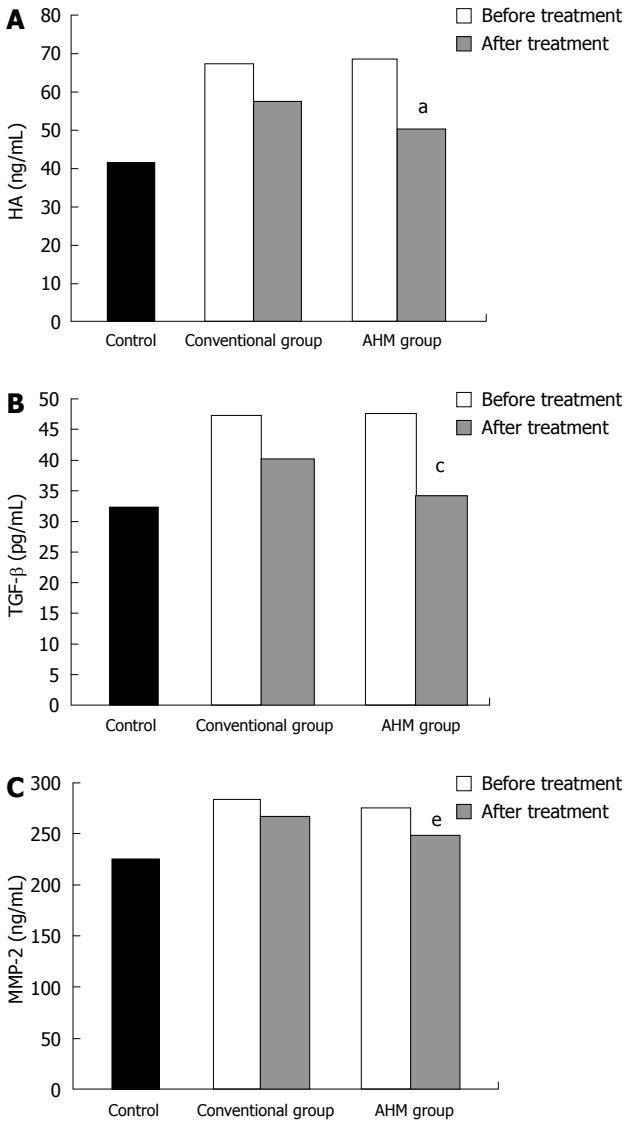

Figure 4 Serum hyaluronic acid, transforming growth factor-β and matrix metalloproteinase-2 of hepatic fibrosis patients and controls. A: Serum hyaluronic acid (ng/mL) of hepatic fibrosis patients and controls; B: Serum transforming growth factor-β (pg/mL) of hepatic fibrosis patients and controls; C: 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-

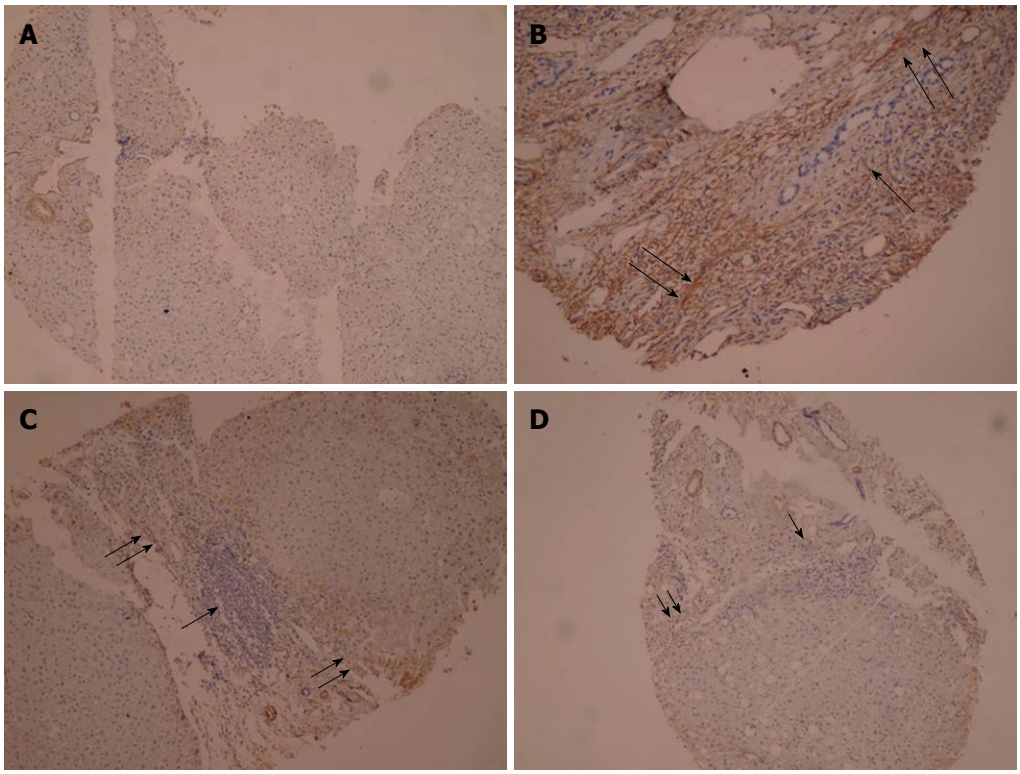

Figure 5 Immunohistochemical staining of α-smooth muscle actin. A: Section of liver from controls showing negative staining (PAP × 125); B: Section of liver from liver fibrosis patients before treatment showing dense fibrotic reaction with strong positivity for α-smooth muscle actin (α-SMA) ↑↑ and dense 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.

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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

Lei Jiang, Yiu-Kay Lai, Jin-Fang Zhang, Chu-Yan Chan, Gang Lu, Marie CM Lin, Ming-Liang He, Ji-Cheng Li, Hsiang-Fu Kung

Lei Jiang, Laboratory of Internal Medicine, The First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, Zhejiang Province, China
Yiu-Kay Lai, Department of Life Science, National Tsing Hua University, Hsinchu 30013, Taiwan
Jin-Fang Zhang, Chu-Yan Chan, Ming-Liang He, Hsiang-Fu Kung, Stanley Ho Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong, Shatin 999077, Hong Kong, China
Gang Lu, Marie CM Lin, Brain Tumor Centre and Division of Neurosurgery, Department of Surgery, The Chinese University of Hong Kong, Shatin 999077, Hong Kong, China
Ji-Cheng Li, Institute of Cell Biology, Zhejiang University, Hangzhou 310058, Zhejiang Province, China
Author contributions: Jiang L, Chan CY, Lin MCM, He ML, Li JC and Kung HF designed the research; Jiang L, Zhang JF and Lu G performed the research; Jiang L, Lai YK, Lin MCM, He ML, Li JC and Kung HF analyzed the data; Jiang L, Lai YK and Chan CY wrote the paper.
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Correspondence to: Hsiang-Fu Kung, Professor, Stanley Ho Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong, Shatin 999077, Hong Kong, China. hkung@cuhk.edu.hk
Telephone: +852-26037743 Fax: +852-29944988
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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.

Key words: Growth inhibition; Hepatocellular carcinoma; Stathmin; Transforming growth factor-β; Transforming growth factor-β-inducible early gene 1

Peer reviewers: Arezoo Aghakhani, MD, PhD, Assistant Professor, Clinical Research Department Pasteur Institute of Iran, No 69, Pasteur Ave., Tehran 13164, Iran; Dr. Andreas Hillenbrand, Department of General Surgery, University of Ulm, 89075 Ulm, Germany

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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 addition, 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'-GTCACATCTGTAGCCACCCA-3' and 5'-CCTCCTTTCACAACCTTTCC-3'; TIEG2: 5'-TCTGACTCTGGGGATGTCAC-3' and 5'-CGGCAATCTGGAGTCTGGA-3'; Smad2: 5'-GCCACGGTAGAAATGACAAG-3' and 5'-CAGACTGAGCCAGAAGAGCA-3'; Smad3: 5'-GAACGGGCAGGAGGAGAAAT-3'and 5'-ACAGGCGGCAGTAGATGACA-3'; Smad4: 5'-CCATTTC-

CAATCATCCTGCT-3' and 5'-ACCTTTGCCTATGTGCAACC-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'-ATGAGCCCCAGCCTTCTCCAT-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'-CCGGTACCTCTAAGGCACGGTCAGACCA-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).

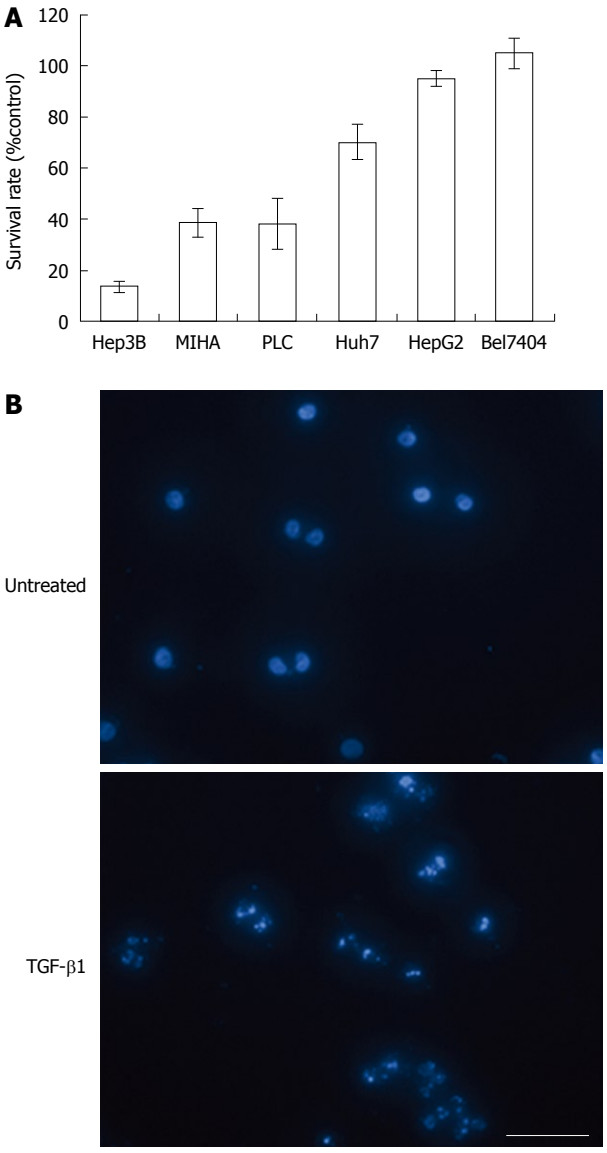

Figure 1 Susceptibilities of various human hepatocyte (MIHA) and hepatocellular carcinoma cells (Hep3B, PLC, Huh7, HepG2, and Bel7404) to transforming growth factor-β. A: Cells were treated with 5 ng/mL transforming growth factor (TGF)-β1 for 96 h and cell survival was then determined by methylthiazoletetrazolium 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-

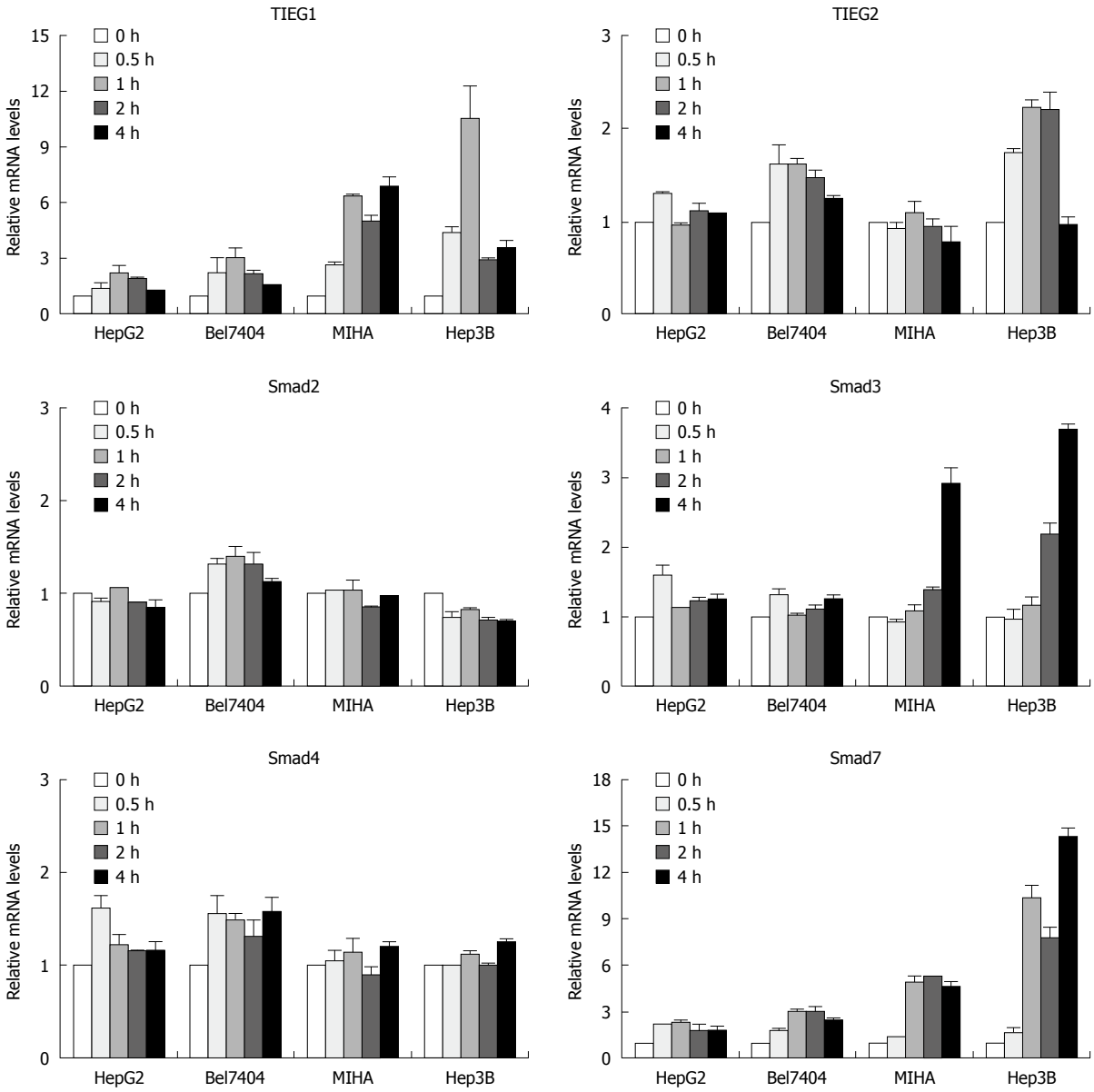

Figure 2 Differential responses of TIEGs and Smads in various TGF-β1-treated cells. Cells were treated with 5 ng/mL TGF-β1 for up to 4 h. At indicated time intervals, the mRNA levels of TIEG1, TIEG2, Smad2, Smad3, Smad4, and Smad7 were determined by quantitative real-time RT-PCRs. Data were presented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

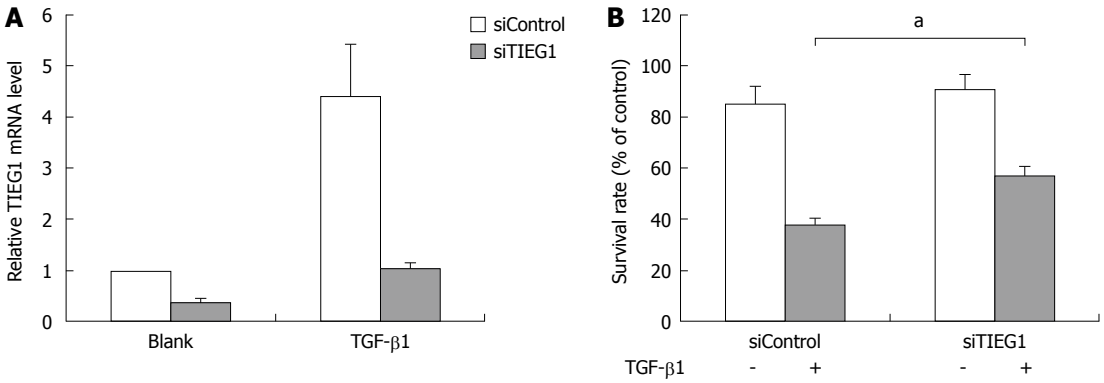

Figure 3 Downregulation of transforming growth factor-β-inducible early gene 1 by RNA interference (siRNA) decreased the transforming growth factor-β1 susceptibility of Hep3B cells. A: Decreases in the transforming growth factor (TGF)-β-inducible early gene 1 (TIEG1) mRNA level in siRNAs targeting TIEG1 (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

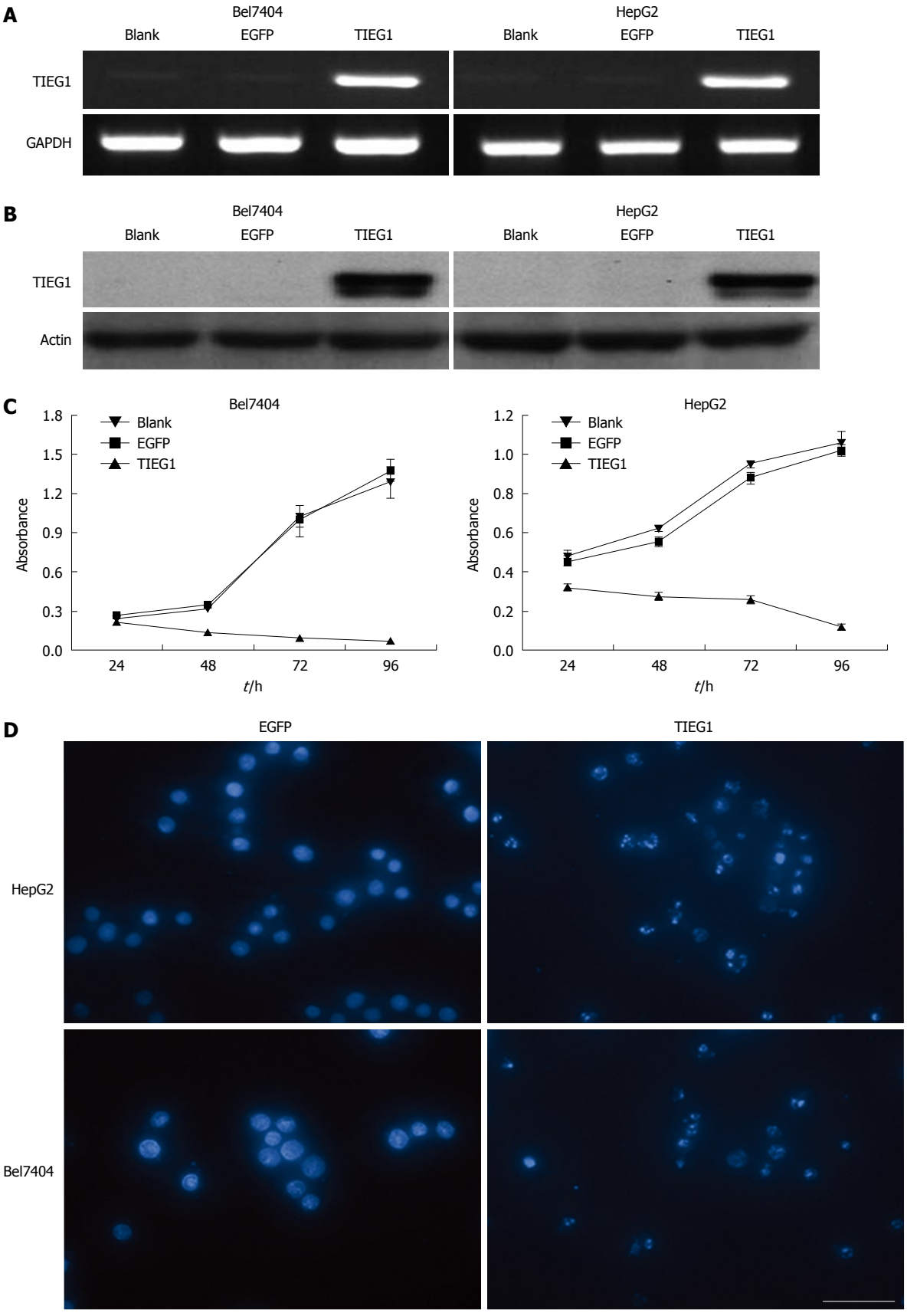

Figure 4 The overexpression of transforming growth factor-β-inducible early gene 1 by lentiviral-mediated transfection-induced growth inhibition and apoptosis in transforming growth factor-β1-resistant hepatoma Bel7404 and HepG2 cells. A and B: Increases in mRNA and protein levels of transforming growth 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 ×.

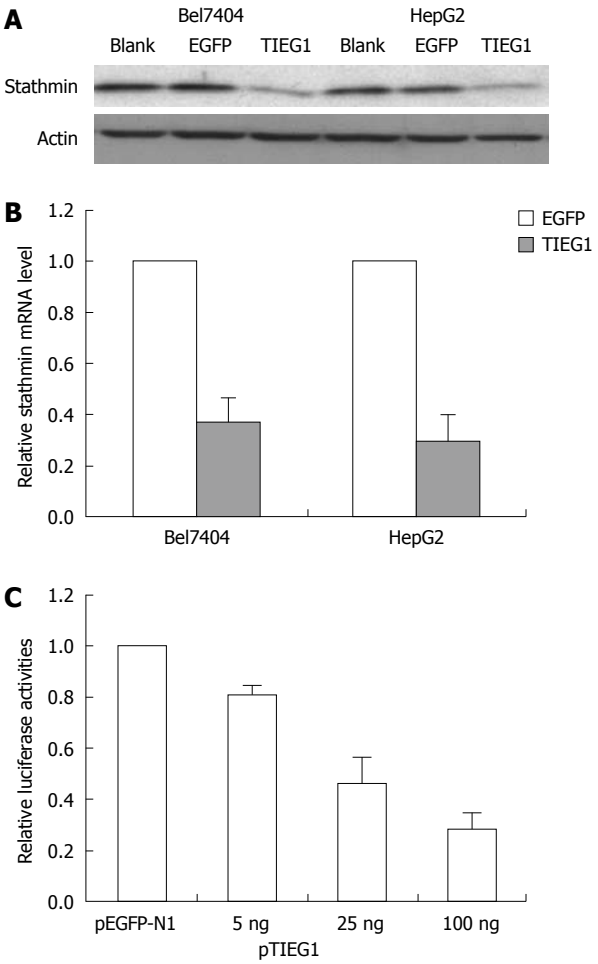

Figure 5 Transcriptional regulation of STMN (stathmin) by transforming growth factor-β-inducible early gene 1. A and B: Decreases in mRNA and protein levels of STMN in Bel7404 and HepG2 cells transfected by lenti-transforming growth factor-β-inducible early gene 1 (TIEG1), revealed by reverse transcriptase-polymerase 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

Ye Zhang, Bin Yang, Zhi Du, Tong Bai, Ying-Tang Gao, Yi-Jun Wang, Cheng Lou, Feng-Mei Wang, Yu Bai

Ye Zhang, Zhi Du, Third Central Clinical College of Tianjin Medical University, Tianjin 300170, China
Ye Zhang, Zhi Du, Tong Bai, Yi-Jun Wang, Cheng Lou, Feng-Mei Wang, Yu Bai, The Third Central Hospital of Tianjin, Tianjin 300170, China
Bin Yang, Ying-Tang Gao, Tianjin Key Laboratory of Artificial Cell, Tianjin 300170, China
Author contributions: Zhang Y performed the experiments and wrote the manuscript; Yang B, Bai T and Gao YT provided vital reagents and were involved in editing the manuscript; Wang YJ, Lou C, Wang FM and Bai Y collected all the human materials; Du Z designed the study and revised the manuscript.
Supported by Tianjin Health Bureau for research projects, No. 09KY04, No. 2010KZ17 and No. 11KG112
Correspondence to: Zhi Du, Professor of Medicine, The Third Central Hospital of Tianjin, Tianjin 300170, China. zhi-du@163.com
Telephone: +86-22-84112148 Fax: +86-22-24315132
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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.

Key words: Biomarker; Diagnosis; Hepatocellular carcinoma; Methylation; Prognosis; Tumor suppressor gene

Peer reviewer: Zenichi Morise, MD, PhD, Professor and Chairman, Department of Surgery Banbuntane Houtokukai Hospital, Fujita Health University School of Medicine, 3-6-10 Otobashi Nakagawa-ku, Nagoya, Aichi 454-8509, Japan

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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 Biotechology, 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

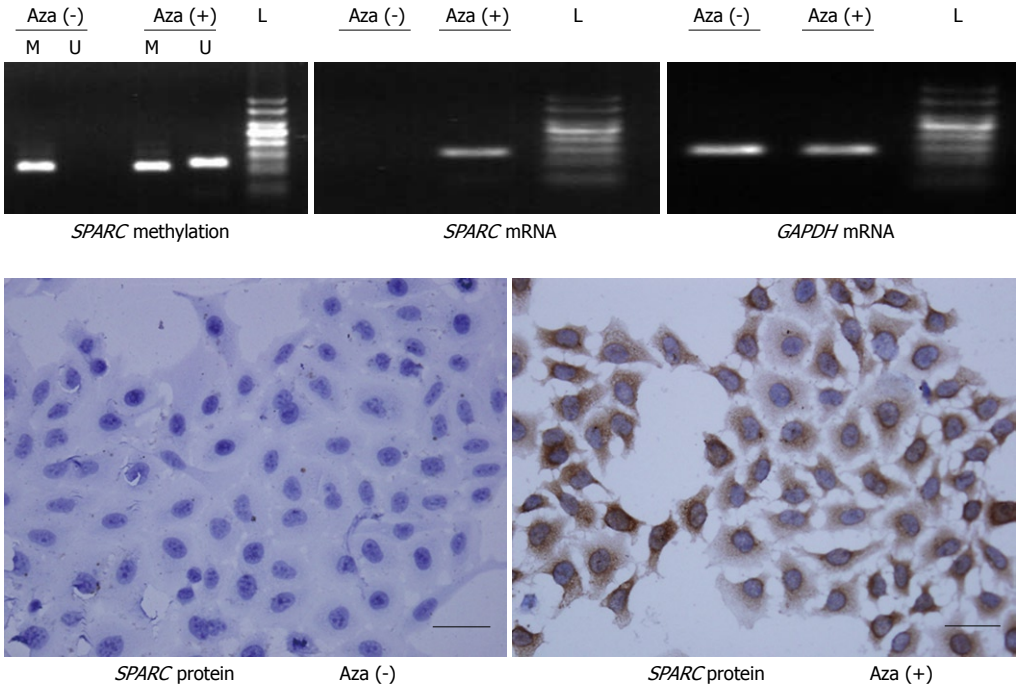

Figure 1 Secreted protein acidic and rich in cysteine methylation and expression in SMMC-7721 cell line. *SPARC*: Secreted protein acidic and rich in cysteine; Aza: 5-aza-2'-deoxycytidine; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase; M: Methylation; U: Unmethylation; L: 50 bp ladder; Scale bar: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

Table 2 Methylation frequencies of secreted protein acidic and rich in cysteine in 60 cases

Tissue	*SPARC* methylation status		*P* value
	Methylated (%)	Unmethylated (%)	
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

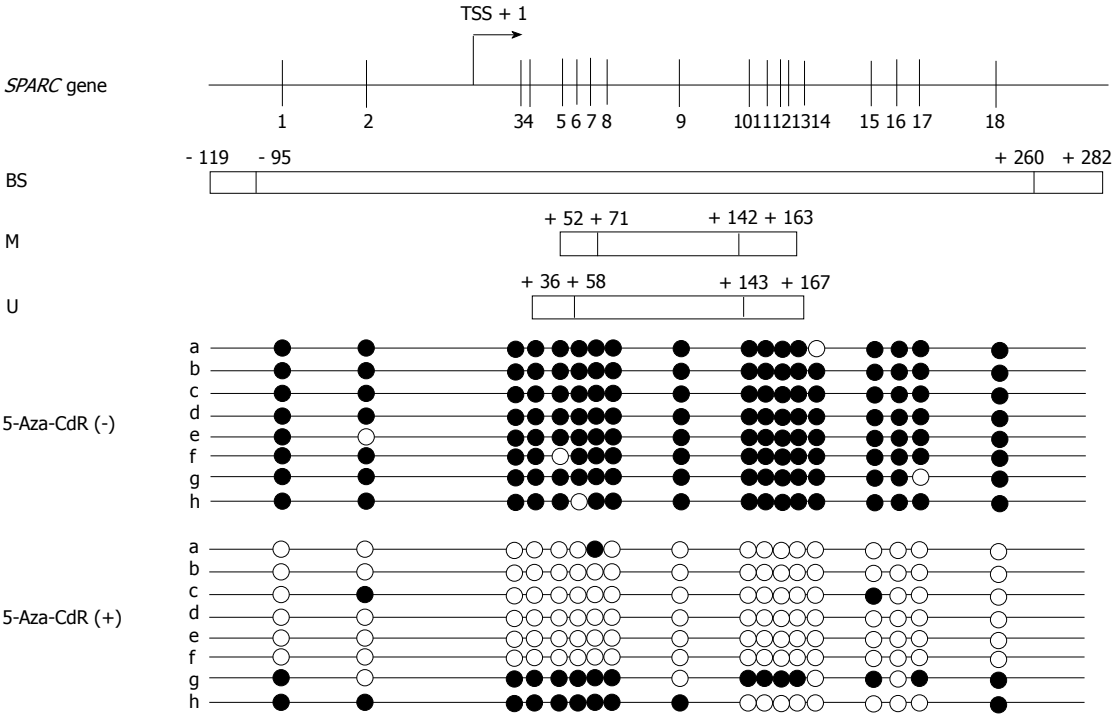

Figure 2 Bisulfite sequencing of secreted protein acidic and rich in cysteine in SMMC-7721 cell line. *SPARC*: Secreted protein acidic and rich in cysteine; TSS: Transcription start site; BS: Bisulfite sequencing; M: Methylation; U: Unmethylation; 5-Aza-CdR: 5-aza-2'-deoxycytidine; 1-18: CpG sites; - 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.

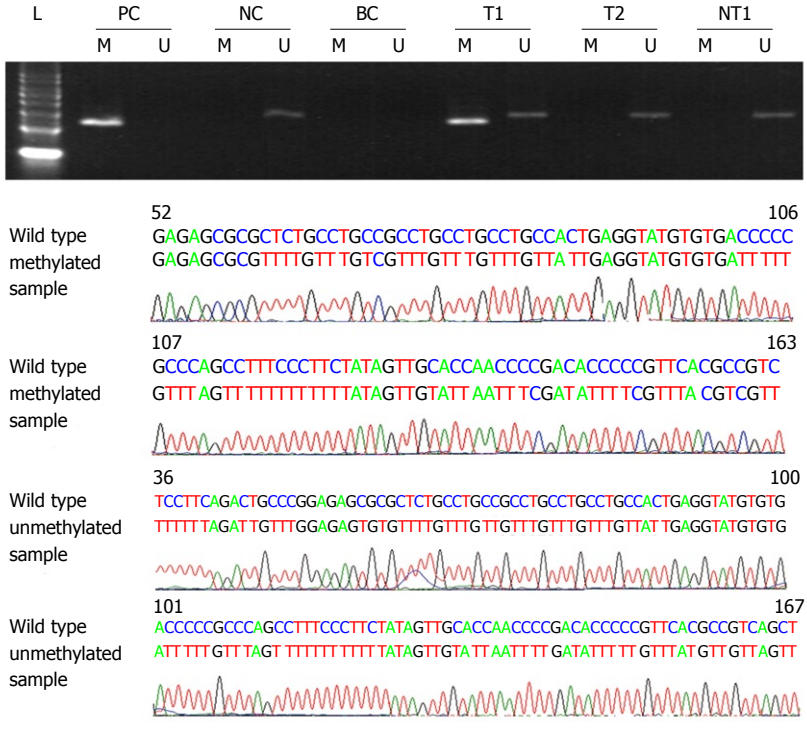

Figure 3 Representative results of methylation specific polymerase chain reaction analysis and sequencing in tissues. L: 50 bp ladder; PC: Positive control; NC: Negative control; BC: Blank control; M: Methylation; U: Unmethylation; T: Hepatocellular carcinoma tissue; NT: Nontumorous tissue.

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

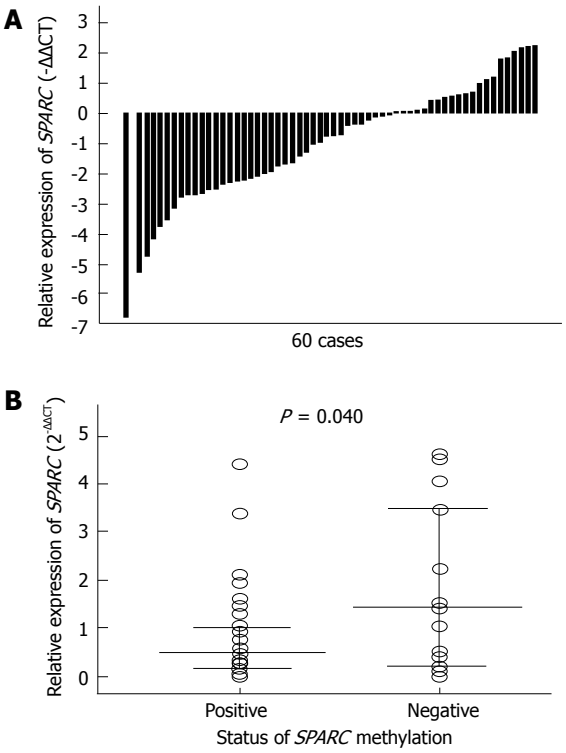

Figure 4 Expression of secreted protein acidic and rich in cysteine mRNA in hepatocellular carcinoma. Horizontal lines represent the median, and range indicates a 25%-75% quartile. *SPARC*: Secreted protein acidic and rich in cysteine.

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 clinicopathological 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

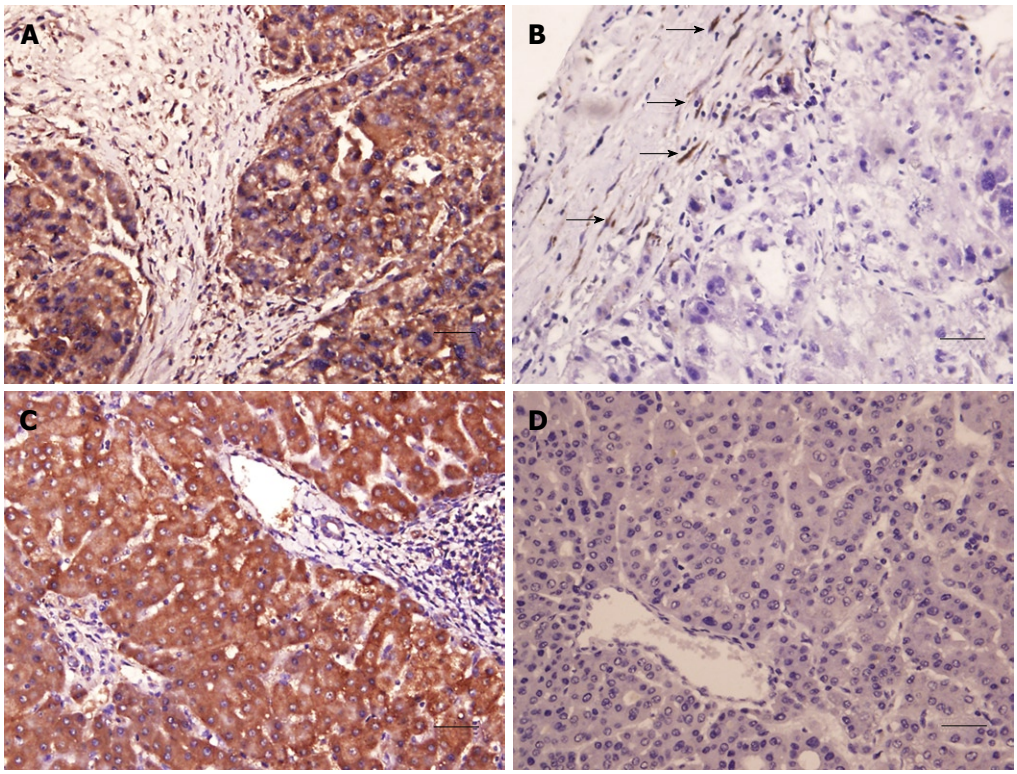

Figure 5 Immunohistochemical analysis of secreted protein acidic and rich in cysteine expression. A: A tumor with positive staining; B: A tumor with negative result, but stromal tissues with positive signal (arrows); C: Nontumorous tissues with positive staining; D: Nontumorous tissues with negative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

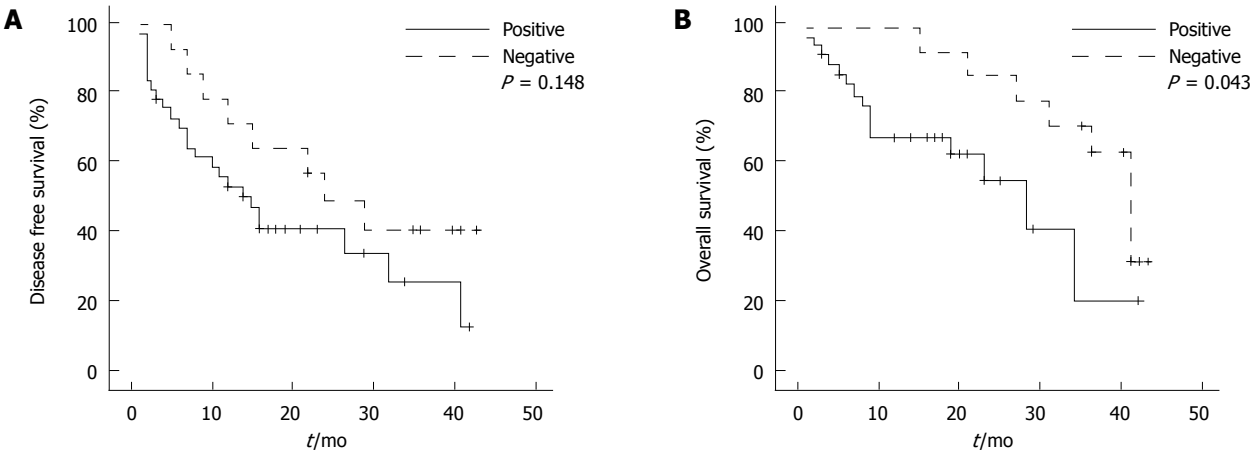

Figure 6 Disease free (A) and overall (B) survival analysis of patients with different secreted protein acidic and rich in cysteine methylation status.

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 prerequisite 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

Wen-Jie Zhang, Yan-Xia Sui, Arun Budha, Jian-Bao Zheng, Xue-Jun Sun, Ying-Chun Hou, Thomas D Wang, Shao-Ying Lu

Wen-Jie Zhang, Yan-Xia Sui, Arun Budha, Jian-Bao Zheng, Xue-Jun Sun, Shao-Ying Lu, Department of General Surgery, The First Affiliated Hospital of Medical School, Xian Jiaotong University, Xi'an 710061, Shaanxi Province, China
Wen-Jie Zhang, The Third Hospital of Chengdu, Chengdu 610031, Sichuan Province, China
Ying-Chun Hou, Shaanxi Normal University, Xi'an 710061, Shaanxi Province, China
Thomas D Wang, Department of Medicine, Division of Gastroenterology and Hepatology, School of Medicine, University of Michigan, Ann Arbor, MI 48109, United States
Author contributions: Zhang WJ, Sun XJ, Hou YC, Wang TD and Lu SY designed the research; Zhang WJ, Sui YX, Budha A, Zheng JB and Lu SY performed the experiments; Zhang WJ and Lu SY analyzed the data; Zhang WJ, Wang TD and Lu SY wrote the paper.
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Correspondence to: Shao-Ying Lu, MD, PhD, Department of General Surgery, The First Affiliated Hospital of Medical School, Xian Jiaotong University, Xi'an 710061, Shaanxi Province, China. robertlu@mail.xjtu.edu.cn
Telephone: +86-29-85323875 Fax: +86-29-85324149
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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.

Key words: Gastric cancer; Peptide; Phage library; Molecular imaging; Early detection; Immunohistochemistry; Enzyme-linked immunosorbent assay

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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 pre-cancerous 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 titering 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

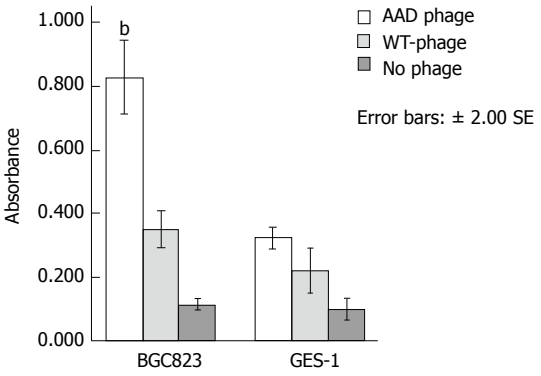

Figure 1 Preferential phage-binding to BGE823 and GES-1 cells. Phage capture enzyme-linked immunosorbent assay revealed a greater optical density at binding sites of AADNAKTKSFPV (AAD) phage to BGE823 cells compared with that of wild type phage ($^bP < 0.01$) or no phage. No significant difference was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

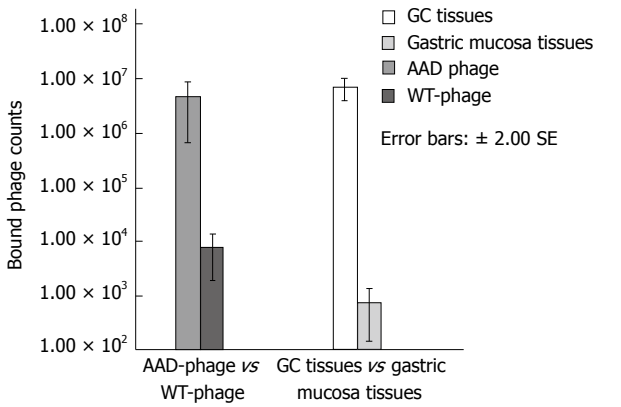

Figure 2 Phage binding affinity. AADNAKTKSFPV (AAD) phage showed an about 615 times higher binding efficiency in gastric cancer (GC) tissues than wild type (WT)-phage, and the binding of AAD phage was about 591 times greater in GC tissues than in gastric mucosa.

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 $(2300 \pm 41) \times 10^4$ bound phage ($P < 0.01$) at 5000 μmol. These

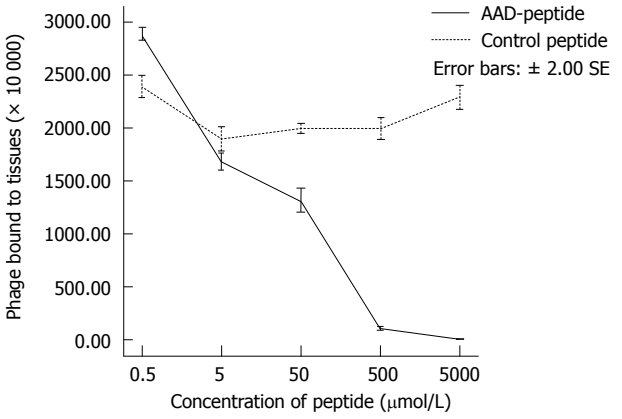

Figure 3 Competition binding assay. Binding of AADNAKTKSFPV (AAD) phage to gastric cancer tissues is reduced by competition with increasing concentrations of AAD peptide ($P < 0.01$) in a dose-dependent manner. The addition of the control peptide at concentrations of 0.5, 5, 50, 500 and 5000 μmol revealed 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 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.

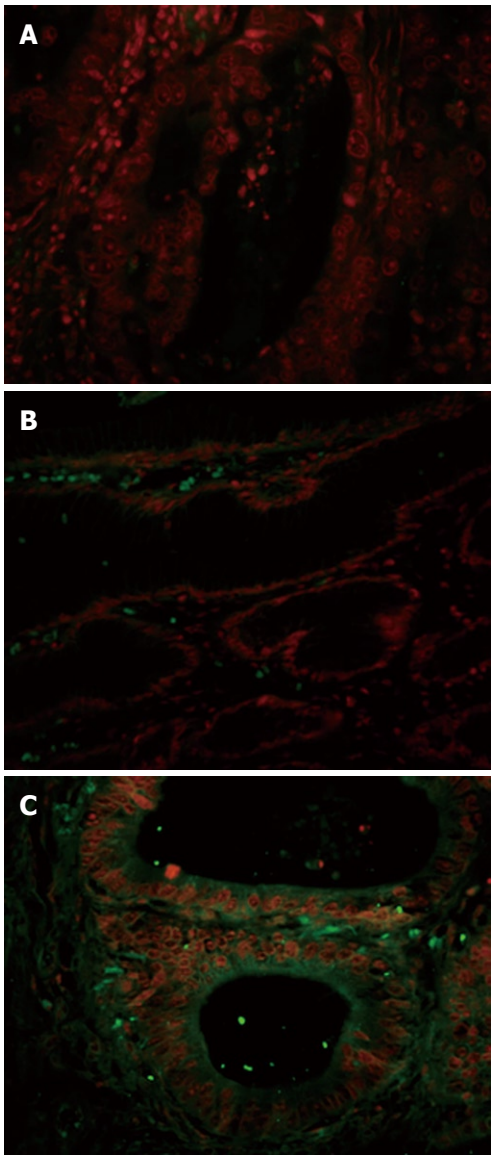

Figure 4 Immunofluorescence analysis of fluorescein isothiocyanate-conjugated AADNAKTKSFPV binding to human gastric cancer tissues. Frozen sections for biopanning were incubated with fluorescein isothiocyanate-conjugated AADNAKTKSFPV (AAD); scrambled peptide PAKFKAANSDVT was used as the control. Immunofluorescence 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).

DISCUSSION

Other investigators have used phage display technology

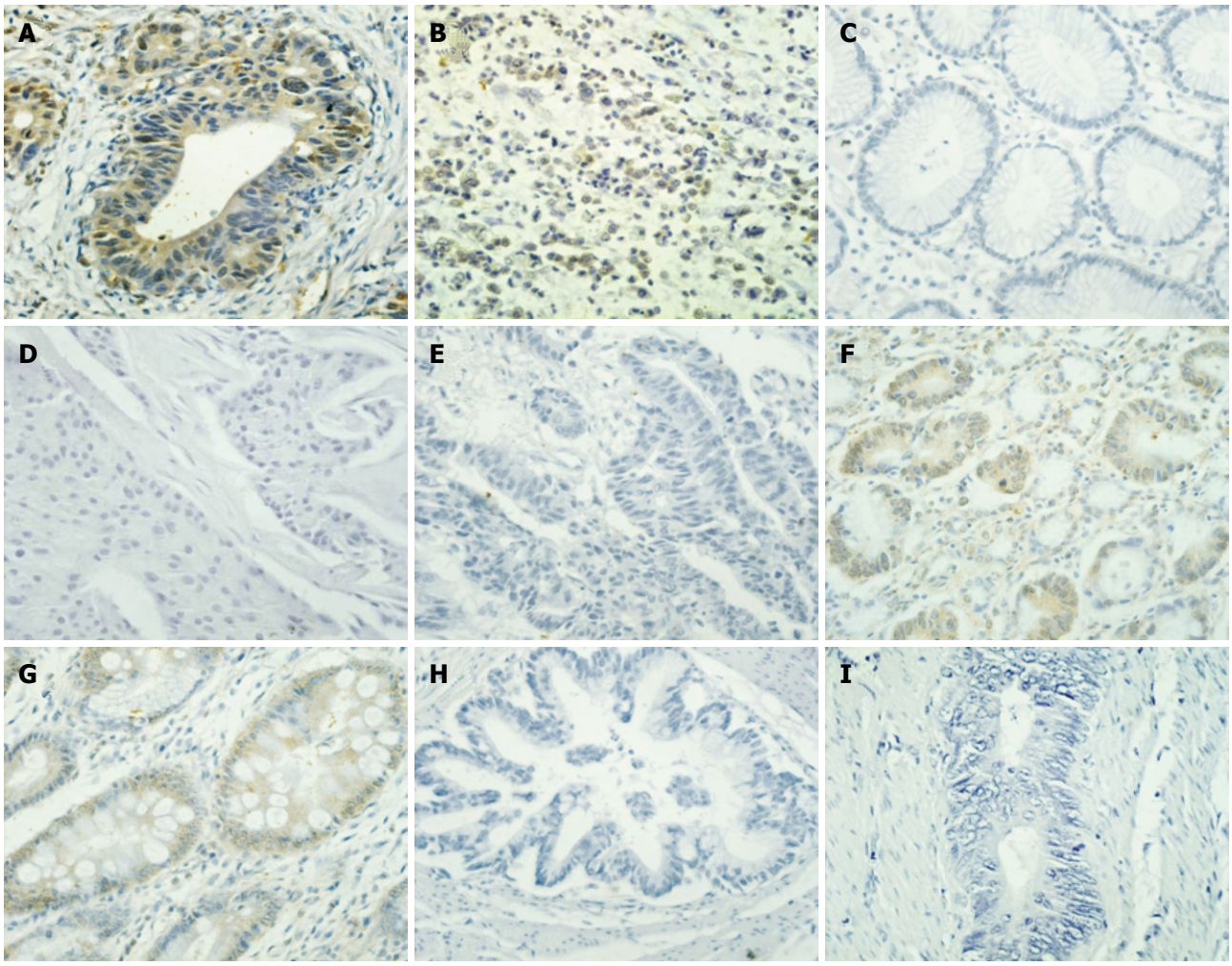

Figure 5 Binding analysis of biotin-AADNAKTKSFPV by immunohistochemistry. The results demonstrate that biotin showed a specific binding affinity to gastric cancer (GC) (A: Intestinal; B: Diffuse). In contrast, no positive staining was observed in gastric mucosae (C). In addition, peptide AADNAKTKSFPV (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).

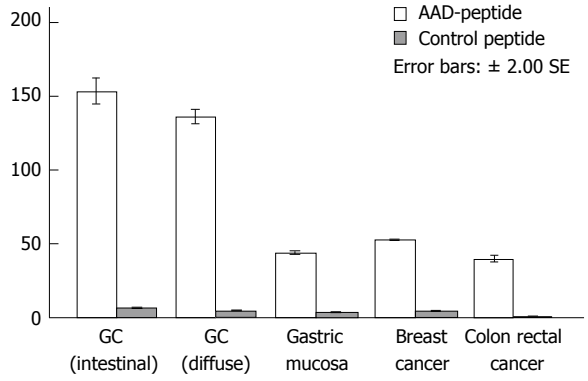

Figure 6 Semi-quantitative image analysis. AADNAKTKSFPV (AAD) peptide is statistically significant for gastric cancer as compared with other histological classifications and control peptide. GC: Gastric cancer.

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

Ingmar Königsrainer, Derek Zieker, Jörg Glatzle, Olivia Lauk, Julia Klimek, Stephan Symons, Björn Brücher, Stefan Beckert, Alfred Königsrainer

Ingmar Königsrainer, Derek Zieker, Jörg Glatzle, Olivia Lauk, Julia Klimek, Björn Brücher, Stefan Beckert, Alfred Königsrainer, Department of Surgery, University of Tübingen, Hoppe-Seyler-Strasse 3, D-72076 Tübingen, Germany
Stephan Symons, Center for Bioinformatics Tübingen, Sand 14, D-72076 Tübingen, Germany
Author contributions: Königsrainer I wrote the paper and designed the study; Beckert S helped to write the paper and contributed to the design; Zieker D and Glatzle J contributed to the design, analysis of the study; Brücher B helped designing the study; Symons S analyzed data; Lauk O and Klimek J helped with study design and literature search; Königsrainer A helped writing the paper, designed the study and approved the final version of the manuscript.
Correspondence to: Ingmar Königsrainer, MD, Department of Surgery, University of Tübingen, Hoppe-Seyler-Strasse 3, D-72076 Tübingen,
Germany. ingmar.koenigsrainer@med.uni-tuebingen.de
Telephone: +49-7071-2985073 Fax: +49-7071-295588
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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.

Key words: Peritoneal carcinomatosis; Single-center experience; Hyperthermic intraoperative chemotherapy; Complications; Risk assessment; Selection criteria

Peer reviewer: Jesus Esquivel, Professor, St Agnes Hosp, 900 Caton Ave, Baltimore, MD 21229, United States

Königsrainer I, Zieker D, Glatzle J, Lauk O, Klimek J, Symons S, Brücher B, Beckert S, Königsrainer A. Experience after 100 patients treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. *World J Gastroenterol* 2012; 18(17): 2061-2066 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i17/2061.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i17.2061

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/kg^2)	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.

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 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

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.

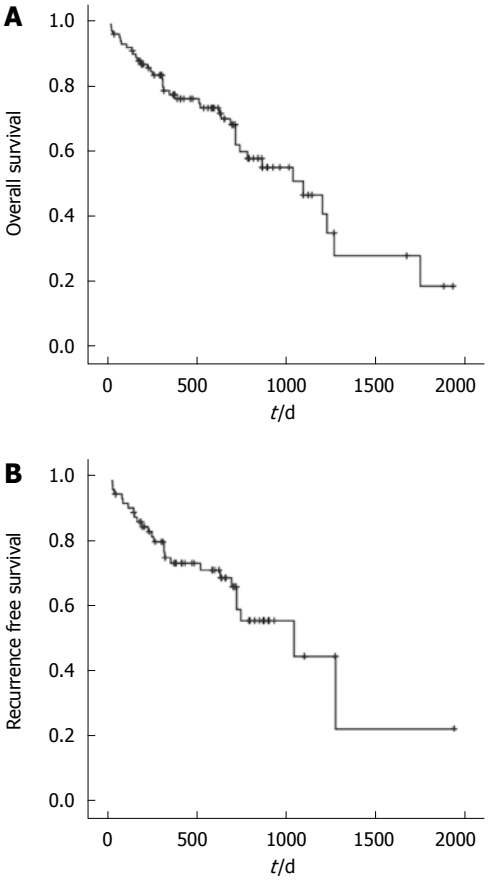

Figure 1 Overall survival (A) and recurrence-free survival (B).

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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BRIEF ARTICLE

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

Michel Dapoigny, Thierry Piche, Philippe Ducrotte, Bernard Lunaud, Jean-Michel Cardot, Annick Bernalier-Donadille

Michel Dapoigny, Médecine Digestive, Centre Hospitalier Universitaire (CHU) Estaing, CHU Clermont-Ferrand, Clermont Université, Inserm UMR 766, 63001 F-Clermont-Ferrand, France
Thierry Piche, Department of Gastroenterology and Inserm 576, Hôpital de l'Archet 2, 06202 Nice, Cedex 3, France
Philippe Ducrotte, Department of Hepatogastroenterology and Nutrition, Hôpital Charles Nicolle, Inserm UMR 1073, 76031 Rouen, Cedex, France
Bernard Lunaud, Department of Gastroenterology, Centre République, 63000 Clermont-Ferrand, France
Jean-Michel Cardot, Biopharmacy Department, Faculty of Pharmacy, 63000 Clermont-Ferrand, France
Annick Bernalier-Donadille, UR454 Microbiology Division, Institut National de la Recherche Agronomique, Research Centre of Clermont-Ferrand-Theix, 63122 Saint Genès-Champanelle, France
Author contributions: Dapoigny M was the coordinator-investigator of the study; Piche T, Ducrotte P and Lunaud B were co-investigators; Cardot JM was the scientific advisor for statistical issues; Bernalier-Donadille A was responsible for the analysis of faeces samples; all authors critically reviewed the manuscript and approved the final version.
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Correspondence to: Michel Dapoigny, MD, PhD, Médecine Digestive, Centre Hospitalier Universitaire (CHU) Estaing, CHU Clermont-Ferrand, Clermont Université, Inserm UMR 766, 63001 F-Clermont-Ferrand,
France. mdapoigny@chu-clermontferrand.fr
Telephone: +33-4-73750523 Fax: +33-4-73750524
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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.

Key words: Irritable bowel syndrome; *Lactobacillus casei rhamnosus*; Probiotics; Symptom severity score

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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

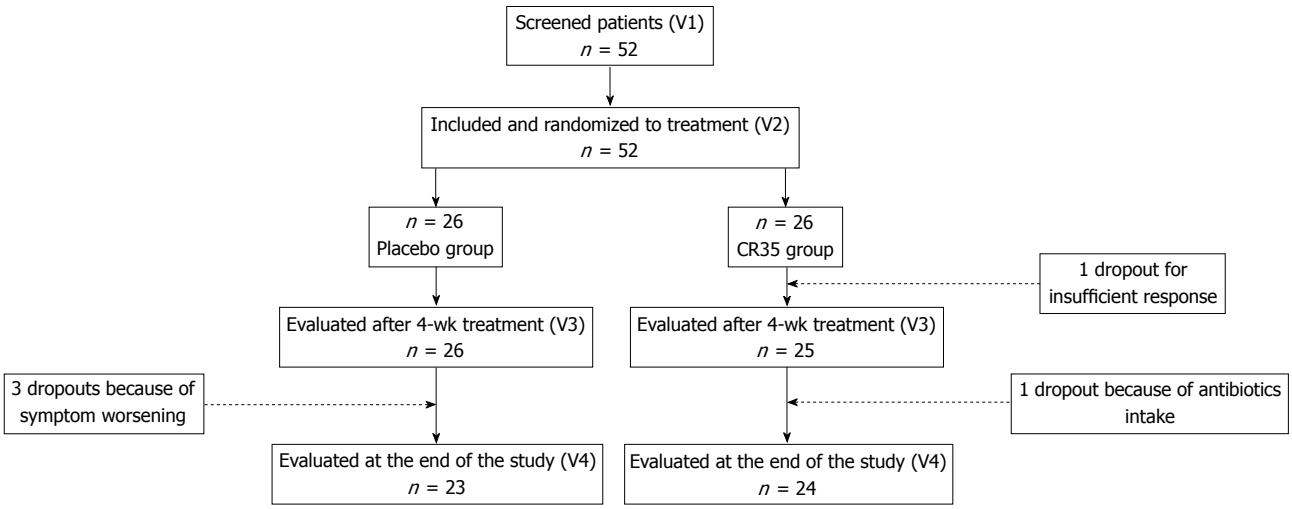

Figure 1 Disposition of patients.

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 unblinding 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.

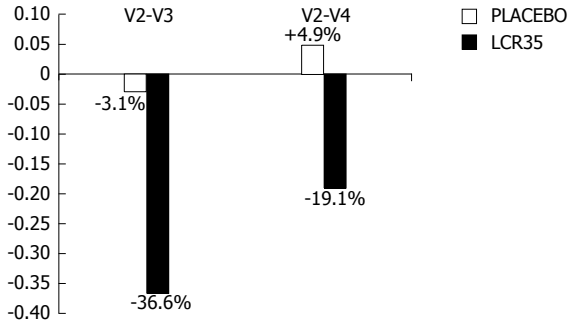

Figure 2 Relative changes in irritable bowel syndrome severity score between V2 and V3, V2 and V4 in irritable bowel syndrome patients with predominance of diarrhoea. V2: Baseline; V3: At the end of the 4-wk treatment period, a more marked decrease in irritable bowel syndrome (IBS) severity score occurred 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 gastroenterologists. 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, probiotics are generating great interest.

Research frontiers

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. 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 symptomatology, 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 absence 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 question 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

Christian Felley, Hanifa Bouzourene, Marianne Bründler G VanMelle, Antoine Hadengue, Pierre Michetti, Gian Dorta, Laurent Spahr, Emiliano Giostra, Jean Louis Frossard

Christian Felley, Pierre Michetti, Gian Dorta, Service of Gastroenterology, University Hospital, 1000 Lausanne, Switzerland
Hanifa Bouzourene, Institute of Pathology, University Hospital, 1000 Lausanne, Switzerland
Marianne Bründler G VanMelle, Institute of Social and Preventive Medicine, University of Lausanne, 1000 Lausanne, Switzerland
Antoine Hadengue, Laurent Spahr, Emiliano Giostra, Jean Louis Frossard, Service of Gastroenterology, Geneva University Hospital, 1211 Geneva, Switzerland
Author contributions: Felley C, Hadengue A, Michetti P, Dorta G, Spahr L and Frossard JL conceived and designed the study, and acquired the data; Felley C, Bouzourene H, VanMelle MBG, Michetti P, Dorta G and Frossard JL analyzed and interpreted the data; Felley C, Hadengue A, Michetti P and Frossard JL drafted the manuscript; VanMelle MBG and Giostra E performed statistical analysis; Felley C, VanMelle MBG, Hadengue A and Frossard JL supervised the study.
Correspondence to: Jean Louis Frossard, MD, Service of Gastroenterology, Geneva University Hospital, 1211 Geneva, Switzerland. jean-louis.frossard@hcuge.ch
Telephone: + 41-22-3729340 Fax: +41-22-3729366
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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.

Key words: Endoscopy; Gastroesophageal reflux disease; Metaplasia; *Helicobacter pylori*; Obesity; Smoking

Peer reviewer: Cesare Tosetti, Department of Primary Care, Health Care Agency of Bologna, via Rosselli 21, Porretta Terme (BO) 40046, Italy

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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 diseases, 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 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 ($P < 0.01$).

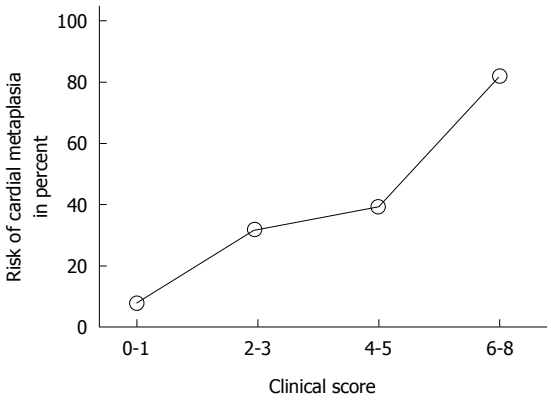

Figure 1 The curve indicates the actual risk (with standard deviation) of cardia metaplasia as a function of the personal clinical score.

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

Gemma Òdena, Mireia Miquel, Anna Serafín, Amparo Galan, Rosa Morillas, Ramon Planas, Ramon Bartolí

Gemma Òdena, Ramon Planas, Ramon Bartolí, Hepatology Unit, Department of Gastroenterology, Germans Trias i Pujol Health Science Research Institute, 08916 Badalona, Spain
Gemma Òdena, Mireia Miquel, Rosa Morillas, Ramon Planas, Ramon Bartolí, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), 08036 Barcelona, Spain
Mireia Miquel, Department of Gastroenterology, Corporació Sanitària Parc Taulí, 08208 Sabadell, Spain
Anna Serafín, Platform of Laboratory Animal Applied Research, Parc Científic de Barcelona, 08028 Barcelona, Spain
Amparo Galan, Department of Biochemistry, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Spain
Rosa Morillas, Ramon Planas, Liver Unit, Department of Gastroenterology, Hospital Universitari Germans Trias i Pujol, 08916 Badalona, Spain
Author contributions: Òdena G and Miquel M contributed equally to this work; Miquel M, Morillas R, Planas R and Bartolí R designed the research; Òdena G, Miquel M and Bartolí R performed the experimental procedures; Serafín A performed the histological analysis; Galan A performed the biochemical analysis; Òdena G, Morillas R, Planas R and Bartolí R analyzed the data; Òdena G, Miquel M and Bartolí R wrote the paper.
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Correspondence to: Ramon Bartolí, PhD, Hepatology Unit, Department of Gastroenterology, Germans Trias i Pujol Health Sciences Research Institute, 08916 Badalona, Spain. rbartolisole@gmail.com
Telephone: +34-93-4978688 Fax: +34-93-4978654
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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.

Key words: Hyperammonemia; Low-grade brain edema; Hepatic encephalopathy; Rifaximin; Insulin-like growth factor 1; Cirrhosis

Peer reviewers: Sanaa Ahmed Ali, Assistant Professor, Department of Theraputic Chemistry, National Research Centre, El Behooth St., Cairo 12622, Egypt; Dr. Lisheng Zhang, Beckman Research Institute, City of Hope/National Medical Center, 1500 E, Duarte, CA 91010, United States

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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 (CCl_4-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 CCl_4-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 CCl_4-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 CCl_4-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 CCl_4 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 (CCl_4 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 $KHCO_3$ (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 CCl_4 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-

Table 1 Biochemical features of groups studied

	Endotoxin (EU)	Glucose (mmol/L)	Bilirubin (μmol/L)	ALT (UI/L)	AST (UI/L)
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.

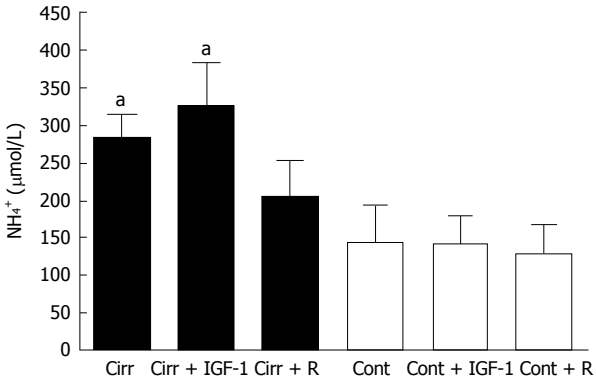

Figure 1 Blood ammonia levels. Comparison of the concentrations of basal plasma ammonia in cirrhotic groups (closed bars) and control groups (open bars). Cirrhosis plus portal vein occlusion resulted in a significant increase in basal ammonia levels. Rifaximin-treated cirrhotic rats showed plasma ammonia 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.

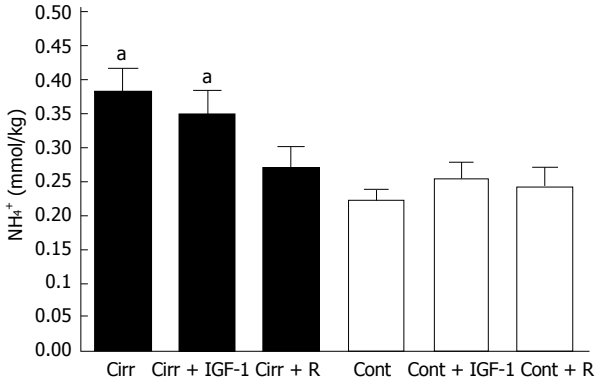

Figure 2 Brain ammonia levels. Comparison of the concentrations of brain ammonia in cirrhotic groups (closed bars) and control groups (open bars). Liver cirrhosis plus portal vein occlusion resulted in an increase in brain ammonia levels compared to controls, whereas in rifaximin-treated cirrhotic rats, 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

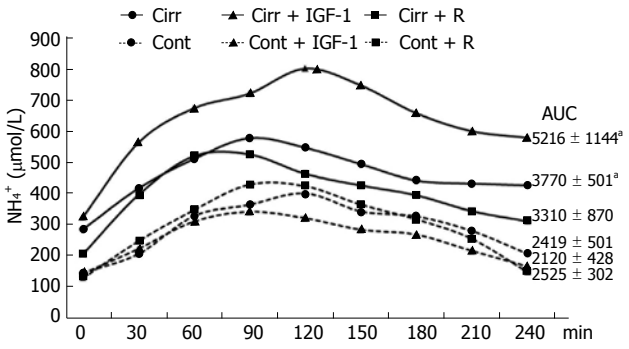

Figure 3 Ammonia response to oral glutamine-challenge tests. The area under the curve (AUC) was significantly increased by both placebo and insulin-like growth factor (IGF)-1-treatment in cirrhotic rats. By contrast, the AUC in rifaximin-treated cirrhotic rats was similar to that observed in controls.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

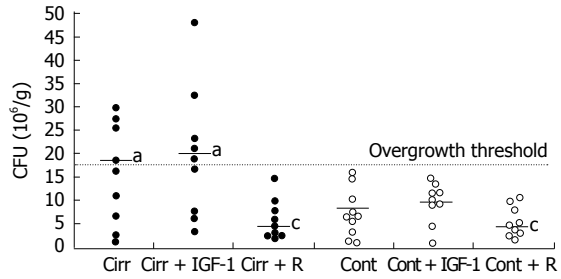

Figure 4 Cecal bacterial populations. Comparison of cecal bacterial content in cirrhotic groups (●) and control groups (○). Bacterial overgrowth was defined as a colony-forming units (CFU) count greater than the mean ± 2SD of the control group (dashed line). CCl_4-induced cirrhosis plus portal vein occlusion resulted in cecal bacterial overgrowth in placebo and insulin-like growth factor (IGF)-1-treated cirrhotic rats ($^aP < 0.05$ *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 ($^cP < 0.05$). 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 ± 3.6 × 10^6 CFU/mL *vs* 8.4 ± 1.3 × 10^6 CFU/mL; $P = 0.042$ and 20.4 ± 4.6 × 10^6 CFU/mL *vs* 8.9 ± 1.4 × 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 ± 1.4 × 10^6 CFU/mL *vs* 4.4 ± 1.3 × 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% ± 0.22% *vs* 78.26% ± 0.04%; $P = 0.002$). 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% ± 0.31% *vs* 78.24% ± 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% ± 0.08% *vs* 78.34% ± 0.19%; $P = 0.009$; Figure 5).

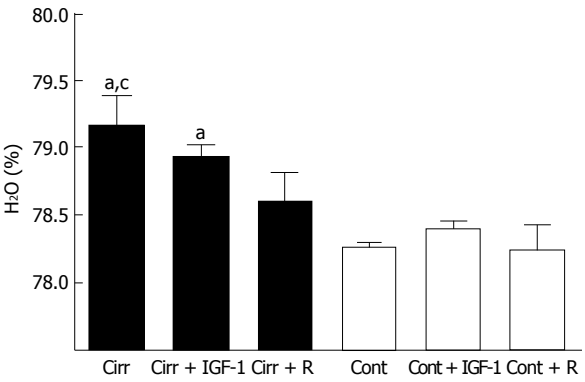

Figure 5 Low-grade brain edema. Low-grade brain edema was determined by measuring the percentage brain water content in cirrhotic groups (closed bars) and control groups (open bars). Placebo-treated cirrhotic rats showed the presence of low-grade brain edema. Brain water content in rifaximin-treated 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. $^aP < 0.05$ *vs* all control groups; $^cP < 0.05$ *vs* cirrhosis + R. Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

Histology

All ascitic cirrhotic rats with portal vein occlusion developed liver cirrhosis with regeneration nodules, necrosis, and steatosis regardless of treatment received. All CCl_4-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

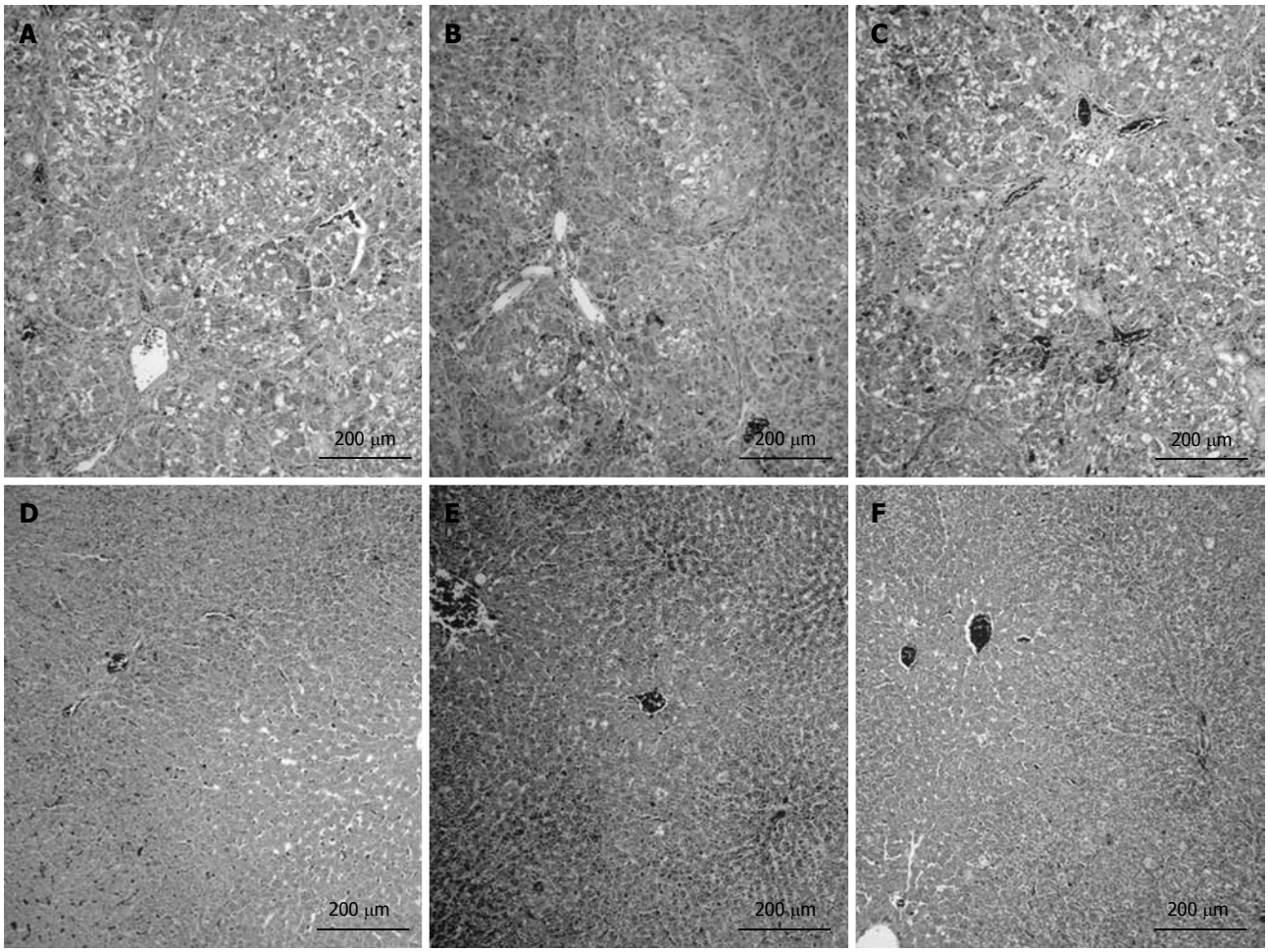

Figure 6 Photomicrographs of liver sections. All cirrhotic rats developed micronodular cirrhosis with regeneration nodules, necrosis and steatosis regardless of treatment (A: Cirrhosis; B: Cirrhosis + IGF-1; C: Cirrhosis + rifaximin). All control rats showed normal cellular architecture (D: Control;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

Yasuo Kakugawa, Yutaka Saito, Shoichi Saito, Kenji Watanabe, Naoki Ohmiya, Mitsuyuki Murano, Shiro Oka, Tetsuo Arakawa, Hidemi Goto, Kazuhide Higuchi, Shinji Tanaka, Hideki Ishikawa, Hisao Tajiri

Yasuo Kakugawa, Division of Screening Technology and Development, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo 104-0045, Japan
Yasuo Kakugawa, Yutaka Saito, Endoscopy Division, National Cancer Center Hospital, Tokyo 104-0045, Japan
Shoichi Saito, Hisao Tajiri, Department of Endoscopy, Jikei University School of Medicine, Tokyo 105-8461, Japan
Kenji Watanabe, Tetsuo Arakawa, Department of Gastroenterology, Osaka City University Graduate School of Medicine, Osaka 545-8685, Japan
Naoki Ohmiya, Hidemi Goto, Department of Therapeutic Medicine, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
Mitsuyuki Murano, Kazuhide Higuchi, Second Department of Internal Medicine, Osaka Medical College, Osaka 569-8686, Japan
Shiro Oka, Shinji Tanaka, Department of Endoscopy, Hiroshima University Hospital, Hiroshima 734-8551, Japan
Hideki Ishikawa, Department of Molecular-Targeting Cancer Prevention, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-0841, Japan
Author contributions: Kakugawa Y participated in the design of the study, data acquisition and interpretation, performed colon capsule endoscopy, and wrote the first draft of the manuscript; Saito Y participated in the design of the study, and in writing the manuscript; Saito S, Watanabe K, Ohmiya N, Murano M and Oka S performed colon capsule endoscopy and contributed to writing the manuscript; Arakawa T, Goto H, Higuchi K and Tanaka S participated in the design of the study, and in writing the manuscript; Ishikawa H participated in the design of the study, data acquisition, and writing the manuscript; Tajiri H participated in the design of the study, data acquisition and interpretation, and writing the manuscript; all authors have read and approved the submission version of the manuscript.
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Correspondence to: Dr. Yasuo Kakugawa, MD, Endoscopy Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. yakakuga@ncc.go.jp
Telephone: +81-3-35422511 Fax: +81-3-35423815
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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.

Key words: Colon capsule endoscopy; Polyethylene glycol electrolyte lavage solution; Colon cleanliness; Reduced volume preparation method; Isotonic magnesium citrate

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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 Maximum, administered twice: Maximum dosage 600 mL	Additional 300 mL PEG-ELS 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

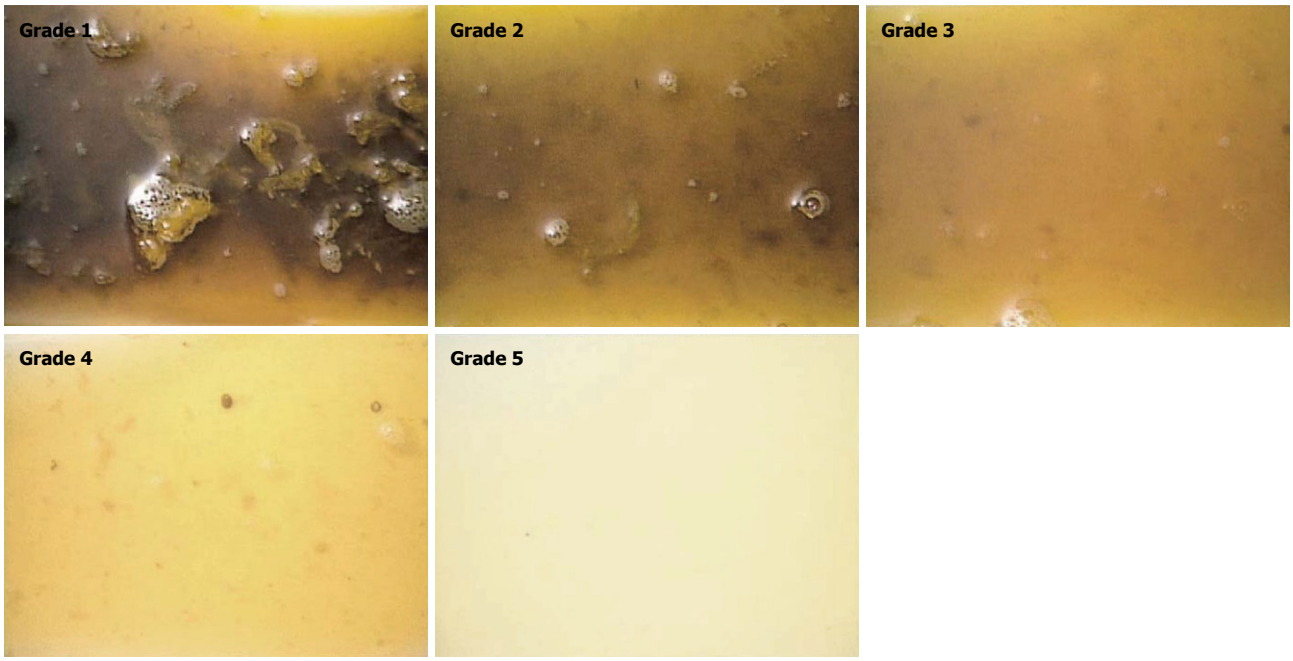

Figure 1 Reference used to evaluate the quality of bowel preparation prior to capsule ingestion. Experienced medical staff assessed the quality of the bowel preparation by checking the clarity of subjects' evacuation before the subjects were given the colon capsule. We define that the capsule is ready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

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

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)

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-

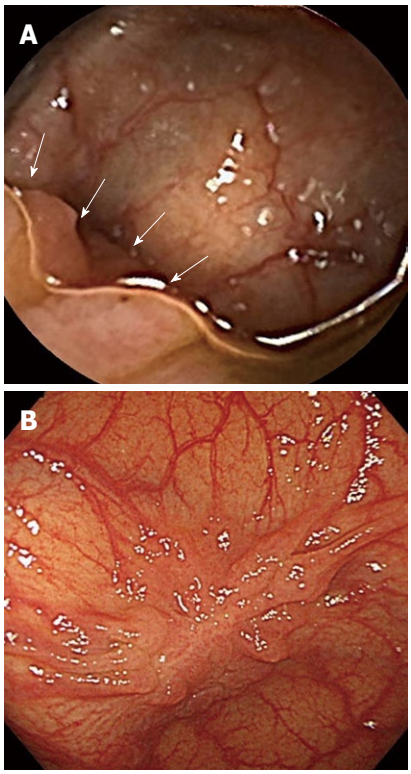

Figure 2 Example of the contribution of dimethicone to improved mucosal visualization by the colon capsule. A: A lesion (arrow) is clearly observed on the transverse colon by colon capsule endoscopy. Dimethicone worked to join numerous microbubbles to form groups of large bubbles, resulting in better 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 acceptance 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

Taku Tabata, Terumi Kamisawa, Kensuke Takuma, Seiichi Hara, Sawako Kuruma, Yoshihiko Inaba

Taku Tabata, Terumi Kamisawa, Kensuke Takuma, Seiichi Hara, Sawako Kuruma, Yoshihiko Inaba, Department of Internal Medicine, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan
Author contributions: Tabata T and Kamisawa T contributed equally to this work, analyzed the data and wrote the manuscript; Takuma K, Hara S, Kuruma S and Inaba Y collected the data.
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Correspondence to: Terumi Kamisawa, MD, PhD, Department of Internal Medicine, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan. kamisawa@cick.jp
Telephone: +81-3-38232101 Fax: +81-3-38241552
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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.

Key words: Autoimmune pancreatitis; Immunoglobulin G 4; Lymphoplasmacytic sclerosing pancreatitis

Peer reviewers: Shoichiro Sumi, MD, PhD, Associate Professor, Department of Organ Reconstruction, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan; Atsushi Masamune, MD, PhD, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

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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,

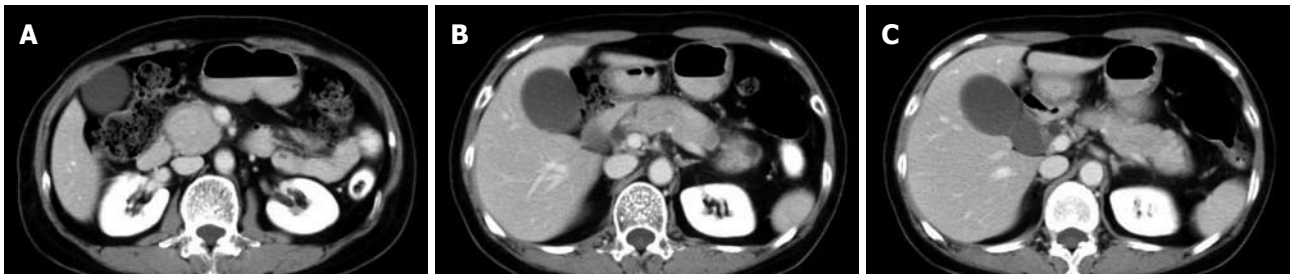

Figure 1 Computed tomography scan showing diffuse type autoimmune pancreatitis. Swelling of the head (A), body (B) and tail (C) of the pancreas was seen.

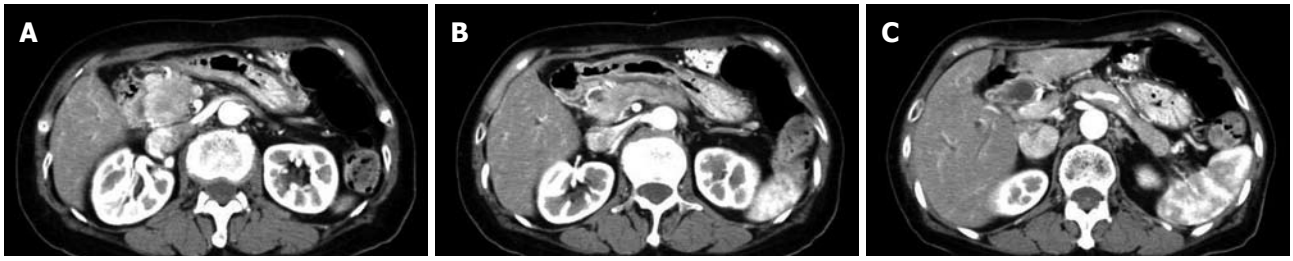

Figure 2 Computed tomography scan showing head type autoimmune pancreatitis. There was swelling of the only pancreatic head (A), but the pancreatic body (B) and tail (C) were a normal size.

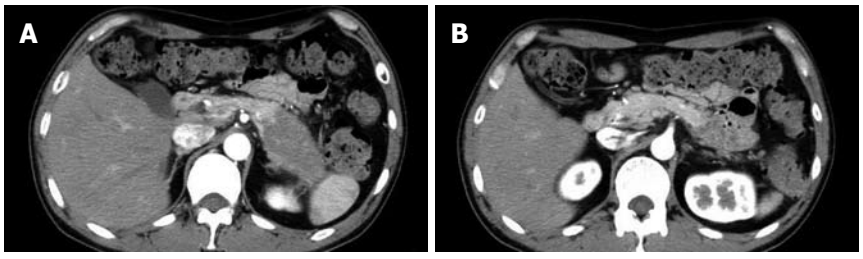

Figure 3 Computed tomography scan showing body and/or tail type autoimmune pancreatitis. Pancreatic body and tail was swollen (A), but the pancreatic head was within a normal size (B).

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) Inadequate material (*n* = 1)	LPSP (*n* = 1) Inadequate material (*n* = 2)	LPSP (*n* = 3) 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

developed to diffuse enlargement in one case.

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

Hassan Momtaz, Negar Souod, Hossein Dabiri, Meysam Sarshar

Hassan Momtaz, Department of Microbiology, ShahreKord Branch, Islamic Azad University, Shahre Kord 166, Iran
Negar Souod, Young Researcher's club, Jahrom Branch, Islamic Azad University, Jahrom 74135-355, Iran
Hossein Dabiri, Department of Medical Microbiology, School of Medicine, Shaheed Beheshti University, Tehran 19835-151, Iran
Meysam Sarshar, Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran 19945-581, Iran
Author contributions: Momtaz H and Souod N defined the research theme; Momtaz H designed methods and experiments; Momtaz H and Sarshar M carried out the laboratory experiments; Souod N and Dabiri H analyzed the data, interpreted the results and wrote the paper.
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Correspondence to: Negar Souod, MSc, Young Researcher's club, Jahrom Branch, Islamic Azad University, Jahrom 74135-355, Iran. negarsouod@yahoo.com
Telephone: +98-381-3361045 Fax: +98-381-3361064
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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.

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, consequently, 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

Peng Wang, Li Zhang, Jian-Ming Jiang, Dan Ma, Hao-Xia Tao, Sheng-Ling Yuan, Yan-Chun Wang, Ling-Chun Wang, Hao Liang, Zhao-Shan Zhang, Chun-Jie Liu

Peng Wang, Li Zhang, Hao-Xia Tao, Sheng-Ling Yuan, Yan-Chun Wang, Ling-Chun Wang, Zhao-Shan Zhang, Chun-Jie Liu, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Biotechnology, Beijing 100071, China
Jian-Ming Jiang, Dan Ma, Hao Liang, Department of Gastroenterology and Hepatology, China People's Liberation Army General Hospital, Beijing 100853, China
Author contributions: Wang P and Zhang L contributed equally to this work; Wang P, Zhang L, Liang H and Liu CJ designed the research; Wang P, Zhang L, Jiang JM, Ma D, Tao HX, Yuan SL, Wang YC and Wang LC performed the research; Wang P and Zhang L analyzed the data; Wang P, Zhang ZS and Liu CJ wrote the paper.
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Correspondence to: Dr. Chun-Jie Liu, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Biotechnology, 20 Feng Tai Dong Da Jie Street, Beijing 100071, China. liucj@nic.bmi.ac.cn
Telephone: +86-10-66948834 Fax: +86-10-63833521
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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.

Key words: Gastric cancer; NOD1; NOD2; Gene polymorphisms; *Helicobacter pylori* infection

Peer reviewer: Dr. Jianyuan Chai, PhD, MS, BS, Assistant Professor, Research (09-151), VA Long Beach Healthcare System, 5901 E. 7th St, Long Beach, CA 90822, United States

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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 becoming 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	ACGTTGGATGTCAAGAGGAGGGTATTAGGC	ACGTTGGATGCTGTGTGCTTGGGCAGTAAC	93	TTTGGCAGTAACAGTGACAAG
rs2907749	ACGTTGGATGGCTGTGAAGAACAGCAAATC	ACGTTGGATGCACACAGCAGGTTGTACCAC	99	GTAGGTTGTACCACATACATCC
rs2075820	ACGTTGGATGAAGCGCAGCAGGAAGGCAAA	ACGTTGGATGACCTGCTCTTCAAGCACTAC	90	CACTCCTGCTACCCAGAGCGGGACCCC
rs7789045	ACGTTGGATGAGCAGACACAGACAGGGTTC	ACGTTGGATGTTGAGATTGCTGACTGGTGG	95	GTGGTCTCTTCCAGC
rs2067085	ACGTTGGATGATCAGGTTGCCGATCTTCAC	ACGTTGGATGCCTTTCCTGAGAACTCTGTG	95	GTGCCTCACCCTCTG
rs1861759	ACGTTGGATGTGACATTTCTCTTGGCTTCC	ACGTTGGATGTGATGTGAAGGAATTCCAGG	100	AAGACACGACACCTTTGGC
rs3135500	ACGTTGGATGGGCCATGTTGTCTATAAGAG	ACGTTGGATGGATGTGTGAAAACTGGTTAA	99	ATTTGTGAAAACTGGTTAATATTTATAG
rs7205423	ACGTTGGATGCTTGGCTCCAGCCCATTTTG	ACGTTGGATGTATGGCTGCTGCAGGAAATG	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 $r^2 \geqslant 0.80$.

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 NOD1and 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

Bin-Bin Su, Hui Shi, Jun Wan

Bin-Bin Su, Hui Shi, Jun Wan, Department of Gastroenterology, South Building, Chinese People's Liberation Army General Hospital, Beijing 100853, China
Author contributions: Su BB and Wan J designed the research; Su BB and Shi H performed the research and analyzed the data; Su BB and Wan J wrote the paper.
Correspondence to: Jun Wan, Professor, Department of Gastroenterology, South Building, Chinese People's Liberation Army General Hospital, Beijing 100853, China. wanjun301@126.com
Telephone: +86-10-66876246 Fax: +86-10-68295664
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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., ≤ 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.

Key words: Colorectal cancer; Carcinoembryonic antigen; Recurrence

Peer reviewers: Francesco Crea, MD, PhD, Division of Pharmacology, University of Pisa, Via Roma 55, 56100 Pisa, Italy; Dr. Kevin J Spring, PhD, Conjoint Gastroenterology Laboratory, The Queensland Institute of Medical Research, the Bancroft Centre, rm H07, PO Royal Brisbane Hospital, Herston, QLD 4029, Australia

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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., ≤ 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 considered 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.

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	
≤ 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)	
≤ 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.

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.

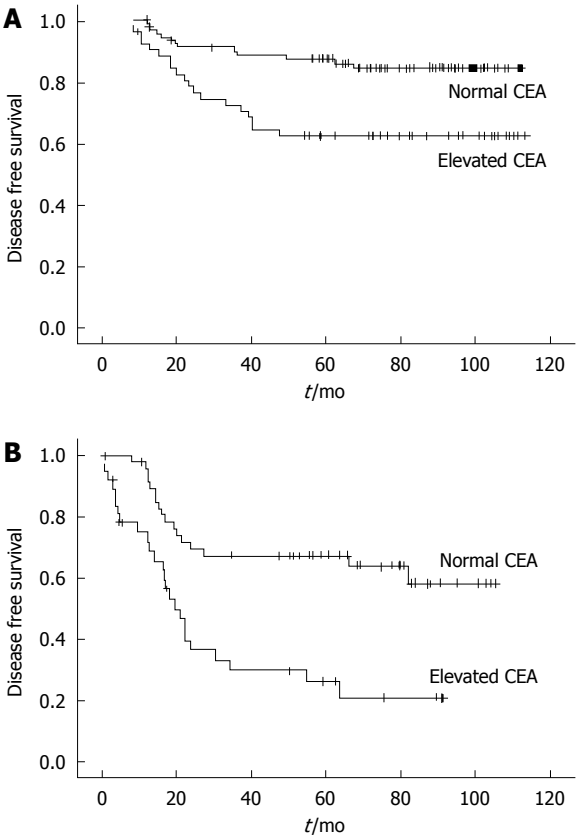

Figure 1 Disease-free survival curves for patients with stage ⅡA colorectal cancer (A) and stage ⅢB colorectal cancer (B) based on preoperative serum levels of carcinoembryonic antigen.

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.

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 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).

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.

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-

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 tumor 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

Jia-Qing Gong, Guo-Hu Zhang, Fu-Zhou Tian, Yong-Hua Wang, Lin Zhang, Yong-Kuan Cao, Pei-Hong Wang

Jia-Qing Gong, Guo-Hu Zhang, Fu-Zhou Tian, Yong-Hua Wang, Lin Zhang, Yong-Kuan Cao, Pei-Hong Wang, Department of General Surgery, the People's Liberation Army General Hospital of Chengdu Command, Chengdu 610083, Sichuan Province, China

Author contributions: Gong JQ performed the research, analyzed the data and wrote the paper; Zhang GH analyzed the data and wrote the paper; Tian FZ played a leading role in this research and designed the research; Wang YH, Zhang L, Cao YK and Wang PH helped collecting and analyzing the datas.

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Correspondence to: Fu-Zhou Tian, Professor, Department of General Surgery, the People's Liberation Army General Hospital of Chengdu Command, Chengdu 610083, Sicuan Province, China. cdgjq123@yahoo.com
Telephone: +86-28-86570351 Fax: +86-28-86570351

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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.

Key words: Intestinal necrosis; Stress; Trauma; Earthquake; Exploratory laparotomy; Fatty acid binding protein

Peer reviewer: Bruno Bonaz, MD, PhD, Professor, Clinique Universitaire d'Hépato-Gastroentérologie, Department of Gastroenterology Grenoble Hospital, CHU de Grenoble, BP 217, France

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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 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.

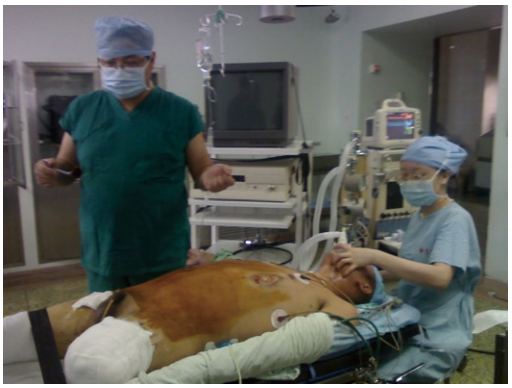

Figure 1 Anesthesia was administrated to the patient who underwent amputation of the bilateral extremities. The surgical area was sterilized in preparation for the operation.

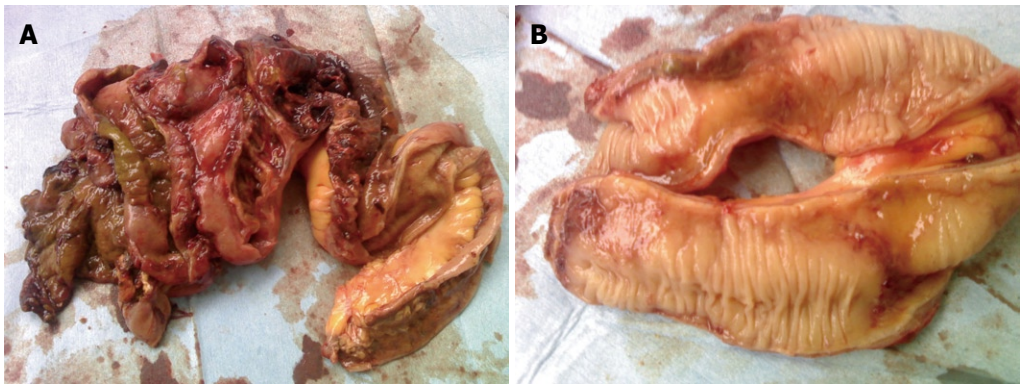

Figure 2 The damaged small intestine was adhered into a mass with a fistula. Thickness and hyperplasia were noted on the small intestinal wall after the part of damaged intestine was cleaned.

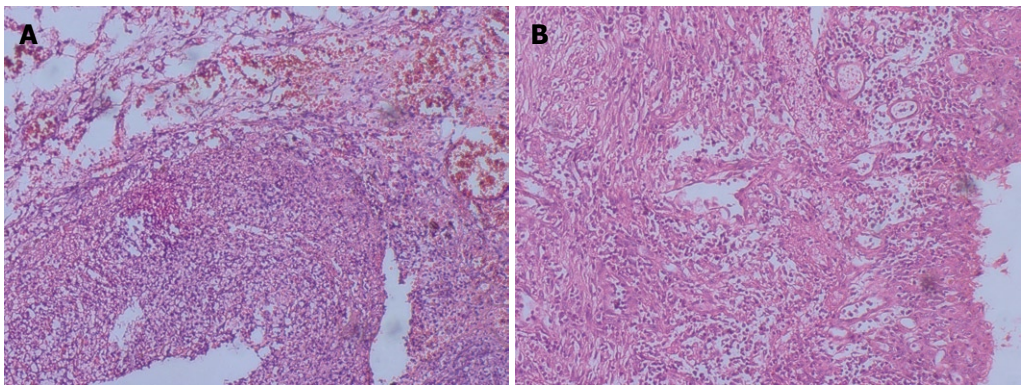

Figure 3 Large necrotic lesions along with macrophage infiltration were observed in the intestinal mucosa. Collagen fiber hyperplasia was also present on the submucosal membrane.

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. Concloud: 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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BRIEF ARTICLE

Emodin promoted pancreatic claudin-5 and occludin expression in experimental acute pancreatitis rats

Xian-Ming Xia, Bang-Ku Li, Shi-Mei Xing, Hai-Ling Ruan

Xian-Ming Xia, Shi-Mei Xing, Hai-Ling Ruan, Department of Gastroenterology and Hepatology, the Fourth Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China
Bang-Ku Li, Department of Gastroenterology and Hepatology, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China
Author contributions: Xia XM and Li BK designed the research; Xia XM, Li BK, Xing SM and Ruan HL performed the experiments; Xing SM analyzed the data; and Xia XM wrote the paper.
Supported by National Natural Science Foundation of China, No. 30500688
Correspondence to: Xian-Ming Xia, Associate Professor, PhD, Department of Gastroenterology and Hepatology, the Fourth Affiliated Hospital of Anhui Medical University, 372 Tunxi Road, Hefei 230022, Anhui Province, China. xiaxm2000@hotmail.com
Telephone: +86-551-2887190 Fax: +86-551-2876038
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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.

Key words: Acute pancreatitis; Paracellular permeability; Emodin; Claudin; Occludin

Peer reviewer: Dr. Naoaki Sakata, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

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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 Hietaranta *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'-TACTCAGCACCAAGGCGAACCAC-3', reverse 5'-GCGGCTT CCCACATCG-GTC-3'), occludin (forward 5'-AGTACATGGCTGCTGCTGATG-3', reverse 5'-CCCACCATCCTCTTGAT GTGT-3'), GAPDH (forward 5'-CA GTGCCAGCC-TCGTCTCATA-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

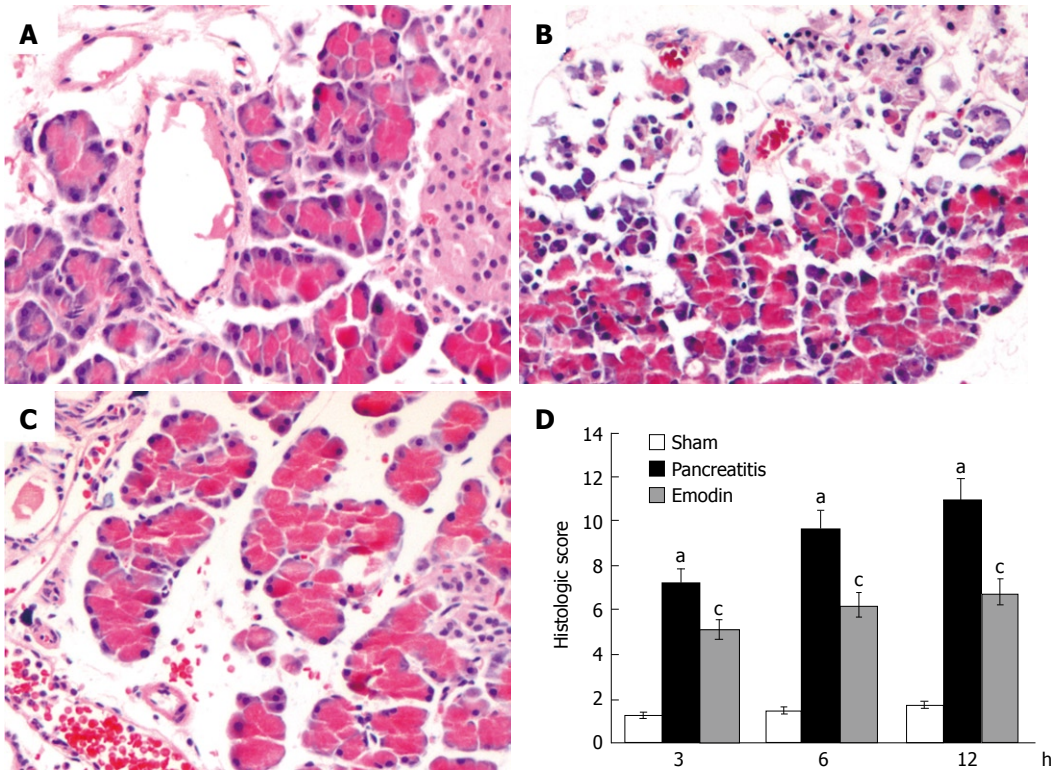

Figure 1 Effects of emodin on pancreatic injury in sham group (A), pancreatitis group (B), emodin group (C) and (D) histological score. (Original magnification, × 200). Six rats were studied in each experimental group at each time point. Results are mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *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

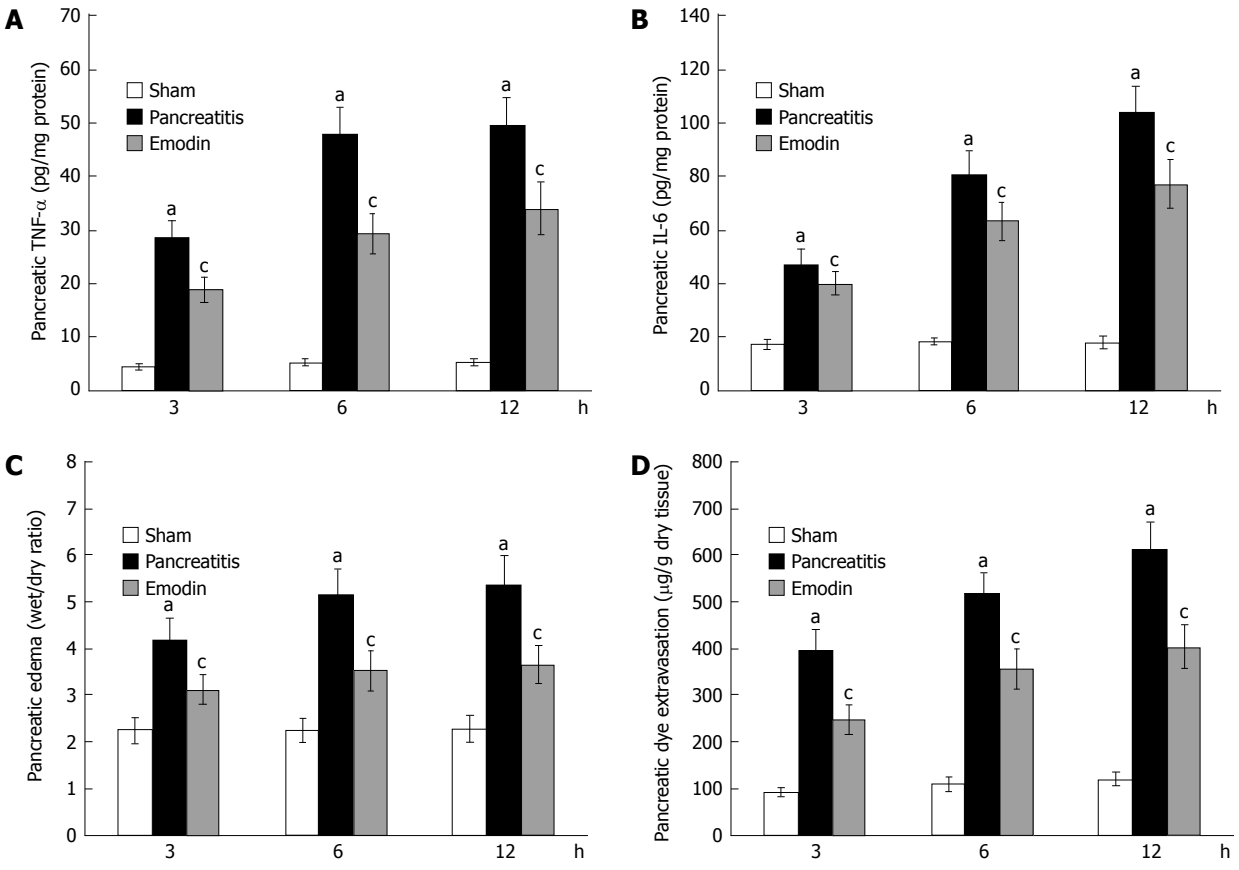

Figure 2 Effects of emodin on pancreatic (A) tumor necrosis factor-α, (B) interleukin-6, (C) edema and (D) dye extravasation. Six rats were studied in each experimental group at each time point. Results are mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* pancreatitis group. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.

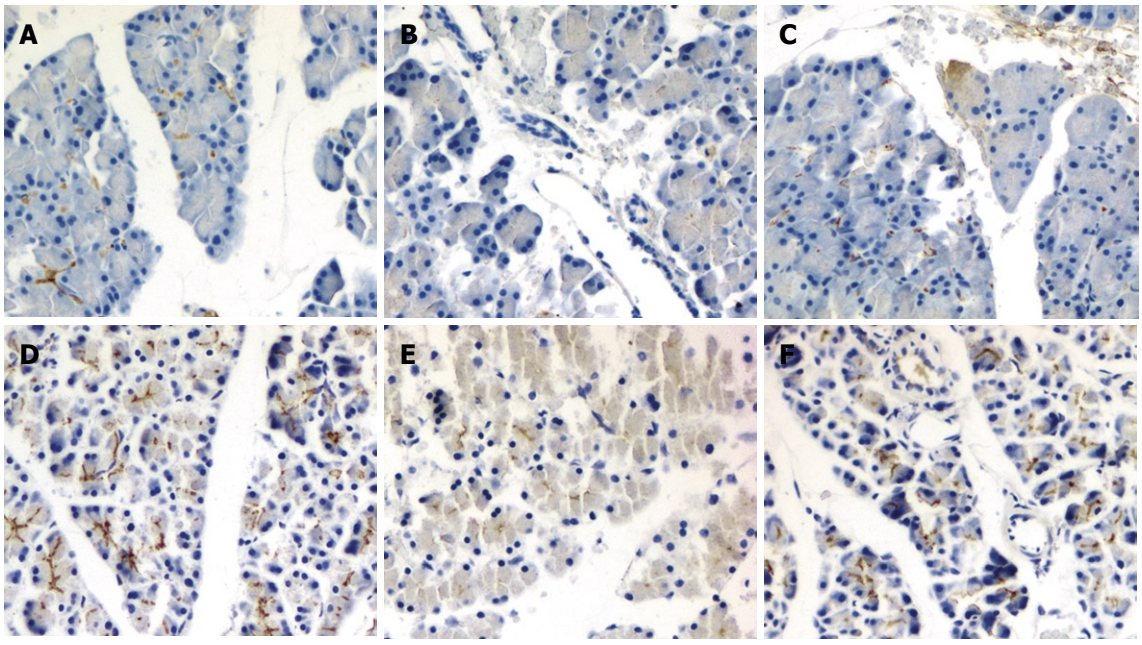

Figure 3 Immunohistochemical staining of claudin-5 (A-C) and occludin (D-F) in sham group (A, D), pancreatitis group (B, E) and emodin group (C, F) (original magnification, × 200).

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-

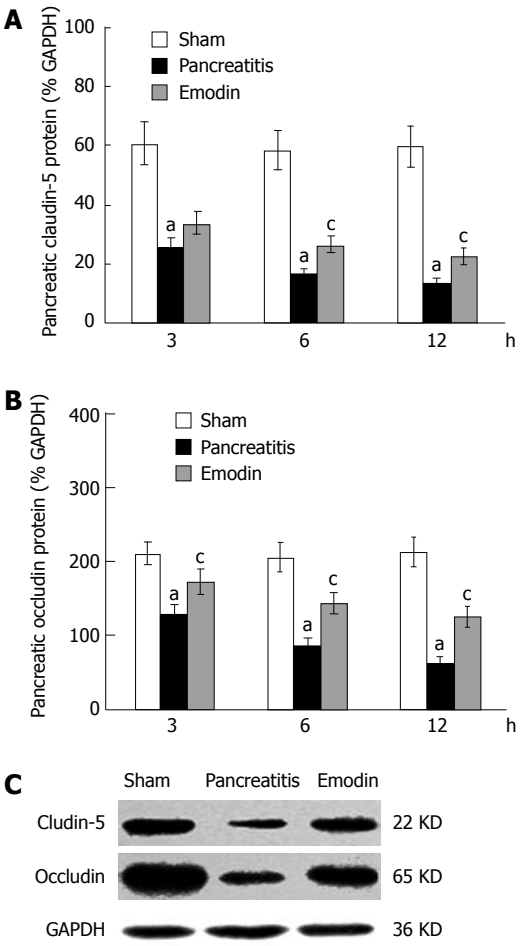

Figure 4 Effects of emodin on claudin-5 (A, C) and occludin (B, C) protein levels in rats. Six rats were studied in each experimental group at each time point. Results are expressed as mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* pancreatitis group. GAPDH: Glyceraldehyde phosphate dehydrogenase.

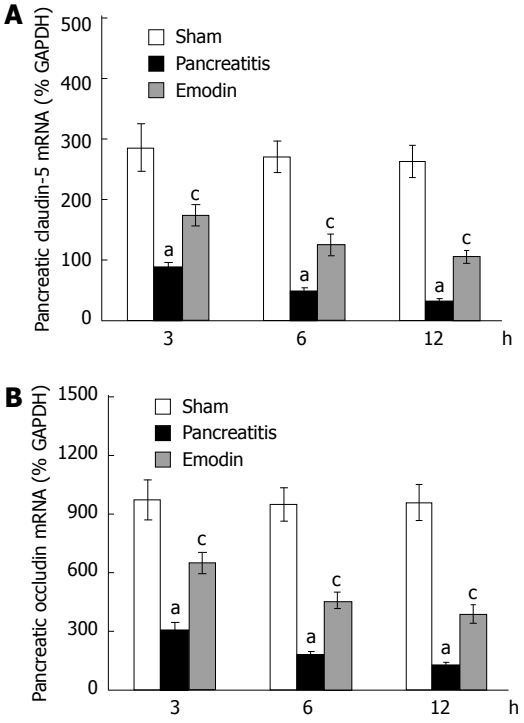

Figure 5 Effects of emodin on claudin-5 (A) and occludin (B) mRNA levels in rats. Six rats were studied in each experimental group at each time point. Results are expressed as mean ± SD. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* pancreatitis group. GAPDH: Glyceraldehyde phosphate dehydrogenase.

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

Tomohiro Terai, Mitsushige Sugimoto, Hiroki Uozaki, Tetsushi Kitagawa, Mana Kinoshita, Satoshi Baba, Takanori Yamada, Satoshi Osawa, Ken Sugimoto

Tomohiro Terai, Mitsushige Sugimoto, Hiroki Uozaki, Takanori Yamada, Satoshi Osawa, Ken Sugimoto, First Department of Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
Tetsushi Kitagawa, Seirei Health Care Division, Seirei Preventive Health Care Center, Hamamatsu 431-3192, Japan
Mana Kinoshita, Satoshi Baba, Department of Diagnostic Pathology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
Author contributions: Terai T, Sugimoto M, Uozaki H, Kitagawa T, Yamada T, Osawa S and Sugimoto K diagnosed and treated the patient; Kinoshita M and Baba S diagnosed the patient pathologically; Terai T and Sugimoto M wrote the paper.
Correspondence to: Mitsushige Sugimoto, MD, PhD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. mitsu@hama-med.ac.jp
Telephone: +81-53-4352261 Fax: +81-53-4349447
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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.

Key words: Gastric lymphomatoid gastropathy; Gastric natural killer/T-cell lymphoma nasal type; *Helicobacter pylori*; Eradication

Peer reviewer: Jian-Zhong Zhang, Professor, Pathology and Laboratory Medicine, Beijing 306 Hospital, 9 North Anxiang Road, PO Box 9720, Beijing 100101, China

Terai T, Sugimoto M, Uozaki H, Kitagawa T, Kinoshita M, Baba S, Yamada T, Osawa S, Sugimoto K. Lymphomatoidgastropathy mimicking extranodal NK/T cell lymphoma, nasal type: A case report. *World J Gastroenterol* 2012; 18(17): 2140-2144 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i17/2140.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i17.2140

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 cytoplasm (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 $CD3^+$, $CD4^-$, $CD5^-$, $CD7^+$, $CD8^-$, $CD20^-$, $CD30^-$, $CD56^+$, $CD79a^-$, $TIA\text{-}1^+$ 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

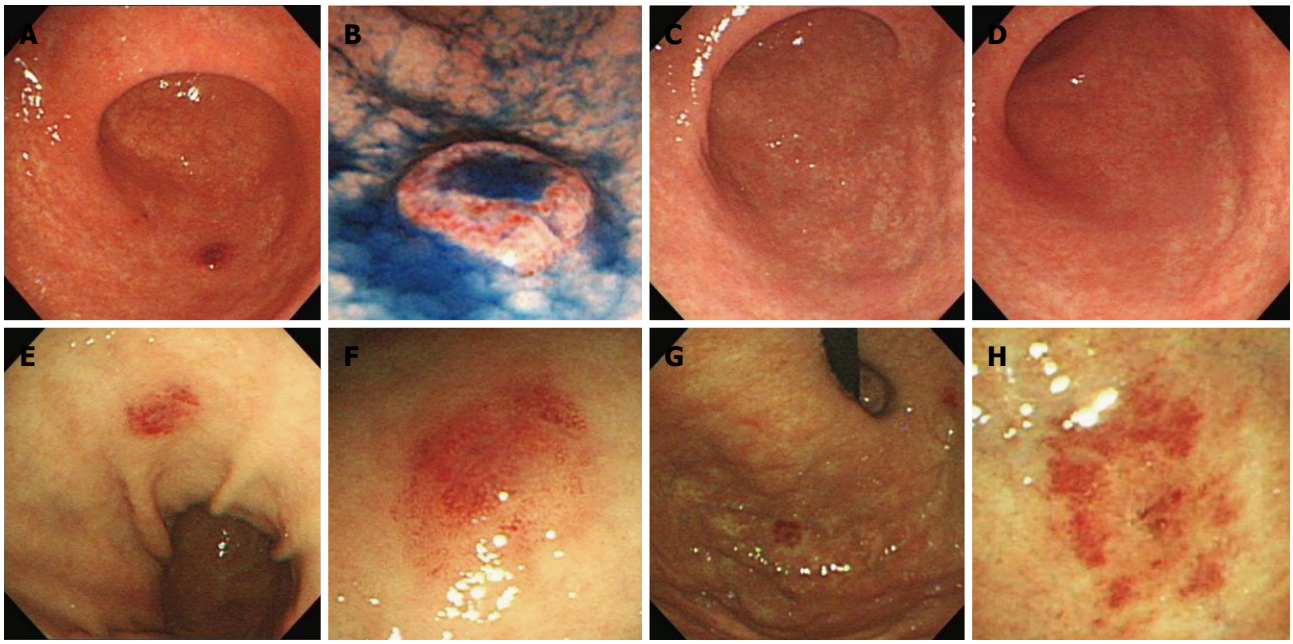

Figure 1 Gastroendoscopy revealed an erythematous dish-like elevated lesion in the greater curvature of the lower body at check-up (A and B), and at one (C) and six months later (D); Twelve months later, endoscopy revealed a similar lesion in the anterior wall of the middlebody (E and F), and an erythematous lesion in the fornix (G and H).

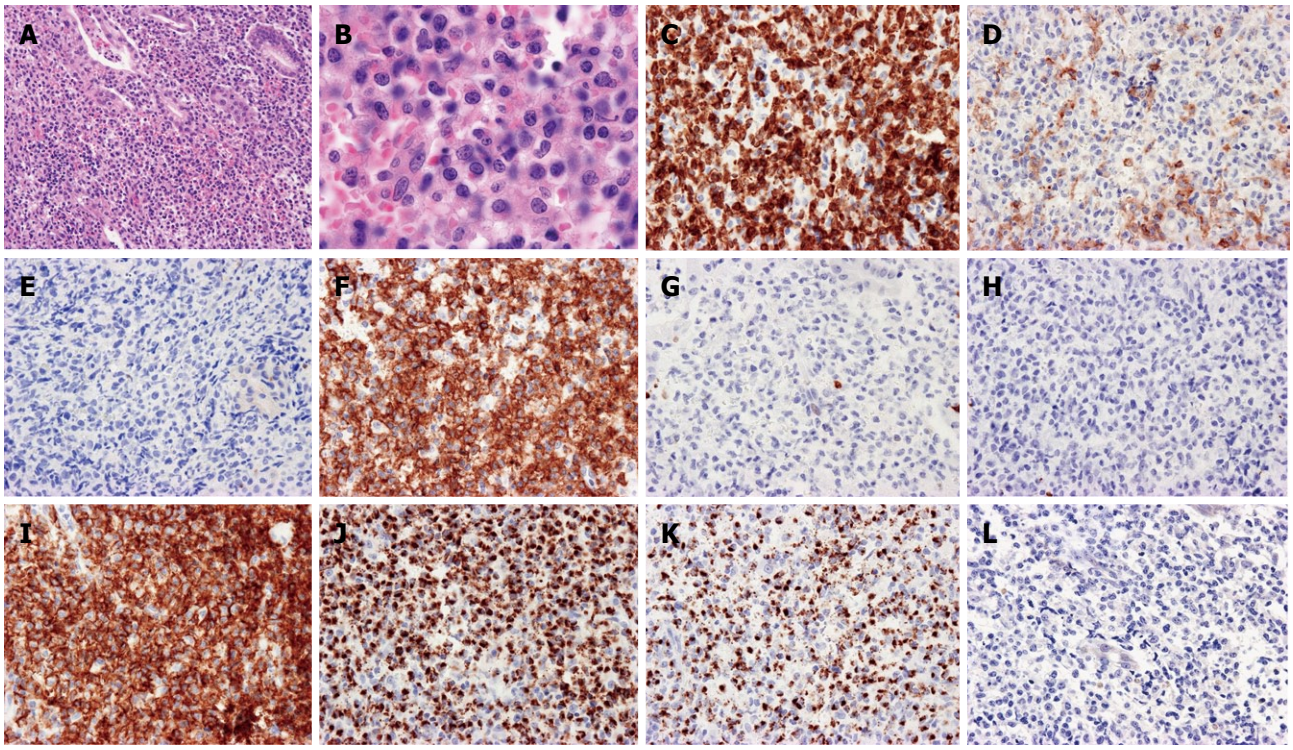

Figure 2 Histological examination showed massive atypical medium- to large-sized natural killer lymphocyte infiltrations with slightly irregular nuclear contours. A dispersed chromatin pattern and clear cytoplasm in the gastric mucosa, × 100 (A) and × 400 (B); Immunohistochemical stains showed $CD3^+$ (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

Yan-Fei Fang, Li-Feng Qiu, Ying Du, Zhi-Nong Jiang, Min Gao

Yan-Fei Fang, Ying Du, Min Gao, Department of Gastroenterology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Li-Feng Qiu, Department of Emergency, The First Municipal Hospital of Hangzhou, Hangzhou 310006, Zhejiang Province, China
Zhi-Nong Jiang, Department of Pathology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Author contributions: Fang YF wrote the paper; Qiu LF collected materials; Jiang ZN contributed figures; Du Y and Gao M revised the language.
Correspondence to: Min Gao, MD, Department of Gastroenterology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou 310016, Zhejiang Province, China. gaomindy@sina.com
Telephone: +86-571-86006788 Fax: +86-571-86006788
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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.

Key words: Hemolymphangioma; Small intestine; Gastrointestinal bleeding; Benign tumor

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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

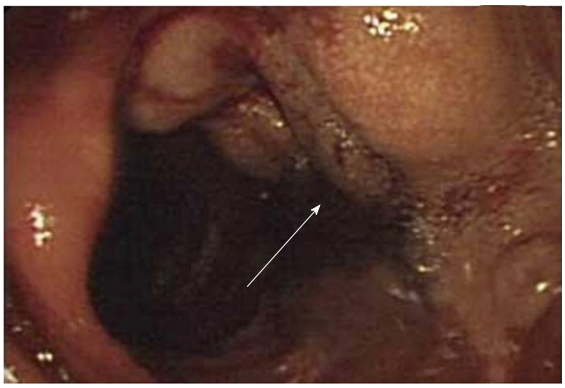

Figure 1 Enteroscopy revealed a mass at the small intestine (arrow).

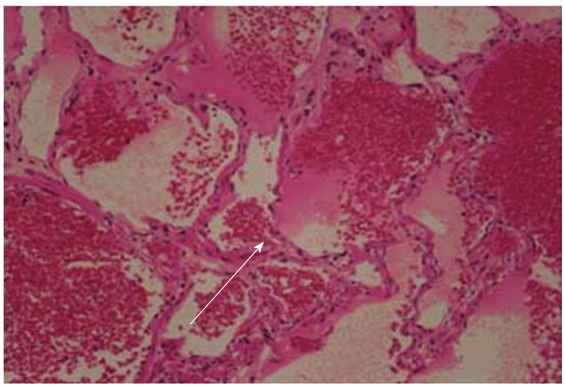

Figure 2 Histological analysis revealed a benign soft tissue mass (arrow) consisting of lymphatic and blood vessels (hematoxylin and eosin, × 100).

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 hemorrhage, 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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Alastair D Burt, Professor, Dean of Clinical Medicine, Faculty of Medical Sciences, Newcastle University, Room 13, Peacock Hall, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, United Kingdom

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Andreas Geier, MD, Division of Gastroenterlogy and Hepatology, Zürich University Hospital, Raemistrasse 100, CH-8901 Zürich, Switzerland

Andrea De Gottardi, MD, PhD, Assistant Professor, Clinic of Visceral Surgery and Medicine-Hepatology, Freiburgstrasse, CH-3010 Berne-Inselspital, Switzerland

Grigoriy E Gurvits, MD, Department of Gastroenterology, St. Vincent's Hospital and Medical Center, New York Medical College, 153 West 11th Street, Smith 2, New York, NY 10011, United States

Salvatore Gruttadauria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

Kevin Cheng-Wen Hsiao, MD, Assistant Professor, Colon and Rectal Surgery, Tri-Service General Hospital, No. 325, Sec. 2, Cheng-Kung Rd, Nei-Hu district, Taipei 114, Taiwan, China

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Toshihiro Mitaka, MD, PhD, Professor, Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan

Jesús Prieto, Professor, Clinica Universitaria, University of Navarra, Avda, Pio XII, 36, 31080 Pamplona, Spain

Philip Rosenthal, MD, Professor of Pediatrics and Surgery, University of California, San Francisco, 500 Parnassus Avenue, Box 0136, MU 4-East, San Francisco, CA 94143-0136, United States

Bronislaw L Slomiany, PhD, Professor, Research Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States

Jon Arne Søreide, Professor, MD, PhD, FACS, Department of Surgery, Stavanger University Hospital, N-4068 Stavanger, Norway

Patricia Sylla, MD, General and Colorectal Surgery, Massachusetts General Hospital, WACC 460, 15 Parkman Street, Boston, MA 02114, United States

Antonello Trecca, MD, Usi Group Digestive Endoscopy and Gastroenterology, Via Machiavelli, 22, 00185 Rome, Italy

Dr. Stefan Wirth, Professor, Children's Hospital, Heusnerstt 40, 42349 Wuppertal, Germany

Dr. Anthony T Yeung, BS, MS, PhD, Fox Chase Cancer Center, Room R404, 333 Cottman Avenue, Philadelphia, PA 19111-2497, United States

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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
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Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

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March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
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March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
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Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
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April 18-20, 2012
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April 19-21, 2012
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Madrid, Spain

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June 2-6, 2012
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June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
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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
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September 20-22, 2012
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Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
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November 3-4, 2012
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Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *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.0000035706.28494.09]

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

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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

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 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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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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