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EDITORIAL

Ursodeoxycholic acid therapy in gallbladder disease, a story not yet completed

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Abstract

Gallstone disease represents an important issue in the healthcare system. The principal non-invasive non-surgical medical treatment for cholesterol gallstones is still represented by oral litholysis with bile acids. The first successful and documented dissolution of cholesterol gallstones was achieved in 1972. Since then a large number of investigators all over the world, have been dedicated in biochemical and clinical studies on ursodeoxycholic acid (UDCA), demonstrating its extreme versatility. This editorial is aimed to provide a brief review of recent developments in UDCA use, current indications for its use and, the more recent advances in understanding its effects in terms of an anti-inflammatory drug.

Key words: Gallbladder; Cholesterol gallstones; Ursodeoxycholic acid

Core tip: Ursodeoxycholic acid can be considered one of the less expensive, best tested and safest of the drugs currently available. This editorial is aimed to provide a brief review of the principal non-invasive non-surgical medical treatments for cholesterol gallstones. Based on the literature and on our experimental and clinical works we try to summarize the recent developments in ursodeoxycholic acid use, current indications for its use and the more recent advances in understanding its effects in terms of an anti-inflammatory drug. For these reasons, the story would not appear to end herewith but deserves further attention and investigation.

Guarino MPL, Cocca S, Altomare A, Emerenziani S, Cicala M. Ursodeoxycholic acid therapy in gallbladder disease, a story not yet completed. *World J Gastroenterol* 2013; 19(31): 5029-5034 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5029.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5029

INTRODUCTION

Gallstone disease still represents a relevant issue for the healthcare system and one of the most common and costly of all digestive diseases if we consider the number of cholecystectomies, which are performed annually all over the world, and the hospital admission rate for complicated gallstone disease[1,2]. A marked variation in overall gallstone prevalence between the different ethnic populations has been reported; native populations from North and South America represent the groups at the highest risk in the world. Symptoms occur in approximately 20% of patients, and this subgroup is at the highest risk of developing serious complications from gallstone disease. These complications can range from simple to severe recurrent biliary colic, ascending cholangitis and/or pancreatitis[3].

Gallstone disease is a complex disorder where both

environmental and genetic factors contribute to the susceptibility to the disease. Risk factors include age, gender, race, parity, dietary factors. A family history of gallstones has also been identified as a risk factor suggesting that genetics play a role in gallstone formation. Genetic factors seems to be responsible for at least 30% of symptomatic gallstone disease[4]. Furthermore, as in atherosclerosis, the risk of cholesterol gallstone disease increases with obesity, type 2 diabetes, insulin resistance and dyslipidaemia, conditions associated with the metabolic syndrome[1,5].

Gallstones are classified as cholesterol and pigment stones. More than 90% of gallstones consist mainly of cholesterol and are formed within the gallbladder[3].

TREATMENT OF GALLSTONE DISEASE

A physician of the Byzantine Empire first described calculi in the human liver, but the earliest evidence of human gallstones is represented by the finding of 30 stones in the intact gallbladder of a mummified Egyptian priestess from around 1500 BC. In the past, a multiplicity of treatments have been used to attempt gallstone dissolution, including prayer, magic, herbs and potions[6].

The modern medical therapeutic management of gallstone disease depends primarily upon the clinical stage: asymptomatic, symptomatic (typical biliary colic pain), and complicated disease.

Asymptomatic gallstones rarely warrant treatment, since they generally have a benign natural course; the progression to symptomatic disease is relatively low, ranging from 10% to 25%. The majority of patients rarely develop gallstone-related complications without having at least one episode of biliary pain. In the pre-laparoscopy era, cholecystectomy was generally performed for symptomatic disease. The minimally invasive laparoscopic cholecystectomy refuelled the controversies regarding the optimal management of asymptomatic or silent gallstones, but most experts agree that the majority of patients should be managed by observation alone (expectant management)[7]. According to the National Institutes of Health Consensus Conference report "the availability of laparoscopic cholecystectomy should not expand the indications for gallbladder removal"[8]. Moreover, follow-up studies on a total of 279 patients with silent gallstone disease reported that the natural history of asymptomatic gallstones is benign and only 20% of these patients developed pain or complications within 24 years[9].

Symptomatic gallstone disease or acute cholecystitis are the primary indications for cholecystectomy that is currently considered the "gold standard" for the treatment of gallstone disease. Cholecystectomy is one of the most commonly performed abdominal surgical procedures, the first carried out in 1882 by Carl von Langenbuch[6]. The credit of establishing surgery of the gallbladder on a firm footing belongs to Langenbuch. The safety and success of this operation was soon established. Laparoscopic cholecystectomy is a minimally invasive surgical technique that was first performed in France, in 1987, and, in the United States, in 1988. This technique has now replaced open cholecystectomy as first-choice treatment for selected types of patients and represents one of the safer surgical procedures[8].

Non-surgical management of gallstones has been widely investigated over the last few decades, including gallstone dissolution both by mechanical and biochemical means[10].

Since its introduction, in 1985, in Germany, extracorporeal shockwave lithotripsy (ESWL) had been shown to be useful for fragmentation of bile duct stones that were not extractable endoscopically and its efficacy was soon established for selected patients at high surgical risk (> 70 years old, high morbidity and mortality rates) presenting gallstone disease (solitary radiolucent calculi < 2 cm in diameter)[11]. ESWL adopts focused shock waves produced by electromagnetic or ultrasound sources to fragment gallstones, but its efficacy depends upon the amount of energy delivered to the stone as well as the emptying and fasting volumes of the gallbladder[6]. Since its introduction in gastroenterology, ESWL had been considered as an adjuvant of oral bile acid in the treatment of gallstones, since it increases the surface for bile salt action fragmenting the stones into smaller particles. The major disadvantage of ESWL is the high post-dissolution recurrence rate (being 11%-26% for a 24-mo period), which had always raised the issue of cost-effectiveness[12]. For this reason, at present, even if advances have been made in lithotripsy technology (*i.e.*, the introduction of pulverization), none of the ESWL machines have been approved by the Food and Drug Administration (FDA) for routine clinical use in the United States, therefore this technique is no longer widely used, except in some European countries[8]. In the early period of the first use of ESWL, much interest was aroused by the application of contact dissolution agents, even if considerably less experience had been recorded. It involved direct entry of a potent cholesterol solvent (such as methyl tertiary-butyl ether, MTBE), either instilled directly into the gallbladder or into the bile duct following endoscopic intubation. Cholesterol prevalent stones could be cleared within hours to days. Interest in this method was soon lost due to the potential side-effects and was therefore limited to patients that were at high surgical risk[13] .

The principal non-invasive non-surgical medical treatment for cholesterol gallstones is still represented by oral litholysis with bile acids[14]. The first successful and documented dissolution of cholesterol gallstones was achieved in 1972 by oral administration of chenodeoxycholic acid (CDCA), a primary trihydroxy bile acid[15]. The use of CDCA due to a dose-dependent increase in aminotransferases, to an increase in serum low-density lipoprotein cholesterol and the development of bile salt-induced diarrhoea, raised concerns[15]. Since the more hydrophilic UDCA appeared to be as effective in gallstone dissolution but practically devoid of side-effects, it rapidly replaced CDCA and represents the most widely recorded experience in the literature[16].

Recently some studies have suggested the possibility of using, as therapeutic agents for gallstone disease, cholesterol-lowering agents such as statins and ezetimibe that inhibit hepatic cholesterol synthesis or reduce the absorption of cholesterol in the small intestine, alone or in combination with other forms of treatment[17-21]. Despite some promising initial data in the literature, there are still some conflicting results, thus suggesting that UDCA is the most suitable of medical treatments for gallstone disease.

URSODEOXYCHOLIC ACID

The use of UDCA in the treatment of liver diseases dates back to the traditional Chinese medicine during the Tang Dynasty. For centuries, the Chinese drug "*shorea spp.*", derived from the bile of adult black bears, has been used to cure various hepatobiliary disorders. Only at the beginning of the 20th century, was UDCA identified from polar bear bile by Hammarsten[22], a swedish research worker, who named this uncharacterized bile acid as ursocholeinic acid. The bile acid he identified was actually CDCA. It is anecdotally said that he ran out of the sample during the course of purification and abandoned its crystallization. Twenty years later, in 1927, Shoda, from Okayama University, isolated UDCA from bear bile imported from China, succeeded in crystallizing it and then called it by its present name, *i.e.*, Urso-deoxycholic ("*urso*", bear in Latin), being the predominant bile acid in bears[22].

Until Makino *et al*[22] clearly demonstrated that treatment with UDCA resulted in dissolution of cholesterol gallstones, UDCA was predominantly used in Japan as a liver tonic being administered in doses that were too small to have any significant therapeutic effect. Thereafter its use spread worldwide following further confirmation of its effectiveness and safety[23].

From the time of marketing to the present day, a large number of investigators all over the world have been involved in biochemical. At present, and clinical studies on UDCA, demonstrating its extreme versatility. UDCA can be used as a therapeutic tool in cholestatic liver diseases, being currently considered the only medical treatment officially approved by the United States FDA, to treat primary biliary cirrhosis. It can also be a therapeutic tool for non-cholestatic diseases and even for non-hepatobiliary ones[24]. For example, it appears to exert an anti-proliferative effect in terms of colon cancer prophylaxis and adenoma recurrence, an immunomodulating effect in patients affected by AIDS and it would appear to play a protective role in idiopathic recurrent pancreatitis[25]. Finally, UDCA, thanks to its biochemical structure, can penetrate the blood-brain barrier, so in the future it may be found an application of UDCA as a cell membrane stabilizer in central nervous system disorders[25].

Despite the extensive evidence accumulated regarding the possible use of UDCA in various types of diseases, the largest amount of evidence still remains the beneficial effect of UDCA in dissolution of cholesterol gallstones.

UDCA IN GALLSTONE DISEASE

UDCA, in pharmacological doses, markedly decreases biliary cholesterol saturation by 40%-60%, by inhibition of cholesterol absorption in the intestine, and cholesterol secretion into bile as indicated by a decrease in the cholesterol fraction of biliary lipids[24]. Moreover, it is well known that UDCA decreases toxicity of bile acids which can damage cell membranes and cause cholestasis, through different means of action: by inhibition of hydrophobic endogenous bile acids absorption from the small intestine, by exerting a choleretic function that induces dilution of endogenous bile salts in the bile ducts and by protecting hepatocytes against toxic bile acids[25,26].

Since Makino *et al*[22] first reported gallstone dissolution with UDCA, it has been used above all in the treatment of gallbladder cholesterol stones as an alternative to cholecystectomy[24,27]. Although gallstones are mainly composed of cholesterol, only a small number of patients (< 10% of total) can be treated with systemic dissolution therapy using UDCA[16]. Candidates for UDCA treatment should have cholesterol-enriched non-calcified gallstones < 20 mm in diameter and a patent cystic duct. The recommended dose of UDCA for gallbladder stones is 8-10 mg/kg per day, larger doses do not offer additional benefits. A dissolution rate of 30%-60% (about 1 mm decrease in stone diameter per mo) has been reported, although the initial gallstone diameter has been shown to be the most important factor affecting the dissolution rate[27-29]. A clinical study demonstrated complete disappearance of small stones (< 5 mm) with UDCA treatment after 6 mo (90% in approximately 90% of cases)[16]. Following complete dissolution, UDCA should be continued for another 3 mo in order to confirm decomposition of microscopic stones that may not be detected by ultrasonography. Absence of, or minimal, change in gallstone diameter within 6 to 12 mo of UDCA treatment represents a poor prognostic sign for dissolution[28]. The chance of reducing, by means of dissolution the size of large (> 20 mm diameter) or multiple stones, is very poor (less than 40%-50% after 1 year of treatment)[16].

Biliary sludge has been considered another therapeutic target of UDCA. Sludge formation in the biliary system can be accelerated for example by rapid weight loss, pregnancy, total parenteral nutrition and solid organ transplantation. The beneficial effect of UDCA in this condition has been shown in a clinical study in which idiopathic acute pancreatitis has been related to microscopic gallstones or biliary sludge. In this study UDCA administration within 3 to 6 mo prevented gallstone recurrence and more episodes of pancreatitis over a follow-up of 44 mo[28].

The greater limit of UDCA therapy for gallstone dissolution can be considered the high recurrence rate. Several studies have reported a recurrence rate of 30%-50% at 5 years and 50%-70% at 12 years, after successful treatment, especially in patients with multiple gallstones[16,28,29].

For these reasons, the therapeutic effect of UDCA in patients with symptomatic gallbladder stones has been controversial over the last few decades but the usefulness of this bile acid, as a therapeutic tool, has been successively reconsidered not only for its dissolution capacity, but also for the anti-inflammatory effect. A long-term follow-up study on UDCA treatment showed a significant decrease in the incidence of gallstone disease complications. In particular, this study showed that UDCA treatment in patients with symptomatic gallstones reduced the incidence of biliary pain and acute cholecystitis compared with no treatment over an 18-year period[30]. Interestingly, this therapeutic effect was independent of gallstone dissolution suggesting that UDCA could achieve these effects by restoring the normal gallbladder environment which more recent studies, on gallstone disease, have clearly shown to be characterized by an inflammatory status. A more recent 3-mo randomized placebo-controlled study showed that UDCA did not exert any beneficial effect on biliary pain or complications[31]. It should be pointed out that, there are significant differences in the recurrence rates of biliary pain and need for cholecystectomy between these two studies. Tomida *et al*[30] reported recurrence rates of < 10% in those patients on UDCA compared to 40% in those on placebo after 4 years. In contrast, in the most recent clinical trial, the need for cholecystectomy after 100 d on UDCA or placebo reached almost 75%[31]. These differences suggest that UDCA may not be effective in patients with more advanced chronic inflammatory gallbladder disease. Our earlier findings showing that UDCA treatment restores gallbladder muscle functions and reduces the biochemical markers of oxidative stress and inflammation may support, and partially explain, the beneficial effects in patients with symptomatic gallbladder stones which were independent of gallstone dissolution[32].

A series of *in vitro* studies have investigated the anti-inflammatory effect of UDCA. Cystic duct ligation in guinea pigs does not to cause acute cholecystitis unless the bile is lithogenic with cholesterol and concentrated bile is injected into the gallbladder[33,34]. Guinea pigs submitted to common bile duct ligation develop acute cholecystitis within 2-3 d together with biochemical and pathologic changes similar to those found in human acute cholecystitis, with or without gallstones[34,35]. Gallbladder muscle cells present increased levels of reactive oxygen species (ROS), lipid peroxidation and prostaglandin E2 (PGE2) levels, their response to cholecystokinin (CCK-8), PGE2 and potassium chloride being impaired, and associated with a significant reduction in receptor binding of these ligands[34]. These abnormalities were reproduced by treating normal human muscle cells with H_2O_2 or with hydrophobic bile acids (tauro-chenodeoxycholic acid, TCDC) and are prevented by pre-treatment with PGE_2 or with the free radical scavenger catalase suggesting that hydrophobic bile acids damage receptors and calcium channels of gallbladder muscle cells by stimulating the generation of ROS[36,37]. Interestingly, *in vitro* studies have shown that muscle cells pre-incubated with UDCA prevent TCDC-induced muscle cell damage and ROS production[36]. This specific beneficial effect of UDCA has been confirmed by the previously mentioned double blind, randomized 4-wk, study, carried out by our group, comparing the effects of UDCA with those of placebo in patients scheduled to undergo cholecystectomy for symptomatic gallbladder stones. In particular, this study revealed that pre-treatment with UDCA restores the normal contraction of gallbladder muscle cells by reducing cholesterol content in the plasma membranes and levels of H_2O_2, lipid per-oxidation, platelet-activating factor-like lipids as well as the production of PGE2 and catalase activity[32]. These results are consistent with data reported in a non-randomized study showing improved gallbladder muscle strip contraction in patients treated with UDCA for 3 wk compared to patients not receiving treatment[38].

These data support the hypothesis that lithogenic bile containing excess cholesterol creates a permissive environment in the gallbladders altering the normal balance between hydrophobic bile acids and gallbladder protective mechanisms. Bile acids stimulate the formation of reactive oxygen species, capable of initiating inflammatory processes and cholecystitis. Thus UDCA, by reducing the excess cholesterol and "neutralizing" the hydrophobic bile acids, restores the balance between aggressive biliary factors and gallbladder protective mechanisms[32].

Hydrophobic bile acids, such as chenodeoxycholic and deoxycholic acid, have also been demonstrated to have a toxic effect on the liver mainly by the generation of reactive oxygen species[39,40]. In particular, hydrophobic bile acids, following hepatic retention, may affect not only the hepatocytes but also the resident macrophages (*i.e.*, Küpffer cells) which generate reactive oxygen species and increase the level of oxidative stress[41]. Therapeutic concentrations of UDCA enrich the bile acid pool with UDCA resulting in a pool profile shifting from hydrophobicity to hydrophilicity[42]. UDCA administration has been shown to prevent and reduce the hydrophobic bile acid damage in the liver; indeed, in addition to displacement of the hydrophobic bile acids, UDCA appears to exert a beneficial effect by preventing hydrophobic bile acid-induced stimulation of macrophage oxidative processes[41].

A study from our group suggests that UDCA appears to exert a prophylactic action on the effects of hydrophobic bile acids on the macrophage oxidative processes in the gallbladder. Data emerging from this study reveal the occurrence, in gallbladders surgically

removed from patients with cholesterol gallstones, of an increased number of macrophages in the muscle layer when compared to the normal gallbladder. Of interest, this double blind randomised 4-wk study comparing the effects of UDCA with those of placebo in patients with symptomatic gallbladder stones, scheduled to undergo cholecystectomy, showed that this hydrophilic bile acid leads to a decrease in the number of activated macrophages in the muscle layer and to the reduced production of PGE2 in the gallbladder muscle[43]. PGs are catalytic products of cyclooxygenase-2 (COX2) and are well-known modulators of gastro-intestinal smooth muscle function[44,45]. In our study, COX2 was mainly expressed in the muscle by macrophages and a direct correlation was found between the number of the COX2 and the CD68 positive cells which represent the macrophages. Although a minor contribution of other cell types, such as mast cells and muscle cells, in which PGE2 production contributes to the mechanisms of cytoprotection[46], cannot be definitely excluded, our findings support the hypothesis that another anti-inflammatory effect of UDCA could result from the decrease in the number of activated macrophages which are the main source of PG production. This finding adds another evidence of the anti-inflammatory effect of this hydrophilic bile acid.

CONCLUSION

The large number of studies concerning the UDCA in gallbladder and liver disease published in the literature, over the last few years, clearly indicates the beneficial effect of this bile acid, supported by the more recent advances in the understanding of its effects in terms of anti-inflammatory drug.

Indeed, as only a small number of patients can benefit from UDCA, in terms of dissolution therapy, its specific beneficial effect is related also to prevention of complications in symptomatic gallstone carriers, which is independent from stone dissolution. In our opinion this hydrophilic bile acid could be an alternative therapeutic approach in high surgical risk patients with symptomatic gallbladder stones.

Furthermore, UDCA is one of the less expensive, best tested and safest drugs currently available. For these reasons, the story would not appear to end herewith but deserves further attention and investigation.

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REVIEW

Portal hypertension and gastrointestinal bleeding: Diagnosis, prevention and management

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Abstract

Bleeding from esophageal varices is a life threatening complication of portal hypertension. Primary prevention of bleeding in patients at risk for a first bleeding episode is therefore a major goal. Medical prophylaxis consists of non-selective beta-blockers like propranolol or carvedilol. Variceal endoscopic band ligation is equally effective but procedure related morbidity is a drawback of the method. Therapy of acute bleeding is based on three strategies: vasopressor drugs like terlipressin, antibiotics and endoscopic therapy. In refractory bleeding, self-expandable stents offer an option for bridging to definite treatments like transjugular intrahepatic portosystemic shunt (TIPS). Treatment of bleeding from gastric varices depends on vasopressor drugs and on injection of varices with cyanoacrylate. Strategies for primary or secondary prevention are based on non-selective beta-blockers but data from large clinical trials is lacking. Therapy of refractory bleeding relies on shunt-procedures like TIPS. Bleeding from ectopic varices, portal hypertensive gastropathy and gastric antral vascular ectasia-syndrome is less common. Possible medical and endoscopic treatment options are discussed.

Key words: Portal hypertension; Esophageal varices; Gastric varices; Portal hypertensive gastropathy; Gastric antral vascular ectasia-syndrome; Variceal bleeding; Endoscopy; Band ligation; Beta-blocker

Core tip: Gastrointestinal bleeding is a life threatening complication of portal hypertension. Primary prevention of bleeding in patients at risk for a first bleeding episode is therefore a major goal. The article gives a concise overview of possible bleeding sites in patients with portal hypertension. The diagnosis, prevention, therapy of acute bleeding and secondary prophylaxis of bleeding from esophageal and gastric varices, portal hypertensive gastropathy gastric antral vascular ectasia and ectopic varices are discussed.

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INTRODUCTION

One of the major complications of portal hypertension is bleeding from esophageal varices. Bleeding from gastric or duodenal varices as well as bleeding from colonic varices or from portal hypertensive gastropathy is less common.

A lot of studies investigating prophylaxis and therapy of bleeding in portal hypertension have been published in the last years. This paper gives a concise overview of the current knowledge.

PRIMARY PROPHYLAXIS OF BLEEDING FROM ESOPHAGEAL VARICES

Definition

Primary prophylaxis of bleeding from esophageal varices

is defined as a therapeutic intervention that aims at the prevention of the first variceal hemorrhage.

Diagnosis

At the time of the first diagnosis, about half of the patients with liver cirrhosis have esophageal varices (Figure 1)[1,2]. During the course of the disease about 90% of the patients develop esophageal varices. Variceal hemorrhage still carries a significant mortality of 7%-15%[3-5]. The identification and prophylactic treatment of patients at risk for esophageal bleeding is therefore mandatory[6].

Risk factors for variceal bleeding are the diameter of the varix, presence of red wale signs and an impaired liver function[7-10]. Hemodynamic studies point at a close association of the hepatic venous pressure gradient (HVPG) and the bleeding risk[9].

Every patient with newly diagnosed liver cirrhosis should underwent upper endoscopy for screening of esophageal and/or gastric varices[6]. In patients with esophageal varices with a diameter of more than 5 mm, prophylactic treatment should be initiated.

Prophylactic treatment is not necessary when only small varices (diameter below 5 mm) are present. Nevertheless, endoscopic follow-up is mandatory[6]. The overall incidence of esophageal varices is 5% per year[11,12]. Esophageal varices tend to increase in size in a linear fashion. One study including 258 patients with small varices and without a history of variceal bleeding found an increase in variceal size in 21%, 45% and 66% of the patients after 1.5, 3 and 4.5 years, respectively[13]. However, it has to be kept in mind that the course of the underlying liver disease is a major determinant of variceal progression[7,13]. The actual recommendation for surveillance in patients with compensated liver disease and small varices at the screening endoscopy is a follow-up examination after 1-2 years[6,14]. If the screening endoscopy showed no varices, a follow-up examination after 2-3 years is sufficient in patients with compensated liver disease[6,13,14].

Prophylaxis/therapy

Non-selective beta-blockers cause vasoconstriction of the splanchnic circulation by β_2-receptor inhibition and decrease cardiac output by β_1-receptor blockade. This leads to a decrease in portal venous inflow and thereby lowers portal pressure.

Beta-blocker therapy is not effective in preventing gastro-esophageal varices in patients with cirrhosis[15]. There is only one study that showed that prophylaxis with a non-selective beta-blocker is effective in preventing the enlargement of small varices[16]. Patients with varices at risk of bleeding (diameter > 5 mm, presence of red-color-signs) should receive prophylactic treatment (see below), since the risk of bleeding is 30%-35% in two years. Effective prophylactic treatment reduces the risk of bleeding by about 50%[17].

A major drawback of beta-blocker therapy is that not all patients respond to beta-blockers with a reduction of the HVPG[18]. Clinical studies have shown that at most

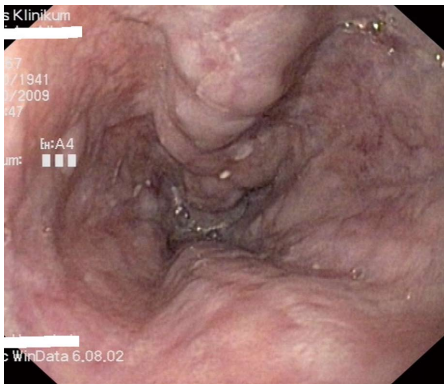

Figure 1 Esophageal varices grade Ⅱ in a patient with liver cirrhosis.

50% of beta-blocker treated patients achieved a reduction of the HVPG below 12 mmHg or > 20% from baseline levels[18]. However, other effects of beta-blocker therapy besides the reduction of HVPG like a decrease in azygos blood flow or a decrease in bacterial translocation from the gut[19] may play a role in the prevention of variceal hemorrhage[20].

Endoscopic sclerotherapy and shunt procedures are obsolete in primary prophylaxis. Standard modalities are drug therapy with non-selective beta-blockers and endoscopic variceal band ligation (VBL) of varices.

Non-selective beta-blockers like propranolol and nadolol were introduced for primary prophylaxis almost 30 years ago[17]. In recent years, the non-cardioselective vasodilating beta-blocker with mild intrinsic anti-$\alpha(1)$-adrenergic activity carvedilol was shown to be at least as effective in lowering HVPG as propranolol[21] or nadolol plus nitrate[22] and to be as effective as VBL for primary prophylaxis of variceal bleeding[23]. A monotherapy with nitrates or a combination of beta-blockers and nitrates compared to beta-blockers alone has no benefit in primary prophylaxis[17,24]. Meta-analysis have shown a reduction of the bleeding risk by a non-selective beta-blocker of about 50%. Around 20% of patients suffer from intolerable side effects that require discontinuation of the drug. After discontinuation, the bleeding risk is not different from an untreated population. That makes an indefinite prophylactic therapy necessary[25]. The most important predictor for variceal bleeding in patients on a therapy with beta-blockers is the dose of the drug[26]. Patients should therefore receive the highest tolerated dose.

An effective alternative treatment for primary prophylaxis is endoscopic VBL[27-30]. One meta-analysis has shown, that compared with untreated controls, prophylactic VBL reduces the risks of variceal bleeding and mortality[31]. Several studies compared endoscopic VBL with propranolol for primary prophylaxis of variceal bleeding[27-30]. Only one study that is controversially discussed because of some methodological flaws found a significant benefit for endoscopic VBL[29]. The other studies found no difference between beta-blockers and VBL concerning prophylaxis of bleeding[27,29,30]. A recently published Cochrane analysis that included 19 ran-

domized trials found a slight beneficial effect for VBL, but that effect was not present when only full published paper articles were analyzed[32]. In terms of efficacy, VBL and non-selective beta-blocker therapy are considered to be equivalent.

Because of the low costs, ease of administration as well as the absence of procedure-related mortality, non-selective beta-blockers are recommended as first-line treatment for the primary prophylaxis of esophageal variceal bleeding[17].

VBL is recommended in patients with serious side effects or intolerability of beta-blocker therapy as well as in patients with contraindications for drug therapy.

ACUTE BLEEDING FROM ESOPHAGEAL VARICES

Definition

Acute variceal bleeding is defined as: (1) active bleeding from esophageal varices at the moment of endoscopy; or (2) non-bleeding varices and blood in the esophagus/stomach are present and no other source of bleeding is found[33]. Recurrent bleeding is defined as rebleeding after 24-h of clinical absence of bleeding.

Therapy

Acute bleeding from esophageal varices is often a dramatic event. Most patients vomit blood but hematochezia and melena might be the only symptoms. Dependent on the amount of lost blood, patients might be hemodynamic instable and present in hemorrhagic shock. Today only 40% of patients die from exsanguinating bleeding. Most deaths are caused by complications of bleeding like liver failure, infections and hepatorenal syndrome[34,35]. Risk factors for an adverse course are the degree of liver dysfunction, creatinine, hypovolemic shock, active bleeding on endoscopy and presence of hepatocellular carcinoma[4,34-37]. Thus, the management of patients with acute variceal bleeding includes not only treatment and control of active bleeding but also the prevention of rebleeding, infections and renal failure[38].

If variceal bleeding is suspected, patients should be hemodynamically stabilized and receive medical treatment with vasopressors and antibiotic treatment[39-43]. In uncomplicated patients antibiotic therapy is done using quinolones[44]. High-risk patients with advanced liver disease (ascites, encephalopathy, jaundice, malnutrition) or previous therapy with quinolones should receive ceftriaxone[41]. Antibiotic treatment of patients with acute variceal bleeding does not only decreases mortality but also decreases the probability of rebleeding[42]. Transfusion of blood should be done with caution with a target hemoglobin level between 7 to 8 g/dL, since higher hemoglobin levels can increase portal pressure[45] and restrictive transfusion strategies are associated with better survival[46]. Patients with massive bleeding and/or patients who are somnolent should undergo endotracheal intubation and mechanical ventilation prior endoscopy to prevent aspiration pneumonia.

Available therapy options include medical and endoscopic treatment, balloon tamponade, placement of fully covered self-expandable metallic stents, transjugular intrahepatic portosystemic shunt (TIPS) and surgical shunts. Nowadays, the initial approach is a combination of vasoactive drugs, antibiotics and endoscopic therapy[47].

Medical therapy

The aim of medical therapy is to reduce splanchnic blood flow and portal pressure. Drugs currently in use are vasopressin, somatostatin and, most important in Europe, terlipressin. Due to its short half-life, vasopressin has to be given as a continuous *iv* infusion. Relevant adverse effects include systemic vasoconstriction with serious implications like mesenteric or myocardial ischemia[48]. Application of vasopressin in combination with nitrates reduces the side effects associated with vasoconstriction[49,50]. Several studies have shown that the vasopressin treatment is effective in terms of bleeding control but does not affect mortality[48,51-53]. Terlipressin is a synthetic vasopressin analogue with a longer half-life and less adverse effects. Several studies have shown that terlipressin is effective in bleeding control and has a positive impact on survival[54-56]. Terlipressin achieves control of bleeding in 75%-80% and 67% of patients at 48 h and at 5 d, respectively[56,57]. It is given at a dose of 2 mg every 4 h for the first 48 h and could be continued for prevention of early rebleeding at a dose of 1 mg every 4 h for up to 5 d[57,58]. A recent study has shown a drop of serum sodium in the range of > 5 mEq/L in 67% of patients and of > 10 mEq/L in 36% of patients treated with terlipressin[59]. Therefore, serum sodium should be monitored in patients receiving terlipressin. Compared to vasopressin, terlipressin is more effective in control of esophageal bleeding[60,61] and compared to vasopressin plus nitrate[62] as well as compared to somatostatin it is comparable effective[63,64].

Somatostatin is given as an initial bolus of 250 μg followed by a 250 to 500 μg/h continuous infusion until a bleed-free period of 24 h is achieved[38]. Octreotide is a synthetic analogue of somatostatin with longer half-life. It is administered as an initial bolus of 25 μg, followed by an infusion of 25 to 50 μg/h[65]. Both, somatostatin and octreotide, have a good safety profile. Possible adverse effects include mild hyperglycemia and abdominal craps. Somatostatin is as effective as vasopressin in control of variceal bleeding; the safety profile is superior to vasopressin[66]. The combination of terlipressin and octreotide is not superior to a monotherapy with terlipressin[67].

In summary, the available data is most convincing for terlipressin, however, the direct comparison of terlipressin and octreotide revealed no superiority of terlipressin[68,69].

Endoscopic therapy

About 80%-90% of acute variceal bleeding episodes are successfully controlled by endoscopic therapy[70]. Nowadays, most important is endoscopic VBL, injec-

tion therapy using sclerosing agents like ethoxysklerol or cyanoacrylate is less commonly used. Ethoxysklerol is injected next to - not into - the varix. It causes local inflammation and scaring and thereby thrombosis and obliteration of the vessel. On the opposite, cyanoacrylate is injected directly into the varix, causing immediate obliteration of the vessel. Endoscopic band ligation is done using a transparent cap that is attached to the tip of the endoscope. By applying suction, the varix is then pulled into the cap and a rubber ring is thrown over the varix causing thrombosis and scaring of the vessel (Figure 2).

Before the introduction of VBL, ethoxysklerol injection was widely used in the treatment of acute esophageal variceal bleeding. Studies have shown that sclerotherapy was at least as effective as balloon tamponade[71,72]. The injection of cyanoacrylate is used as a second line therapy when VBL of variceal bleeding fails.

Endoscopic VBL was first carried out in 1988[73]. The method is now widely available and complications are - compared to sclerotherapy - less common[74]. The most frequent complications are superficial ulcerations and esophageal strictures. Bleeding after the rubber rings have been fallen off is less common. A disadvantage of the method is the impaired sight that is caused by the ligation system. Costs are - compared to sclerotherapy - higher. Mortality rates after VBL are lower as compared to sclerotherapy[75,76].

Balloon tamponade

The use of balloon tamponade for the treatment of acute esophageal variceal bleeding was introduced by Sengstaken *et al*[77] The Minnesota-tube is a modified version with an aspiration channel above the esophageal balloon. For uncontrolled bleeding from gastric varices, the Linton-Nachlas tube is preferred[78]. In the hand of the experienced user the method allows control of bleeding in most patients[79]. A major drawback of the method is the high amount of possible serious complications like necrosis and/or rupture of the esophagus as well as aspiration pneumonia[80]. Deflating of the balloon after six hours reduces the risk of complications. Due to the serious risks, balloon tamponade should only be applied by an experienced physician under fluoroscopic control. After all, balloon tamponade is only a bridging procedure until other, definite therapy options are available.

Self-expandable metal stents

The placement of fully covered self-expandable metal stents (SEMS) is an alternative to balloon tamponade. The SEMS is inserted over an endoscopic placed guidewire using a stent delivery device without the need of fluoroscopy[81]. SEMS controls bleeding by compression of the bleeding varices[81]. The stent can be left in place for up to two weeks and can be easily removed by endoscopy. The effectiveness in the control of refractory esophageal variceal bleeding has been shown in four case series[82-85]. The procedure is save with minor complications like esophageal ulcerations, compression of the bronchial system and stent migration into the stomach being described[82-85]. Like balloon tamponade, the procedure is reserved for patients with bleeding refractory to medical and endoscopic treatment. It does not allow definite treatment of variceal bleeding due to the high percentage of patients with rebleeding after the SEMS has been removed, but has to be considered as an effective and safe bridging procedure that allows stabilization of the patient until definite treatment is possible.

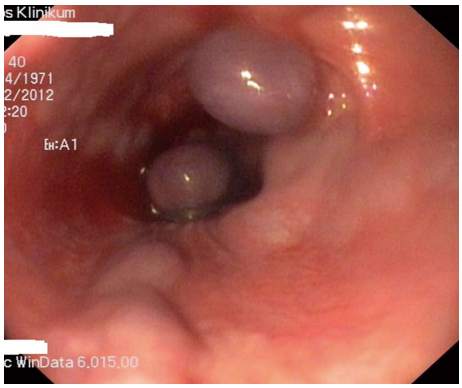

Figure 2 Variceal band ligation of esophageal varices.

Transjugular intrahepatic portosystemic shunt

By TIPS placement a functional portacaval side-to-side shunt is established. TIPS is indicated in patients with refractory acute variceal bleeding that could not be sufficiently controlled by endoscopic and/or medical therapy and in patients with recurrent bleeding despite optimal endoscopic therapy. After TIPS insertion, bleeding is stopped in almost all of the affected patients[86-88]. The rate of recurrent bleeding after one year is 8%-18%[89-91]. However, TIPS insertion is a problem in patients with multi-organ failure and/or in patients with decompensated liver disease. In these patients, the 30-d-mortality rate is as high as 100%[86,88,92]. Disadvantages of the procedure are the risk of hepatic encephalopathy as well as TIPS dysfunction with the risk of recurrent bleeding[93,94]. A major improvement was the introduction of polytetrafluoroethylene (PTFE) covered stents. These stents have higher rates of patency over time and mortality rates are lower[95]. A recently published trial has investigated the role of early TIPS in high-risk patients[96,97]. The multicenter study including 63 patients with esophageal hemorrhage and a high risk of treatment failure (Child B with active bleeding or Child C < 14 points) demonstrated that insertion of a PTFE covered TIPS within 72 h (preferable within 24 h) compared to combined endoscopic and vasoactive drug treatment decreased rebleeding (50% patients without rebleeding in the non-TIPS *vs* 97% in the TIPS group) and 1-year mortality (86% survival in the TIPS *vs* 61% in the non-TIPS group)[96].

Surgery

Surgical procedures in patients with acute or recurrent variceal bleeding are limited to a very small portion of

patients in whom medical and/or endoscopic control of bleeding was not achievable and TIPS was no option because of technical or anatomical problems (*e.g.*, complete thrombosis of the portal vein). Possible procedures are porto-systemic shunt operations[98] or staple transection of the esophagus[99]. Survival of patients who have undergone surgery is dependent on liver function but the mortality rate is as high as 80%.

SECONDARY PROPHYLAXIS OF ESOPHAGEAL VARICEAL BLEEDING

In patients who survive the first episode of esophageal hemorrhage, the risk of recurrent bleeding is as high as 60% with a mortality rate of up to 33%[100]. Prevention of rebleeding is therefore a major goal in patients in whom the initial bleeding episode has been successfully controlled.

Definition

Secondary prophylaxis of variceal bleeding is defined as the prevention of rebleeding from varices.

Medical therapy

Several studies are available that compared the non-selective beta-blockers propranolol or nadolol with no prophylaxis after initial bleeding[101-107]. Most of the studies found a reduction of the rebleeding risk as well as a reduction in mortality. Addition of nitrates further increased this positive effect[108]. Essential is a reduction of the HVPG of at least 20%, even if a reduction below 12 mmHg could not be achieved[26,109-111].

Endoscopic therapy

Several groups studied the effect of sclerotherapy for secondary prophylaxis of variceal bleeding[105,112-114]. The comparison of sclerotherapy to medical therapy with a non-selective beta-blocker found a benefit for patients treated with sclerotherapy in two studies[115,116] and a slight but statistically not significant benefit for beta-blocker therapy[105,117,118]. Three more studies did not find a difference between the two treatment modalities[115,116,119].

For prophylaxis of recurrent bleeding, sclerotherapy is now replaced widely by VBL. Several studies have shown the superiority of VBL over sclerotherapy[74,76,120-124].

Comparing VBL to medical therapy with non-selective beta-blockers in combination with nitrates, two studies found medical therapy to be as effective[110] or more effective[125] than VBL. In contrast, one study found VBL to be advantageous over medical therapy[126]. From the pathophysiological point of view, the combination of VBL and medical therapy is an even more promising approach for secondary prophylaxis. This has been investigated in five studies[127-131]. Whereas two studies found combination therapy to be more effective than VBL alone[127,131] two more recent studies, that compared nadolol plus nitrates with combination treatment of drugs and VBL failed to demonstrate superiority of combination treatment[128,130]. Therefore, it seems that a clear recommendation for medial treatment alone, VBL alone or combination treatment of drugs and VBL cannot be made at the moment. A reasonable approach is to perform VBL alone in patients with contraindications for beta-blocker therapy or in patients who suffer from side effects of beta-blocker therapy. Patients who tolerate drug treatment well should be placed on a combination therapy.

Transjugular intrahepatic portosystemic shunt

TIPS was compared to sclerotherapy[90,132-137] as well as to VBL[89]. In all but two studies[136,138] patients treated with TIPS had lower rates of recurrent bleeding. Three meta-analysis[139-141] summarized the available studies and found a significant lower probability of rebleeding in the TIPS treated patients. The incidence of hepatic encephalopathy was higher in the TIPS-group. A difference in mortality was not evident.

Surgery

Shunt surgery has been shown to be effective in the prophylaxis of rebleeding from esophageal varices. This has been shown for non-selective as well as for selective shunts (*e.g.*, distal spleno-renal shunt) comparing operative shunts with no therapy or endoscopic sclerotherapy[99,142-147]. As in TIPS, the most important side effect was the incidence of hepatic encephalopathy.

One study[148] compared non-covered TIPS with a small diameter prosthetic porta-caval H-shunt. Both shunts led to an adequate reduction in portal pressure, but patency rates of the operative shunts were higher over time. This led to a lower rate of rebleeding as well as to a decrease in mortality in patients with the surgical shunt. A meta-analysis compared different porto-systemic shunts (TIPS, diverse surgical shunts) with endoscopic treatment[149]. All shunts were equally effective in reducing the risk of rebleeding. The incidence of hepatic encephalopathy was higher in patients who received a shunt procedure. TIPS was complicated by a high incidence of shunt dysfunction. Comparing the different shunt procedures, there was no difference in survival.

GASTRIC VARICES AND HYPERTENSIVE GASTROPATHY

In contrast to esophageal variceal bleeding, prevention and treatment of bleeding from gastric varices and from portal gastropathy is less well evaluated in clinical studies.

Definition

According to Sarin *et al*[150] gastric varices (Figure 3) are endoscopically classified as gastro-esophageal varices type I (lesser curvature), gastro-esophageal varices type II (greater curvature), isolated gastric varices type I (located in the gastric fundus) or isolated gastric varices type II (any location in the stomach except the gastric fundus).

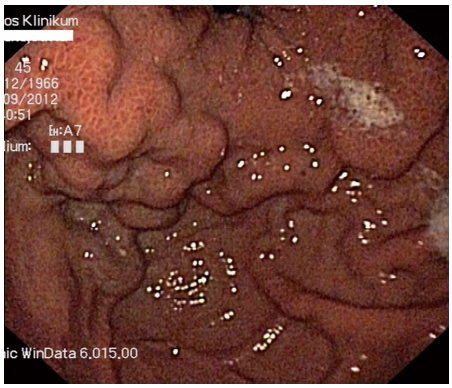

Figure 3 Isolated gastric varices type Ⅰ and portal hypertensive gastropathy in a patient with liver cirrhosis.

Gastric varices

The diagnosis of gastric varices is made by endoscopy. In case of doubt of the diagnosis, endosonography with Doppler sonography allows further differentiation. If only isolated gastric varices are present, the exclusion of portal or splenic vein thrombosis as the underlying cause is mandatory.

About on fifth of the patients with portal hypertension develop gastric varices[150]. In patients with gastrointestinal bleeding due to portal hypertension, bleeding from gastric varices is the cause in 5%-10% of patients[151]. The risk of the first bleeding from gastric varices is lower than the risk of first bleeding from esophageal varices (4% in one and 9% in three years)[152]. The risk of recurrent bleeding is dependent on the localization of the varix: isolated varices in the gastric fundus (53%) bear the highest risk of recurrent bleeding, followed by varices of the greater curvature (19%) and lesser curvature (6%)[150]. The prophylactic treatment of esophageal varices by VBL does not increase the risk of secondary gastric varices compared to propranolol[153].

Almost no data is available whether medical treatment for the primary prophylaxis of bleeding from gastric varices is effective. Pathophysiological considerations warrant the use of non-selective beta-blockers for this indication[151]. One trial including 27 patients with gastric varices studied the injection of cyanoacrylate for primary prophylaxis of bleeding from large gastric varices and found the injection of cyanoacrylate to be safe and effective in primary prophylaxis[154]. However, before recommending cyanoacrylate injection as prophylactic therapy, more studies are necessary.

Data for the treatment of acute bleeding from gastric varices is sparse. Therapy with terlipressin or somatostatin is recommend although controlled studies are lacking. The endoscopic treatment of choice is injection with cyanoacrylate[155-157]. Control of bleeding is as high as 90% and more effective than sclerotherapy or band ligation in one trial[158], whereas another study found VBL and cyanoacrylate injection equally effective in terms of control of acute bleeding but reported higher rebleeding rates in the VBL group[159]. Known complications of cyanoacrylate injection include mucosal ulcerations as well as thromboembolism. TIPS insertion is highly effective with control of bleeding in more than 90% of patients[160,161] and should be considered in patients in whom endoscopic therapy fails.

The use of non-selective beta-blockers and nitrates for prophylaxis of rebleeding was shown in one study to be not effective[162]. The comparison of cyanoacrylate with propranolol for secondary prophylaxis has shown no difference between the two treatment modalities in terms of rebleeding or mortality but found more complications in the cyanoacrylate group[163]. Another study compared TIPS with cyanoacrylate in patients with bleeding from gastric varices. TIPS was shown to be more effective for prevention of recurrent bleeding, with no difference in mortality[164]. These results are in contrast to a retrospective analysis that found TIPS and cyanoacrylate equally effective in controlling and preventing gastric variceal hemorrhage with no significant differences in survival[165]. Patients who received TIPS experienced significantly more long-term morbidity[165]. Nevertheless, the above mentioned studies have to be interpreted with caution, since they included patients with different types of gastric varices.

PORTAL HYPERTENSIVE GASTROPATHY

The diagnosis of portal hypertensive gastropathy is made by endoscopy. Typical signs are mosaic, also called "snakeskin", pattern of erythema. More severe forms present with red punctuate erythema, diffuse hemorrhagic lesions and/or brown spots that indicate submucosal hemorrhage[166]. Histopathologic features of portal hypertensive gastropathy are vascular ectasia of the mucosal and submucosal veins and capillaries[166]. The exact pathogenesis of portal hypertensive gastropathy is unknown. Important factors in the pathogenesis are the presence of portal hypertension as well as hyperemia of the gastric mucosa. Several authors assumed that the endoscopic treatment of esophageal varices aggravates portal hypertensive gastropathy[167]. The worsening is often transient and portal hypertensive gastropathy shows regression in more than 40% of patients after VBL[168]. The incidence of portal hypertensive gastropathy is around 80% in patients with liver cirrhosis[169]. Acute bleeding from portal hypertensive gastropathy (Figure 4) is a rare event, with an incidence of less than 3% in three years. One study that evaluated the cause of GI-bleeding in 1496 patients found bleeding from portal hypertensive gastropathy the cause in 0.8% of patients, accounting for 8% of non-variceal bleeding in patients with liver disease[170]. The probability of chronic bleeding is around 10%-15% in three years[6].

There is only one small trial that studied the effect of non-selective beta-blockers on portal hypertensive gastropathy[171]. Twenty-four patients with non-bleeding portal hypertensive gastropathy received 160 mg propranolol per day in a double-blind placebo controlled cross-

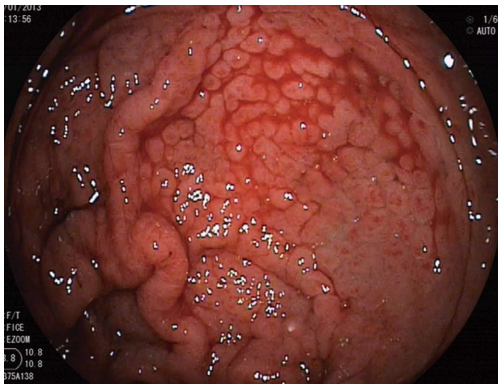

Figure 4 Acute diffuse bleeding from portal hypertensive gastropathy in a patient with decompensated liver cirrhosis.

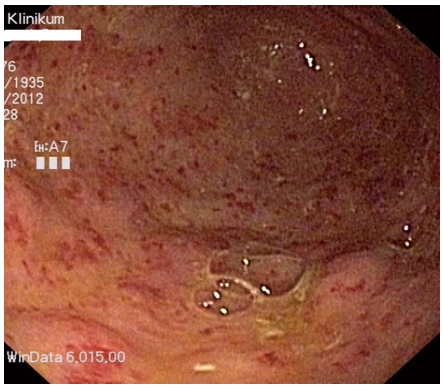

Figure 5 Typical appearance of a watermelon stomach in a patient with gastric antral vascular ectasia-syndrome and compensated liver cirrhosis.

over trial. Endoscopic grading of portal hypertensive gastropathy improved after propranolol in nine patients compared to three after placebo[171].

The therapy of acute bleeding from portal hypertensive gastropathy is mainly based on drugs that decrease portal pressure. In one study, 14 portal hypertensive patients with heavy diffuse bleeding from portal hypertensive gastropathy received propranolol in a dose of 24 to 480 mg per day. Within 3 d, bleeding ceased in 13 (93%) of patients[171]. Since the study did not have a control group of untreated patients, the results have to be interpreted with caution. A small study compared octreotide, vasopressin and omeprazole for therapy of acute bleeding. In this setting, octreotide was more effective than omeprazole or vasopressin[172]. Terlipressin was also shown to be effective in acute bleeding from portal hypertensive gastropathy[173].

No studies that investigated the role of endoscopic treatment using argon-plasma-coagulation in acute or recurrent bleeding from portal hypertensive gastropathy are available. If medical therapy fails, TIPS insertion or surgical shunt are an option[6,174,175].

In the secondary prophylaxis of bleeding from portal hypertensive gastropathy, one study including 54 patients showed that propranolol is effective in the prevention of rebleeding[176]. In the group of the propranolol treated patients 65% were free of rebleeding after one year compared to 38% in the control group. After 30 mo of follow-up, 52% of the patients in the propranolol group were free of rebleeding compared to 7% of the untreated patients[176].

In summary, the risk of bleeding from portal hypertensive gastropathy is low and primary prophylaxis is therefore not necessary. In patients with recurrent bleeding from portal hypertensive gastropathy, propranolol should be considered for secondary prophylaxis.

Gastric antral vascular ectasia-syndrome

Bleeding from gastric antral vascular ectasia (GAVE) is an uncommon but sometimes severe cause of upper gastrointestinal bleeding. It accounts for 4% of non-variceal upper GI-bleeding[177].

Gastric antral vascular ectasia (GAVE-syndrome, also known as "watermelon stomach" or "honeycomb stomach") is endoscopically as well as histologically distinguished form portal hypertensive gastropathy. In most patients, the diagnosis of GAVE is easily made on endoscopy. In case of diagnostic uncertainty, the so called GAVE-score that defines histological changes helps to distinguish the both entities[177]. GAVE-syndrome is most often found in older women and is associated with autoimmune disorders in about 60% of patients[178]. Liver disease is a risk factor for the development of GAVE-syndrome, but only 30% of affected patients suffer from liver cirrhosis[179]. On endoscopy (Figure 5), linear red streaks running longitudinally in the gastric antrum are apparent ("watermelon stomach"). In patients with liver cirrhosis the mucosal pattern is often more diffuse ("honeycomb stomach")[180]. The lesion consists of ectatic vessels of the mucosa with focal thrombosis surrounded by fibromuscular hyperplasia[181]. The pathogenesis of GAVE is not well known. Hypothesis for the pathogenesis include mechanical stress[182], humoural[183] and autoimmune factors[184]. Portal hypertension per se does not seem to be a risk factor for GAVE[185,186].

Different drugs have been used in the treatment of bleeding from GAVE. A small controlled cross-over trial has shown estrogen-progesterone to be highly effective in GAVE related bleeding[187]. Another study confirmed these findings[188]. However, the therapy has to be maintained on a long-term basis since a dose reduction results in recurrent bleeding[189]. Moreover, long-term hormonal treatment is associated with an increased risk for breast and endometrial cancer[190]. One small trial showed octreotide to be effective in bleeding from GAVE[191], but another study failed to confirm the efficacy of octreotide[192].

Treatment consists mainly of endoscopic measures like argon plasma coagulation (APC) (Figure 6), or laser photoablation of the lesions[193,194]. Endoscopic treatment using (Nd: YAG) laser has been shown to be effective in bleeding from GAVE in several studies[195-198]. The treatment is relatively safe, complications like perforation or pyloric stenosis are infrequent[199]. Disadvantages of the method are the high costs and the need for a long

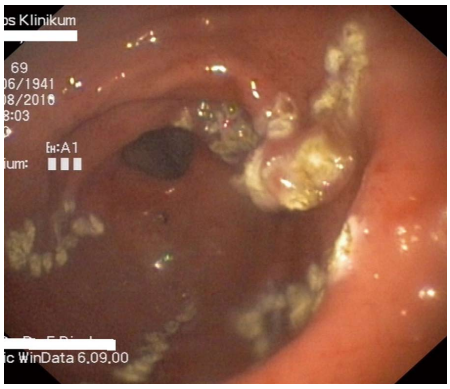

Figure 6 Endoscopic treatment of gastric antral vascular ectasia with argon plasma coagulation therapy.

training period. Argon plasma coagulation has therefore widely replaced laser therapy in the treatment of GAVE related bleeding. The procedure is easy to use, relatively cheap and widely available as well as safe. The efficacy of APC in the treatment of bleeding from GAVE is very high (90%-100% in two studies[194,200]). On average, 2.5 sessions are necessary for successful eradication of the lesions[193,194,201]. Three studies using endoscopic band ligation for the treatment of GAVE related bleeding are available[202-204]. Band ligation was shown to be effective in all trials but a study with sufficient patient numbers comparing band ligation to APC treatment is missing. Lowering portal pressure by TIPS-insertion is not effective in chronic bleeding from GAVE[179,205]. Surgery (antrectomy) is efficient in bleeding from GAVE[206] but bears a significant morbidity and mortality and is therefore reserved for patients with recurrent bleeding despite therapy with argon plasma coagulation.

ECTOPIC VARICES

Definition

Ectopic varices are dilated porto-venous vessels of the gastrointestinal mucosa that are located outside of the esophagus or the stomach.

Ectopic varices have their origin from preexisting small veins of the gastrointestinal mucosa that are porto-systemic collaterals between the portal vein and the inferior vena cava. In the majority of cases, portal hypertension or an extrahepatic obliteration of the portal vein are the cause for the development of ectopic varices.

Diagnosis

Bleeding from ectopic varices is a rare event. It accounts for 1%-5% of all gastrointestinal bleeding episodes in patients with portal hypertension[207,208]. Endoscopy is the most important diagnostic tool. In patients with portal hypertension, acute bleeding and negative findings on upper endoscopy, bleeding from ectopic varices has to be considered. In these patients, accurate examination of the duodenum is mandatory. Examination of the jejunum makes double-balloon enteroscopy necessary.

Colonoscopy is the principal method for the diagnosis of colonic varices. One study found rectal varices *via* endoscopy in 43% and *via* EUS in 75% of patients with portal hypertension, pointing out that rectal varices might be overlooked by conventional endoscopy[209].

In patients in whom bleeding from ectopic varices is assumed but endoscopy was negative, nuclear magnetic resonance (NMR) with NMR-angiography is the diagnostic tool of choice and allows the identification of ectopic varices in most patients.

Therapy

Sclerotherapy/injection therapy: Therapy of ectopic varices is mainly based on sclerotherapy or injection therapy. Controlled studies which method is best are not available but case reports showed that both sclerotherapy with ethoxysklerol as well as injection of the varix with cyanoacrylate are feasible[210-213].

Band ligation may be useful for temporary hemostasis[209,214] in duodenal varices but rebleeding of duodenal varices is a problem with ligation therapy. Additional treatment following band ligation for duodenal varices is therefore mandatory.

Surgery and TIPS: Porta-caval shunts are effective therapy measures in recurrent bleeding from ectopic varices[147,215,216]. Another option in patients without portal vein thrombosis is TIPS-insertion. Several case reports that show that TIPS is an effective option in the treatment of ectopic varices have been published[217-220].

Interventional radiology: Balloon-occluded retrograde transvenous obliteration (B-RTO) was successfully performed for patients with duodenal varices[221,222]. B-RTO can obliterate not only varices but also the afferent and efferent veins and should be considered for treating duodenal varices.

Medical therapy: From a pathophysiological point of view the application of beta-blockers does makes sense in patients with ectopic varices, but no data from controlled trials that investigate the role of non-selective beta-blockers and/or nitrates are available.

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REVIEW

Role of bevacizumab in colorectal cancer growth and its adverse effects: A review

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Abstract

Angiogenesis affects both wound healing and malignant cell growth through nutrients and oxygen. Vascular endothelial growth factor (VEGF) is the most important element involved in this complex process. Inhibition of VEGF influences angiogenesis and may restrict tumor growth and metastatic ability. Modern anti-angiogenic therapy is based on this theory. Bevacizumab is a recombinant humanized monoclonal antibody (immunoglobulin G1) which binds with VEGF-A forming a large molecule. It can not be bound with VEGF tyrosine kinase receptors preventing VEGF-A incorporation; thus its activity is inhibited inducing blockage of VEGF-mediated angiogenesis. Bevacizumab, in combination with chemotherapy or other novel targeted therapeutic agents, is currently used more frequently in clinical practice, mainly for managing advanced colorectal cancer. It is also used for managing other malignancies, such as breast cancer, pancreatic cancer, prostate cancer, non small-cell lung cancer, metastatic renal carcinoma and ovarian tumors. Although it is generally considered a safe treatment, there are reports of some rare side effects which should be taken into account. Recent experiments in rats and mice show promising results with a wider therapeutic range.

Key words: Angiogenesis; Vascular endothelial growth factor; Anti-angiogenic agents; Bevacizumab; Avastin; Cancer targeted therapy; Colorectal cancer

Core tip: Modern targeted therapy with anti-angiogenic agents is based on inhibition of angiogenesis, as the formation of new vessels is crucial for the growth and metastasis of malignant cells. Recent studies on the biological agent, bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor activity, have shown improved outcome in advanced colorectal cancer. The combination of irinotecan, capecitabine and bevacizumab is currently the most frequently used regime in the treatment of metastatic colorectal cancer with improved response rates. However, the rare side-effects of bevacizumab should always be considered.

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INTRODUCTION

Angiogenesis is a complex process responsible for the formation of new vessels originating from pre-existing vessels. It is necessary for the proliferation and growth of normal cells and tissues during the fetal and neonatal period, but also for the proliferation and growth of cancer cells. Its physiological role in adult life is limited in wound healing and the reproductive cycle of females. The development of such vessel networks, or even col-

lateral circulation, aim to supply the tissues with oxygen and nutrients, remove carbon dioxide and waste products of cell metabolism and transfer hormones. A variety of factors are involved in the regulation of angiogenesis. Vascular endothelial growth factor (VEGF) is one of the main growth factors involved in vessel formation[1,2].

The current targeted therapy of cancer with anti-angiogenic agents is based on angiogenesis inhibition and restriction of tumour spread, as neo-angiogenesis has a crucial effect on the growth and invasion of malignant cells[3-7].

The topic of this study has attracted much interest in clinical oncology and experimental research. VEGF by promoting angiogenesis favours tumor growth, while its inhibition results in tumor limitation. The novel anti-angiogenic agent, bevacizumab, is a recombinant humanized monoclonal antibody against VEGF activity. This targeted therapy is currently combined with chemotherapy and used mainly in the treatment of metastatic colorectal cancer.

ANGIOGENESIS AND ITS INHIBITION

VEGF and its receptor (VEGFr) play important roles in the neo-angiogenesis process in physiological growth and healing as well as in pathological states such as malignancy. VEGF levels are known to be increased, particularly in the most malignant tumors, such as colorectal cancer, and are associated with an increased ability of the malignancy to spread and with poorer prognosis. Thus, inhibition of angiogenesis results in growth restriction or even a reduction in malignant cells[8]. A variety of events and factors at the molecular level have been evaluated for application in novel anti-cancer drugs. VEGF is one of these factors. Targeting VEGF with bevacizumab, a humanized monoclonal immunoglobulin G (IgG) antibody, in combination with adjuvant chemotherapy has been proved to effectively manage advanced colorectal cancer[5,6,8].

Malignant tumors require nutrients for growth, and tumors more than 1-2 mm^3 in size ensure independent blood flow for continuing growth. These new vessels develop *via* angiogenesis. Inadequate blood flow leads to hypoxia, the main stimulus for angiogenesis initiation. Proteins such as hypoxia inducible factor are activated resulting in over-expression of pro-angiogenic factors including VEGF and fibroblastic growth factors. The number of cancer cells is reduced in parallel with the expression of anti-angiogenic factors, such as thrombospondin I. Through the over-expression of pro-angiogenic factors, as opposed to anti-angiogenic factors, endothelial cells are activated, thus triggering the initiation of angiogenesis[8].

In spite of the similarities in the angiogenesis process between wound healing and malignancy, there are differences in the structure of new vessels.

Several angiogenic factors derived from platelets and inflammatory cells are involved in the stages of wound healing through various mechanisms. They include phosphorylation of tyrosine kinase receptors, activation and proliferation of epithelial cells, migration and creation of tubular formations and finally new vessel formation. VEGF initiates angiogenesis by abruption of cell walls and protein lysis of vessel walls, proliferation and migration of endothelial cells and formation of new vessels. This vessel network is derived from endothelial tip cells, which have phenotypic and functional differentiation from other endothelial stalk cells[3,4].

Six subtypes of VEGF have been reported, *i.e.*, VEGF-A, VEGF-B, VEGF-C, VEGF-D, virus VEGF-E and placental VEGF (PIGF). VEGF-A increases vascular permeability, degeneration of the extracellular matrix and cell aggravation. VEGF-B and PIGF are involved mainly in the normal angiogenesis process. However, an increase in PIGF levels promotes angiogenesis in pathological conditions, such as tumors and inflammation. VEGF-C and VEGF-D have a predominant role in lymphatic angiogenesis; VEGF (PIGF) regulates placental angiogenesis[8].

Four isomers of VEGF-A have been reported in humans ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{184}$, $VEGF_{206}$). The isomer $VEGF_{165}$ is over-expressed in the majority of human malignancies. This over-expression enhances growth, invasiveness and metastatic ability.

VEGF is derived from malignant cells and promotes the growth of colorectal cancer[9]. However, a recent study has shown that the expression of EGFR and VEGF are not prognostic factors in the survival of patients with colorectal cancer and the expression of EGFR does not determine lymphatic metastasis; however, this issue remains controversial[10]. It is the over-expression of VEGF and not the density of microvasculature or vein invasion that plays the important role; it is also responsible for hematogenous dissemination after curative resection for gastric cancer[11].

VEGFr is a receptor of tyrosine kinase and has three forms, VEGFr-1, 2, 3. They are expressed in vessel endothelial cells as well as in cancer cells (VEGFr-1 and 2). VEGFr-1 is also found in monocytes and macrophages. VEGFr-3 is found in endothelial cells of the lymphatic system. VEGF-A correlates with receptors VEGFr-1 and 2, VEGF-B and PIGF with receptor VEGFr-1, and VEGF-C and D correlate with receptor VEGFr-3. VEGFr-2 plays an important role in the angiogenesis process in physiological as well as in pathological conditions. VEGFr-2 stimulation promotes cell growth and migration, the creation of tubular formations (endothelial cells) and the increase in vascular permeability[1,2].

The role of VEGF in other diseases such as allergic and immune-mediated diseases has been well-established[12,13]. The potential positive effect of other biological drugs (specific immunotherapy) such as tumor necrosis factor-α inhibitors on the mechanisms of action of VEGF has also been debated[14].

BEVACIZUMAB-ACTION MECHANISM

As mentioned above, angiogenesis plays a pivotal role in cell proliferation and tumor growth. Malignant cells

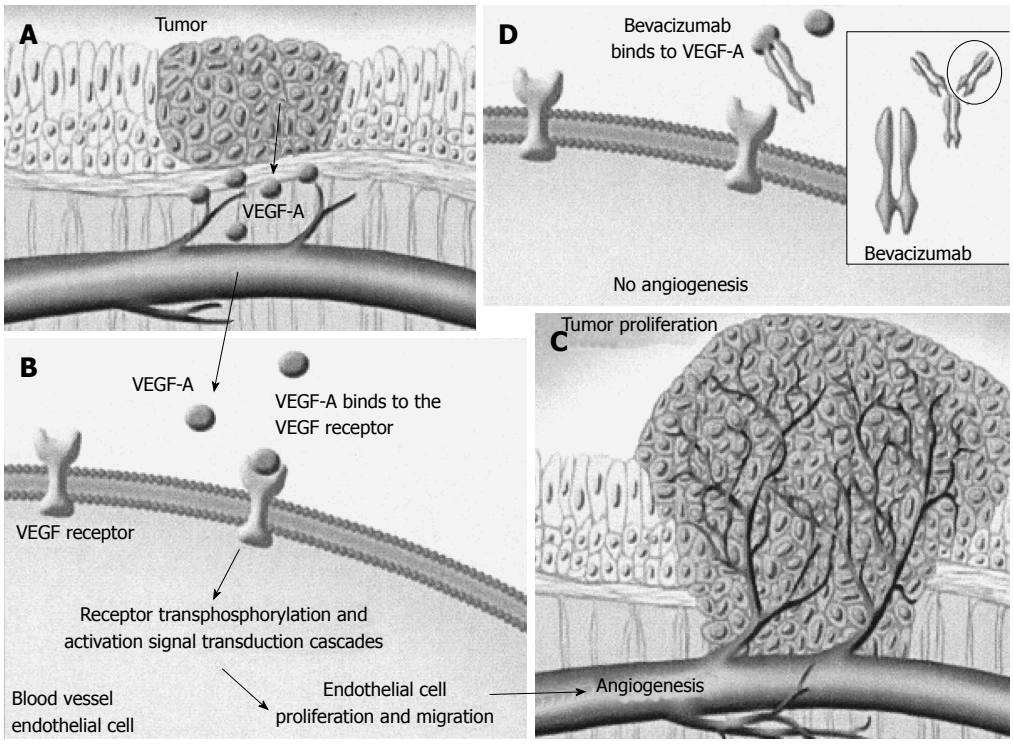

Figure 1 The process of angiogenesis and the mechanism of action of bevacizumab. A: The malignant cells secrete vascular endothelial growth factor (VEGF)-A; B: It is incorporated with its tyrosine kinase receptors (VEGFRs), promoting endothelial cell proliferation and migration; C: It leads to increased angiogenesis inducing tumor growth; D: Bevacizumab is combined with VEGF-A forming a new large molecule that lacks the ability to bind with its receptors; thus avoiding its incorporation and action, it then inhibits angiogenesis. Taken from Shord *et al*[15].

secrete VEGF-A, a growth factor responsible for neoangiogenesis. This action is accomplished by incorporation of its tyrosine kinase receptors, VEGFrs, which are located on the surface of epithelial cells. An increase in angiogenesis facilitates blood flow to malignant cells permitting their growth and spread by ensuring a supply of oxygen and nutrients. Bevacizumab, a recombinant humanized monoclonal antibody, combines with VEGF-A forming a new molecule that lacks the ability to bind with its receptors, VEGFrs, thus avoiding its incorporation and action. This restriction of VEGF-A receptors activity induces a reduction in small vessel growth, inhibits new vessel formation and restores normal tumor blood supply[15].

Bevacizumab is an IgG1 that inhibits the activity of VEGF and its isomers. This monoclonal antibody has been derived from murine antihuman VEGF and is 93% human and 7% murine[16]. The absence of VEGF influences epithelial cells resulting in destruction of neoplastic capillaries. Although it has been reported that malignant cells continue to grow despite the absence of VEGF, they exhibit reduced invasion ability resulting in reduced metastatic activity. Furthermore, their reduced intracellular pressure makes them more vulnerable to chemotherapy and radiotherapy.

The half-life time of bevacizumab ranges from 11 to 50 d (mean half-life time 20 d). As a result, even small doses of the drug (0.3 mg/kg *bw*) may be bound with VEGF preventing incorporation with its receptors, and thus inactivating VEGF efficiency. Bearing in mind that the acceptable dose is 5 mg/kg *bw* every 2 wk, it has been suggested that active levels of the drug may be detected for 12 wk[8] (Figure 1).

CLINICAL APPLICATION

Bevacizumab in colorectal cancer

The current data on the management of colorectal cancer indicate that angiogenesis and its inhibition are key factors. Bevacizumab remains the most important and well-studied drug among the known anti-angiogenic agents. The use of bevacizumab (Avastin, Roche Pharma AG) has been widely accepted as first-line therapy in the management of advanced colorectal cancer in combination with other classic chemotherapy agents such as 5-fluorouracil (5-FU) or novel agents[17-22]. This combination improves the response rates to treatment, progression-free survival and overall survival, in patients with advanced disease, as opposed to chemotherapy alone[23-25]. Its licence was granted in 2004 in the United States and in 2005 in Europe[26]. Currently, the combination of the novel targeted therapy agents irinotecan, capecitabine and bevacizumab is the most widely used in metastatic colorectal cancer resulting in increased response rates[23,24,27,28].

Bevacizumab is the first agent to affect survival in patients with metastatic colorectal cancer, improving survival by 30%[16]. Furthermore, it has been established as the first- and second-line therapy for this cancer, due to its advantages compared with routine chemotherapy,

which include less resistance and toxicity[23]. Its beneficial effect has been proved in phases Ⅱ and Ⅲ clinical trials[25].

Conclusions have been drawn from a variety of trials investigating its safety and efficacy. It has been suggested that surgery should be performed at least 6-8 wk after drug cessation to minimize complications; post-operatively, re-initiation should be after 28 d and/or complete wound healing[29].

The usual dose of bevacizumab is 5 mg/kg *bw* every two weeks in combination with other chemotherapeutic agents such as irinotecan and 5-fluorouracil/leucovorin (LV). It is administered by intravenous (IV) injection which must last 90 min initially and is gradually reduced to 60 min and 30 min; IV bolus injection is contraindicated[16].

Bevacizumab has been used postoperatively 6 wk after colorectal cancer resection for the management of synchronous liver metastasis at a dose of 5 mg/kg *bw* every 2 wk or 7.5 mg/kg *bw* every 3 wk[30].

The usual dose of bevacizumab is 5 mg/kg *bw* every 2 wk for 5 cycles and even the uncommon dose of 10 mg/kg *bw* has been combined with 5-FU/LV or capecitabine in advanced colorectal cancer[31-34].

Recent trials have confirmed the effectiveness of bevacizumab in combination with other chemotherapeutic agents in metastatic colorectal cancer showing its increasing application in clinical practice. A large randomized multi-center controlled trial showed that the addition of bevacizumab to capecitabine plus or minus mitomycin significantly improved progression-free survival (PFS) without inducing further major toxicity; only expected modest adverse events including proteinuria, hypertension, arterial thromboembolism and hemolytic uremic syndrome were observed. However, it did not improve response rate or overall survival (OS), and overall quality of life was similar. Furthermore, there were 11 treatment-related deaths: one in the capecitabine group (sepsis); seven in the capecitabine-bevacizumab group (hemorrhage, myocarditis, bowel perforation, sepsis); and three in the capecitabine-bevacizumab-mitomycin group (hemorrhage, pulmonary embolism, neutropenic colitis)[35]. A meta-analysis of 5 randomized controlled trials showed that the addition of bevacizumab to first-line chemotherapy significantly increased both the PFS and OS. Females and patients with primary rectal tumors seemed to benefit most[36].

Based on a pivotal study, the United States Food and Drug Administration (FDA) in February 2004 approved bevacizumab for the first-line treatment of patients with metastatic carcinoma of the colon and rectum. In this study, 833 patients were randomly allocated to irinotecan, 5-FU, and LV either alone (the IFL regimen) or with bevacizumab (5 mg/kg every 2 wk). In the group treated with bevacizumab, OS was significantly longer (median, 20.3 mo *vs* 15.6 mo) as were PFS and response rate[24]. Subsequently on June 20, 2006, the FDA approved bevacizumab administered in combination with 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) as a second-line treatment for metastatic carcinoma of the colon or rectum. This was based on the Eastern Cooperative Oncology Group open-label, multicenter, randomized, three-arm, active-controlled trial. In this study, 829 patients with recurrence following prior chemotherapy were randomly allocated to bevacizumab (10 mg/kg, as a 90-min *iv* infusion on day 1, every 2 wk) with FOLFOX4, or FOLFOX4 alone. In the group treated with bevacizumab, there was a statistically significant and clinically meaningful improvement in OS (13.0 mo *vs* 10.8 mo) in patients whose disease had progressed after adjuvant chemotherapy with 5-FU and irinotecan and in patients with advanced or metastatic disease who had received prior 5-FU and irinotecan. The administration of bevacizumab was beneficial in these sub groups, well tolerated and with no impact on quality of life[37].

In a recent phase Ⅱ study, bevacizumab was added to capecitabine plus irinotecan (XELIRI) as first-line treatment for metastatic colorectal cancer and acceptable tolerability and improved outcome were observed[38].

An updated meta-analysis and systematic review of 10 randomized controlled trials including 1366 patients with metastatic colorectal cancer identified the additional benefits of bevacizumab to cytotoxic chemotherapy regarding OS and PFS[39].

However, there was controversy regarding the aforementioned findings in a large phase Ⅲ trial of 2672 patients with stage Ⅱ to Ⅲ colon cancer. The addition of bevacizumab to modified FOLFOX6 (mFOLFOX6; *i.e.*, infusional/bolus fluorouracil, leucovorin, and oxaliplatin) as adjuvant treatment for 1 year, did not significantly prolong disease free survival[40].

The development of bevacizumab-induced hypertension as a biomarker did not predict radiological response or survival in patients with poor-risk colorectal liver-only metastases unsuitable for upfront resection[41].

Overall survival, disease-free survival, and local control showed favourable trends in patients with stage Ⅱ/Ⅲ rectal cancer treated with neo-adjuvant bevacizumab with chemoradiotherapy followed by surgery[42]. Another study of neo-adjuvant oxaliplatin, bevacizumab, continuous infusion 5-FU, and radiation in rectal cancer was terminated early because of significant gastrointestinal toxicity[43].

Bevacizumab has been used as first-line treatment early in advanced cancer and in patients with stage Ⅲ unresectable or stage Ⅳ adenocarcinoma of the colon or rectum[44,45].

A retrospective analysis of a large United States managed database estimated that the cost of treatment containing bevacizumab was lower than that containing cetuximab[46].

BEVACIZUMAB IN OTHER MALIGNANCIES

Several clinical trials have confirmed the effectiveness

of bevacizumab in other malignancies, *i.e.*, breast cancer, pancreatic cancer, prostate cancer, non-small cell lung cancer, metastatic renal carcinoma, and ovarian tumors.

Recently, targeted therapy with various anti-angiogenic agents including sunitinib, sorafenib, temsirolimus, everolimus and bevacizumab has been used as first-line systemic therapy with impressive success in patients with metastatic renal cell carcinoma, which otherwise has a poor prognosis[47].

The combination with another anti-angiogenic agent enhances activity and decreases toxicity[48].

Bevacizumab has been accepted in combination with taxanes for the treatment of metastatic breast cancer in unselected patients[49]. Its combination with paclitaxel showed a statistically significant difference in outcome compared to treatment with paclitaxel alone.

Results of trial E2100 led to the initial approval of bevacizumab as first-line therapy for patients with metastatic breast cancer in the United States in February 2008. However, based on results from subsequent trials, the United States FDA Oncologic Drugs Advisory Committee revoked its approval in July 2010[50-52]. The drug costs about $90000 (£58000; €68000). Bevacizumab has not been shown to be safe and effective in metastatic breast cancer, as several studies showed no influence on overall survival or benefits in overcoming the drug's serious and potentially life-threatening side effects.

Despite the FDA decision, it was not withdrawn in Europe by the European Medicines Agency, however, the prescribing practice has been reduced[50]. A recent survey highlighted the discord between the opinion of oncologists and the FDA's recent decision[53]; similarly there is controversy over the FDA decision[54].

Bevacizumab has also been used in primary and metastatic brain tumors, mainly in glioblastomas[55]. It has been extensively studied in patients with primary malignant gliomas and has been approved as second-line chemotherapy alone or in combination with irinotecan following first or second recurrence after radiotherapy and temozolomide[56-58]. Furthermore, the efficacy and safety of combining bevacizumab with standard-of-care therapy in patients with newly diagnosed glioblastoma multiforme is currently being studied by the AVAGLIO phase Ⅲ randomized trial[59].

Bevacizumab has also been proved to be effective as mono-therapy in recurrent ovarian stromal tumors[60].

Chemotherapy plus targeted therapy with bevacizumab had better efficacy than chemotherapy alone in patients with non-small cell lung cancer, which otherwise has a poor prognosis[61]. The combination of paclitaxel/carboplatin with bevacizumab showed increased efficacy (27% *vs* 10% with chemotherapy alone) and raised overall survival to 12.5 mo *vs* 10.2 mo, respectively.

Bevacizumab is currently being used more frequently in the management of breast, ovarian and cervical cancer[62-64]. It has also been used in advanced pancreatic cancer in phase Ⅱ clinical trials alone or combined with other therapeutic agents, but without improved outcome[21,65,66].

BEVACIZUMAB SIDE EFFECTS AND REPORTED COMPLICATIONS

Despite the documented benefits of bevacizumab use in the treatment of colorectal cancer, there have been reports of rare side effects, *i.e.*, thrombosis, arterial hypertension, proteinuria, perforation of the gastrointestinal tract or nasal septum, wound healing abnormalities, irreversible leuco-encephalopathy syndrome, allergic skin rash and hypersensitivity reactions[15,67,68]. Wound healing abnormalities include wound dehiscence, ecchymosis, bleeding and wound infection. Hypersensitivity reactions include flashing, pruritus, arterial hypertension, rigors, broncho-constriction, chest pain, and sweats. The risk of postoperative bleeding is statistically significant[25,29] as well as the risk of thromboembolic events, *i.e.*, deep vein thrombosis, pulmonary embolism, transient ischemic attack, and acute mesenteric ischemia[69-71]. Due to the aforementioned side effects, continuous monitoring of patients receiving bevacizumab treatment is mandatory to achieve the best outcome[72].

The contraindications of bevacizumab use include hypersensitivity to its active components or to recombinant monoclonal antibodies, pregnancy, lactation, brain metastasis without treatment due to bleeding risk, gastrointestinal tract perforation, wound healing complications, persistent arterial hypertension, proteinuria, arterial thromboembolic episodes, hemorrhage and congestive heart failure or cardiomyopathy[16].

The reported wound healing complications include bowel perforation, external abdominal fistula, anastomotic dehiscence, intraperitoneal bleeding, gastrointestinal hemorrhage and cellulitis. In oncoplastic surgery for advanced breast cancer, failure of free flaps due to increased thrombotic risk as well as bleeding episodes increase the morbidity and mortality rate[67].

The risks of GI-tract perforation including free perforation, fistula formation and intra-abdominal abscess are rare, but these are serious complications, which may be fatal[73,74]. These risks depend on the drug dose and increases in cancer patients. The use of non-steroidal or other anti-inflammatory drugs, peptic ulcer and colon diverticular disease are also risk factors. It should be stressed that there have been isolated reports of spontaneous delayed (several months or even one year after operation) leakage from previous colon or rectal anastomosis after treatment with bevacizumab[75-78].

An interesting case reported skin flap necrosis in a female undergoing preoperative bevacizumab and paclitaxel plus 5-FU, epirubicin, and cyclophosphamide treatment for locally advanced breast cancer[78]; we should also mention the case of Fournier's gangrene in a male during bevacizumab treatment 4 mo after chemotherapy with 5-FU/LV/oxaliplatin for advanced colorectal cancer[79].

However, in a recent study of 57 cancer patients who received bevacizumab and underwent immediate insertion of a central venous access port, there were no side-effects such as delayed wound healing, bleeding, infection

or ulceration[80].

The reported long-term anastomotic complications attributed to the use of the anti-angiogenic agent refer to 18 cases[81]. They occurred more than a year or even 78 mo following bevacizumab treatment. The risk factors included low anterior recto-sigmoid resection for rectal cancer, perioperative radiotherapy and healed early anastomotic leakage.

For the aforementioned reasons, it has been recommended that a period of 6 wk should elapse following drug cessation before hepatectomy; post-operatively, a 4-wk period is required before therapy is re-initiated[82]. However, there has recently been a debate based on experimental findings[83,84] and clinical data. The safety and effectiveness of bevacizumab were proved in a large meta-analysis of randomized controlled trials, which found no statistically significant difference in wound healing[85].

BEVACIZUMAB EXPERIMENTAL USE-PERSPECTIVES

Bevacizumab at an IV dose of 5 mg/kg *bw* has been used in combination with irinotecan in an experimental model of implanted colon cancer cells in rats[86].

Intraperitoneal administration of bevacizumab in combination with other novel targeted agents has been proven to be effective in reducing tumor size in an experimental cancer model (colon, renal) in mice[87].

Bevacizumab at an IV dose of 10 mg/kg *bw* per week was effective in reducing tumor size and vasculature in an experimental model of breast cancer with bone metastasis in rats using volumetric computed tomography and magnetic resonance imaging (MRI)[88]. Also, the effectiveness of intraperitoneal administration of bevacizumab at different doses has been documented in an experimental model of implanted breast cancer cells in rats using MRI[89,90].

Intraperitoneal administration of bevacizumab has been used with encouraging results in several experimental tumor models in mice, *i.e.*, tuberous sclerosis[91], glioblastoma[92], medullary thyroid carcinoma[93], gastric cancer[94-96], malignant fibrous histiocytoma[97], ovarian cancer[98], and endometrial cancer[99].

Furthermore, it has been used in lung cancer xenografts[100], immune-mediated vascular remodeling[101], tumor angiogenesis assessment using positron emission tomography (PET) imaging in experimental models of colorectal and ovarian cancer in mice[102], an experimental model of schwannoma[103] and in a mouse model of hepatocellular carcinoma with promising results[104]. PET imaging and VEGF bio-distribution with radio-labeled bevacizumab in colorectal cancer xenografts has been performed[105].

These experimental data on the use of bevacizumab or other novel anti-angiogenic agents in cancer models using rats or mice open new horizons broadening its targeted therapeutic application with promising results.

CONCLUSION

The promotion of angiogenesis by VEGF favors tumor growth. Bevacizumab, which is a recombinant humanized monoclonal antibody against VEGF activity, inhibits angiogenesis restricting the growth of malignant cells and thus prevents tumor spread. It has recently been used as targeted therapy in combination with chemotherapy, mainly in advanced colorectal cancer with hepatic or other metastasis, and in breast cancer despite the debate surroundings its use for this disease, and occasionally in pancreatic cancer (but without proven efficiency), ovarian tumors, small-cell lung cancer, renal cancer and prostate cancer. A number of experimental studies have also attracted great interest on its use in other advanced malignancies. This novel biological agent is generally safe and well-tolerated. However, there are rare, although serious side effects and complications that should be considered.

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MINIREVIEWS

Eosinophilic gastroenteritis: An unusual type of gastroenteritis

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Abstract

Eosinophilic gastroenteritis (EGE) is a rare disorder characterized by eosinophilic infiltration of the bowel wall with various gastrointestinal manifestations. Till date only 280 cases have been described in the literature. A high index of suspicion, by excluding other causes of peripheral eosinophilia, is a pre requisite for accurate diagnosis. EGE is an uncommon gastrointestinal disease affecting both children and adults. It was first described by Kaijser in 1937. Presentation may vary depending on location as well as depth and extent of bowel wall involvement and usually runs a chronic relapsing course. This condition can respond to low dose steroid therapy, thereby preventing grave complications like ascites and intestinal obstruction that might need surgical intervention. The natural history of EGE has not been well documented. Eosinophilic gastroenteritis is a chronic, waxing and waning condition. Mild and sporadic symptoms can be managed with reassurance and observation, whereas disabling gastrointestinal (GI) symptom flare-ups can often be controlled with oral corticosteroids. When the disease manifests in infancy and specific food sensitization can be identified, the likelihood of disease remission by late childhood is high. GI obstruction is the most common complication. Fatal outcomes are rare.

Key words: Eosinophilic gastroenteritis; Unusual type; Review of literature

Core tip: Eosinophilic gastroenteritis is a rare disorder characterised by eosinophilic infiltration of the bowel wall and various gastrointestinal manifestations. Diagnosis requires a high index of suspicion and exclusion of various disorders that are associated with peripheral eosinophilia. Corticosteroids are the mainstay of therapy with a 90% response rate.

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INTRODUCTION

Eosinophilic gastroenteritis is a rare disorder that can present with various gastrointestinal manifestations depending on the specific site and specific layer of the gastrointestinal tract involved. Majority of the cases involve stomach and proximal small bowel. The diagnostic criteria include demonstration of eosinophilic infiltration of bowel wall, lack of evidence of extra intestinal disease and exclusion of other causes of peripheral eosinophilia[1-4].

Eosinophilic gastroenteritis is characterized by the presence of abnormal gastrointestinal (GI) symptoms, most often abdominal pain, eosinophilic infiltration in one or more areas of the GI tract, defined as 50 or more eosinophils per high-power field, the absence of an identified cause of eosinophilia and the exclusion of eosinophilic involvement in organs other than the GI tract.

It can be classified into mucosal, muscular and serosal types based on the depth of involvement[5,6]. The

stomach is the organ most commonly affected, followed by small intestine and colon[7,8]. The anatomical locations of eosinophilic infiltrates and the depth of GI involvement determine clinical symptoms. The therapeutic role of steroids and antihelminthic drugs in the treatment of eosinophilic gastroenteritis is not established. In a few cases, steroids have produced symptomatic improvement in controlling malabsorption syndrome[1,9].

EPIDEMIOLOGY

Eosinophilic gastroenteritis occurs over a wide age range from infancy through the seventh decade, but most commonly between third to fifth decades of life[10,11]. A slight male preponderance has been reported[12].

Although cases have been reported worldwide, the exact incidence of eosinophilic gastroenteritis is unclear. After first described by Kaijser[10], a little less than 300 cases have been reported in the literature. Kim *et al*[2] reported 31 new cases of eosinophilic gastroenteritis in Seoul, Korea, between January 1970 and July 2003.

Venkataraman *et al*[5] reported 7 cases of eosinophilic gastroenteritis over a 10-year period in India[5]. Chen *et al*[3] reported 15 patients including 2 children, with eosinophilic gastroenteritis in 2003. In eosinophilic enteritis the morbidity is mainly due to combination of chronic nonspecific GI symptoms which include abdominal pain, nausea, vomiting, diarrhea, weight loss, and abdominal distension and more serious complications like intestinal obstruction and perforation[13,14].

PATHOPHYSIOLOGY

Eosinophilic gastroenteritis can involve any part of gastrointestinal tract from esophagus down to the rectum. The stomach and duodenum are the most common sites of involvement[1,13-17]. The etiology and pathogenesis is not well understood. There is evidence to suggest that a hypersensitivity reaction may play a role. The clinical presentations of eosinophilic gastroenteritis vary according to the site and depth of eosinophilic intestinal infiltration. The presence of peripheral eosinophilia, abundant eosinophils in the gastrointestinal tract and dramatic response to steroids provide some support that the disease is mediated by a hypersensitivity reaction[1,18]. Moreover, a study at Mayo clinic showed that 50% of patients with eosinophilic gastroenteritis give history of allergy such as asthma, rhinitis, drug allergy and eczema[1]. Peripheral blood eosinophilia and elevated serum immunoglobulin E (IgE) are usual but not universal. The damage to the gastrointestinal tract wall is caused by eosinophilic infiltration and degranulation[19]. Eosinophils are normally present in gastrointestinal mucosa as a part of host defense mechanism, though the finding in deeper tissue is almost always pathologic[20]. In eosinophilic gastroenteritis (EGE) cytokines interleukin (IL)-3, IL-5 and granulocyte macrophage colony stimulating factor may be responsible for the recruitment and activation of eosinophils and hence the pathogenesis. They have been observed immunohistochemically in diseased intestinal wall[21]. In addition eotaxin has been shown to have an integral role in regulating the homing of eosinophils into the lamina propria of stomach and small intestine[22]. Indeed, many patients have history of food allergy and other atopic conditions like eczema, asthma etc. In this allergic subtype of disease, it is thought that food allergens cross the intestinal mucosa and trigger an inflammatory response that includes mast cell degranulation and recruitment of eosinophils[23,24].

CLINICAL PRESENTATIONS

The clinical presentations of eosinophilic gastroenteritis vary according to the site and depth of inflammatory involvement of different layers of the intestinal wall. Approximately 80% have symptoms for several years[25]. Occasionally, the disease may manifest itself as an acute abdomen or bowel obstruction[13,14]. Children and adolescents can present with growth retardation, failure to thrive, delayed puberty or amenorrhea. Adults have abdominal pain, diarrhea or dysphagia. Mucosal disease is the commonest variety that presents with features of protein losing enteropathy, bleeding or malabsorption. Failure to thrive and anaemia may also be present. Lower gastrointestinal bleeding may imply colonic involvement[1,26,27]. Involvement of muscle layer may cause bowel wall thickening and intestinal obstruction. Cramping and abdominal pain associated with nausea and vomiting occurs frequently. It can also present as an obstructing caecal mass or intussusception. The subserosal form, which is least common but can cause more morbidity, usually presents as eosinophilic ascites, which is usually an exudate, with abundant peripheral eosinophilia. Serosal and visceral peritoneal inflammation leads to leakage of fluids but has a more favourable response to corticosteroids. In literature features like cholangitis, pancreatitis[28], eosinophilic splenitis, acute appendicitis and giant refractory duodenal ulcer are also mentioned.

DIAGNOSTIC EVALUATION

Four criteria are required for the diagnosis of eosinophilic gastroenteritis namely-presence of gastrointestinal symptoms, eosinophilic infiltration of gastrointestinal tract, exclusion of parasitic disease and absence of other systemic involvement. The presence of peripheral eosinophilia is not a universal phenomenon[1,29].

A thorough evaluation of the patient is necessary, starting with laboratory evaluation.

After a detailed history and physical examination, a complete blood count plays an important role. Peripheral blood eosinophilia is found in 20%-80% of cases. Average count is 2000 eosinophils (eos)/μL in patients with mucosal layer involvement, 1000 eos/μL in patients with muscle layer involvement, and 8000 eos/μL in patients with serosal involvement. Iron-deficiency anemia may be evident on mean corpuscular volume. Serum albumin may

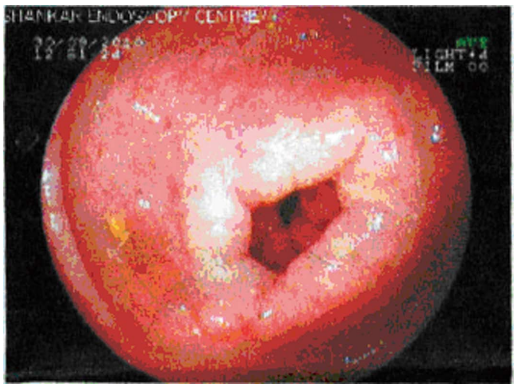

Figure 1 Endoscopy showing small superficial ulcers in stomach.

be low, especially in patients with mucosal involvement.

Fecal protein loss can be assessed by measuring alpha1-antitrypsin in a 24-h feces collection. It is used to identify the inability to digest and absorb proteins in the GI tract. The normal value is 0-54 mg/dL. Patients with eosinophilic gastroenteritis have elevated alpha1-antitrypsin in their feces. Protein loss can also result in low levels of total immunoglobulins, but serum IgE could be elevated, which then strongly supports the diagnosis of eosinophilic gastroenteritis in conjunction with other findings. The erythrocyte sedimentation rate can be elevated in few cases.

Stool examination should be performed to rule out parasitic infestation. Mild-to-moderate steatorrhea is present in approximately 30% of patients. This can be measured by qualitative and quantitative stool tests. Skin prick tests help to identify sensitization to specific ingestant and/or inhalant allergens.

Computed tomography (CT) scan may show nodular and irregular thickening of the folds in the distal stomach and proximal small bowel, but these findings can also be present in other conditions like Crohn's disease and lymphoma. On ultrasonography ascitic fluid is usually detected in patients with serosal involvement.

Radiographic changes are variable, nonspecific, and/or absent in at least 40% of patients. Gastric folds can be enlarged, with or without nodular filling defects. In extensive disease strictures, ulceration or polypoid lesions may occur and valvulae conniventes may be thickened and flattened. In eosinophilic gastroenteritis involving the muscle layer, localized involvement of the antrum and pylorus may occur, causing narrowing of the distal antrum and gastric retention. The small intestine may be dilated, with an increase in the thickness of the mucosal folds. Prominent mucosal folds may be observed in the colon. Other tests like exploratory laparotomy may be indicated in patients with serosal eosinophilic gastroenteritis.

The endoscopic appearance is nonspecific. It includes erythematous, friable, nodular, and occasional ulcerative changes[3] (Figure 1). Sometimes diffuse inflammation results in complete loss of villi, involvement of multiple layers, submucosal oedema and fibrosis[30,31]. When performing endoscopy, it is necessary to obtain at least 6

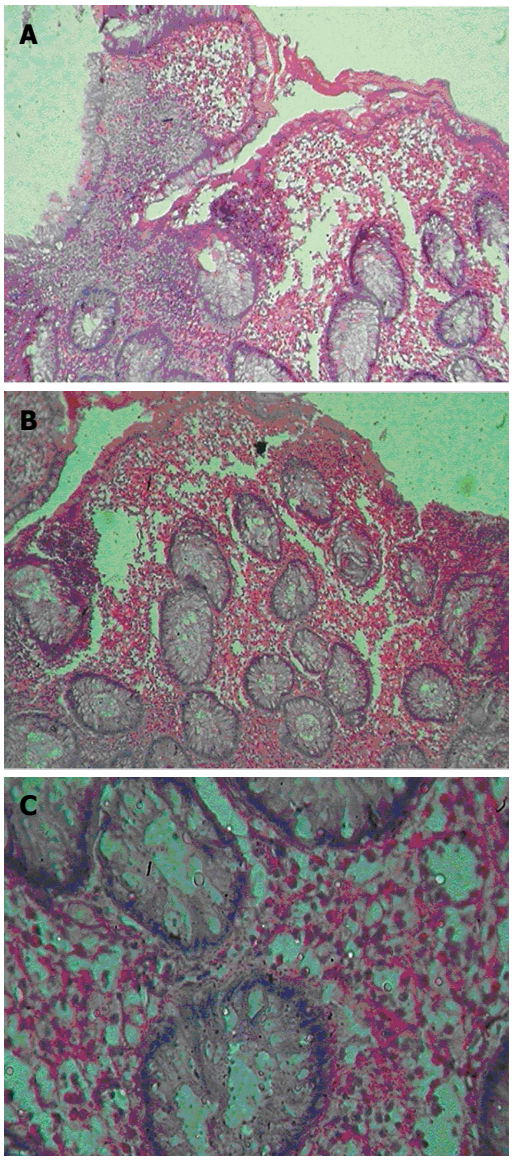

Figure 2 Large numbers of eosinophils are often present in the muscularis and serosa. A, B: Showing dense eosinophilic infiltrates in the lamina propria and mucosa (× 10); C: Showing dense eosinophilic infiltrates in the lamina propria and mucosa (× 40).

biopsy specimens from normal and abnormal areas of the bowel to exclude the possibility of sampling error. In patients with esophageal or colonic symptoms, additional biopsy specimens may be obtained from the relevant sites to aid the diagnosis.

Patients with serosal disease present with ascites. Abdominal paracentesis demonstrates a sterile fluid with a high eosinophil count. Pleural effusion also may be present.

The diagnosis can be confirmed on histopathological examination of gastric and duodenal biopsies. The gross appearance of eosinophilic gastroenteritis upon endoscopy shows erythematous, friable, nodular, and often ulcerated mucosa. Microscopy demonstrates increased numbers of eosinophils (often > 50 eos per high-power field) in the lamina propria. Large numbers of eosinophils are often present in the muscularis and serosa (Figure 2). Localized eosinophilic infiltrates may cause crypt

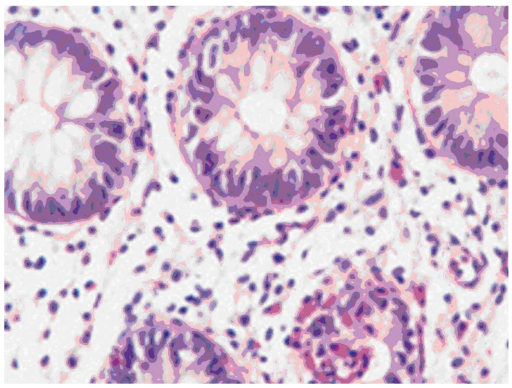

Figure 3 Post treatment (low dose steroid) biopsy showing resolution of disease.

hyperplasia, epithelial cell necrosis, and villous atrophy. Diffuse enteritis with complete loss of villi, submucosal edema, infiltration of the GI wall, and fibrosis may be apparent. Mast cell infiltrates and hyperplastic mesenteric lymph nodes infiltrated with eosinophils may be present[1,27,31,32]. Infiltration is often patchy, can be missed and laparoscopic full thickness biopsy may be required.

Histologic analysis of the small intestine reveals increased deposition of extracellular major basic proteins and eosinophilic cationic proteins.

Radio isotope scan using technetium (^{99m}Tc) exametazime-labeled leukocyte single-photon emission CT may be useful in assessing the extent of disease and response to treatment but has little value in diagnosis, as the scan does not help differentiating EGE from other causes of inflammation[33,34].

When eosinophilic gastroenteritis is observed in association with eosinophilic infiltration of other organ systems, the diagnosis of idiopathic hypereosinophilic syndrome should be considered[35].

Differential diagnosis

The main differential diagnoses are: (1) eosinophilic esophagitis; (2) eosinophilic ascites; (3) coeliac disease; (4) protein losing enteropathy from intolerance to cow milk protein; (5) infantile formula protein intolerance; and (6) idiopathic hypereosinophilic syndrome.

A diagnosis of idiopathic hypereosinophilic syndrome can be ruled out when there is absence of eosinophilic infiltration in all other organs except the bowel[35].

In celiac disease, biopsy of small bowel shows blunting of villi, crypt hyperplasia, and predominantly lymphocyte infiltration of crypts. Coeliac disease is caused by a reaction to gliadin, a prolamin (glutenprotein) found in wheat, and similar proteins found in other grains[36].

In eosinophilic esophagitis only the eosophagus is involved and not the whole bowel. A minimum of 15 eosinophils per high power field is required to make the diagnosis. Typically, eosinophils can be found in superficial clusters near the surface of the epithelium. An expansion of the basal layer is also seen in response to the inflammatory damage to the epithelium. At the time of endoscopy, ridges or furrows may be seen in the esophageal mucosa. Presence of white exudates in esophagus is also suggestive of the diagnosis[37,38].

Treatment

The role of steroids and antihelminthic drugs is not well established. However, in a few cases, steroids have been reported to produce symptomatic improvement in controlling diarrhea and protein losing enteropathy[9].

Corticosteroids are the mainstay of therapy with a 90% response rate in some studies (Figure 3). Appropriate duration of steroid treatment is unknown and relapse often necessitates long term treatment. Various steroid sparing agents, *e.g.*, sodium cromoglycate (a stabilizer of mast cell membranes), ketotifen (an antihistamine), and montelukast (a selective, competitive leukotriene receptor antagonist) have been proposed, centering around an allergic hypothesis, with mixed results[24,39,40].

Corticosteroids

Fluticasone inhaled (Flovent): Decreases recruitment of inflammatory cells including eosinophils and decreases the release of eotaxins and other inflammatory mediators. Dosage required is higher than that used in asthma.

Prednisolone (AK-Pred, Delta-Cortef): Decreases inflammation by suppressing migration of polymorphonuclear leukocytes and reducing capillary permeability. Equivalent dosages of prednisone or methylprednisolone may be used.

Budesonide (Pulmicort Respule) oral viscous suspension: Decreases inflammation, reduces capillary permeability[6].

MAST CELL STABILIZERS

Cromolyn (Intal, Gastrocrom): Inhibits release of histamine, leukotrienes, and other mediators from sensitized mast cells. It also inhibits the influx of neutrophils, as well as the formation of the active form of NADPH oxidase, which in turn prevents tissue damage caused by oxygen radicals.

Leukotriene receptor antagonists

Prevent or reverse some of the pathologic features associated with the inflammatory process mediated by leukotrienes C4, D4 and E4. Successful treatment of eosinophilic gastroenteritis has been reported in few cases, mainly with Montelukast (Singulair) which is a potent and selective antagonist of leukotriene D4 at the cysteinyl leukotriene receptor, CysLT1[41].

Role of surgical care

Surgery is avoided, except when it is necessary to relieve persistent pyloric or small bowel obstruction. Most patients respond to conservative measures and oral glucocorticosteroids. Recurrence is possible, even after surgical excision.

Prognosis

The natural history of EGE has not been well documented. Eosinophilic gastroenteritis is a chronic, waxing and waning condition. Mild and sporadic symptoms can be managed with reassurance and observation, whereas disabling GI symptom flare-ups can often be controlled with oral corticosteroids. When the disease manifests in infancy and specific food sensitization can be identified, the likelihood of disease remission by late childhood is high. GI obstruction is the most common complication. Fatal outcomes are rare.

Preventive and diet therapy

The strong association of eosinophilic gastroenteritis with food allergies has prompted the use of restrictive or elemental diets. Initially, a trial elimination diet that excludes milk, eggs, wheat and/or gluten, soy, and beef may be helpful. Skin testing can identify food hypersensitivity. If a prohibitive number of food reactions are found, an amino-acid-based diet or elemental diet may be considered. Educate patients to avoid foods that they cannot tolerate and to seek medical care when needed.

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ORIGINAL ARTICLE

Serum proteins in chronic hepatitis B patients treated with peginterferon alfa-2b

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Abstract

AIM: To study the differential protein profile in serum of hepatitis B patients.

METHODS: Serum samples were obtained from patients with chronic hepatitis B who were receiving peginterferon alfa-2b. The serum samples were subjected to albumin depletion and analyzed by two-dimensional gel electrophoresis (2-DE). Differentially expressed protein spots were identified by electrospray ionization-quadrupole time-of-flight mass spectrometry. Alpha-2-HS-glycoprotein, complement component C3c and CD5 antigen were further analyzed by an enzyme-linked immunosorbent assay and immunonephelometry.

RESULTS: Nineteen patients with HBeAg-positive chronic hepatitis B (CHB) were studied. These patients were followed for at least 1 year after treatment and were classified according to their treatment response: responders (n = 9) and non-responders (n = 10). 2-DE and MS/MS analysis were performed to compare the serum proteins before initiating peginterferon alfa-2b. From the quantitative analysis of the 2-D gel, 7 proteins were detected between the two groups at different levels before treatment. Among these potential candidates, serum levels of alpha-2-HS-glycoprotein, complement component C3c and CD5 antigen-like precursor were further analyzed. In the validation phase, 23 subjects, 9 sustained responders and 14 non-responders, were recruited. Interestingly, the levels of alpha-2-HS-glycoprotein and complement component C3c were elevated in the serum of the non-responders compared to the responders.

CONCLUSION: Serum alpha-2-HS-glycoprotein and

complement component C3c may be potential serum biomarkers in predicting the treatment response of peginterferon alfa-2b in patients with CHB prior to treatment.

Key words: Proteomics; Peginterferon alfa-2b; Chronic hepatitis B; Alpha-2-HS-glycoprotein; Serum

Core tip: Serum proteins serve as non-invasive biomarkers for several diseases. This is the first report on the potential use of common protein levels in the serum of chronic hepatitis B (CHB) patients to predict treatment responsiveness to peginterferon alfa-2b. We identified 2 potential serum biomarkers, alpha-2-HS-glycoprotein and complement component C3c, that can be used to predict treatment outcome in patients with CHB receiving peginterferon alfa-2b. The identification of these biomarkers prior to treatment is preferable in order to avoid systemic side effects due to interferon therapy.

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INTRODUCTION

One of the most common health care problems encountered worldwide is hepatitis B virus (HBV) infection which can progress to liver fibrosis, liver cirrhosis and liver cancer (also known as hepatocellular carcinoma). Treatment for chronic hepatitis B includes immunomodulatory agents and antiviral drugs such as nucleoside or nucleotide analogs (NAs). NAs inhibit the replication process of the virus by inhibiting its DNA polymerase[1], whereas immunomodulating therapy mainly includes treatment with interferon-α and pegylated interferon-α. The treatment duration, eradication and lack of drug resistance strains make type 1 interferons ideal for the treatment of chronic HBV[2]. Unfortunately, only 30% of patients will respond to treatment with interferon type 1[3]. The reason for this is because other factors, including the virus and the host, can significantly influence the treatment outcome. Viral factors such as the level of HBV DNA, HBV genotype, levels of hepatitis B surface antigen and hepatitis B core antigen (HBeAg), and HBV viral mutants can affect the outcome of therapy[4,5]. Other factors such as low levels of viral HBV DNA, higher levels of alanine aminotransferase (ALT), older age, being female, and naive to interferon therapy have been shown to be significantly associated with sustainable virological response among HBeAg-positive chronic hepatitis B (CHB) patients[6]. In HBeAg-negative patients, younger age, being female, having high levels of ALT and low levels of viral HBV DNA have been associated with sustainable virological response[7]. In addition, genetic host factors such as human leukocyte antigen (HLA) class Ⅱ (HLA-DRB1*14 allele), presence of polymorphism A (MxA)-88, levels of interleukin-10 and interleukin-12 have been proposed to predict the patient's treatment response after therapy[8-10]. The use of biomarkers is invaluable in predicting treatment response as well as being cost-effective in managing patients with CHB.

Various biomarkers for hepatocellular carcinoma (HCC)[11-13], HBV inflammation, HBV liver cirrhosis[14,15], and hepatitis C virus treatment response[16] have been investigated using proteomics, however, there are no predictive data for the treatment of CHB. One report from MA Hui and colleagues identified a potential serum biomarker for detecting changes after treatment, but was not able to predict the treatment outcome prior to treatment[17]. In the present study, albumin and immunoglobulin G (IgG) depleted serum was subjected to 2-dimensional gel electrophoresis and mass spectrometry. We identified 2 potential serum biomarkers, alpha-2-HS-glycoprotein and complement component C3c, and found that they can be used to predict treatment outcome in patients with CHB receiving peginterferon alfa-2b.

MATERIALS AND METHODS

Serum samples

Serum samples were obtained from patients with CHB who were followed at the King Chulalongkorn Memorial Hospital. All patients received peginterferon alfa-2b (1.5 mg/kg per week) subcutaneously for 48 wk and their responses to this treatment were assessed. These patients were followed for at least 1 year after treatment and were classified as sustained responders or non-responders. Sustained virological response among HBeAg-positive patients was characterized by undetectable HBeAg, detectable anti-HBe (HBeAg seroconversion) and HBV viral load < 2000 IU/mL 48 wk after treatment[18]. Patients without sustained virological response were classified as non-responders. Serum samples were obtained before initiating peginterferon alfa-2b treatment and at 24 wk after treatment.

Study population used to screen for biomarkers

Nineteen patients with HBeAg-positive CHB (9 sustained responders and 10 non-responders) were included in the proteomic study before initiating treatment. After 24 wk of treatment, 6 patients (3 sustained responders and 3 non-responders) were included.

Study population used to validate the system

Another 23 subjects, 9 sustained responders and 14 non-responders, were enrolled in the validation phase of the proteomic study using an enzyme-linked immunosorbent assay (ELISA) and immunonephelometry.

All studies were approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University,

Bangkok, Thailand. Informed consent forms were collected from all patients from both phases of the study before any of the procedures were initiated.

Optimization of 2D-gel electrophoresis for pretreatment serum

Albumin and IgG were removed from the patient's serum using the ProteoPrep Blue Albumin Depletion Kit (Sigma: PROTBA) according to the company's protocol. Protein concentrations were measured by the Bio-Rad Bradford total protein assay kit (Biorad Laboratories, Inc., Redmond, WA, United States)[19] using bovine serum albumin (BSA) as the standard curve.

Two-dimensional gel electrophoresis and image analysis

The Immobiline Dry strip (pH 4-7, length 7 cm, Amersham Biosciences, Uppsala, Sweden) was rehydrated with 150 μg protein in 125 μL rehydration buffer containing 9 mol urea, 2% CHAPS, 0.002% w/v bromophenol blue, 0.8% (w/v) DTT, 1% IPG buffer for 14 h at room temperature. Iso-electric focusing (IEF) was performed by IPG ph or IEF apparatus (Amersham Biosciences, Uppsala, Sweden) with a total of 8000 Vhrs. The strip was then equilibrated in equilibration buffer containing 6 mol/L urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 50 mmol/L Tris-HCl (pH = 8.8) with 135 mmol/L DTT for 15 min followed by incubation, but replacing with 130 mmol/L iodoacetamide for 15 min. Next, the equilibrated strips were placed on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) composed of 12.5% acrylamide and sealed with 0.5% (w/v) agarose. The SDS-PAGE was run on constant electric field, 15 mA per gel, using the SE 260 Mini-Vertical Units (GE Healthcare, Uppsala, Sweden) until the bromophenol blue tracking dye reached the bottom of the gel. Protein spots were stained with Coomassie Brilliant Blue G-250 stain[20]. The stained gels were scanned with an ImageMaster scanner (GE Healthcare; Uppsala, Sweden). Intensity analysis was carried out using the software, Image Master 2D Platinum (GE Healthcare, Uppsala, Sweden).

Tryptic digestion of the gels

Differentially expressed protein spots were excised from the 2-DE gels and subjected to in-gel tryptic digestion according to the method modified from Katayama *et al*[21]. The gel pieces were destained with 50% methanol and 50 mmol ammonium bicarbonate, and dehydrated with 100% acetonitrile (ACN). The gel pieces were reduced and alkylated in 10 mmol/L of DTT and 100 mmol/L iodoacetamide at room temperature for 1 h. They were then dehydrated twice with 100% ACN for 5 min after alkylation. The gel pieces were subsequently digested in 10 μL trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI, United States) solution (20 ng in 10 mmol/L ammonium bicarbonate in 50% ACN) and incubated at room temperature overnight. The peptides were extracted twice by adding 30 μL of solution containing 50% ACN and 0.1% formic acid. The extracted solutions were dried in a heat box at 40 ℃ and kept at -80 ℃ for further analysis by mass spectrometry. Prior to mass spectrometry analysis, the peptide mixtures were reconstituted in 10 μL of 0.1 % formic acid.

Protein identification by LC/MS/MS analysis

Peptide mixtures were analyzed by ultra-performance liquid chromatography (UPLC) (Ultimate 3000, Dionex, united states) coupled to the micrOTOF-Q Ⅱ ™ ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an online nanoESI source. The peptide mixture was injected onto a μ-precolumn cartridge (C18 PepMap; 300 μmol/L × 5 mm; 5 μmol/L particle size) composed of peptides, concentrated and then directly separated using a PepMap100 C18 analytical column (5 μm particle size, with 100 Å pore size). The mobile phase was run for each sample using a linear gradient of 10%-55% of 80% ACN in high performance liquid chromatography (HPLC) water for 30 min, with a hold of 15 min at 90% of 80% ACN in HPLC water, followed by a step to 10% of 80% ACN in HPLC water, hold of 20 min. The Q-TOF instrument was operated in positive ionization mode to switch automatically between MS and MS/MS acquisition. The precursor ion (MS) and fragmentation ion (MS/MS) with a mass range were 400-1600 m/z and 50-3000 m/z, respectively. The source parameters were as follows: capillary 2.0 kV, dry gas 0.3 L/min and dry temperature at 150 ℃ . The MS and MS/MS spectrometry data were processed using data analysis software (Bruker Daltonics, Germany) and searched against the NCBInr database using the MASCOT search engine. The parameters were identified using the following set up: species-homo sapiens; enzyme-trypsin; allowed up to 1 missed cleavage; fixed modification-carbamidomethylation on cystenine; variable modification-oxidation on methionines. The peptide mass tolerance and fragment mass tolerance were set at 1.2 Da and 0.6 Da, respectively[15]. A probability-based Mowse score of more than 43 was considered significant ($P < 0.05$).

Validation of the proteomic data by ELISA and immunonephelometry

Validation of the proteomic study was performed in a different population ($n = 23$) composed of 9 sustained responders and 14 non-responders. ELISA was performed according to the company's protocol using the Alpha 2 HS Glycoprotein Human ELISA kit (Abcam, Cambridge, United Kingdom) and human CD5 antigen like (CD5L) ELISA kit (Cusabio Biotech., Ltd., China). Complement component C3c was further validated using immunonephelometry and the BN ProSpec system (Siemens Healthcare Diagnostics Products GmbH, Germany).

Statistical analysis

SPSS version 17.0 (SPSS Inc., Chicago, IL, United States) was used for all statistical analyses. The values of the intensities of the spots are shown as the mean ± SE. In-

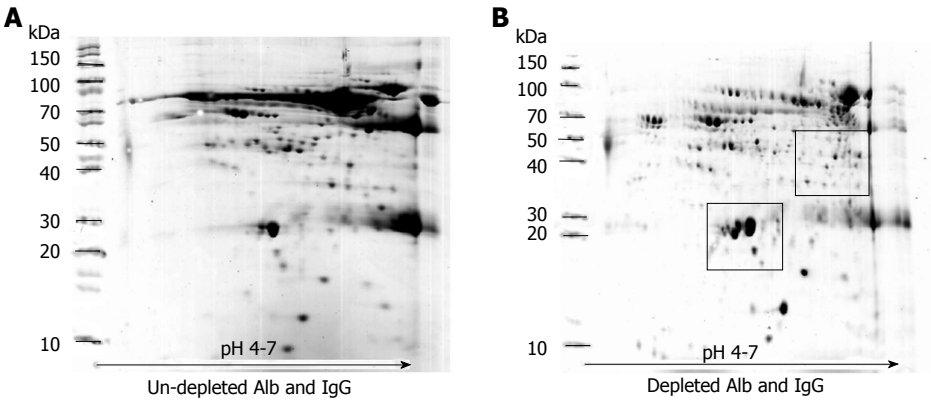

Figure 1 Serum samples from chronic hepatitis B virus-infected patients were run on two-dimensional gels (linear immobilized pH gradients; pH 4-7; 7 cm length). The pictures of the gels show the results before (A) and after (B) treatment using the ProteoPrep Blue Albumin Depletion kit. IgG: Immunoglobulin G; Alb: Albumin.

Table 1 Baseline characteristics of the patients used for screening the biomarkers before initiating chronic hepatitis B therapy (mean ± SE)

	Sustained virological responders (n = 9)	Non-responders (n = 10)	P value
Age (yr)	29.67 ± 8.29	36.90 ± 6.40	0.047
Sex (male:female)	7:2	8:2	0.912
ALT level (U/L)	103.88 ± 105.04	130.50 ± 75.77	0.609
HBV DNA (copies/mL)	(11.07 ± 8.52) × 10^6	(14.35 ± 8.44) × 10^6	0.511
HBeAg	Positive	Positive	NS

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

dependent sample t test was used to evaluate the baseline characteristics of the patients and compare the intensity data of each matched protein spot between the sustained responders and non-responders; the P value cut-off for the independent sample t test was 0.05. Mann-Whitney U test was used to evaluate the different protein expressions between the two groups 24 wk after treatment. For the Mann-Whitney U test, any proteins identified with $P <$ 0.05 were considered significant. The independent sample t test was performed during the validation phase to compare the different levels of alpha-2-HS-glycoprotein. The Mann-Whitney U test was also performed in the validation phase to compare the different levels of complement component C3c and CD5 antigen like proteins. The Pearson correlation was carried out on age and each protein expression value to determine if age had an influence on the expression of the proteins.

RESULTS

Characteristics of the study population in the screening phase

Basic characteristics of the patients are shown in Table 1. The number of men and women, levels of serum ALT and HBV DNA, and presence of HBeAg were comparable between the two groups. However, non-responders were older than the sustained responders.

Optimization of 2D-gel electrophoresis for pretreatment serum

Before performing electrophoresis on the collected serum, the efficiency of the ProteoPrep Blue Albumin Depletion Kit was determined. Figure 1 shows the two representative maps of the serum samples (chronic HBV infection) before and after treatment with the ProteoPrep Blue Albumin Depletion Kit. In the untreated sample, levels of albumin and IgG in serum were approximately 60%-70% and 10%-20%, respectively (Figure 1A). When an equal quantity of protein was pre-treated with ProteoPrep Blue Albumin Depletion Kit, the resolution of the 2D-gels dramatically improved and several spots of other less abundant proteins became visible (Figure 1B).

Comparisons of the expressed proteins between the sustained responders and non-responders before initiating peginterferon alfa-2b treatment

The results of the protein separation by 2D-gel electrophoresis, gel digestion and protein identification by LC/MS/MS are shown in Figure 2. Seven protein spots were detected with various intensities in the patients (Table 2 and Figure 2). Four proteins were significantly detected among the sustained responders: (1) chain A, alpha-1-antitrypsin; (2) albumin, isoform CRA-b; (3) CD5 antigen-like precursor; and (4) albumin. Three proteins were significantly detected among the non-responders: (1) chain A, crystal structure of lipid-free human apolipoprotein A-I; (2) chain C, human complement component C3c; and (3) alpha-2-HS-glycoprotein. Since the sustained responders and non-responders showed significant age differences, the Pearson correlation was used for age and each protein expression value. No significant correlation between age and protein expression was observed; therefore, it is unlikely that age affected protein expression in this study. The identified proteins have the following functions: alpha-1-antitrypsin is a protease inhibitor, serum albumin is a transport and binding protein, alpha-2-HS-glycoprotein is an acute phase response protein, CD5 antigen precursor and complement component C3c are immune protection proteins, and the human apolipoprotein A-I has a role in lipid metabolism

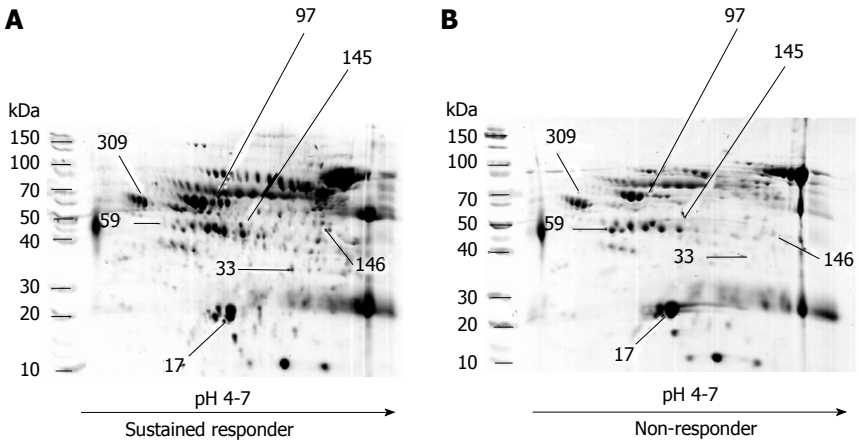

Figure 2 Serum protein spots from the 2-DE gels were significantly different. A: Sustained responders; B: Non-responders before chronic hepatitis B treatment.

Table 2 Proteins found to be significantly different between the sustained responders and non-responders before initiating peginterferon-Interferon alfa-2b treatment

Spot	Protein	NCBI ID	MS score	%cov	pI	MW	Relative intensity (mean ± SE)		SVR/NR	*P* value
							SVR	NR		
Protease inhibitor										
97	Chain A, alpha-1-antitrypsin	Gi|157831596	399	64	5.37	44.28	0.2764 ± 0.0327	0.4275 ± 0.0609	2.34	0.038
Transport protein and protein binding										
33	Albumin, isoform	Gi|119626065	539	38	6.96	61.12	0.0330 ± 0.0121	0.0764 ± 0.0144	2.31	0.033
146	Albumin	Gi|332356380	243	41	5.73	68.48	0.0602 ± 0.0182	0.1516 ± 0.0333	2.52	0.024
Acute phase protein										
309	Alpha-2-HS-glycoprotein	Gi|112910	507	40	5.43	40.09	0.7165 ± 0.0238	0.4782 ± 0.0851	0.67	0.012
Immunity protection										
59	Chain C, human complement C3c	Gi|78101271	513	70	4.79	40.20	0.3969 ± 0.0391	0.2675 ± 0.0403	0.67	0.034
145	CD5 antigen-like precursor	Gi|5174411	443	67	5.28	39.60	0.1086 ± 0.0192	0.1903 ± 0.0197	1.75	0.009
Lipid metabolism										
17	Chain A, crystal structure of lipid-free human apoliprotein A-I	Gi|90108664	1347	78	5.27	28.06	9.4993 ± 0.5044	6.0364 ± 1.0047	0.64	0.005

SVR: Sustained virological response; NR: Non-responder.

Characteristics of the patients from the validation phase of the proteomic study and immunonephelometry

In the validation phase, nine and 14 patients with sustained virological response and nonresponders were enrolled, respectively. The sex ratio, levels of serum and HBV DNA, and HBeAg were not significantly different, but there was a significant difference in age between the groups (Table 3).

Validation of proteins associated with the immune response

We selected 3 proteins (alpha-2-HS-glycoprotein**,** complement component C3c, and CD5 antigen-like proteins) that were significantly different between the 2 groups, and have functions related to immune response for further validation (Figure 3). According to the ELISA results, the serum levels of alpha-2-HS-glycoprotein were significantly elevated among the non-responders when compared to the sustained responders at baseline (Figure 4A). Similarly, the immunonephelometry results showed that serum levels of complement component C3c were significantly elevated in the non-responders when compared to the sustained responders at baseline (Figure 4B), however, the serum levels of CD5 antigen like proteins were comparable between the two groups (Figure 4C). No significant correlation between age and protein expression was observed.

Comparison of the identified proteins after 24 wk of peginterferon alfa-2b treatment

A total of 6 patients, divided into 2 groups, were included in the analysis 24 wk post-treatment. The clinical data were not significantly different between the 2 groups (Table 3). The samples were separated by 2D-gel electrophoresis, and identified by LC/MS/MS. Thirteen protein spots were significantly changed at 24 wk after treatment among the patients in the sustained response group, whereas 6 were found in the non-responders (Table 4). Interestingly, all 13 proteins in the sustained response group which were increased at 24 wk were composed of cholesterol metabolites, proteins from the acute phase response, protease inhibitors, transport proteins and immune protection. Of these, alpha-2-HS-glycoprotein was higher than the baseline level in the responder group. In the non-responder group, levels of proapoliprotein, chain A of human antithrombin Ⅲ complex and alpha-1-B-glycoprotein were higher than the baseline level, whereas

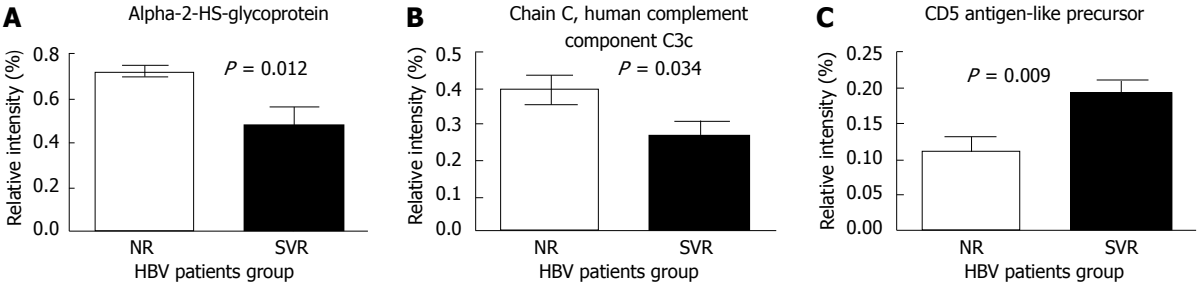

Figure 3 Histograms of the proteomic analysis at different levels. A: Alpha-2-HS-glycoprotein; B: Complement component C3c; C: CD5 antigen-like proteins in sustained responders and non-responders before chronic hepatitis B treatment. The intensity data are presented as mean ± SE (n = 19 gels from all patients and groups). SVR: Sustained virological response; NR: Non-responder.

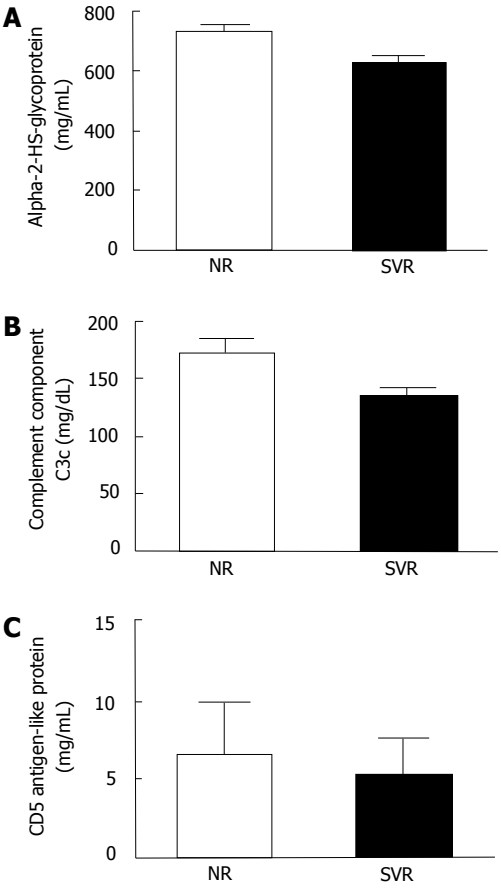

Figure 4 Validation of proteins associated with the immune response. A: Validation by enzyme-linked immunosorbent assay showed that there were elevated levels of alpha-HS-glycoprotein in non-responders before chronic hepatitis B treatment. n = 19 gels from all patients and groups; B: Validation by immunonephelometry showed elevated levels of complement component C3c in non-responders before chronic hepatitis B treatment; C: Validation by immunonephelometry showed elevated levels of CD5 antigen-like proteins in non-responders before chronic hepatitis B treatment. SVR: Sustained virological response; NR: Non-responder.

levels of albumin and alpha-2-HS-glycoprotein decreased 24 wk post-treatment.

DISCUSSION

The proteomic approach is usually used to analyze protein expression. It can be used with various specimens such as tissue, serum, plasma or body fluids. For this study, serum samples were used to identify potential biomarkers that can be further applied to predict the outcome of CHB therapy. Serum was selected because the collection process is non-invasive and proteins from the liver are secreted into the serum. Therefore, serum is an ideal specimen to screen for new proteins or biomarkers. In addition, serum proteomics can be used to detect post-translational modified proteins. 2-DE was used to separate and identify the proteins between 10-200 kDa. The high sensitivity and high throughput of mass spectrometry has resulted in the detection of several new biomarkers in ovarian cancer, prostate cancer, breast cancer and hepatocellular carcinoma. However, it should be noted that 2-DE does have limitations. 2-DE cannot detect low abundant proteins because high abundant proteins such as albumin and IgG can suppress the detection of low abundant proteins. To overcome this obstacle, albumin and IgG were removed from the serum samples before electrophoresis using the ProteoPrep Blue Albumin Depletion Kit. Albumin (-45 mg/mL) and IgG (-10 mg/mL) are the two major protein components of serum, representing 60%-70% and 10%-20% of the total serum protein, respectively[22]. When the high abundant

Table 3 Baseline characteristics of the patients in the validation phase and at 24 wk of treatment (mean ± SE)

	Sustained virological responders	Non-responders	*P* value
Validation phase	(n = 9)	(n = 14)	
Age (yr)	29.56 ± 2.78	37.71 ± 1.97	0.023
Sex (male:female)	7:2	11:3	0.966
ALT level (U/L)	106.88 ± 36.26	130.50 ± 30.93	0.644
HBV DNA (copies/mL)	(11.06 ± 2.84) × 10^6	(13.12 ± 2.45) × 10^6	0.593
HBeAg	Positive	Positive	NS
24 wk	(n = 3)	(n = 3)	
Age (yr)	29.33 ± 6.89	35.67 ± 6.06	NS
Sex (male:female)	Male	Male	NS
ALT level (U/L)	52.33 ± 13.78	253.67 ± 187.74	NS
HBV DNA (copies/mL)	(6.70 ± 6.65) × 10^6	(13.65 ± 6.35) × 10^6	NS
HBeAg	Positive	Positive	NS

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

Table 4 Serum levels of proteins after 24 wk of treatment in the sustained virological responders and non-responders

Spot	Protein Name	NCBI ID	MW/pI	No. of match peptide	MS score	Fold change	Δ relative intensity	Biological function
Sustained virological response								
38	Proapolipoprotein	Gi|178775	28.94/5.50	63	798	↑ 5.17	0.1525[a]	Cholesterolmetabolism
26	Chain A, the structure of pentameric human serum amyloid P	Gi|576259	23.36/6.12	12	174	↑ 6.65	0.0783[a]	Acute phase protein
86	Alpha-2-HS-glycoprotein	Gi|112910	40.098/5.43	32	494	↑ 2.39	0.3773[a]	
309	Alpha-2-HS-glycoprotein	Gi|112910	40.09/5.43	29	507	↑ 2.04	0.4987[a]	
171	Chain A, the intact and cleaved Ⅲ complex as a model for serpin-proteinase interaction	Gi|999513	49.35/5.95	37	396	↑ 9.66	0.1481[b]	
173	Chain A, the intact and cleaved Ⅲ complex as a model for serpin-proteinase interaction	Gi|999513	49.35/5.95	26	210	↑ 8.01	0.0694[a]	Protease inhibitor
149	Serotransferin precursor	Gi|4557871	79.28/6.81	58	650	↑ 3.43	0.1165[a]	
201	PRO2619	Gi|11493459	58.51/5.96	36	278	↑ 4.95	0.0594[a]	Transport protein and proteinbinding
207	Albumin	Gi|332356380	68.48/5.73	48	781	↑ 6.97	0.1653[a]	
280	Albumin	Gi|332356380	68.48/5.73	31	201	↑ 7.33	0.1206[a]	
202	CD5 antigen-like precursor	Gi|5174411	39.60/5.28	11	70	↑ absent at baseline	0.0348[a]	
229	Ig J-chain	Gi|532598	16.04/4.62	3	42	↑ absent at baseline	0.0923[b]	Immunity protection
299	Immunoglobulin light	Gi|218783338	24.16/5.95	26	747	↑ absent at base line	0.1709[b]	
Non-responder								
20	Proapolipoprotein	Gi|178775	28.94/5.45	63	798	↑ absent at base line	0.2383[b]	Cholesterol metabolism
171	Chain A, the intact and cleaved Ⅲ complex as a model for serpine-proteinase interaction	Gi|999513	49.35/5.95	37	396	↑ 2.39	0.0896[a]	Acute phase protein
4	Albumin isoform	Gi|119626066	27.67/6.39	20	660	↓ 0.04	-0.2832[a]	Transport protein and protein binding
264	Serum albumin	Gi|62113341	71.09/5.85	21	115	↓ absent at 24 wk	-0.1196[a]	
123	Alpha-1-B-glycoprotein	Gi|69990	52.47/5.69	27	247	↑ 1.38	0.1610[a]	Serum protein
310	Alpha-2-HS-glycoprotein	Gi|112910	40.09/5.43	30	475	↓ 0.46	-0.4119[a]	Acute phase protein

Relative intensity, [(volume of spot/volume of all the spots in the gel) × 100]. [a]$P < 0.05$, [b]$P < 0.01$ *vs* relative intensity.

proteins were depleted from the serum, this allowed the low abundant protein spots to become visible. However, it is also possible to miss certain low abundant proteins when the high abundant proteins are removed[23]. The reason for this is that low abundant proteins sometimes bind themselves to high abundant proteins. Hence, only albumin and IgG were removed in order to prevent the loss of other important proteins[24]. As expected, in the untreated sample, albumin dominated the gel, obscuring signals from other less abundant proteins. When an equal quantity of protein was pre-treated with the ProteoPrep Blue Albumin Depletion Kit, the resolution of the 2D-gels significantly improved. This process cannot completely eliminate all albumin, but can eliminate enough to allow several protein spots to be clearly visible. Using these techniques, a total of seven protein spots were found to be differentially expressed in the serum of CHB patients, 9 sustained responders and 10 non-responders, before starting peginterferon alfa-2b treatment. Interestingly, four of the proteins were higher in the sustained responders. Of these proteins, only 3 were involved in immune responses: alpha-2-HS-glycoprotein, complement component C3c and CD5 antigen-like proteins. We further validated the proteomic results using the sensitive ELISA and nephelometry assay. In the validation phase of the study, the serum levels of alpha-2-HS-glycoprotein and complement component C3c were significantly higher in the non-responders when compared to the sustained responders. However, the level of CD5 antigen-like protein was not statistically different between the two groups. Since the band intensity of this protein was lower than the other proteins and the level was detected only in the validation phase of the study when the abundant proteins were not depleted, it is possible that the level of CD5 antigen-like protein may have altered when albumin and IgG were removed.

Based on these findings, the authors believe that alpha-2-HS-glycoprotein and complement component C3c are potential serum pre-treatment biomarkers in predicting sustained virological response in CHB patients treated with peginterferon alfa-2b. In this study, the levels of serum alpha-2-HS-glycoprotein were elevated in the non-responders before treatment initiation, whereas the levels were much lower in the sustained responders. In addition, we performed a subsequent serum proteomic analysis in a subset of samples at 24 wk after peginterferon alfa-2b treatment. Serum alpha-2-HS-glycoprotein was also up-regulated in the sustained virological responders, but downregulated in the non-responders. This finding is consistent with the results reported earlier by Ma *et al*[17]. However, a previous study only detected changes after treatment, but was not able to predict the treatment out-

come prior to treatment. The identification of biomarkers prior to treatment is preferable in this case to avoid systemic side effects due to interferon therapy.

Alpha-2-HS-glycoprotein is a high abundant protein produced by the liver and osteoblasts and is usually concentrated in the mineralized tissues. This protein belongs to the cystatin super family[25]. Several studies have reported various levels of alpha-2-HS-glycoprotein in patients with liver diseases[26-28]. Some have found low levels of serum alpha-2-HS-glycoprotein in patients with acute drug-induced hepatitis, alcoholic hepatitis, chronic autoimmune hepatitis, primary biliary cirrhosis, fatty liver and HCC[26,28]. Aside from its multiple functions and ability to affect metabolic diseases, tumor and sepsis[29,30], Dai *et al*[31] reported that it was an independent marker for liver injury and a prognostic marker for CHB. They suggested that the protein may decrease liver inflammation by inhibiting the release of inflammatory factors from activated peripheral blood mononuclear cells[31]. Similarly, Patel *et al*[32] found reduced levels of alpha-2-HS-glycoprotein in chronic hepatitis C patients identified as sustained virological responders before initiating treatment. The reduced levels of alpha-2-HS-glycoprotein in chronic hepatitis C[32] and B patients identified as sustained virological responders before treatment suggest that this protein may potentially be used as a biomarker in predicting the outcome of peginterferon alfa-2b treatment.

Another protein detected in this study was complement component C3c which is the degradation product of complement C3. This protein is important for both the acquired and innate immune systems, especially against microbial infection as it switches the cellular responses from cell death to opsonization[33]. 80%-90% of complement is produced by hepatocytes and is associated with the pathogenesis of many chronic human diseases such as autoimmune diseases, complement-mediated hemolytic anemia, vascular and liver diseases[33,34]. Interestingly, low levels of C3 fragments were detected in HBV and HCC patients compared to normal, healthy controls[11]. According to the results obtained from the screening and validation phases of this study, the expression and levels of complement component C3c were elevated in non-responders before treatment. Thus, complement component C3c may be a possible biomarker in predicting the outcome of peginterferon alfa-2b treatment in patients with CHB.

In conclusion, alpha-2-HS-glycoprotein and complement component C3c proteins were elevated in non-responders indicating that these proteins may be potential biomarkers in predicting the response to peginterferon alfa-2b treatment in patients with CHB prior to treatment. However, external validation is needed to assess the clinical applicability of these two proteins as predictors of anti-HBV treatment outcome.

COMMENTS

Background

The treatment duration, eradication and lack of drug resistance strains make type 1 interferons ideal for the treatment of chronic hepatitis B virus. Unfortunately, only 30% of patients will respond to treatment with interferon type 1. To date, there is no effective predictor of interferon responsiveness.

Research frontiers

The authors identified 2 potential serum biomarkers, alpha-2-HS-glycoprotein and complement component C3c, and determined that they can be used to predict treatment outcome in patients with chronic hepatitis B (CHB) receiving peginterferon alfa-2b.

Innovations and breakthroughs

Serum proteins serve as non-invasive biomarkers for several diseases. This is the first report of the potential use of common protein levels in the serum of CHB patients to predict responsiveness to peginterferon alfa before treatment. The identification of biomarkers prior to treatment is preferable in this case to avoid systemic side effects due to interferon therapy.

Applications

These 2 serum proteins can be added to the list of potential biomarkers to predict responsiveness to peginterferon alfa. However, these findings require further validated using a larger sample size and other independent studies.

Peer review

The authors examined the differential protein profile in serum of hepatitis B patients before treatment with peginteferon-2b. From the quantitative analysis of the 2D-gel and validation step using Enzyme-linked immunosorbent assay and immunonephelometry, serum levels of alpha-2-HS-glycoprotein and complement component C3c were elevated in the serum of the non-responders compared to the responders. This indicated that these 2 serum proteins may be potential serum biomarkers in predicting the treatment response in CHB patients on peginterferon alfa-2b therapy.

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ORIGINAL ARTICLE

Impact of mesocaval shunt on safe minimal liver remnant: Porcine model

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Abstract

AIM: To investigate the capacity of shunts to relieve portal hypertension and decrease the safe minimal liver remnant in pigs.

METHODS: A subtotal hepatectomy with < 60 mL blood loss and without hepatic pedicle occlusion was performed. The mesenteric venous inflow was diverted through a mesocaval shunt (MCS) constructed using the prepared left renal vein with an end-to-side running suture of 5-0 proline. All 21 animals that underwent subtotal hepatectomy and/or MCS were divided into three groups. In the 15% group, the residual volume was 14%-19% of total liver volume (TLV); in the 15%+ S group, the residual volume was also 14%-19% of TLV with a mesocaval shunt (MCS); and in the 10%+ S group, the residual volume was 8%-13% of TLV with an MCS. In the three groups, the intraoperative portal vein pressure (PVP) and portal vein flow (PVF) were monitored and compared at laparotomy and 1 h post-hepatectomy. The survival rate, sinusoidal endothelial damage, tissue analysis, and serum analysis were investigated among the three groups.

RESULTS: The percentage residual liver volume was 15.9%, 16.1% and 11.8% in the 15%, 15%+ S, 10%+ S groups, respectively. After hepatectomy, PVF and portal-to-arterial flow ratio in the 15%+ S group significantly decreased and hepatic artery flow (HAF) per unit volume significantly increased, compared to those in the 15% group. The PVP in the 15%+ S group and 10%+ S group increased slightly from that measured at laparotomy; however, in the 15% group, the PVP increased immediately and significantly above that observed in the other two groups. The 14-d survival rates were 28.5%, 85.6%, and 14.2% in the 15%, 15%+ S, and 10%+ S groups, respectively. In the 15%+ S group, the shunts effectively attenuated injury to the sinusoidal endothelium, and the changes in the serum and tissue analysis results were significantly reduced compared to those in the 15% and 10%+ S groups.

CONCLUSION: MCS can decompress the portal vein and so attenuate liver injury from hyperperfusion, and make extreme or marginal hepatectomy safer.

Key words: Hepatectomy; Safe minimal remnant volume; Mesocaval shunt; Pigs

Core tip: When the residual liver volume is extremely small after extended hepatectomy or living-donor liver transplantation, postoperative hepatic failure (PHF) or small-for-size syndrome (SFSS) may result from portal hypertension or hyperperfusion. We demonstrated that mesocaval shunt attenuated portal overflow injury, however, it is unknown how much the shunt can decrease, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy. We showed that the residual volume was the determinant factor of

PHF or SFSS after subtotal hepatectomy, and the shunt attenuated injury from hyperperfusion, and made marginal hepatectomy safer.

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INTRODUCTION

Currently, there is no definitive answer to the question "How much liver excision is too much?"[1-4]. When the residual liver volume or graft is extremely small after extended hepatectomy or living-donor liver transplantation (LDLT), postoperative hepatic failure (PHF) or small-for-size syndrome (SFSS) may ensue, and portal hypertension or hyperperfusion is regarded as the determinant factor of liver failure or SFSS. It has been demonstrated the portal decompression, such as portacaval or mesocaval shunt (PCS/MCS), splenic artery ligation or splenectomy, can attenuate portal overflow injury, and result in smaller graft or liver remnant generated successfully in animal experiments and clinical studies[5-8]. However, it is unknown how much the shunt can decrease portal overflow, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy[7]. Large-animal models provide a clinically relevant means of investigating the pathophysiology of a disease process that can be more readily applied in the human setting[9-11]. We investigated the capacity of MCS to relieve sinusoidal microcirculatory injury and to decrease the safe minimal liver remnant (MLR) value in massive hepatectomy.

MATERIALS AND METHODS

Animals and husbandry

Twenty-five male Bama miniature pigs (15-20 kg) were obtained from the Pig and Poultry Production Institute (Guangxi Province, China). The pigs were raised from a closed herd and kept under strict quarantine. The study was approved by the Chinese PLA General Hospital Clinic Committee on Ethics in Animal Experimentation. All animals in this study were treated humanely and in accordance with institutional and national guidelines for ethical animal experimentation.

Anesthesia

The pigs were food-deprived for 8 h before the operation. All pigs were anesthetized in the following way: initial sedation was obtained with a deep intramuscular injection of ketamine (15-20 mg/kg) and chlorpromazine (6-8 mg/kg) 15 min after atropine (0.01 mg/kg). Oxygen saturation and heart rate were monitored throughout the operation. A size 4 laryngeal mask airway was inserted, and anesthesia was maintained using 1.5% halothane in oxygen titrated to provide anesthesia. Central venous access was established with a tunneled catheter from the right femoral vein. Intraoperatively, 1 L of normal saline and 500 mL 5% dextrose were administered intravenously. No attempt was made to lower central venous pressure.

Surgical technique

An upper-midline incision with right or bilateral subcostal extensions (inverse "L" shape or Mercedes incision) was performed. A subtotal hepatectomy with < 60 mL blood loss and without hepatic pedicle occlusion was performed as previously described described[12-14]. A 16-gauge catheter was inserted into the main portal vein via the gastroduodenal vein to measure the portal vein pressure (PVP). Another catheter was advanced into the suprahepatic inferior vena cava (IVC) through one of the phrenic veins to monitor the pressure in the IVC. Ultrasonic flow probes were connected to a flow meter (TS420; Transonic Systems, Ithaca, NY, United States) to measure hepatic artery flow (HAF) and portal vein flow (PVF).

MCS

The mesenteric venous inflow was diverted through an MCS constructed using the prepared left renal vein with an end-to-side running suture of 5-0 proline (Qiangsheng, Shanghai, China), while the mesenteric vein was partly occluded (Figure 1). After the shunt, its patency was examined, and the size of shunt was adjusted to preserve PVF.

Postoperative management

After the operation, the pigs were monitored for 14 d: every 2 h in the first day and every 24 h thereafter, and one dose of 375 mg penicillin/375 mg streptomycin was given intramuscularly to all pigs. This dose was repeated daily every morning until euthanasia. They were given free access to water. Food and water intake and serum glucose levels were evaluated at each postoperative assessment, and animals that had limited or no intake per os and/or low serum glucose levels (< 70 mg/dL) were administered 50 g intravenous glucose (500 mL 10% glucose solution). Every dead or euthanized pig was necropsied to examine the patency of the shunt.

Experimental protocols

Twenty-five pigs were included and four were excluded for the obliteration of MCS or other surgery-related complications. Based on previous studies[13,14], the remaining 21 animals, which were submitted to massive hepatectomy with different liver mass removed, were divided into three groups: 15% group (n = 7), which was submitted to massive hepatectomy with a residual volume of approximately 15% (range 14%-19%, median: 15.9%) of TLV (Table 1); 15%+ S group (n = 7), which was subjected to MCS (Figure 1) and then massive hepatectomy with a residual volume of approximately 15% (range 14%-19%,

Table 1 Study characteristics and evolution of hemodynamic parameters

	15% group	15%+ S group	10%+ S group	[1]*P* value	[2]*P* value
Body weight (kg)	19.6 ± 2.9	18.4 ± 3.0	18.9 ± 3.9	NS	NS
Left-trilobes (g)	347.51 ± 18.2	334.6 ± 16.4	340.7 ± 17.2	NS	NS
ETL (g)	434.4 ± 22.5	418.1 ± 21.4	425.8 ± 21.2	NS	NS
WRL (g)	362.1 ± 17.3	355.0 ± 16.6	368.1 ± 16.9	-	-
ERL (g)	69.3 ± 4.5	67.8 ± 4.8	47.7 ± 3.1	-	-
Rate of RL (%)	15.9	16.1	11.8	-	-
PVF, mL/min per 100 g					
BAS	61.3 ± 7.1	62.9 ± 5.9	59.1 ± 4.3	NS	NS
PH	312.4 ± 24.1	215.4 ± 20.3	231.4 ± 31.2	0.001	NS
HAF, mL/min per 100 g					
BAS	21.1 ± 4.6	19.4 ± 4.5	18.6 ± 3.4	NS	NS
PH	8.3 ± 3.4	15.5 ± 4.1	14.1 ± 3.4	0.001	NS
P/A					
BAS	2.9 ± 0.3	3.2 ± 0.4	3.3 ± 0.3	NS	NS
PH	36.3 ± 4.1	14.1 ± 2.6	16.4 ± 3.6	0.000	NS

All flow values are reported in mL/min per 100 g hepatic tissue. Data expressed as mean ± SD. Estimated total liver weight = (weight of left trilobes) × 100/80. [1]Indicating difference between 15%+ S and 15% groups; [2]Indicating difference between 15%+ S and 10%+ S groups. ETL: Estimated total liver weight; RL: Residual liver volume; ERL: Estimated residual liver volume; WRL: Weight of resected liver; NS: Not significant; BAS: Baseline; PH: Post-hepatectomy; HAF: Hepatic artery flow; PVF: Portal vein flow; P/A: Portal-to-arterial flow ratio.

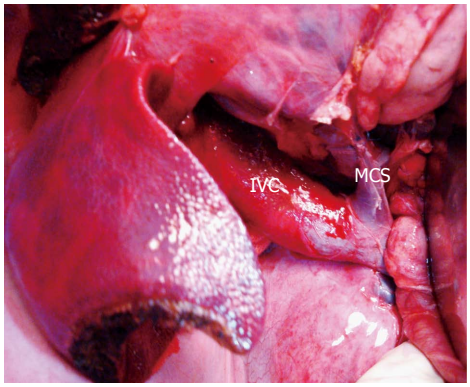

Figure 1 Photograph of the vascular anastomosis with the renal vein in the experimental group. IVC: Inferior vena cava; MCS: Mesocaval shunt.

median: 16.1%) of TLV; 10%+ S group (n = 7), with the same surgical procedure as the 15%+ S group, but with a residual volume of approximately 10% (range 8%-13%, median: 11.2%) of TLV (Table 1). In the 15%+ S group and 10%+ S group, there was a portal inflow of 3.0-3.5 times baseline per unit volume, which was regarded as a optimum flow for liver regeneration based on our previous study and other studies[15]. This was maintained through regulating the size of the MCS after hepatectomy. In the three groups, the intraoperative PVP and PVF were monitored and compared at laparotomy and 1 h post-hepatectomy (PH). The survival rate and tissue and serum analysis among the three groups were also investigated.

Blood and serum analysis

Blood sampling was performed preoperatively, and 1 h PH, then daily for 7 d or until death. During the follow-up period, levels of alanine aminotransferase (ALT) and total bilirubin (TB) and the international normalized ratio (INR) were determined. Hyaluronic acid (HA) is a polysaccharide synthesized by mesenchymal cells and eliminated chiefly by receptor-mediated endocytosis in the hepatic sinusoidal endothelium, and increased serum HA levels reflect sinusoidal endothelial damage[16]. HA was measured by a radiometric assay with the Pharmacia HA test (Yihua BioScience, Shanghai, China) in pre-reperfusion and post-reperfusion serum samples. The arterial ketone body ratio (acetoacetate/β-hydroxybutyrate, AKBR) is a useful tool for the estimation of liver functional reserve. Liver mitochondrial redox state (liver mitochondrial free NAD+/NADH ratio), which indicates hepatic energy charge, is known to reflect the ketone body ratio (acetoacetate/3-hydroxybutyrate) in liver tissue[15,17]. Ozawa *et al*[17] first demonstrated that the AKBR was correlated with the ketone body ratio in liver tissue, and it has been reported as a useful tool for the estimation of liver functional reserve in hepatic surgery. The AKBR was measured preoperatively and at 2 h and 48 h PH.

Tissue analysis

Hepatic tissue specimens were obtained from the edges of the liver at laparotomy and from the edges of the remnant liver at 2 h PH, and then divided into two sections. One was preserved in 10% formaldehyde for subsequent fixation in paraffin, and the other was immediately cut into 1-mm cubes and fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L sodium cacodylate-HCl buffer, pH 7.4) overnight at 4 ℃ prior to sectioning for transmission electron microscopy to study hepatocyte and sinusoidal ultrastructure. Platelet endothelial cell adhesion molecule-1 (CD31) helps maintain endothelial stability by interacting with other CD31 molecules at the extracellular border of adjacent cells. Sections of hepatic

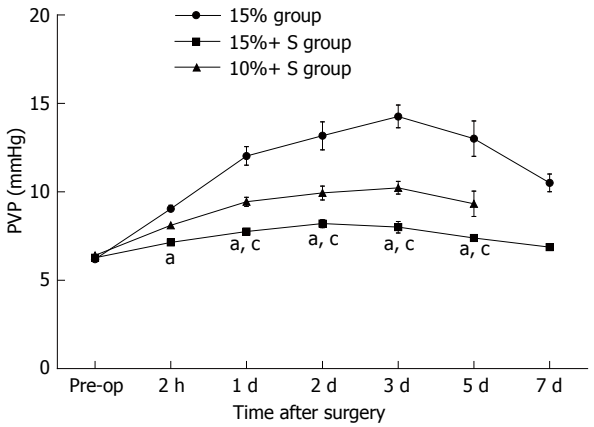

Figure 2 Serial changes in portal vein pressure in the three groups. There was a significant difference in changes in portal vein pressure (PVP) among the three groups. [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group.

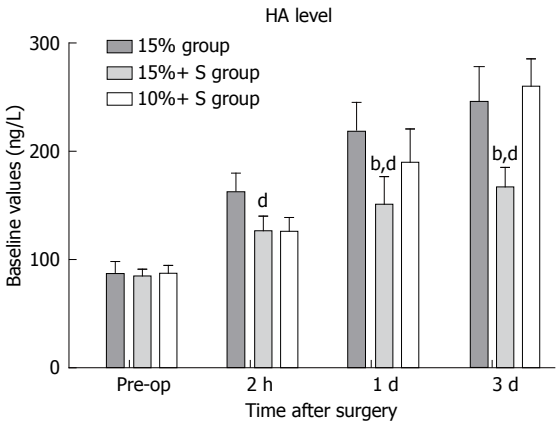

Figure 3 Baseline values of hyaluronic acid among the three groups were not significantly different, but in the 15%+ S group it was significantly decreased at 1 and 3 d post-hepatectomy compared to that in the 15% group. [b]*P* < 0.01 *vs* 10% group; [d]*P* < 0.01 *vs* 15% group. HA: Hyaluronic acid.

tissue were immunostained with porcine anti-CD31 antibody (Serotec, Oxford, United Kingdom) to evaluate the integrity of the endothelial cells in the hepatic sinusoid, as previously described[18].

Lipopolysaccharides and inflammation response

The lipopolysaccharides (LPS) level was quantitated by a limulus amebocyte lysate (LAL) assay based on the methods first introduced by Iwanaga *et al*[19] using the commercially available chromogenic LAL Endpoint Kit (Yihua BioScience, Shanghai, China) following the manufacturer's instructions. A calculated value of 0.1 EU/mL (10 pg/mL) was considered the threshold for LPS positivity. Standards and samples were analyzed in duplicate. Serum levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured using commercial ELISA kits (Jingmei Biotech Co. Ltd., Shenzhen, China) following the manufacturer's instructions.

Statistical analysis

The survival rates in the three groups were calculated using a generalized Wilcoxon test. The biochemical results were compared by Student's *t* test, comparing mean values among the three groups. Parameters are presented as mean ± SD. Statistical significance was determined by Student's *t* test (SPSS, Chicago, IL, United States). $P < 0.05$ was regarded as significant.

RESULTS

Study characteristics and hemodynamic studies

The characteristics of the study and the evolution of hemodynamic parameters are shown in Table 1. The percentage RLV was 15.9%, 16.1%, and 11.8% in the 15%, 15%+ S, and 10%+ S groups, respectively. After hepatectomy, PVF and portal-to-arterial flow ratio in the 15%+ S group significantly decreased and HAF per unit volume significantly increased, compared to those in the 15% group.

PVP

Serial changes in PVP in the three groups are shown in Figure 2. PVP in the 15%+ S and 10%+ S groups increased slightly from that measured at laparotomy; however, in the 15% group, PVP increased immediately and significantly compared to that observed in the other two groups ($P < 0.05$ for all comparisons).

Hepatic endothelial cell injury

Changes in HA concentration are shown in Figure 3. Two hours after subtotal hepatectomy, serum HA concentration increased in all pigs. In the 15%+ S group, HA level was significantly reduced compared to that in the 15% group. At other time points, the values were significantly lower than those observed in the 15% and 10%+ S groups ($P < 0.01$). The histological changes in tissue samples taken at 1 h PH in the three groups are shown in Figure 4.

Hepatocellular injury

The serial measurements of serum ALT, TB, and INR are shown in Figure 5, in which significant differences were noted. There were significant differences between the 15% and 15%+ S groups, and between the 10%+ S and 15%+ S groups ($P < 0.05$ for two comparisons).

Survival rate

The animals were followed-up for 14 d. An observation period of 14 d was chosen because liver function recovered to normal within 14 d after major hepatectomy[20]. The survival rate was calculated by the Kaplan-Meier method. Survival in the 15%+ S group with a shunt was better than in the 15% and 10%+ S groups (85.7% *vs* 28.5% *vs* 14.3%, $P < 0.01$). In the 15% group without a shunt, all pigs survived for > 4 d, and only two pigs survived until 14 d. In the 10%+ S group, all pigs survived > 3 d, but only one pig survived until 14 d.

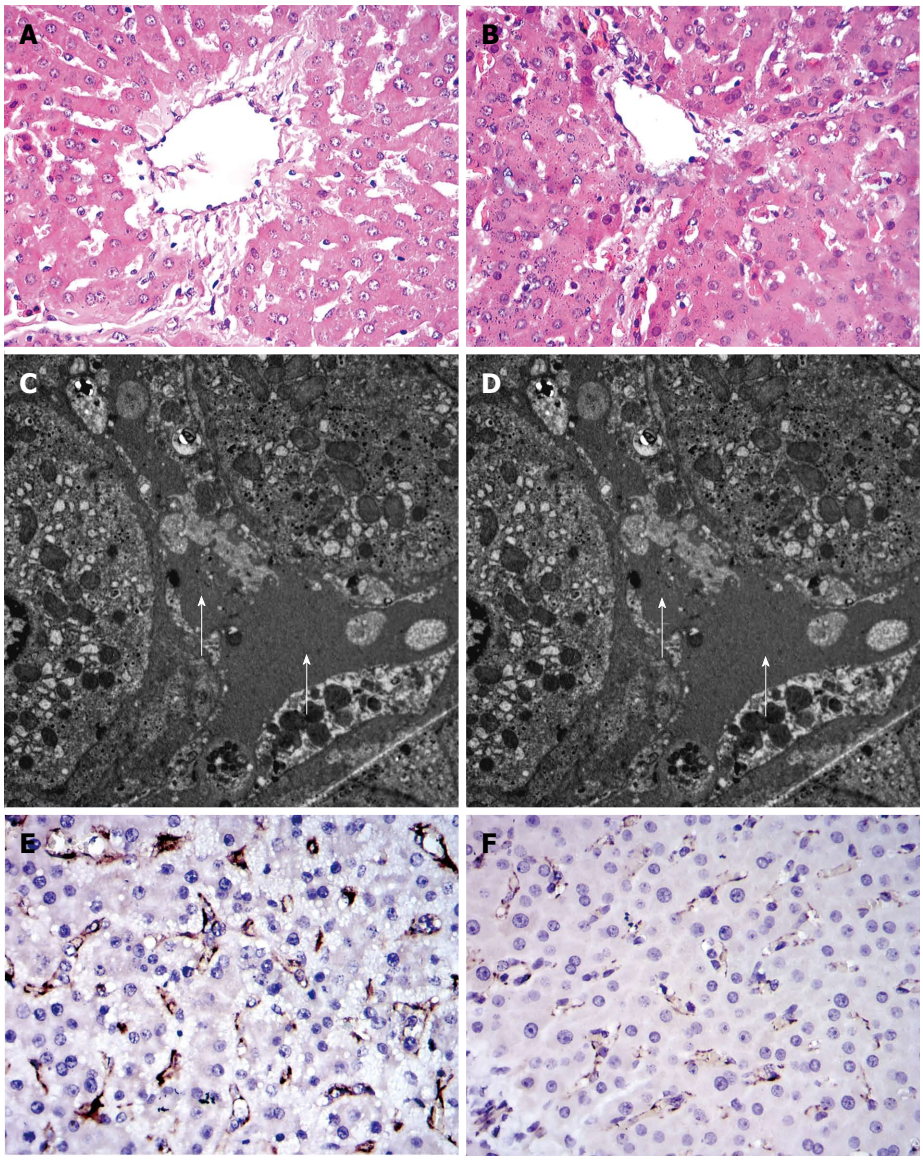

Figure 4 The histological changes in tissue samples were taken at 1 h post-hepatectomy in the three groups. Hematoxylin and eosin (magnification × 400); Transmission electron microscopy (magnification × 6000) and CD31 immunohistochemical staining in the three groups. In the 15% group, there was significant endothelial denudation, sinusoidal dilation, hydropic changes in hepatocytes, and hemorrhage into the perivenular connective tissue (A); the sinusoidal endothelial lining was slightly damaged and detached into the sinusoidal space, with enlargement of the Disse's spaces (C, arrow); CD31 immunostaining also revealed destruction of the endothelial lining (E); whereas in the 15%+ S or 10%+ S group, there was no intraparenchymal hemorrhage present (B); Transmission electron microscopy demonstrated the sinusoidal endothelial cells, and the structure of the endothelial lining can also be seen (arrow) (D); CD31 immunostaining also revealed mild sinusoidal microarchitecture injury (F).

DISCUSSION

In hepatectomy, when the RLV decreases below a certain threshold, the liver vascular bed immediately decreases, and vascular resistance in the residual liver increases. This leads to portal hypertension or hyperperfusion and a steady decrease in liver function; the liver remnant cannot sustain metabolic, synthetic, and detoxifying functions; and SFSS or PLF ensues[1,3]. The portal hypertension or hyperperfusion is regarded as the determinant factor of liver failure or SFSS. Portal decompression, such as PCS/MCS, splenic artery ligation or splenectomy, is often used to improve the prognosis of an SFSS graft in LDLT when the graft-to-recipient weight ratio is < 0.8% or the graft volume/standard liver volume (GV/SLV) is < 30%[5-7,21-23]. PCS/MCS could make a GV/SLV < 30%, or as low as 20%-25% a viable option with a fair prognosis[24-26], indicating shunts can make small grafts successfully regenerate or make them safe. However, it is unknown how much the shunt can decrease portal pressure, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy as it does in LDLT[7].

In our previous study, we showed that the survival rate in pigs with approximately 15% residual liver volume was 24.8%, which was similar to the present study; whereas it was 100% in pigs with approximately 20% RLV. We established that the safe MLR should be >

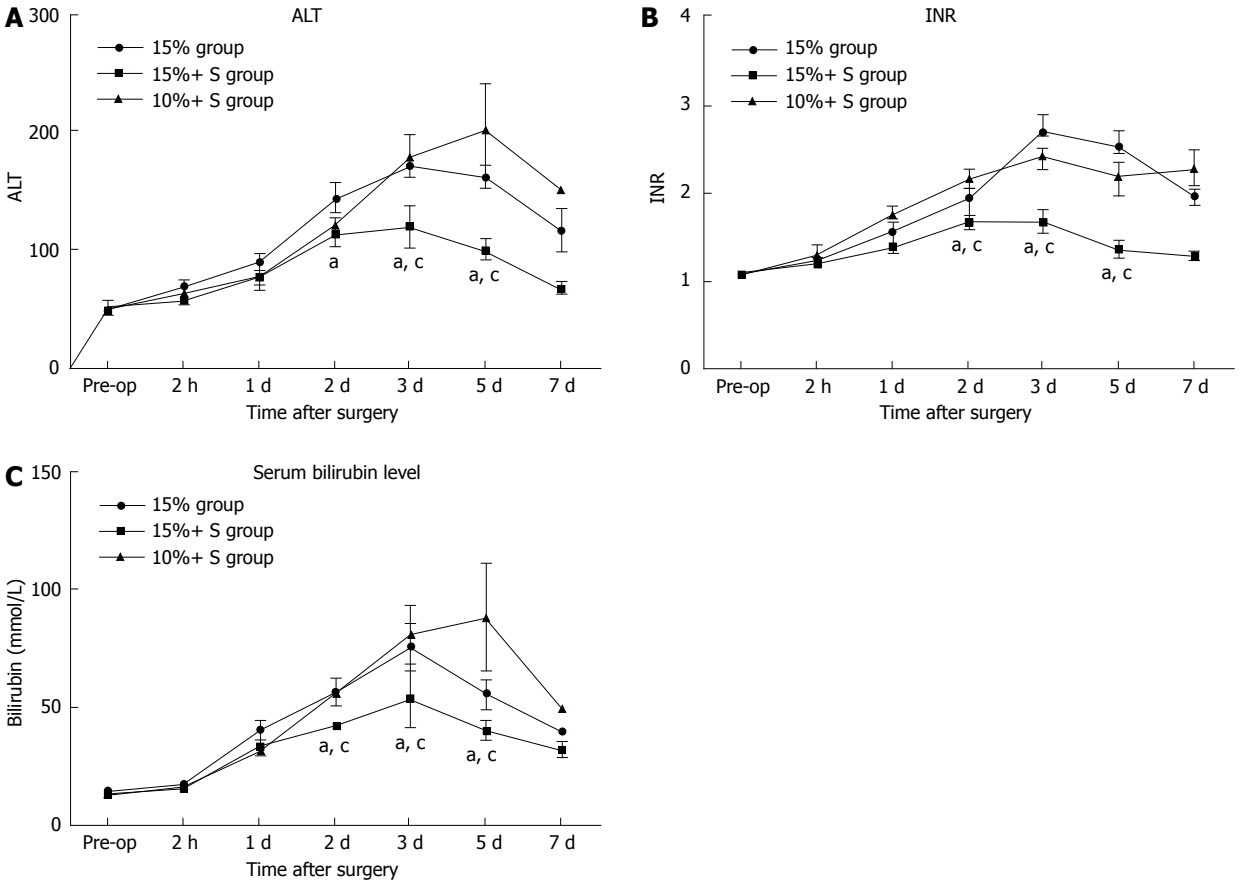

Figure 5 Changes in serum alanine aminotransferase and bilirubin level, and international normalized ratio in the three groups. A: Serum alanine aminotransferase (ALT); B: International normalized ratio (INR); C: Serum bilirubin level. [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group.

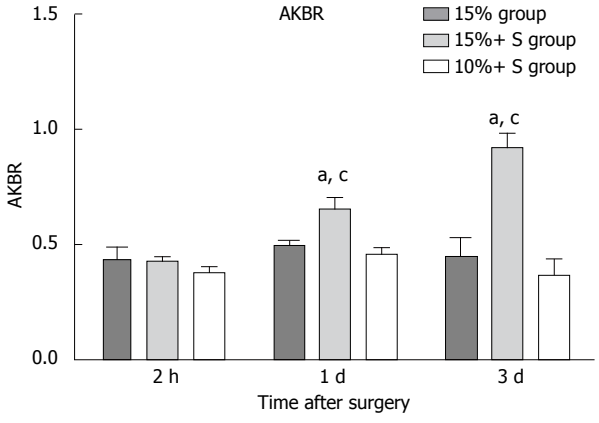

Figure 6 Changes in the arterial ketone body ratio level in the three groups. [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group. AKBR: Arterial ketone body ratio.

15% of TLV in a porcine model. However, in the present study, we found that MCS could decrease the degree of sinusoidal injury, protect liver function, regenerate liver with approximately 15% RLV, and increase the survival rate up to 85.7%. Nonetheless, none of the pigs with 10% RLV in the 10%+ S group could sustain metabolism and failed to regenerate even though portal decompression was performed. These data also indicate that portal decompression can make extreme liver resection or marginal size liver remnant (approximately 15% of TLV) safe, but it cannot make LRV < 5% (10% of TLV) viable.

In the normal state, PVF and HAF are linked by the hepatic artery buffer response[27,28], which induces a decrease in hepatic artery diameter and flow if PVF increases, and is synonymous with liver microcirculation failure. In the present study, it also showed the HAF in the 15% group was significantly decreased (Table 1), and this insufficient HAF might be another important contributor to the failure of liver remnant regeneration. However, the MCS prevented injury from excess PVF and significantly decreased PVF, resulting in a significant increase in HAF in the 15%+ S group; successfully regenerated liver in animals with approximately 15% RLV; but it could not regenerate 10% RLV. This is probably due to the liver remnant being too small to sustain the metabolic, synthetic and detoxifying functions, despite the presence of sufficient arterial flow. AKBR is a predictor of liver viability and responds to disorders of energy metabolism in the mitochondria[16]. The present study also demonstrated that there was no significant difference among the three groups at 2 h PH. At other time points, the 15%+ S group showed significant differences from the 15% and 10%+ S groups ($P < 0.05$),

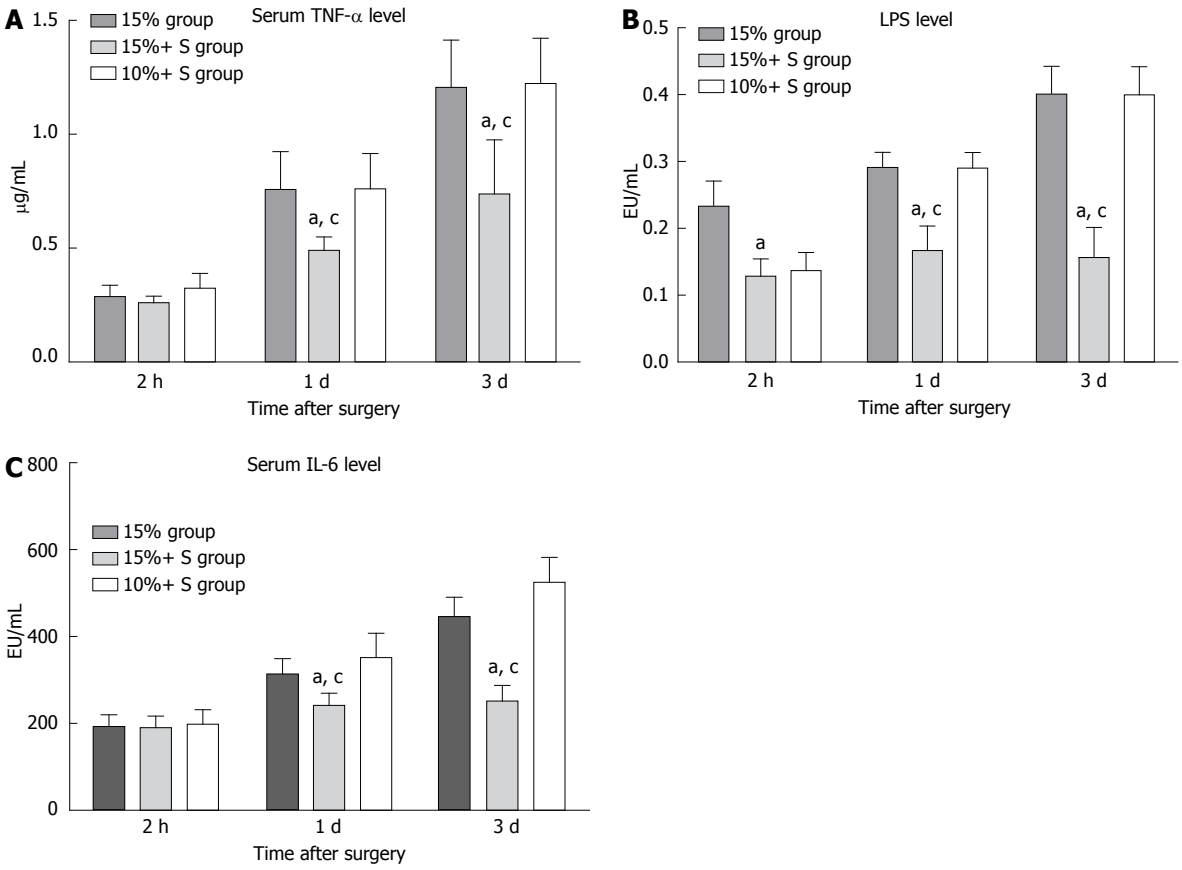

Figure 7 Serial changes in the serum lipopolysaccharides, tumor necrosis factor-α, and interleukin-6 level in three groups, in which significant differences were noted. A: Serum level of tumor necrosis factor-α (TNF-α); B: Serum level of lipopolysaccharides (LPS); C: Serum level of interleukin-6 (IL-6). [a]*P* < 0.05 *vs* 15% group; [c]*P* < 0.05 *vs* 10%+ S group.

indicating the optimum portal inflow and safe MLR in the 15%+ S group were important for recovery of liver energy metabolism (Figure 6).

However, MCS was a "double-edged sword". Excessive diversion of portal flow results in a portal pressure that is insufficient to promote liver regeneration[24,25,29,30]. It is well known that vascular shear stress in the portal vein is a major determinant factor of regeneration[25]. Therefore, diversion by MCS should be controlled. Hessheimer *et al*[29] demonstrated twice-baseline portal inflow was necessary for the functional recovery of a small-for-size liver graft. In the present study, the portal flow was preserved at approximately 3.2 times baseline to avoid portal hypoperfusion and benefit liver remnant regeneration. The portal flow was similar to that in 70% hepatectomy model which was supposed to be the optimum portal flow for liver regeneration[31]. It was also indentified that a portal inflow 3.2 times the baseline value can greatly stimulate the regeneration of the liver remnant without causing hyperperfusion injury.

In addition, the hepatic parenchyma contains an abundance of reticuloendothelial cells; after subtotal resection, the reticuloendothelial function declines, and portal hyperperfusion further promotes endotoxin absorption and bacterial translocation. Bacterial infection and bacteremia are serious complications that are frequently encountered in patients with subtotal hepatectomy. In this study, severe endotoxin or bacterial translocation in the 15% group was significantly elevated compared to the 15%+ S group, and serum TNF-α or IL-1 level was significantly elevated (Figure 7), also indicating that optimum portal decompression relieves portal overflow injury, and decreases the endotoxin/bacterial translocation[32], which play important roles in delaying liver remnant regeneration.

In summary, the decompression of portal vein can decrease the hyper-reperfusion injury, and make the marginal size hepatectomy safer. Therefore, the portal decompression modality should be considered when the risk of PHF or SFSS in hepatectomy is high or one-stage resection is adopted for the small future residual liver volume, for which portal venous embolism or two-stage resection is usually adopted.

COMMENTS

Background

Currently, there is no definitive answer to the question "How much liver excision is too much?". When the residual liver volume or graft is extremely small after extended hepatectomy or living-donor liver transplantation, postoperative hepatic failure (PHF) or small-for-size syndrome (SFSS) may ensue, and portal hypertension or hyperperfusion is regarded as the determinant factor of liver failure or SFSS.

Research frontiers

The authors demonstrated that mesocaval shunt could attenuate portal overflow injury, however, it is unknown how much the shunt can decrease, and whether the shunt can do the same for small liver remnants following subtotal hepatectomy.

Innovations and breakthroughs

The authors showed that portal vein decompression decreased hyper-reperfusion injury, and made "marginal size" hepatectomy safer, but did not reduce the safe value of the minimal residual volume (MRV) to < 5% of TLV.

Applications

The portal decompression modality should be considered when the risk of PHF or SFSS in hepatectomy is high, or one-stage resection is adopted for small future residual liver volume, in which portal venous embolism or two-stage resection is usually adopted.

Peer review

This study demonstrated that portal vein decompression decreased hyper-reperfusion injury, and made marginal size hepatectomy safer, but did not reduce the safe value of the MRV to < 5% of TLV. Therefore, the portal decompression modality should be considered when the risk of PHF or SFSS in hepatectomy is high, or one-stage resection is adopted for small future remnant liver volume.

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ORIGINAL ARTICLE

Effects of radix curcumae-derived diterpenoid C on *Helicobacter pylori*-induced inflammation and nuclear factor kappa B signal pathways

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Abstract

AIM: To study effect of diterpenoid C extracted from radix curcumae on *Helicobacter pylori* (*H. pylori*)-infected inflammation, intestinal metaplasia, and nuclear factor kappa B (NF-κB) signaling pathway *in vitro*.

METHODS: We used I-type *H. pylori* to infect human gastric epithelial gastric epithelium cell line (GES-1) cell lines, and then *H. pylori*-infected GES-1 cells were treated with radix curcumae (RC)-derived diterpenoid C of different concentrations (5, 10, 20 μg/mL) and amoxicillin. The expression of p65, IκB kinase (IKK) α and IKKγ proteins was detected with Western blotting, and the expression of interleukin (IL)-8, IL-6 and IL-4 was determined with enzyme-linked immunosorbent assay method. Data were analyzed using SPSS software ver18.0. For comparisons between groups of more than two unpaired values, one-way analysis of variance (ANOVA) was used. If an ANOVA *F* value was significant, *post hoc* comparisons were performed between groups. If results were not normally distributed, the Mann-Whitney *U* test was used to compare two groups of unpaired values, whereas for comparisons between groups of more than two unpaired values, the Kruskal-Wallis *H* test was used. Statistical significance was established at $P < 0.05$.

RESULTS: The MTT assay results revealed the inhibited rate of GES-1, and indicated that the IC5 of RC-derived diterpenoid C and amoxicillin all were 5 μg/mL for gastric GES-1 cells. The expression of IL-8 was significantly increased, especially at 12 h time point; and the expression of IL-4 was decreased in *H. pylori*-infected GES-1 cells. After *H. pylori*-infected GES-1 cells were treated with RC-derived diterpenoid C of different concentrations and amoxicillin, the expression of IL-8 was decreased at 12, 24, 48, 72 h points ($P < 0.01$), especially in high-concentration diterpenoid C (20 μg/mL) group; and the expression of IL-4 was increased, especially in moderate and high-concentration diterpenoid C (10 and 20 μg/mL) groups. RC-derived diterpenoid C had the inhibitory effects on *H. pylori*-induced p65 translocation from cytoplasm into cell nucleus, *H. pylori*-stimulant IkBα degradation, the phosphorylation of p65 and IkBα, and the expression of IKKα and IKKβ proteins.

CONCLUSION: RC-derived diterpenoid C can block NF-κB signal pathway, effectively reducing the secretion of *H. pylori*-induced proinflammatory cytokine and increasing the secretion of anti-inflammatory cytokine.

Key words: Radix curcumae-derived diterpenoid C; *Helicobacter pylori*; Nuclear factor-κB; Inflammatory cytokine

Core tip: Radix curcumae (RC), a common Chinese crude drug, has a wide range of pharmacological activity including hypolipidemic effect, hepatoprotective effect, anti-tumor, anti-radiation and anti-anaphylaxis. RC-derived diterpenoid C is recently obtained from RC ether extract by us, and its chemical properties and constitution are different from curcumin and β-elemene. Our results showed that RC-derived diterpenoid C can block nuclear factor kappa B signal pathway, effectively reducing the secretion of *Helicobacter pylori*-induced proinflammatory cytokine and increasing the secretion of anti-inflammatory cytokine. RC-derived diterpenoid C may become an effective drug for treatment of chronic gastritis.

Huang X, Lv B, Zhang S, Dai Q, Chen BB, Meng LN. Effects of radix curcumae-derived diterpenoid C on *Helicobacter pylori*-induced inflammation and nuclear factor kappa B signal pathways. *World J Gastroenterol* 2013; 19(31): 5085-5093 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5085.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5085

INTRODUCTION

Gastric carcinogenesis is usually believed to undergo the process including *Helicobacter pylori* (*H. pylori*) infection, chronic gastritis, atrophy, intestinal metaplasia, atypical hyperplasia abd gastric cancer[1]. *H. pylori* infection can bring to inflammation continuing through activating nuclear factor kappa B (NF-κB) signal pathway[2]. As *H. pylori* drug resistance becomes strong, it is difficult to eradicate *H. pylori*. How early to block the progression of chronic gastritis and to reduce gastric carcinogenesis is a main problem for us[3]. At present, there are no effective drugs for treatment of chronic gastritis. Our previous review has indicated that the total effective rate and pathological improvement (atrophy and intestinal metaplasia) are better in Chinese medicine group than in Western medicine group in the treatment of chronic gastritis[4]. But the mechanism of Chinese medicine is still unclear.

Radix curcumae (RC), a common Chinese crude drug, has a wide range of pharmacological activity including hypolipidemic effect, hepatoprotective effect, anti-tumor, anti-radiation and anti-anaphylaxis. RC-derived diterpenoid C is recently obtained from RC ether extract by us, and its chemical properties and constitution are different from curcumin and β-elemene. Our previous experiments have shown that RC-derived diterpenoid C has better anti-tumor activity and RC-derived diterpenoid C of high concentration can induce apoptosis[5,6]. Inflammation is strongly associated with tumor and the activation of some signal pathways occur in both inflammation and tumor[7,8], so we investigated the role of RC-derived diterpenoid C in anti-inflammation. Since biological properties are similar in gastric epithelium cell line (GES-1) cells and normal gastric epithelial cells, GES-1 cells were used in this study. The purpose of this study was to observe the effects of RC-derived diterpenoid C on inflammation, intestinal metaplasia and the expression of NF-κB signal pathway-related proteins in *H. pylori*-treated GES-1 cells.

MATERIALS AND METHODS

Materials

H. pylori strain, (CagA$^+$, VacA$^+$) NCTCl 1637 consistent with international standards, was purchased from China Disease Control and Prevention Center (Beijing, China). Human gastric epithelial GES-1 cells were purchased from the Institute of Cancer Research, Peiking University. RC-derived diterpenoid C (molecular weight: 380; molecular formula: C22H36O5) was provided by the College of Pharmacy, Zhejiang University (Hangzhou, China). Amoxicillin (molecular weight: 365.4) dispersible tablets with the batch number 63-110604 were from Xiansheng (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits was purchased from Nanjing KeyGey Biotech Co., Ltd. Primary antibodies were used. Horseradish peroxidasecoupled secondary antibodies were bought from Promega (Promega). The protein bands were detected employing electrochemi-luminescence chemiluminescence (Thermo Scientific).

Preparation of RC-derived diterpenoid C

Extraction of RC-derived diterpenoid C: RC-dried rhizome (10 kg) was used in extraction with 80 L of 95% ethanol, which was repeated four times to obtain 247 g of crude extract. After dispersion with 500 mL of water, the crude extract was respectively extracted with 500 mL of petroleum ether, dichloromethane and n-butanol to obtain 95.1 g of methylene bichloride. The methylene bichloride underwent silice gel column chromatography with petroleum ether/acetic ether (100:0, 100:10, 100:20, 100:30, 100:40, 100:50, 100:60, 100:70, 100:80 and 100:90), respectively, to obtain fractions A-J. The fraction E underwent chromatograph with acetonitrile/water (7:3) for 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70 and 70-80 min, respectively, to obtain subfractions E1-E8. The subfraction E8 underwent RP-HPLC with acetonitrile/water (45:55) as eluant to obtain diterpenoid C (5.0 mg, tR: 43.7 min). Its molecular structure was shown in Figure 1.

Preparation of diterpenoid C of different concentrations: RC-derived diterpenoid C was made into 10 mg/mL of stock solution with dimethyl sulfoxide (DMSO), and then stored at -20 ℃. The stock solution was diluted with fetal calf serum-free Dulbecco's Modification of Eagle's Medium (DMEM) containing high glucose for use in the experiment. DMSO concentration was controlled at 0.1% (volume percentage).

Cell culture

The tube containing frozen cells was placed in 37 ℃

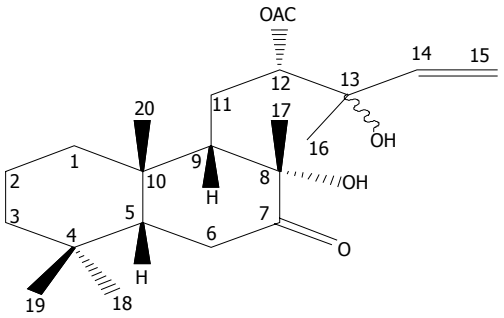

Figure 1 The molecular structure of radix curcumae-derived diterpenoid C. Originated from Huang *et al*[9], with permission.

water bath with constant shaking, and the frozen cells were melted within one minute. The tube was sterilized with 75% alcohol, and then quickly placed on a sterile bench for operation. After the tube was opened, cells were placed in high glucose-DMEM containing 10% fetal calf serum for incubation at 37 ℃ in an atmosphere of 5% CO_2. Next day, the medium was changed. When cells reached 80% confluence, cells were digested with 0.25% trypsin for passage. One passage was performed every 2-3 d and the cells after passage 3 were used in this experiment.

Preparation of viable H. pylori suspensions

NCTCI 1637 was incubated in Bushi-modified selective plating medium containing 10% yolk, 10% fetal calf serum, soluble amylum, vancomycin, trimethoprim, amphotericin and polymyxin B at 37 ℃ in an atmosphere of 85% nitrogen, 5% oxygen and 10% CO_2 for 3 d for future use. *H. pylori* was placed in 0.01 mol/L of PBS followed by quantitation with 752 type-spectrophotometer, and then diluted to 3.2×10^4-2.0×10^7 CFU/mL with RPMI1640 containing 2% fetal calf serum. The assays of Gram's stain, urease, katalase and oxidase were performed to confirm the presence of *H. pylori* before application.

Cell infection and intervention

Gastric epithelial GES-1 cells were cultured in an incubator containing antibiotics-free RPMI1640 with 10% fetal calf serum. Gastric epithelial GES-1 cells in logarithmic phase were digested with 0.25% trypsin for counting, and then were seeded in 96-well plate at 5×10^4/mL-1×10^5/mL. When cells reached 80% confluence, *H. pylori*-negative control group without *H. pylori* was set. After adherence of viable *H. pylori* suspensions, *H. pylori*/GES-1 cells (200:1) were incubated at 37 ℃ in an atmosphere of 5% CO_2 for 2 h, and then RC-derived diterpenoid C of different concentrations were added to incubate for 12, 24, 48 and 72 h, respectively, followed by observation on cell morphology under an electron microscopy. Three wells were set for each group. There were 3 RC-derived diterpenoid C groups with different concentrations, negative control group with 100 μL of RPMI1640 containing GES-1 cells, model group with *H. pylori* and positive control group with amoxicillin.

Inhibitory effects of RC-derived diterpenoid C and amoxicillin on GES-1 cell proliferation (MTT assay)

After GES-1 cells were incubated for 24 h, RC-derived diterpenoid C and amoxicillin (0, 5, 10, 20, 40, 80 ng/mL) were added for 24 h-culture. Three wells were set for each group. MTT (20 μL, 5 mg/mL) was added in each well for 3 h-incubation, and then the supernatant was taken followed by addition of 150 μL of DMSO. At the same time, the blank control group without RC-derived diterpenoid C and amoxicillin was set. Absorbance values were measured with a microplate reader (490 nm) for calculating inhibition rates. The inhibitory concentration 5% (IC_5) was adopted in the following experiments, and inhibitory rate (IR) was calculated as follows: IR = (A of control group - A of experimental group/A of control group) × 100%.

Cell morphology

The status of cell growth was observed under an optical microscope after GES-1 cells were incubated for 12, 24, 48 and 72 h, respectively.

Levels of IL-8 and IL-4 in cell supernatant determined with ELISA

We detect the level of IL-8 and IL-4 with ELISA methods according to the manufacturer's instructions.

Effects of RC-derived diterpenoid C on NF-κB signal pathways in H. pylori-induced GES-1 cell inflammation (Western blotting)

The effects of RC-derived diterpenoid C on the nuclear localization of NF-κB p65 were analyzed with Western blotting. Cells were divided into blank control group, model (*H. pylori*) group in which cells were treated for 60 min, and RC-derived diterpenoid C (20 μg/mL) + *H. pylori* group in which cells were first treated with RC-derived diterpenoid C for 2 h, and then infected with *H. pylori*. After nuclear proteins and cytoplasmic proteins were extracted, p65 protein in them was respectively determined. The effects of RC-derived diterpenoid C on the key proteins of NF-κB signal pathways were analyzed.

Statistical analysis

The experiments were repeated three times independently. Data were presented as the mean ± SD. Data were analyzed using SPSS software ver18.0. If the results were distributed normally, the two independent samples t test was used for comparison. For comparisons between groups of more than two unpaired values, one-way analysis of variance (ANOVA) was used. If an ANOVA F value was significant, *post hoc* comparisons were performed between groups. If results were not normally distributed, the Mann-Whitney U test was used to compare two groups of unpaired values, whereas for comparisons between groups of more than two unpaired values, the Kruskal-Wallis H test was used. Statistical significance was established at $P < 0.05$.

Table 1 Inhibition rates of radix curcumae-derived diterpenoid C on human gastric gastric epithelium cell line cell proliferation (n = 3)

Drug level (μg/mL)	Action time		
	24 h	48 h	72 h
Radix curcumae-derived diterpenoid C			
0 (negative control)	-	-	-
5	4.320% ± 0.056%	5.695% ± 0.657%	9.043% ± 0.121%
10	8.409% ± 0.879%	11.734% ± 0.547%	20.512% ± 1.098%
20	10.537% ± 1.098%	19.96% ± 2.093%	29.841% ± 2.345%
40	13.273% ± 0.897%	28.473% ± 5.093%	45.723% ± 5.876%
80	15.805% ± 0.975%	65.056% ± 6.098%	79.527% ± 6.879%
Amoxicillin			
0 (negative control)	-	-	-
5	6.671% ± 0.987%	7.935% ± 0.567%	10.769% ± 1.087%
10	8.325% ± 0.765%	14.769% ± 0.897%	19.130% ± 1.098%
20	9.731% ± 0.345%	18.530% ± 1.876%	29.154% ± 1.543%
40	12.929% ± 1.098%	25.691% ± 1.786%	31.832% ± 1.346%
80	14.953% ± 1.876%	38.427% ± 2.765%	43.790% ± 2.983%

RESULTS

Effects of RC-derived diterpenoid C and amoxicillin on GES-1 cell proliferation

As shown in Table 1 and Figure 1, RC-derived diterpenoid C and amoxicillin inhibited human gastric GES-1 cell proliferation in time and dose-dependent manners, namely that with the increase in drug concentration and the extension in drug action time, the inhibition rate was increased. The maximum un-cytotoxic concentration (IC_5) was 5 μg/mL. We adopted 5, 10, 20 μg/mL of RC-derived diterpenoid C as low, moderate and high-concentration diterpenoid C groups, and 5 μg/mL of moxicillin as drug-intervention group in the following experiments. The highest inhibition rate was 79.527% ± 6.879% obtained by 80 μg/mL of diterpenoid C with 72 h action time.

Effects of RC-derived diterpenoid C on human gastric GES-1 cell morphology

In bland group, GES-1 cells were polygon-shaped or spindle-shape with pseudopodia and island-like growth. Cells gradually were adherent. With prolonged incubation time, the number and density of cells were increased with a few floating cells (Figure 2A). In the GES-1 cells treated with *H. pylori* for 12 (Figure 2B), 24 (Figure 2C), 48 (Figure 2D) and 72 h (Figure 2E), cells became round; adherent cells were decreased and floating cells were increased; fragments occurred around cells; cell junction was reduced; the boundaries between cell nucleus and cytoplasm were obscure, and nucleus-cytoplasm fusion was seen. In the GES-1 cells treated with RC-derived diterpenoid C (5, 10, 20 μg/mL), adherent cells increased and cell morphology gradually recovered at 24 h (Figure 2F-I, respectively). Amoxicillin had no marked effects on cell morphology.

Effects of RC-derived diterpenoid C on H. pylori-induced human gastric GES-1 cell inflammation

Effects of RC-derived diterpenoid C on the secretion of IL-8: As shown in Figure 3A, after human gastric GES-1 cells were infected with *H. pylori*, IL-8 in the supernatant was significantly increased, especially at 12 h time point. With prolonged time, IL-8 level was gradually decreased. There were statistical differences in IL-8 levels at 12, 24, 48 and 72 h time points (all P = 0.000). After human gastric GES-1 cells were treated with diterpenoid C of different concentrations and amoxicillin, compared with model group, IL-8 level at each time point was significantly decreased with statistical significance.

Effects of RC-derived diterpenoid C on the secretion of IL-4: As shown in Figure 3B, after human gastric GES-1 cells were infected with *H. pylori*, IL-4 in the supernatant was significantly decreased with statistical differences compared with that at each time point of blank control group. After human gastric GES-1 cells were treated with diterpenoid C of low concentration, IL-4 level at each time point was increased, but P values at 12, 24, 48 and 72 h time points were 0.472, 0.550, 0.446 and 0.067, respectively, without statistical differences. After human gastric GES-1 cells were treated with diterpenoid C of moderate and high concentrations, IL-4 level at each time points was increased with statistical differences. After human gastric GES-1 cells were treated with amoxicillin, IL-4 level at each time point was increased, but their P values at 12, 24, 48 and 72 h time points were 0.092, 0.245, 0.446 and 0.053, respectively, without statistical differences. The results above suggest that the diterpenoid C of moderate and high concentrations can promote GES-1 cells to secrete IL-4, while amoxicillin has no the similar effect.

Effects of RC-derived diterpenoid C on NF-κB signal pathway activated by H. pylori in human gastric GES-1 cells

Nucleic localization of NF-κB p65: Our results indicated that 60 min after *H. pylori* infected human gastric GES-1 cells, p65 expression was increased in cell nucleus, but decreased in cytoplasm, suggesting that *H. pylori* can allow p65 translocation from cytoplasm to cell nucleus. In blank control group, there was a lot of p65 expression in cytoplasm. In high-concentration group of RC-derived diterpenoid C, p65 translocation was reduced, demonstrating that RC-derived diterpenoid C can inhibit p65 translocation from cytoplasm into cell nucleus induced by *H. pylori* (Figure 4).

Effects of RC-derived diterpenoid C on IkBα degradation caused by H. pylori

After GES-1 cells were respectively treated with *H. pylori* for 0, 15, 30, 60 and 90 min, cytoplasm was isolated to be used for determination of IkBα degradation with

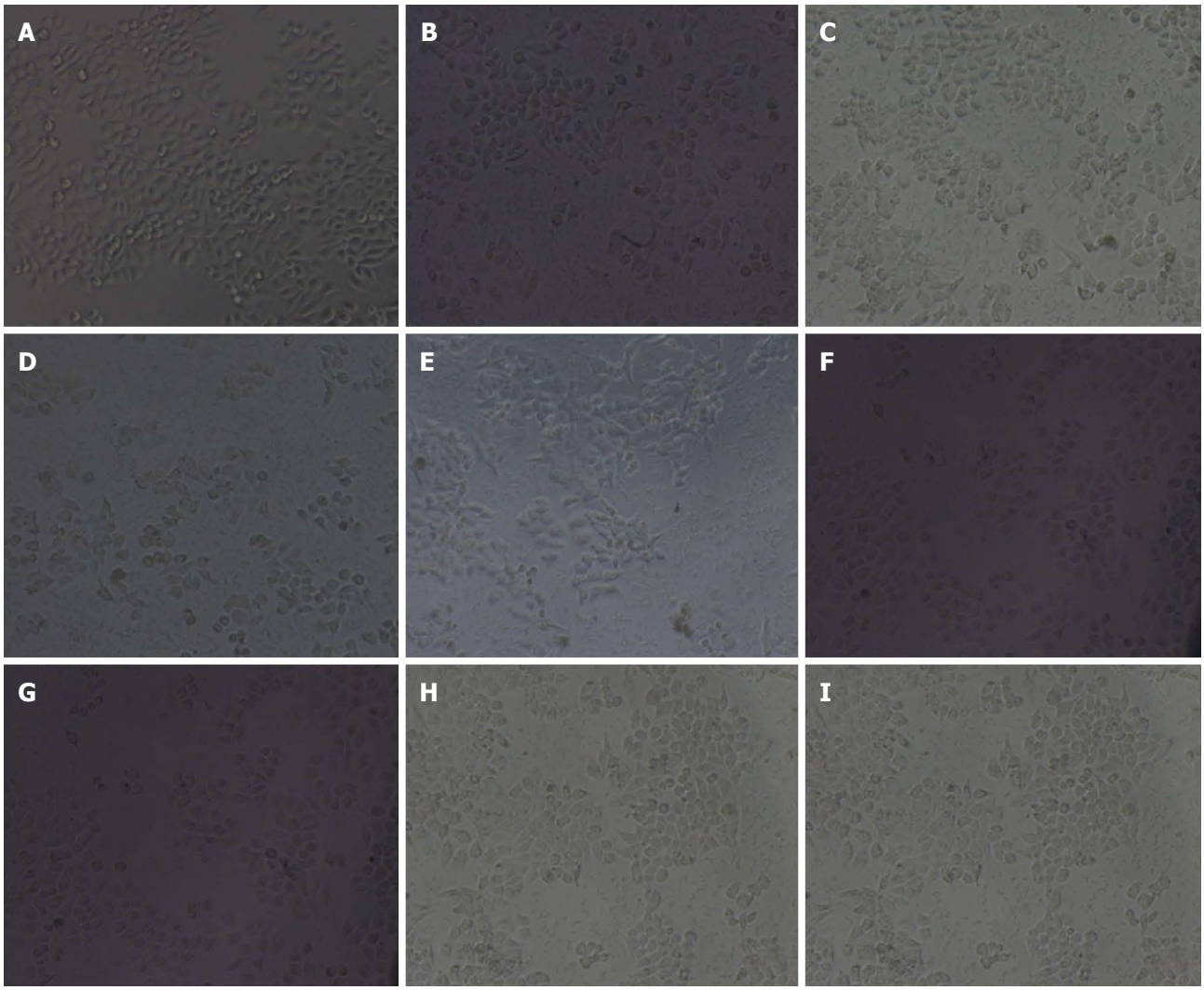

Figure 2 Gastric epithelium cell line cell morphology (× 200). In bland group, gastric epithelium cell line (GES-1) cells were polygon-shaped or spindle-shape with pseudopodia and island-like growth. Cells gradually were adherent. With prolonged incubation time, the number and density of cells were increased with a few floating cells (A). In the GES-1 cells treated with *Helicobacter pylori* for 12 (B), 24 (C), 48 (D) and 72 (E), cells became round; adherent cells were decreased and floating cells were increased; fragments occurred around cells; cell junction was reduced; the boundaries between cell nucleus and cytoplasm were obscure, and nucleus-cytoplasm fusion was seen. In the GES-1 cells treated with radix curcumae-derived diterpenoid C (5, 10, 20 μg/mL), adherent cells increased and cell morphology gradually recovered at 24 h (F-I, respectively). Amoxicillin had no marked effects on cell morphology.

Western blotting. Results indicated that IkBα began reducing at 15 min time point and was the lowest at 30 min time point; 60 min later, the decreased IkBα gradually recovered (Figure 5A and B). These results suggest that *H. pylori* can lead to IkBα degradation. Based on this, we observed the effects of RC-derived diterpenoid C on IkBα degradation caused by *H. pylori*, and foud that IkBα was basically unchanged. This suggests that RG-derived diterpenoid C can inhibit IkBα degradation caused by *H. pylori* (Figure 5C).

Expression of IkBα and p65 phosphorylated proteins, and IκB kinase α, IκB kinase β and p65 proteins

H. pylori rapidly induced phosphorylation of p65 and IkBα proteins. p65 phosphorylation was clearly seen at 5 min time point, and was the most strong between 15 and 30 min, and then gradually weakened. IkBα phosphorylation was seen at 5 min time point, and was the most strong at 15 min time point, and then gradually weakened. In a short time, the expression of p65, IκB kinase (IKK)α and IKKβ proteins was not markedly changed in *H. pylori* group. These results suggest that *H. pylori* is a good activator of NF-κB signal pathways. RC-derived diterpenoid C inhibited *H. pylori*-induced p65 and IkBα phosphorylation, decreased the expression of p65, IKKα and IKKβ proteins (Figure 6). These results indicated that RC-derived diterpenoid C decreased IkBα protein degradation through inhibiting phosphorylation of p65 and IkBα and the expression of IKKα and IKKβ proteins. RC-derived diterpenoid C may be an effective inhibitor of NF-κB.

DISCUSSION

Recent studies indicate that *H. pylori* activates NF-κB through two pathways. One pathway is dependent on Cag

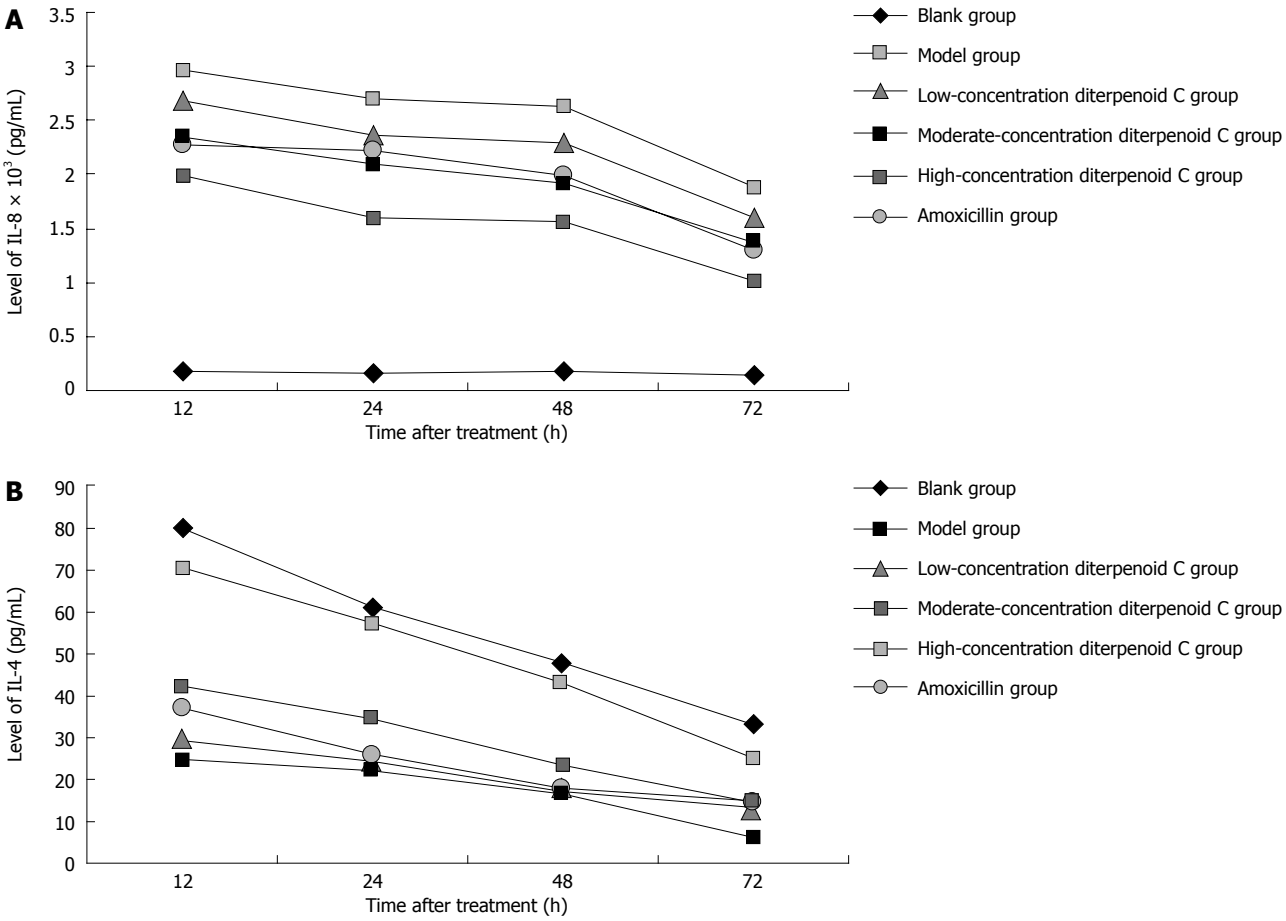

Figure 3 Effects of radix curcumae-derived diterpenoid C on *Helicobacter pylori*-induced human gastric epithelium cell line cell inflammation. A: The changes in the level of interleukin (IL)-8 in cell supernatant; B: The changes in the level of IL-4 in cell supernatant.

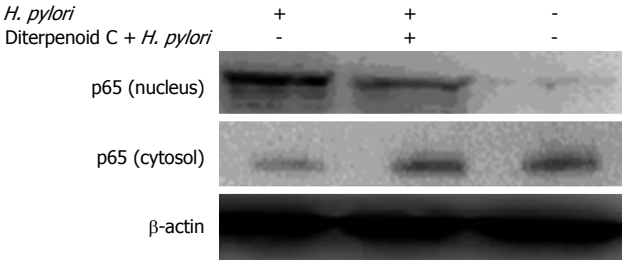

Figure 4 Effects of radix curcumae-derived diterpenoid C on nucleic localization of nuclear factor kappa B p65. *H. pylori*: *Helicobacter pylori*.

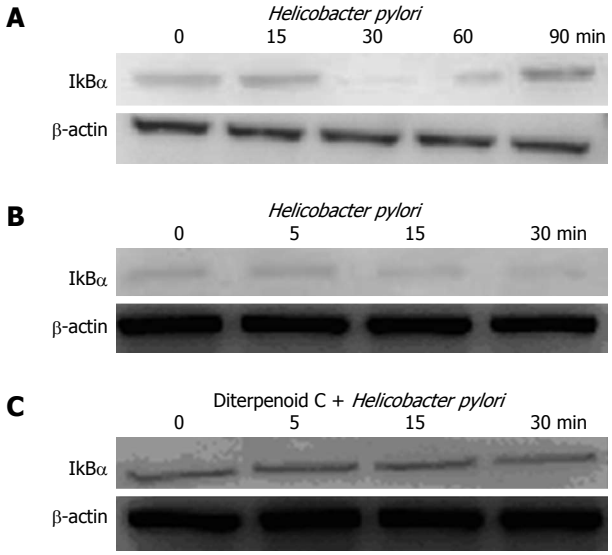

Figure 5 Effects of radix curcumae-derived diterpenoid C on IkBα degradation caused by *Helicobacter pylori*. A: After gastric epithelium cell line cells were respectively treated with *Helicobacter pylori* for 0, 15, 30, 60 and 90 min, cytoplasm was isolated to be used for determination of IkBα degradation with Western blotting; B: *Helicobacter pylori* for 0, 5, 15 and 30 min; C: Diterpenoid C + *Helicobacter pylori* for 0, 5, 15 and 30 min.

pathogenicity island (CagPAI), but independent of CD14 and interleukin-1 receptor-associated kinase. Another pathway is dependent on CD14 and toll-like receptor 4, but independent of CagPAI. *H. pylori* chiefly activates NF-κB classics approach. So it is important to p53 moving nuclear and IkBα degradation in NF-κB classics approach. In addition, *H. pylori* infection induces IkB-β attenuation. In gastric cancer cells, the activities of IkB-α and IkB-β are increase, and the phosphorylation of serine residues of IkB-α and IkB-β induces the degradation of regulatory proteins of NF-κB, activating NF-κB. *H. pylori* infection may induce gastric mucosal inflammatory, and increase the release of PGE2, IL-8 and ROS[10-12], the possible mechanism of which may be related to NF-κB pathways[13].

NF-κB, an important nuclear factor, is involved in cell

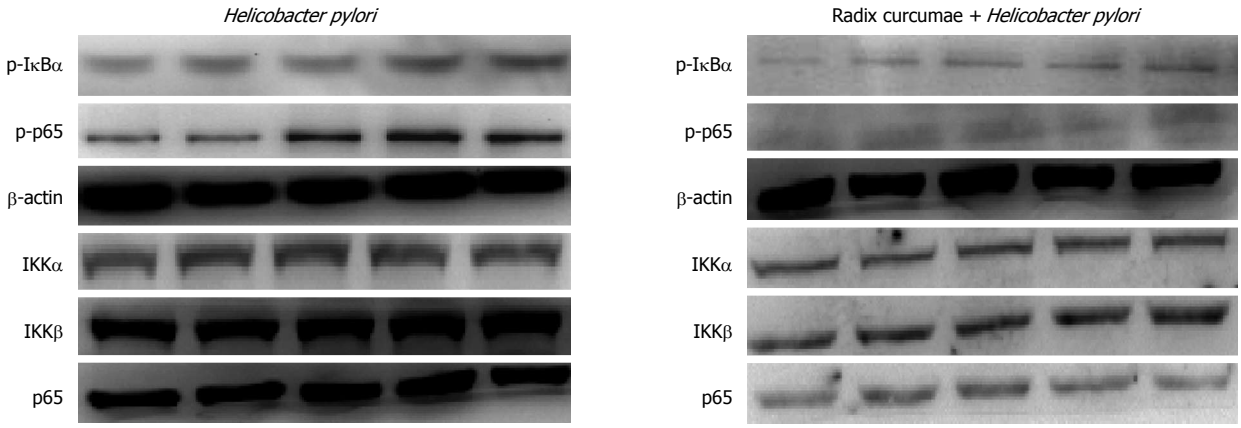

Figure 6 Effects of radix curcumae-derived diterpenoid C on the expression of nuclear factor kappa B proteins. p-IκBα: Phosphorylated IκBα; IKK: IκB kinase.

proliferation[14], immune response[15] and inflammation[16] through regulating the transcription of many genes[17]. In recent years, a great deal of attention has been paid to its role in inflammation and cancer[18,19]. Kim *et al*[20] believes that chronic inflammation is the seventh feature of tumor, chronic inflammation is strongly associated with tumor, and carcinogenesis is from the site of chronic inflammation. In some chronic inflammation-related tumors such as ulcerative colitis and colon cancer, chronic hepatitis and liver cancer, and chronic cervicitis and cervical cancer, NF-κB is found to be super-activated. NF-κB is an important molecule between chronic inflammation and tumor, and is regarded as a bridge between chronic inflammation and tumor.

Many studies have found that the curcumin, a main component of RC-ethanal extract, has highly effective anti-cancer activity with tumor cells[21-24], tumor-associated proteins[25,26], tumor-associated genes[27] and tumor-associated signal transduction pathways[28,29] as targets. It has been classified as the third-generation cancer-chemoprophylactic drug by United States National Cancer Institute. The elemene, a main component of RC-ether extract, can induce cancer apoptosis through down-regulating the expression of Bcl-2 and vascular endothelial growth factor, increasing the levels of cytochrome C and caspase-3 and blocking cell cycle progression[30-32]. Elemene emulsion with β-elemene as the main raw material has been widely used in the treatment of solid tumors, malignant hydrothorax and ascites, and metastasis tumor of brain[33,34]. However, the bioavailability of curcumin is lower, and elemene can produce vein injury, so their clinical application is limited. Therefore, due to this, we successfully obtained a new diterpenoid C from RC-ether extract, and its chemical constitution and properties are different from curcumin and elemene[35,36]. In this study, we explored the inhibitory effects of RC-derived diterpenoid C on *H. pylori*-induced GES-1 cell inflammation.

In this study, in the absence of stimulus, GES-1 cells secrete a tille cytokine. After GES-1 cells were treated with *H. pylori*, the levels of proinflammatory cytokins including IL-8 and IL-6 were significantly increased, and the the level of anti-inflammatory cytokine IL-4 was significantly decreased. RC-derived diterpenoid C was conducive to the balance between proinflammatory cytokines and anti-inflammatory cytokines. The possible mechanism is that RC-derived diterpenoid C has the cascaded inhibitory effects on the expression of IKKα and IKKβ, *H. pylori*-induced IkBα degradation, *H. pylori*-induced p65 translocation from cytoplasm into cell nucleus, the combination of p65 with inflammatory target genes and the release of inflammatory cytokins. Therefore, we infer that RC-derived diterpenoid C is an effective inhibitor of NF-κB.

In summary, RC-derived diterpenoid C, a newly effective anti-inflammatory factor, plays its role in *H. pylori*-infected GES-1 cells possibly through inhibiting NF-κB pathway. In view of the complexity of human life control and cell-signal transduction network, there may be more potential mechanisms about the anti-inflammatory effects of RC-derived diterpenoid C. Exploring RC-derived diterpenoid C to block the combination of NF-κB with its target gene with a reduction or elimination of cytokines has become a new idea to interrupt the progression of chronic gastritis into gastric cancer. This has important values in research and application.

COMMENTS

Background

Gastric carcinogenesis is usually believed to undergo the process including *Helicobacter pylori* (*H. pylori*) infection, chronic gastritis, atrophy, intestinal metaplasia, atypical hyperplasia abd gastric cancer. *H. pylori* infection can bring to inflammation continuing through activating nuclear factor kappa B (NF-κB) signal pathway. As *H. pylori* drug resistance becomes strong, it is difficult to eradicate *H. pylori*. How early to block the progression of chronic gastritis and to reduce gastric carcinogenesis is a main problem for them.

Research frontiers

At present, there are no effective drugs for treatment of chronic gastritis. Their previous experiments have shown that radix curcumae-derived diterpenoid C has better anti-tumor activity and radix curcumae (RC)-derived diterpenoid C of high concentration can induce apoptosis. Inflammation is strongly associated with tumor and the activation of some signal pathways occur in both inflammation and tumor, so the authors investigated the role of RC-derived diterpenoid C in anti-inflammation.

Innovations and breakthroughs

Since biological properties are similar in gastric epithelium cell line (GES-1) cells and normal gastric epithelial cells, GES-1 cells were used in this study. The purpose of this study was to observe the effects of RC-derived diterpenoid

C on inflammation, intestinal metaplasia and the expression of NF-κB signal pathway-related proteins in *H. pylori*-treated GES-1 cells. However, prior study is rare.

Applications

The study demonstrated RC-derived diterpenoid C to block the combination of NF-κB with its target gene with a reduction or elimination of cytokines has become a new idea to interrupt the progression of chronic gastritis into gastric cancer. This has important values in research and application.

Terminology

RC, a common Chinese crude drug, has a wide range of pharmacological activity including hypolipidemic effect, hepatoprotective effect, anti-tumor, anti-radiation and anti-anaphylaxis. RC-derived diterpenoid C is recently obtained from RC ether extract by us, and its chemical properties and constitution are different from curcumin and β-elemene.

Peer review

This paper showed that RC-derived diterpenoid C can block NF-κB signal pathway, effectively reducing the secretion of *H. pylori*-induced proinflammatory cytokine and increasing the secretion of anti-inflammatory cytokine. RC-derived diterpenoid C may become an effective drug for treatment of chronic gastritis.

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BRIEF ARTICLE

Acute effects of rotavirus and malnutrition on intestinal barrier function in neonatal piglets

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Abstract

AIM: To investigate the effect of protein-energy malnutrition on intestinal barrier function during rotavirus enteritis in a piglet model.

METHODS: Newborn piglets were allotted at day 4 of age to the following treatments: (1) full-strength formula (FSF)/noninfected; (2) FSF/rotavirus infected; (3) half-strength formula (HSF)/noninfected; or (4) HSF/rotavirus infected. After one day of adjustment to the feeding rates, pigs were infected with rotavirus and acute effects on growth and diarrhea were monitored for 3 d and jejunal samples were collected for Ussing-chamber analyses.

RESULTS: Piglets that were malnourished or infected had lower body weights on days 2 and 3 post-infection ($P < 0.05$). Three days post-infection, marked diarrhea and weight loss were accompanied by sharp reductions in villus height (59%) and lactase activity (91%) and increased crypt depth (21%) in infected compared with non-infected pigs ($P < 0.05$). Malnutrition also increased crypt depth (21%) compared to full-fed piglets. Villus:crypt ratio was reduced (67%) with viral infection. There was a trend for reduction in transepithelial electrical resistance with rotavirus infection and malnutrition ($P = 0.1$). ^{3}H-mannitol flux was significantly increased (50%; $P < 0.001$) in rotavirus-infected piglets compared to non-infected piglets, but there was no effect of nutritional status. Furthermore, rotavirus infection reduced localization of the tight junction protein, occludin, in the cell membrane and increased localization in the cytosol.

CONCLUSION: Overall, malnutrition had no additive effects to rotavirus infection on intestinal barrier function at day 3 post-infection in a neonatal piglet model.

Key words: Rotavirus gastroenteritis; Kwashiorkor; Occludin; Ussing chamber; Villus

Core tip: We are quite excited about these results which suggest involvement of intestinal tight-junction proteins in the pathology of rotaviral gastroenteritis. The work further examines the interplay of malnutrition superimposed on viral infection. ^{3}H-mannitol flux was significantly increased in rotavirus infected piglets compared to non-infected piglets, but there was no effect of nutri-

tional status. Furthermore, rotavirus infection reduced localization of the tight junction protein, occludin, in the cell membrane and increased localization in the cytosol. This extends work on the molecular mechanisms of rotavirus in the neonatal intestine that we previously published.

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INTRODUCTION

Pediatric diarrheal diseases are the second-leading cause of childhood mortality, and responsible for about 1.34 million deaths each year in children under 5 years of age[1]. Rotaviruses are the most common causes of acute, severe gastroenteritis and dehydrating diarrhea. Furthermore, rotavirus-associated enteritis represents a class of zoonotic diseases that cause major health concerns not only for humans, but also most domestic livestock species[2]. The food animal livestock industry estimated a multi-million dollar annual economic loss due to diarrheal diseases associated with a reduction in weight gain, treatment and death of young animals[2]. In addition to the mortality rates associated with diarrheal disease there is a about 60% increase in pediatric patient mortality rates when diarrheal disease is compounded with malnourishment[3]. In the neonatal piglet model, Zijlstra *et al*[4] demonstrated that malnutrition extends rotavirus infections up to a week longer in malnourished piglets compared with well nourished infected piglets.

Rotaviruses infect the differentiated epithelial cells of the mid- to upper-villus of the small intestine[5]. The infection is associated with cell death, reduced villus surface area, loss of absorptive capacity, osmotic deregulation, and infiltration of the lamina propria by mononuclear cells[6,7]. In pigs, acute viral injury to enterocytes leads to increased epithelial cell loss and intestinal lesions leaving the intestinal epithelial barrier compromised[6]. Rapid restoration of epithelial continuity is important following injury and depends on the migration of uninjured enterocytes from the crypts to cover the compromised barrier. Protein-energy malnutrition (PEM) also decreases intestinal barrier function and integrity, increasing bacterial translocation with subsequent enteritis and diarrhea[8]. Moreover, PEM also inhibits epithelial crypt cell proliferation which delays cellular migration along the crypt-villus axis and results in longer repair periods[9].

Intestinal barrier function is maintained in part by actual physical links between enterocytes by intercellular junction complexes. Tight junctions are located on the uppermost basolateral surface of polarized enterocytes and regulate diffusion between cells. They allow the epithelia to form a cellular barrier separating the luminal content of the intestine from the lamina propria. In cell culture models using Madin-Darby Canine Kidney and Caco-2 cells, studies have demonstrated dysregulation of the paracellular pathways[10,11]. In fact, rotavirus infection in these cells caused alterations in tight junction structure and function related to epithelial cell resistance and permeability. The authors determined that there was a time dependent disruption in localization of tight junction proteins claudin-1, occludin and zonula-occuluden when Caco-2 cells were infected with rotavirus[10,11]. Claudin-1 was the first tight-junction protein to become solubilized in the cytosol of the epithelial cells[11].

Nutritional factors have been shown to impact neonatal intestinal health[12]. In particular, our laboratory has investigated how dietary components impact intestinal health in neonatal piglets with rotavirus infection[13-15]. We have demonstrated supplemental dietary arginine activates mammalian target of rapamycin, mitogen-activate protein kinase, and ribosomal p70S6 kinase signaling in rotavirus infected enterocytes[15]. These cell signaling mechanisms lead to increase jejunal protein synthesis, cell migration and intestinal restitution in rotavirus infected piglets[13,14]. Moreover, we have demonstrated the value of dietary plasma protein because it maintained growth rates, reduced diarrhea, and maintained enzymatic activity in the small intestine of neonatal pigs with rotavirus infection[14]. Additionally, others have shown soy-based infant formula isoflavones are effective in reducing rotavirus infectivity in cell culture models of rotavirus infection[16]. These reports demonstrate the importance of nutritional factors involvement in modulation of host immune response and repair mechanisms associated with rotaviral infection. Therefore, the pathophysiological mechanisms of rotavirus infection and its diarrheal mechanism are the focus of much work toward developing effective vaccines and nutritional treatments for the virus. Understanding the viral interruption of paracellular pathways and the impact of nutritional status on these pathways is critical in the development of adequate medical treatment.

MATERIALS AND METHODS

Animals and experimental design

All protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University. The full experimental protocol was previously reported in Rhoads *et al*[15]. Briefly, 24 piglets were collected directly from the birth canal, colostrum deprived, cleaned with 70% ethanol and transported to a biosecure rearing facility. Piglets were individually housed and contained in two rooms with a temperature of 32 ℃. Pigs were fed milk diet *via* a gravity flow feeding system, adapted from Oliver *et al*[17]. The formula composition was previously reported by Rhoads *et al*[15]. A liquid colostrum diet (La-Belle Associates, Inc., WA, United States) was fed for the first 24 h to provide passive immunity. Feedings (about 300 mL/kg body weight per day) were offered four times

per day (8:00 am, 1:00 pm, 6:00 pm and 11:00 pm), and non-infected pigs were pair-fed to the level of their infected counterparts.

We compared well-nourished and malnourished piglets (n = 16) in a 2 × 2 factorial design examining effects of malnutrition and viral infection as follows: (1) full-strength formula (FSF) (180 g/L), non-infected (positive control); (2) FSF, rotavirus infected; (3) half-strength formula (HSF, 90 g/L), non-infected; or (4) HSF, rotavirus infected (negative control). Intestinal samples from this study were collected only on day 3 post-infection.

Rotavirus inoculation and clinical measurements

Rotavirus inoculation and clinical measures were previously described by Rhoads *et al*[15]. Briefly, the rotavirus inoculum, initially isolated by Lecce *et al*[18], was passaged through colostrum-deprived pigs and prepared as a bacteria-free intestinal supernatant. Approximately 10^7 particles of rotavirus or sham inoculants were suspended in full strength milk formula, and piglets were gastrically intubated at 10:00 am on day 0.

Piglet weights, feed intakes, and fecal consistency were recorded daily. Feces were given a diarrhea score of 0, 1, 2 or 3, corresponding with firm, soft but formed, runny, and severe watery diarrhea, respectively, by a single individual blinded to treatments. A rectal swab was collected daily from each piglet for the detection of rotavirus shedding (Virogen Rotatest; Wampole Laboratories, Cranbury, NJ, United States).

Intestinal sampling

On day 3 post-infection, pigs were anesthetized with isoflurane and killed by the AVMA approved electrocution followed by exsanguination. Intestinal samples from the mid-jejunum area were collected, snap frozen and stored at -80 ℃ until analysis by Western blotting. Intestinal segments were collected and fixed for histological analysis of intestinal morphology[17]. Intestinal morphology and lactase measurements were performed as previously described[17].

Ussing chamber measurements

Segments of mid-jejunum were harvested from the pigs and the mucosa was stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) Ringer's solution. Tissues were mounted in 1.14 cm^2 aperture Ussing chambers, as described previously[19]. Tissues were bathed on the serosal and mucosal sides with 10 mL Ringer's solution. The serosal bathing solution contained 10 mmol/L glucose, which was osmotically balanced on the mucosal side with 10 mmol/L mannitol. Bathing solutions were oxygenated (95% O_2/5% CO_2) and circulated in water-jacketed reservoirs maintained at 37 ℃. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Transepithelial electrical resistance ($\Omega \cdot cm^2$) was calculated from the spontaneous PD and the short-circuit current (I_{sc}), as previously described[19].

Mucosal permeability was assessed following experimental treatments by adding 0.2 μCi/mL ^{3}H-mannitol on the mucosal side of the Ussing chamber-mounted tissues and measuring the flux to the serosal compartment. Following a 15 min equilibration period samples were collected from the mucosal side of the chamber and following a 60 min flux period samples were collected from the serosal side of the chamber as previously described[20]. The concentration of ^{3}H-mannitol was quantified using a liquid scintillation counter (LKB Wallac, model 1219 Rack Beta, Perkin Elmer Life and Analytical Sciences, Boston, MA, United States). The directional flux of ^{3}H-mannitol from the mucosal to serosal chamber were determined by using the mannitol specific activity added to the mucosal bathing solution and calculating the net appearance of ^{3}H-mannitol over time in the serosal bathing solution.

Protein isolation and Western blotting analysis

Intestinal mucosa scrapings from all animals were snap frozen and stored at -80 ℃ for SDS-PAGE analysis. Triton X-soluble and X-insoluble fractions were prepared as previously described[21]. Briefly, samples were homogenized in Triton X-soluble buffer and allowed to rest on ice for 30 min with intermittent vortexing. Thereafter, the homogenates were centrifuged at 400 g for 10 min at 4 ℃ to remove cell debris. The supernatant was removed to a new tube and centrifuged at 9000 g for 10 min at 4 ℃ to separate the soluble and insoluble protein fractions. The insoluble fraction pellet was dissolved in Triton X-insoluble fraction extraction buffer by heating at 95 ℃ for 5 min with intermittent vortexing. Protein concentrations of tissue extracts were determined using the DC protein assay (Bio-Rad; Hercules, CA, United States). Tissue extracts of equal protein concentrations were mixed with equal volumes of 2 × Laemmli Sample Buffer (Bio-Rad; Hercules, CA, United States) and boiled for 5 min. Protein lysates were loaded on a 12% SDS polyacrylamide gel, and electrophoresis was completed as recommended for Bio-Rad Criterion™ gels. Proteins were transferred to polyvinylidene diflouride membrane (Immobilon-S; Millipore, Billaerica, MA, United States) by Criterion™ Blotter (Bio-Rad; Hercules, CA, United States). Membranes were blocked and incubated with primary and secondary antibodies as previously reported by Moeser *et al*[21].

Statistical analysis

Data were analyzed using the general linear models procedure of SAS (Cary; NC 27513, United States) appropriate for a 2 ×2 factorial design, with feeding level (FSF *vs* HSF) and infection (± rotavirus) as the factors.

RESULTS

Animal observations

Body weight for 1-d-old pigs was 1.4 ± 0.2 kg. The pigs were adapted to the feeding system until day 5 when they were switched to either FSF (well-nourished) or

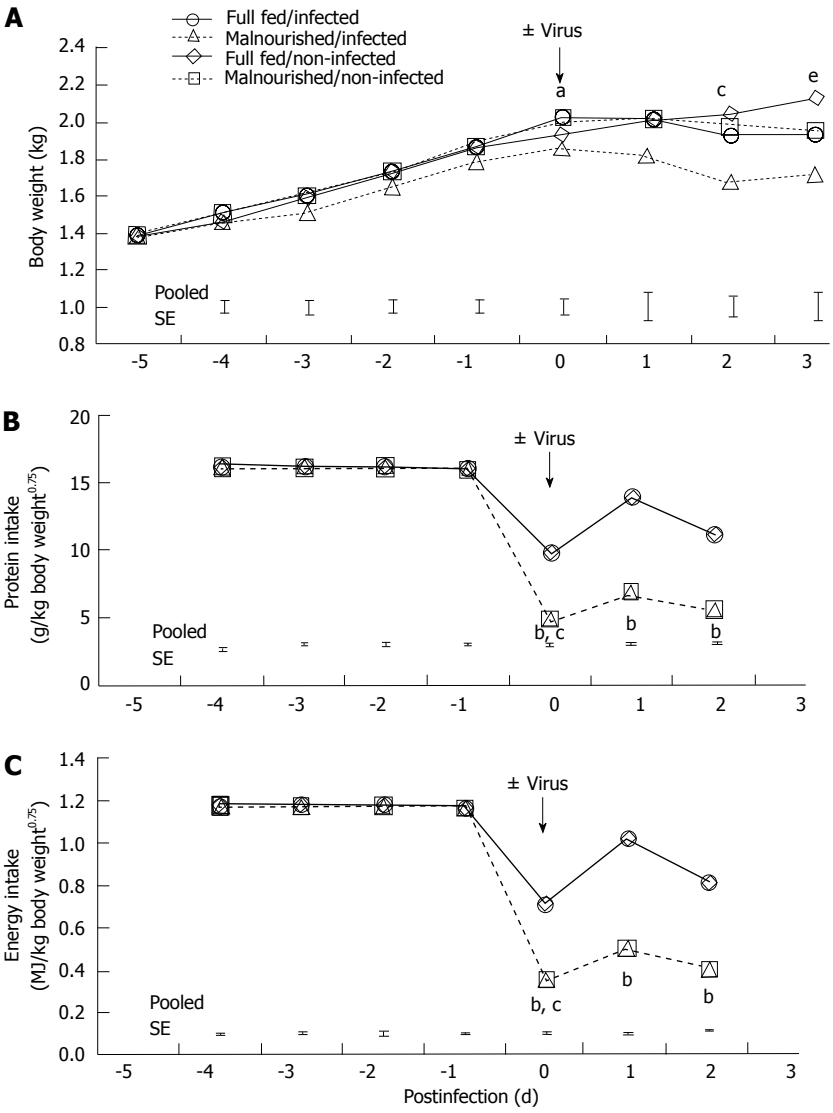

Figure 1 Growth, protein intake and metabolizable energy intake of newborn pigs fed full-strength formula (full fed) or half-strength formula (malnourished) and inoculated with rotavirus or vehicle (non-infected) as indicated. Values are reported as least-square means (n = 6/treatment), and error bars represent ± pooled SE per day. A: Piglet growth curves, calculated using initial body weight as a covariate. [a]$P < 0.05$, feeding-level/rotavirus interaction; [c]$P < 0.05$, feeding-level; [e]$P < 0.05$, rotavirus effect; B, C: Protein intakes (B) and metabolizable energy intakes (C) of newborn pigs over time. Non-infected pigs were pair-fed to their rotavirus-infected counterparts. [b]$P < 0.01$, feeding level effect; [c]$P < 0.05$, rotavirus effect.

HSF (malnourished) diets. Twenty-four hours after pigs were assigned to dietary treatments they were inoculated with rotavirus. There was a feeding level by infection interaction on day 0 (Figure 1A; $P < 0.05$). The interaction was due to the drop in body weight of the HSF/infected piglets compared with pigs in other dietary treatment groups. On days 1-3 post-infection there was no interaction of feeding level and infection. However, on days 2 and 3 post-infection there were main effects of both feeding level and infection. Full-strength formula/non-infected pigs maintained a higher body weight compared HSF/infected pigs ($P < 0.05$). Feed intake of non-infected pigs was pair-fed to the level of their infected counterpart, so there were no major differences in protein and energy intake between the non-infected and infected pigs from the same dietary treatment (Figure 1B and C). Malnourished pigs received a 50% reduction in nutrient intake, but daily intakes of water, sodium, potassium and chloride were similar to full-fed pigs, because electrolyte solution was used for formula dilution. The significant effect of viral infection on body weight on day 3 could be related to multiple factors, however, we have controlled nutrition by pair feeding, and measured growth, so the most likely cause of weight loss was diarrheic water loss. Pigs had no diarrhea prior to rotavirus inoculation (data not shown). However, viral infection resulted in diarrhea scores of 3 (severe, watery diarrhea) for both rotavirus infected groups. On day 3 post-infection there was a main effect of virus and feeding level on diarrhea score ($P < 0.01$ and $P < 0.05$, respectively; Figure 2A); however, there was no additive effect of malnutrition. Additionally, rotavirus-inoculated pigs had a significant increase in viral shedding from days 1 to 3 post-infection (Figure 2B).

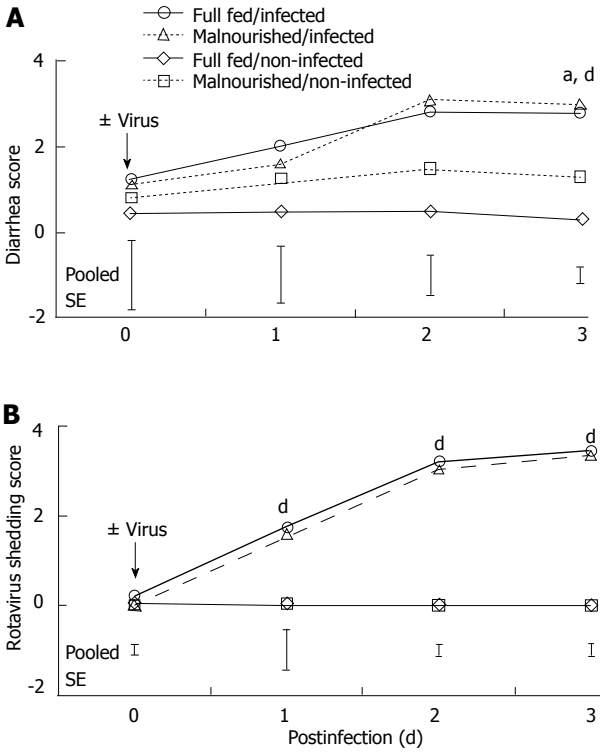

Figure 2 Daily diarrhea (A) and rotavirus shedding scores (B) measured in newborn pigs fed full-strength formula (full fed) or half-strength formula (malnourished) and inoculated with rotavirus or vehicle (non-infected) as indicated. Values are reported as least-square mean (n = 6/treatment), and error bars represent ± pooled SE per day. [a]$P < 0.05$, feeding level effect; [d]$P < 0.01$, rotavirus effect.

Intestinal lactase activity and morphology

Rotavirus infected pigs had significantly reduced lactase activity (Table 1; $P < 0.05$) on day 3 post-infection. In addition, there was a main effect of virus on villus height, crypt depth and villus height:crypt depth ratio (Figure 3 and Table 1; $P < 0.05$). There also was a main effect of feeding level on crypt depth with malnourished pigs having a greater depth than FSF pigs (Figure 3 and Table 1; $P < 0.05$). However, there was not a significant interaction between rotavirus and feeding level on intestinal lactase or morphology.

Intestinal barrier function

Transepithelial electrical resistance (TER) data were recorded on mid-jejunum tissues from pigs on each dietary and viral treatment (Figure 4A). There was no significant interaction of feeding-level by virus and no main effects on TER data. However, there was a trend for FSF/rotavirus infected pigs to have decreased TER compared with FSF/non-infected pigs (Figure 4A; $P = 0.1$). Malnourished animals had similar TER readings regardless of rotavirus infection.

To assess the effect of nutritional status and rotavirus infection on intestinal permeability, ^{3}H-manitol flux was measured on mid-jejunum tissues from pigs. The feeding-level by rotavirus infection interaction was not significant (Figure 4B). However, there was a main effect of rotavirus infection on intestinal permeability ($P < 0.05$). Pigs infected with rotavirus had 50% greater intestinal permeability than non-infected pigs. Conversely, there was no main effect of feeding level on intestinal permeability.

Western blotting for the tight-junction protein, occludin, demonstrated that rotavirus infected pigs had greater quantity of occludin protein in the soluble fraction of protein than in the insoluble fraction (Figure 5). Additionally, non-infected pigs had no occludin protein

Table 1 Jejunal lactase activity and morphology of piglets fed full-strength formula (fully fed) or half-strength formula (malnourished) and infected or non-infected with rotavirus

	Fully fed		Malnourished			
	Non-infected	Infected	Non-infected	Infected	SE	Effect[1]
Lactase activity [mmol/(min·g protein)]	169.8	9.6	160.3	19.7	21.0	V
Intestinal morphology						
Villus height (mm)	0.87	0.37	0.85	0.34	0.13	V
Crypt depth (mm)	0.10	0.13	0.13	0.16	0.01	V, F
Height:depth ratio	8.96	3.12	6.86	2.12	1.24	V

Values are least-square means for n = 6 pigs per group, measured 3 d post-inoculation. [1]V, main effect ($P < 0.05$) of rotavirus infection; F, main effect ($P < 0.05$) of feeding level; Interaction of rotavirus and feeding level (V, F) was not detected ($P > 0.05$).

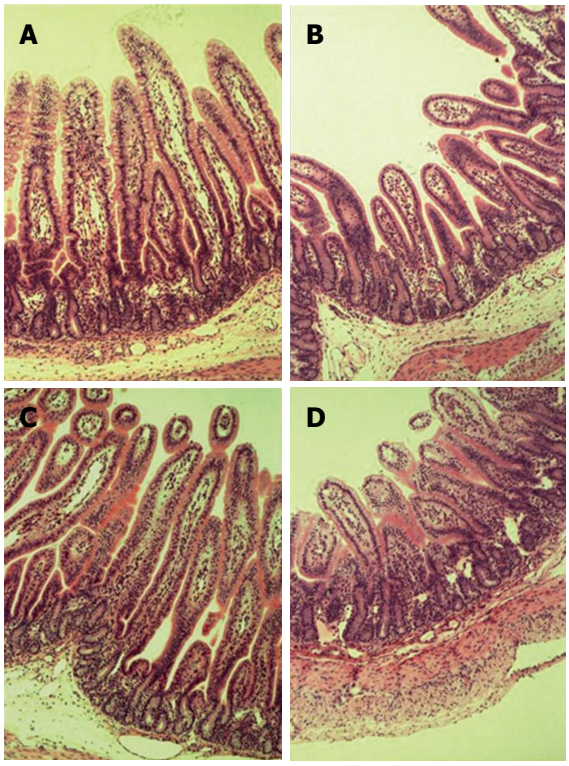

Figure 3 Hematoxylin and eosin stained intestinal sections from newborn pigs fed full-strength formula (full fed; A and B) or half-strength formula (malnourished; C and D) and inoculated with rotavirus (B and D) or vehicle (non-infected; A and C) as indicated (× 10 magnification). Numerical measurements and statistical analysis of the intestinal morphology are reported in Table 1.

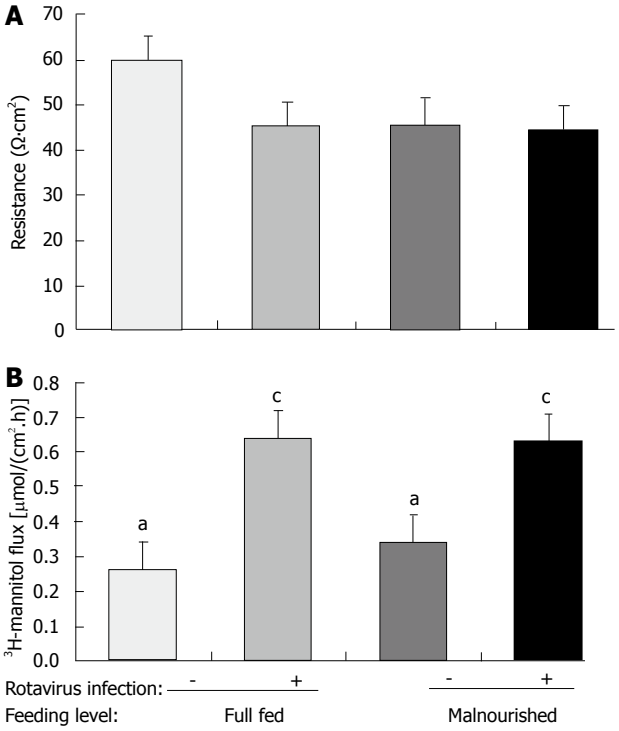

Figure 4 Transepithelial electrical resistance (A) and ^{3}H-mannitol flux (B) in jejunal mucosa from new-born pigs fed full-strength formula (full fed) or half-strength formula (malnourished) and inoculated with rotavirus or vehicle (non-infected) as indicated. Measurements were made 3 d post-inoculation. Values are reported as least-square means (n = 6/treatment), and error bars represent pooled SE. $^{a}P < 0.05$, $^{c}P < 0.05$ between groups.

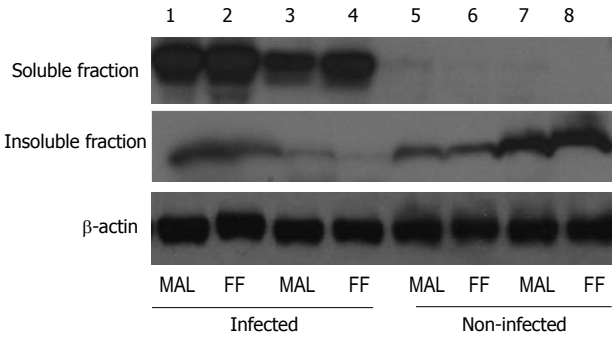

Figure 5 Occludin Western blotting analysis of jejunal tissues from new-born pigs fed full-strength formula or half-strength formula and inoculated with rotavirus or vehicle as indicated. Tissues were collected 3 d post-inoculation. Full-strength formula [full fed (FF)]: Lanes 2, 4, 6, 8; Half-strength formula [malnourished (MAL)]: Lanes 1, 3, 5, 7; Infected rotavirus: Lanes 1-4; Non-infected vehicle: Lanes 5-8; Upper panel: Soluble protein.

in the soluble fraction and higher levels of occludin in the insoluble fraction. As was observed in TER and flux measures of barrier function, feeding level did not alter the cellular localization of the tight-junction protein occludin (Figure 5).

DISCUSSION

Rotavirus gastroenteritis accounts for 30%-40% of pediatric diarrheal deaths worldwide[22], and PEM is also a primary cause of childhood morbidity and mortality especially in developing nations[1,23]. While rotavirus vaccine safety and efficacy has improved over the last 10 years for children in developed countries, the efficacy of rotavirus vaccine for children in poor settings is compromised due to environmental factors associated with reduction in vaccine effectiveness[24]. Our laboratory and others working in neonatal piglet models have sought to determine possible nutritional therapies which could reduce the severity of the infection or enhance the effectiveness of vaccines[13-16]. Nutritional therapies will be affected by previous nutritional status of individuals, and nutritional deprivation is a key component to overcome for treatment of enteric diseases afflicting impoverished children. In mouse models of rotavirus infection and malnutrition there is a significant increase in gut permeability to environmental macromolecules, as well as a significant decrease in minimal infectious dose needed to produce diarrhea[25,26]. Therefore, understanding the mechanisms associated with gut barrier function under normal rotavirus infection as well as rotavirus infection compounded by malnutrition is an important component for developing efficacious treatment and prevention strategies in young children and animals.

The purpose of this study was to examine the effects of PEM and rotavirus infection on intestinal barrier function in neonatal piglets. Diarrhea and malnutrition are two major problems in pediatric patients and a better understanding of the interaction and underlying mechanisms may lead to improved treatment. The study design was a 2 × 2 factorial with two levels of nutrition and either non-infected or infected with rotavirus.

Rotavirus infection caused decreased body weight, reduced protein intake, reduced energy intake, diarrhea, decreased lactase activity, trends for decreased jejunum TER, increased jejunum permeability, and decreased cell membrane localization of occludin. However, malnutrition did not have a significant additive effect to rotavirus infection on intestinal barrier function measured 3 d post-infection. The lack of additive effects on malnutrition is most likely related to the time line of the study. The 3 d post-infection time point may have been too short to evaluate repair of the intestine in this model. It is also possible that if we had applied the nutritional treatment prior to rotavirus infection there might have been a significant effect of PEM in the piglets.

Previously, we have shown that rotavirus infection causes intestinal damage and diarrhea within 2 d post-infection in the neonatal piglet model[4]. Additionally, the effects of the viral infection began to subside nearly 1 wk earlier in full-fed pigs than in the malnourished, infected pigs[4]. Herein, we report similar results of rotavirus infection causing significant weight loss and increased diarrhea by day 2 post-infection. Although there was a significant interaction of feeding level and infection on weight loss on day 0 of inoculation the interaction was not sustained for the next 3 d post-infection. However, the main ef-

fects of rotavirus and feeding level on weight loss were significant on days 2 and 3 post-infection. The reduced weight gain, increased diarrhea scores and increased viral shedding were expected following inoculation. However, we did anticipate there would be additive effects of malnutrition on all three outcomes, which we did not observe. Others have shown that PEM alters physiological and immunological properties of the intestine leaving individuals more susceptible to diarrhea associated illnesses[25-29]. Nevertheless, this bidirectional relationship between PEM and susceptibility to enteric pathogens in pediatric patients is related to the type of pathogen and many environmental factors playing a significant role in susceptibility to infection[30]. We may have underestimated the time needed to detect a plain of nutrition response with rotavirus infection. In fact, in mouse models of malnutrition and intestinal permeability the dietary treatments were applied to the mice a minimum of 5 d before rotavirus inoculation, and the research showed increased ovalbumin absorption in malnourished, infected mice compared to well nourished, infected mice[25].

Dietary nutrients are essential for gastrointestinal growth and health[12]. Malnutrition reduces the integrity of intestinal epithelium, facilitating bacterial translocation with subsequent enteritis and diarrhea[8], and it can also impair epithelial cell proliferation in crypts of the small intestine, resulting in delayed cellular migration along the crypt-villus axis[9]. This impairment is inhibitory to intestinal repair processes associated with gastrointestinal enteritis. We found that PEM did not further reduce lactase activity or villus blunting in the small intestine beyond the reduction seen with rotavirus infection alone. Additionally, crypt depth was increased by PEM and viral infection, but there was no additive effect. Previously, Zijlstra *et al*[4] reported a reduction in crypt depth with infected, malnourished pigs compared to infected, full-fed pigs. However, our data suggest that HSF/infected pigs had increased crypt depth compared to all other treatment groups. This may be related to the exact location of sampling between our study and Zijlstra *et al*[4]. Zijlstra *et al*[4] found the reductions in crypt depth were more distal in the small intestine on day 2 post-infection, and the reduction in crypt depth in the proximal to mid small intestine was not significant until day 9 post-infection. Additionally, in piglet models of transmissible gastroenteritis, increased jejunal crypt depth following infection have been reported[31-33].

Maintenance of migrating crypt cells is essential in maintaining gut barrier function following intestinal insult to seal the basement membrane and close leaky epithelial intercellular spaces and tight junctions. Rotavirus is known to disrupt tight junctions and decrease TER in Caco-2 cells between 8 and 24 h post-infection. We found *in vivo* treatments of rotavirus showed a trend for reduced TER in mid-jejunal tissue from neonatal pigs. TER data showed similar resistance between infected pigs and HSF/uninfected pigs; however there was not an additive effect of malnutrition and rotavirus infection in the neonatal pigs. Serosal to mucosal flux of ^{3}H-mannitol was significantly greater for infected pigs with no effect of PEM on mucosal paracellular permeability. The numerical reduction in TER for all treatment groups except FSF/non-infected piglets does not completely align with the differences in mannitol flux we observed, and this could be explained by recent understandings of tight junction pore and leak pathways[34]. Piglets in the full-fed/infected groups had diarrhea accompanied by alterations in tight junctions, TER, and mannitol flux and we reason that virus infection in the well-nourished state caused tight junction pores to open (allowing electrolytes and water passage) together with a pore/shunt pathway allowing macromolecule passage. In contrast, malnourished piglets, regardless of infection, had numerically reduced TER, but mannitol flux was only increased in malnourished/infected piglets suggesting that malnutrition alone was sufficient to alter passage of small ions across the barrier associated with reductions in TER and diarrhea, but rotavirus infection altered the tight junctions to allow increased macromolecule flux through pore/shunt pathways.

Expression of the tight junction protein, occludin, revealed that although virus infection impacted the proportion of occludin in the membrane (insoluble fraction) versus the cytosolic (soluble) fraction there was no effect of feeding level on occludin localization. Rotavirus infection significantly increased occludin in the soluble fraction of the protein extraction compared to non-infected pigs which had greater occludin in the membrane fraction of the protein extraction. This finding corroborates the effects seen in the TER and flux data showing no additive effects of PEM to the rotavirus infected pigs intestinal barrier function. Other models of starvation and injury in rats suggest an additive effect of malnourishment and intestinal insult on gut barrier permeability[35]. However, our results are consistent with previously reported data showing that PEM did not affect jejunal tissue protein synthesis rates and phosphorylation of $p70^{S6K}$, a key enzyme activated by mammalian target of rapamycin in regulating protein synthesis[15]. Zijlstra *et al*[4] showed that the growth factors, insulin-like growth factor (IGF)- I and IGF-Ⅱ, were not significantly reduced in the malnourished, infected pigs until 9 d post-infection. These growth factors are known trophic factors in the intestine and have been shown to increase jejunal uptake of glucose in the intestine[36]. Because IGF- I and IGF-Ⅱ concentrations were probably not decreased in our pigs by 3 d post-infection the effects of PEM on gut barrier function may not have been detectable at this early time point post-infection.

In conclusion, the present study provides clear evidence that rotavirus infection significantly affects small intestinal TER, and is the first report of increased paracellular permeability in neonatal pigs resulting from altered tight junction protein localization in enterocytes. However, additive effects of PEM on intestinal TER, paracellular permeability, and tight junction protein lo-

calization are not seen by 3 d post-infection in neonatal pigs. Though it is likely that during a more extended timeline wherein metabolic hormone responses decrease following viral infection and PEM there is potential for reduction in crypt cell proliferation[37]. This reduction in proliferation may potentially exacerbate the effect of viral infection in PEM neonates. Further identification of paracellular permeability mechanisms associated with rotavirus and PEM would be useful in developing treatments for pediatric patients facing environments where malnutrition and diarrhea are intertwined.

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COMMENTS

Background

Rotavirus enteritis and malnutrition are common problems for children in the developing countries. Rotavirus infections are common for all children under the age of five. However, children in less than desirable circumstances must deal with rotavirus infection compound by malnourishment. There is limited information on the interactions between the mechanisms of rotavirus and malnutrition on gut barrier function.

Research frontiers

In the present study, investigation of the interactions between rotavirus enteritis and malnutrition on gut barrier function were studied in the neonatal piglet model to determine the mechanisms involved in barrier function failure.

Innovations and breakthroughs

Rotavirus infection significantly blunted villus height in neonatal piglets, increased gut barrier permeability and reduced localization of tight junction proteins in the cell membrane of intestinal enterocytes during acute infection. Determination of the mechanisms of rotavirus and malnutrition interaction could lead to development of new nutritional or pharmacological treatments in high risk children in developing countries.

Applications

The effects of rotavirus and malnutrition in the neonatal piglet experimental model demonstrated the virus significantly effects gut barrier function and could potentially be used to study effective nutritional or pharmacological treatments for rotavirus in children. Further investigation into signaling mechanisms controlling intestinal tight junctions may provide insights into protein targets for development of effective therapies.

Terminology

Protein energy malnutrition is a common problem that compounds gastroenteritis in developing countries and neonatal health.

Peer review

This study is well constructed and it is based on previous work on nutritional impacts on viral infections especially in developing countries. This is an interesting and relevant study which confirms that rotavirus acutely impacts gut barrier function in the neonatal pig making animals more susceptible to flux across intestinal epithelial layer. Moreover, this study suggests that longer term studies should be completed to investigate the interaction of malnutrition with rotavirus infection in the developing neonate.

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BRIEF ARTICLE

Colonic preparation before colonoscopy in constipated and non-constipated patients: A randomized study

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Abstract

AIM: To compare the efficacy of different doses of sodium phosphate (NaP) and polyethylenglicol (PEG) alone or with bisacodyl for colonic cleansing in constipated and non-constipated patients.

METHODS: Three hundred and forty-nine patients, older than 18 years old, with low risk for renal damage and who were scheduled for outpatient colonoscopy were randomized to receive one of the following preparations (prep): 90 mL of NaP (prep 1); 45 mL of NaP + 20 mg of bisacodyl (prep 2); 4 L of PEG (prep 3) or 2 L of PEG + 20 mg of bisacodyl (prep 4). Randomization was stratified by constipation. Patients, endoscopists, endoscopists' assistants and data analysts were blinded. A blinding challenge was performed to endoscopist in order to reassure blinding. The primary outcome was the efficacy of colonic cleansing using a previous reported scale. Secondary outcomes were tolerability, compliance, side effects, endoscopist perception about the necessity to repeat the study due to an inadequate colonic preparation and patient overall perceptions.

RESULTS: Information about the primary outcome was obtained from 324 patients (93%). There were no significant differences regarding the preparation quality among different groups in the overall analysis. Compliance was higher in the NaP preparations being even higher in half-dose with bisacodyl: 94% (prep 1), 100% (prep 2), 81% (prep 3) and 87% (prep 4) (2 *vs* 1, 3 and 4, $P < 0.01$; 1 *vs* 3, 4, $P < 0.05$). The combination of bisacodyl with NaP was associated with insomnia ($P = 0.04$). In non-constipated patients the preparation quality was also similar between different groups, but endoscopist appraisal about the need to repeat the study was more frequent in the half-dose PEG plus bisacodyl than in whole dose NaP preparation: 11% (prep 4) *vs* 2% (prep 1) ($P < 0.05$). Compliance in this group was also higher with the NaP preparations: 95% (prep 1), 100% (prep2) *vs* 80% (prep 3) ($P < 0.05$). Bisacodyl was associated with abdominal pain: 13% (prep 1), 31% (prep 2), 21% (prep 3) and 29% (prep 4), (2, 4 *vs* 1, 2, $P < 0.05$). In constipated patients the combination of NaP plus bisacodyl presented higher rates of satisfactory colonic cleansing than whole those PEG: 95% (prep 2) *vs* 66% (prep 3) ($P = 0.03$). Preparations containing bisacodyl were not associated with adverse effects in constipated patients.

CONCLUSION: In non-constipated patients, compliance is higher with NaP preparations, and bisacodyl is related to adverse effects. In constipated patients NaP plus bisacodyl is the most effective preparation.

Key words: Colonic cleansing; Sodium phosphate; Polyethylenglicol; Bisacodyl constipation; Colonoscopy

Core tip: Colonoscopy has become the standard procedure for the diagnosis and treatment of colon diseases. Adequate bowel cleansing is essential for a high-quality effective and safe colonoscopy. In non-constipated patients, compliance is higher with sodium phosphate (NaP) preparations, and bisacodyl is related to adverse effects. In constipated patients NaP plus bisacodyl is the most effective preparation.

Pereyra L, Cimmino D, González Malla C, Laporte M, Rotholtz N, Peczan C, Lencinas S, Pedreira S, Catalano H, Boerr L. Colonic preparation before colonoscopy in constipated and non-constipated patients: A randomized study. *World J Gastroenterol* 2013; 19(31): 5103-5110 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5103.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5103

INTRODUCTION

Colonoscopy has become the standard procedure for the diagnosis and treatment of colon diseases[1]. An adequate colonic cleansing is necessary for a proper evaluation of the entire colonic mucosa and therefore for achieving a high quality colonoscopy[2]. Sodium phosphate (NaP) is a small volume hyperosmotic solution that provides effective colonic cleansing in preparation for colonoscopy. In the past years the popularity of orally administered NaP has increased because of its superior tolerance by patients compared with large-volume cleansing agents such as polyethylene glycol electrolyte solutions[3-5]. Although it presents a safety profile similar to other colonic cleansing agents, serious adverse events have been reported when administered in high volume or in patients with contraindications to NaP[6]. Polyethylenglicol (PEG) solutions are the most commonly used laxatives for colonic cleansing because of their safety profile and lack of contraindication. However, unpleasant taste and large volume of PEG lead to poor compliance and result in patient dissatisfaction. The two aforementioned agents are the most frequently used for colonic cleansing in many countries and despite the significant heterogeneity between different studies comparing them for colonic preparation, a systematic review showed similar adequate preparation rates, 75% for NaP and 71% for PEG[7,8]. Numerous clinical trials have also assessed prokinetic (metoclopramide, cisapride and tegazerod)[9-13] and laxative agents (magnesium citrate and bisacodyl)[14-16] associated with standard or lower volumes of this colon cleansing agents. Sharma *et al*[14] found that pretreatment with magnesium citrate or bisacodyl in addition to half-dose of PEG was associated with better preparation quality and patient satisfaction than full-dose of PEG. To our best knowledge, there is no study directly comparing whole and half-dose of PEG and NaP alone or in combination with bisacodyl in constipated and non-constipated patients. The aim of this study was to compare the efficacy and tolerability of whole doses of NaP and PEG and half-doses of those agents in combination with bisacodyl for colonic cleansing in constipated and non-constipated patients.

MATERIALS AND METHODS

This was a randomized, double-blind, four-arm study stratified by constipation. The study was carried out in accordance with the declaration of Helsinki. All patients included in the study signed an informed consent form. The human ethics committee from our institution approved the protocol.

Study population

All patients older than 18 years old who were scheduled for an elective outpatient's colonoscopy were eligible for participating in the study and were randomized in a 6-mo period (June-December 2011). As safety issues about NaP solutions have emerged, we only included healthy patients following the United States Food and Drug Administration recommendation to avoid renal damage. Patients were excluded if they presented one or more of the following characteristics: age younger than 18 years old, were hospitalized for any reason, hypersensibility to any of the components of PEG, NaP or bisacodyl, were under more than one antihypertensive medication, presented history of diarrhea (more than 3 bowel movements a day), acute or chronic renal failure, cardiovascular disease (history of myocardial infarction, congestive heart failure, unstable angina pectoris, unstable hypertension and/or cardiac arrhythmia), ascites, electrolite inbalance (hiponatremia, hipokalemia, hipocalcemia, hipomagnesemia or hyperphosphatemia), inflammatory bowel disease, partial or subtotal colectomy, ileus or suspected intestinal obstruction and pregnancy or breastfeeding, childbearing potential without contraception.

Study design

Patients who met all the inclusion criteria and no exclusion criteria were randomly assigned to receive one of the four colonic preparations according to a computer-generated randomization list. Randomization was stratified by constipation in order to make a subgroup analysis of constipated and non-constipated patients at the end of the study. Constipation was defined according to Thompson *et al*[17] criteria. Allocation was concealed using same color, size and weight closed boxes. The nurses that provided the patients with colonic preparation, the endoscopy assistant that evaluated the preparation compliance, tolerance and adverse reactions, the data analysts; and the endoscopists who evaluated bowel cleansing quality were blinded. If the patients had doubts about the preparation they could make a telephone call to a physician that was not blinded, was not present during

Table 1 Bowel preparation quality grading score used by the endoscopists

Excellent	No fecal matter or nearly none in the colon, small-to-moderate amounts of clear liquids
Good	Small amounts of thin liquid fecal matter seen and easily suctioned, mainly distal to splenic flexure, small lesions may be missed, > 90% mucosa seen
Fair	Moderate amounts of thick liquid to semisolid fecal matter seen and suctioned, included proximal to splenic flexure, small lesions may be missed, 90% mucosa seen
Poor	Large amounts of solid fecal matter found, precluding a satisfactory study, unacceptable preparation; < 90% mucosa seen

Table 2 Characteristics of the included patients *n* (%)

Characteristics	Prep 1	Prep 2	Prep 3	Prep 4	*P* value
Patients	78	78	84	84	
Age (yr), mean ± SD	59 ± 13.2	57 ± 11.1	60 ± 13.8	59 ± 10.9	NS
Sex					
Male	37 (47)	40 (51)	41 (49)	45 (53)	NS
Female	41 (53)	38 (49)	43 (51)	39 (47)	NS
Constipation	21 (27)	16 (21)	15 (12)	24 (29)	NS
Successful cecal intubation	78 (100)	78 (100)	84 (100)	84 (100)	NS

NS: Not significant.

colonoscopy nor participated in the endoscopic quality assessment, tolerability questionnaire, or statistical analysis. To reassure that endoscopists were blinded, a blinding challenge was performed after finishing the colonoscopy by asking them which of the four different colonic cleansing agents they thought the patients had received. A kappa coefficient of agreement was used for this purpose. A kappa under 0.3 and a non-significant *P* value was considered as an adequate blinding.

Prep 1 consisted of 90 mL of NaP alone (Gadolax®, Gador Laboratory, Argentina) 45 mL with four glasses of water at 4:00 pm and the other 45 mL at 8:00 pm of the day before the study. Prep 2 consisted of 45 mL of NaP with four glasses of water and 20 mg of bisacodyl at 4:00 pm the day before the study. Prep 3 consisted of 4 L of PEG (Barex®, Dominguez Laboratory, Argentina) alone starting at 4:00 pm the day before the study at a rate of 250 mL every 15 min until finishing the solution.

Prep 4 consisted of 2 L of PEG starting at the same time and with the same rate as mentioned before for prep 3 plus 20 mg of bisacodyl. Patients in all groups were encouraged to go through the same low fiber diet during the three days before the study and to adhere to a clear liquid diet from 8:00 am to midnight on the day before colonoscopy. Before colonoscopy the patients were asked to answer a questionnaire to assess patient satisfaction, tolerability, and compliance to the preparation. The questionnaire included yes/no responses for tolerance, preparation completed, and specific symptoms (nausea, vomiting, abdominal or chest pain, dizziness, bloating, and poor sleep). Before entering the Endoscopy Unit patients were asked not to reveal their assigned preparation to the Endoscopy Unit staff. Colonoscopies were done by four colonoscopists from the Endoscopic Unit and all studies were done between 7:30 am and 1:00 pm. All studies were performed using the same Storz Videocolonoscope. The quality of colonic cleansing was graded according to a previously reported scale[13] (Table 1). All endoscopists were trained on the scale using previously selected videos of colonoscopy with different colonic cleansing quality. Endoscopists were also asked if they thought there was a need to repeat the study due to inadequate preparation.

Statistical analysis

Statistical analysis were performed using statistical software SPSS for windows 10.0. Knowing that 70% of colonic cleansings are excellent or very good[1-3], a sample size of 88 patients in each group was calculated to detect a 20% difference in primary outcome with 80% of power at a standard level of significance $a = 0.05$. Categorical variables were compared using the Fisher exact test or χ^2 test. A *P* value of less than 0.05 was considered significant. Results were analyzed according to the intention-to-treat principle. Handling of loss to follow-up: We evaluated different assumptions about the incidence of events among participants lost to follow-up and the impact of those assumptions on the estimate of effect for the primary outcome. For this purpose, we used the $RI_{LTFU/FU}$ as proposed by Akl *et al*[18]. The $RI_{LTFU/FU}$ is defined as the event incidence among those lost to follow-up relative to the event incidence among those followed up. The assumptions we evaluated by combining a range of $RI_{LTFU/FU}$ values (1, 1.5, 2, 3.5 and 5) in the intervention group and control group.

RESULTS

A total of 349 patients scheduled for outpatient colonoscopy participated in the study and were randomized to receive one of the four colonic cleansing preparations. Three patients were excluded post-randomization because they met one or more exclusion criteria, 15 patients failed to present to the procedure and 7 presented incomplete colonoscopy because of fixed angulations (4 patients) or colonic neoplasia (3 patients). Finally, of the 346 randomized patients, information about the primary outcome was obtained from 324 patients (93%) (Figure 1). There were no significant differences among the four preparation groups with respect to: age, sex, cecal intubation, and constipation (Table 2).

Blinding challenge

There was no significant concordance between the endoscopists presumption and the colonic preparation group that the patients had been assigned to ($P = 0.56$, $\kappa = 0.019$). This observation reassures that the endoscopists were unaware of the assigned groups (blinding).

Quality of colonic cleansing

We obtained information about this outcome for 93% of

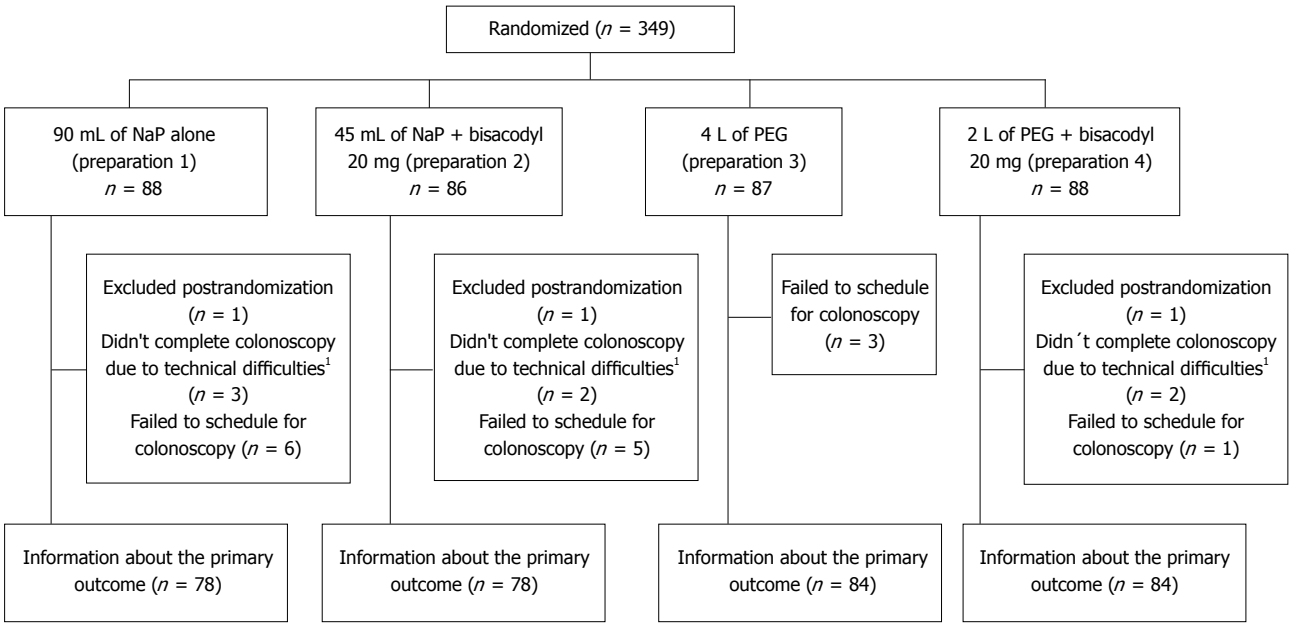

Figure 1 Flow chart of the included patients. [1]Fixed angulations or colonic neoplasia. NaP: Sodium phosphate; PEG: Polyethylenglicol.

patients. The quality of colonoscopic visualization was similar in the four different groups (Figure 2A).

Results were dichotomized into satisfactory colonic cleansing (excellent and good) and unsatisfactory (fair and poor). Satisfactory preparations were achieved in similar proportion in the different groups: prep 1, 82%, prep 2, 80%, prep 3, 79% and prep 4, 78% ($P > 0.05$) (Figure 2B). Endoscopists thought that only 6% of all the patients in this study needed to repeat the study because of inadequate colonic preparation. This was also similar between different preparations: prep 1, 3.4%, prep 2, 4.7%, prep 3, 6.8% and prep 4, 6.8% ($P > 0.05$) (Figure 2C).

We conducted a separate analysis of constipated and non-constipated patients. In the non-constipated patients, we didn´t find differences in the quality of colonic cleansing (Figure 2B) but the necessity to repeat colonoscopy was more frequent in prep 4 compared to prep 1 (11% *vs* 2%, $P < 0.05$) (Figure 2C). In constipated patients, NaP plus bisacodyl preparation (prep 2) achieved higher rate of satisfactory colonic cleansing than those receiving whole dose of PEG (prep 3): 95% *vs* 66% ($P = 0.03$) (Figure 2B).

Compliance

Both preparations containing NaP, presented better compliance than those containing PEG. Preparation was completed by 94% of the patients in prep 1, 100% of patients in prep 2, 81% of the patients in prep 3 and 87% of the patients in prep 4. Therefore, half-dose of NaP plus bysacodyl achieved the highest compliance (prep 2 *vs* 1, 3 and 4, $P < 0.01$) followed by full-dose of NaP (prep 1 *vs* 3 and 4, $P < 0.05$) (Figure 2D). In non-constipated patients, compliance was also higher in those preparations containing NaP compared to full-dose PEG: 95% (prep 1), 100 % (prep 2) *vs* 80% (prep 3) ($P < 0.05$) (Figure 2D). In constipated patients compliance was similar between different preparations.

Tolerability

The preparation was reported as tolerable in 77% of the patients in prep 1, 81% in prep 2, 82% in prep 3 and in 84% in the prep 4, there was no significant difference between the different preparations ($P > 0.05$). There was also no significant difference in tolerability between preparations in constipated and non-constipated patients (Table 3).

Symptoms profile

The most frequent adverse effects reported were: nausea (33%), bloating (30%) and abdominal pain (23%). There were no significant differences among different groups with respect to: nausea, vomiting, chest pain, bloating and dizziness (Table 3). Abdominal pain was more frequent in patients that received both preparations containing bisacodyl, prep 1, 16%, prep 2, 27%, prep 3, 19%, prep 4, 28%, but this difference didn´t reach statistical significance in the overall analysis ($P = 0.2$) (Table 3). The patients receiving NaP and bisacodyl preparations (prep 2) presented more frequently poor sleep than the other groups ($P < 0.05$) (Table 3). In non-constipated patients, abdominal pain was more frequent in those preparations containing bisacodyl: prep 2 (31%) and prep 4 (29%); compared to those without it: prep 1 (14%) and prep 3 (20%) ($P < 0.05$) (Table 3). The symptoms profile was similar between different preparations in constipated patients.

Patient preferences

Only 21% of all the patients would refuse to take the same colonic preparation in the future and almost 37% would like to try a different preparation. This finding was similar in the different groups. There was also no significant differences in patients perception in different groups

Table 3 Symptoms profile of different preparations *n* (%)

Adverse effects	Prep 1			Prep 2			Prep 3			Prep 4			*P* value		
	Overall	Non-constipated (*n* = 59)	Constipated (*n* = 22)	Overall	Non-constipated (*n* = 58)	Constipated (*n* = 20)	Overall	Non-constipated (*n* = 69)	Constipated (*n* = 15)	Overall	Non-constipated (*n* = 65)	Constipated (*n* = 20)	Overall	Non-constipated	Constipated
Tolerability	62 (77)	47 (80)	15 (68)	63 (81)	47 (81)	16 (80)	69 (82)	57 (83)	12 (80)	71 (84)	53 (82)	18 (90)	NS	NS	NS
Nausea	27 (33)	17 (29)	10 (13)	30 (38)	24 (41)	6 (30)	26 (31)	2 (32)	4 (26)	25 (29)	18 (28)	7 (35)	NS	NS	NS
Vomiting	6 (7)	2 (3)	4 (18)	3 (4)	6 (10)	3 (15)	9 (11)	2 (3)	1 (7)	6 (7)	5 (8)	3 (15)	NS	NS	NS
Abdominal pain	13 (16)	8 (14)	5 (23)	21 (27)	18 (31)	3 (15)	16 (19)	14 (20)	2 (13)	24 (28)	19 (29)	5 (33)	0.2	< 0.05[1]	NS
Bloating	25 (31)	17 (29)	8 (36)	21 (27)	15 (26)	6 (30)	27 (32)	22 (32)	5 (33)	24 (28)	16 (25)	8 (40)	NS	NS	NS
Insomnia	10 (12)	9 (15)	1 (5)	17 (21)	14 (24)	3 (15)	5 (6)	4 (6)	1 (7)	11 (13)	10 (15)	1 (5)	< 0.05[2]	NS	NS
Dizziness	12 (15)	10 (17)	2 (9)	7 (9)	6 (10)	1 (5)	7 (8.3)	5 (7)	2 (13)	8 (9)	5 (8)	3 (15)	NS	NS	NS
Chest pain	1 (1)	1 (2)	0 (0)	2 (3)	1 (2)	1 (5)	1 (1)	1 (1)	0 (0)	1 (1)	0 (0)	1 (5)	NS	NS	NS

[1]Prep 2 and 4 *vs* prep 1 and 3; [2]prep 2 *vs* prep 1, 3 and 4. NS: Not significant.

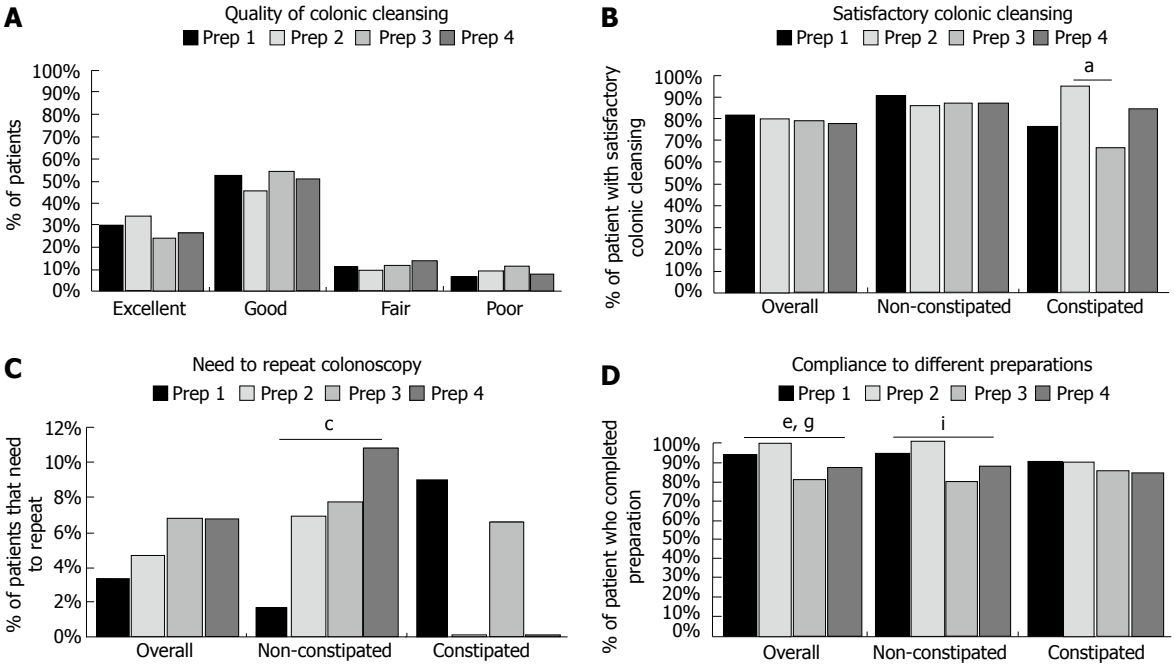

Figure 2 Efficacy and compliance of different preparations. A: Preparation quality score obtained with different preparations (no statistical difference between groups). Values are expressed as the percentage of patients. Prep 1, 90 mL of sodium phosphate (NaP); Prep 2, 45 mL of NaP followed by 20 mg of bisacodyl; Prep 3, 4 L of polyethylenglicol (PEG); Prep 4, 2 L of PEG followed by 20 mg of bisacodyl; B: Percentage of patients who had satisfactory and unsatisfactory colonic cleansing in the overall analysis and in the subgroup of constipated and non-constipated patients. Constipated patients obtained a higher rate of satisfactory colonic cleansing with prep 2, (45 mL of NaP followed by 20 mg of bisacodyl) when compared to preparation 3 (4 L of PEG) (prep 2 *vs* 3, $^{a}P = 0.03$); C: Endoscopist appraisal on the necessity to repeat colonoscopy due to inadequate preparation in the overall analysis and in the subgroup of constipated and non-constipated patients. Non-constipated patients assigned to prep 4 (2 L of PEG followed by 20 mg of bisacodyl) needed to repeat colonoscopy due to inadequate preparation more often when compared to patients assigned to prep 1 (90 mL NaP) (prep 4 *vs* 1, $^{c}P < 0.05$); D: Compliance to different preparations in the overall analysis and in the subgroup of constipated and non-constipated patients. Prep 2 (45 mL of NaP followed by 20 mg of bisacodyl) *vs* 1 (90 mL NaP), 3 (4 L of PEG) and 4 (2 L PEG followed by 20 mg of bisacodyl), $^{e}P < 0.05$; prep 1 (90 mL NaP) *vs* 3 (4 L of PEG) and 4 (2 L of PEG followed by 20 mg of bisacodyl), $^{g}P < 0.05$; prep 1 (90 mL NaP) and 2 (45 mL of NaP followed by 20 mg of bisacodyl) *vs* prep 3 (4 L of PEG), $^{i}P < 0.05$.

in constipated and non-constipated patients.

Loss to follow up

None of the different assumptions of incidence of events in loss to follow up patients changed significantly the estimate of the effect in the different outcomes.

DISCUSSION

There is a growing acceptance of colorectal cancer screening with colonoscopy. Its goal is to identify and remove neoplastics polyps; therefore a high-quality preparation that lends to a clear visualization is crucial. Inadequate colonic cleansing could lead to a diminished adenoma detection rate[19-21]. This has been recently shown to be the strongest predictor of interval colorectal cancer[22,23]. However none of the different preparation agents are ideal for colonic cleansing. They present historic rates for adequate cleansing that ranges from 70% to 82%[24-26]. Tolerability and side effects are probably the main issues and represent some of the most important reasons for patient's refusal to the study[25]. In an attempt

to decrease these sides effects, many studies have evaluated different doses of conventional preparation agents and pretreatment with prokinetics or laxative agents, but there is little information about the effect of these preparations in subgroups of constipated and non-constipated patients[7]. In this study we compared two of the most used colonic cleansing agents, PEG and NaP. As in past years, there has been a strong tendency to prepare patients with half doses of this previously mentioned agents associated with bisacodyl because of commercially available preparation kits. We decided to carry out a direct comparison between whole dose of PEG and NaP alone and half doses of these two agents associated with bisacodyl in constipated and non-constipated patients. Our studies main limitations include, single centre study and the use of non-validated scale for the evaluation of primary outcome (quality of colonic preparation) and patient related outcomes (tolerability, adverse events, preferences). Nevertheless, the randomized, double-blind, four-arm study design and the constipated and non-constipated subgroup analysis could provide useful information on how to manage patients that might undergo colonoscopy. Similar to the results reported by previous studies, almost 80% of patients presented to colonoscopy with satisfactory colonic cleansing (excellent or very good). We did not find any difference with respect to quality of colonic cleansing in the different groups, even in those with half doses of NaP and PEG. Preparation quality was also similar in different groups in non-constipated patients, but endoscopists thought that there was a greater necessity to repeat the study due to an inadequate colonic cleansing in prep 4 (half dose of PEG plus bisacodyl) compared to prep 1 (whole dose of NaP) (11% *vs* 2%, $P < 0.05$). Although this is a non-validated and subjective outcome; we think it´s interesting to know endoscopist perception, because it represents what they really do in the daily practice and is a patient important outcome. In constipated patients, preparations containing bisacodyl presented higher rates of satisfactory colonic cleansing: 95% (prep 2) and 85% (prep 4) *vs* 67% (prep 3) and 77% (prep 1). Only NaP plus bisacodyl reached a statistically significant difference compared to whole dose of PEG (95% *vs* 66%, $P = 0.03$). The prokinetic effect of the bisacodyl may explain the high rates of satisfactory colonic preparations. Even though a statistical significant difference was only obtained with NaP plus bisacodyl and not with PEG plus bisacodyl, we think that this may be related to the small sample size of the constipated patients subgroup. In the overall analysis, compliance was higher in groups with preparations containing NaP, reaching 100% in the half dose NaP plus bisacodyl group and 94% in the whole dose of NaP. In non-constipated patients, compliance with NaP preparations was higher than whole doses PEG preparation. We were not able to demonstrate higher compliance rates with NaP preparations in constipated patients. However, the observed tendency to higher compliance in these groups along with evidence of previous studies lead us to believe that we were unable to find statistically significant difference due to the small sample size. Tolerability (taste, nausea, *etc.*) was similar in the different groups. Consequently, we believe that the differences in compliances were related to the volume of the preparations and probably not to tolerability. The most frequent adverse effect was nausea followed by bloating and abdominal pain. None of the different preparations were associated with an antiemetic medication, so we do not know if nausea and probably tolerance could be optimized with this association. Bisacodyl has been previously associated with abdominal cramping. In this study both groups with preparations containing bisacodyl presented higher incidence of abdominal pain: prep 1, 16%, prep 2, 27%, prep 3, 19%, prep 4, 28%, but the difference was not statistically significant. The difference was statistically significant when we analyzed the subgroup of non-constipated patients: prep 1, 14%, prep 2, 31%, prep 3, 21%, prep 4, 29% ($P < 0.05$). Curiously, constipated patients that received preparation with bisacodyl did not have higher incidence of abdominal pain. We think that constipated patients can present a motility dysfunction that could be optimized with the administration of the bisacodyl and that could explain the difference perception of abdominal pain in constipated and non-constipated patients. In the overall analysis, the combination of NaP with bisacodyl was also associated with higher rates of poor sleep than other preparations. We did not find any previous reports of this association and we do not have a specific explanation for this finding. However, it seems that the bisacodyl adverse effects profile is different in constipated and non-constipated patients, suggesting that constipated patients are less affected by these effects. Although the evaluated preparations presented a high rate of satisfactory colonic cleansing, compliance and a low profile of side effects, almost 37% of all the patients when asked, would prefer to try a different preparation in next colonoscopy. This study shows that none of the preparations agents is ideal, and highlights the need to improve bowel cleansing methods not only to get high quality colonic cleansing, but also to achieve a higher adherence to colonoscopy screening and surveillance programs. In summary, the quality of colonic cleansing and side effects profile of evaluated preparations are different in constipated and none-constipated. In non-constipated patients, preparation quality is similar with whole or half doses of NaP or PEG, alone or in combination with bisacodyl and compliance is higher with NaP preparations. Bisacodyl addition is associated with a higher incidence of adverse events. In constipated patients, the combination of NaP with bisacodyl is the most effective preparation. In this subgroup of patients, bisacodyl addition is not associated with higher incidence of adverse effects as noticed in non-constipated patients.

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COMMENTS

Background

Colonoscopy has become the standard procedure for the diagnosis and treatment of colon diseases. Adequate bowel cleansing is essential for a high-quality effective and safe colonoscopy.

Research frontiers

Numerous clinical trials have assessed the efficacy of whole or low doses of sodium phosphate (NaP) and polyethylenglicol (PEG) alone or with bisacodyl. There is no information about which is the most suitable preparation regimen for constipated and non-constipated patients.

Innovations and breakthroughs

Their randomized clinical trial compared the efficacy and tolerability of whole and half doses of NaP and PEG alone or associated with bisacodyl preparations and explored the different effect on constipated and none-constipated patients.

Applications

Compliance was higher with NaP preparations in non-constipated patients and the addition of bisacodyl was associated with higher incidence of adverse effects. Half-dose of NaP plus bisacodyl was the most effective preparation in constipated patients. Bisacodyl was not associated with adverse effects in constipated patients as noticed in non-constipated patients.

Peer review

This is a good study in which authors compare the efficacy of different doses of NaP and PEG alone or with bisacodyl for colonic cleansing in constipated and non-constipated patients. The results are interesting and suggest that in non-constipated patients, compliance is higher with NaP preparations, and bisacodyl is related to adverse effects. In constipated patients NaP plus bisacodyl is the most effective preparation.

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BRIEF ARTICLE

Diagnostic accuracy of a new point-of-care screening assay for celiac disease

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Abstract

AIM: To determine the diagnostic accuracy of a new point-of-care assay detecting anti-deamidated gliadin peptides in celiac disease (CD) patients.

METHODS: One-hundred-and-twelve patients (age range: 1.8-79.2 years old) with clinical symptoms suggestive of CD and/or first-degree relatives (FDR) of CD patients (n = 66), and confirmed CD on a gluten-free diet (GFD) (n = 46), were prospectively enrolled in the study at Gastroenterology outpatient clinics for adult patients and from the Gastroenterology Consultation Ward at the Pediatric Department of the University Hospital of Geneva. Written informed consent was obtained from all subjects enrolled. The study received approval from the local ethics committee. The original CD diagnosis had been based on serum-positive IgA anti-tissue transglutaminase enzyme-linked immunosorbent assay (ELISA) (QuantaLite™, Inova Diagnostics, San Diego, CA, United States) and on biopsy results. Serum samples from all study participants were tested by the new CD lateral flow immunochromatographic assay (CD-LFIA) device, Simtomax® Blood Drop (Augurix SA, BioArk, Monthey, Switzerland) to detect immunoglobulin (Ig)A and IgG antibodies against deamidated gliadin peptides. The diagnostic performance was evaluated using receiver operating characteristic curves with 95%CIs. A cut-off of 2 on the Rann colorimetric scale was used to calculate the device's sensitivity and specificity.

RESULTS: CD-LFIA was highly accurate in detecting untreated celiac patients. In the group of patients with CD symptoms and/or FDR, eight new cases of CD were detected by ELISA and biopsy. All of these new cases were also correctly identified by CD-LFIA. The test yielded four false positive and four false negative results. The false positive results were all within the groups with clinical symptoms suggestive of CD and/or FDR, whereas the false negative results were all within the GFD group. The test yeld a sensitivity of 78.9% (95%CI: 54.4-93.9) and specificity of 95.7% (95%CI: 89.4-98.8), and the area under the curve reached 0.893 (95%CI: 0.798-0.988). The Kappa coefficient, calculated according to the values obtained by two readers from the same device, was of 0.96 (SE: 0.06). When the GFD patients were excluded from the analysis, the area under the curve reached 0.989 (95%CI: 0.971-1.000) and the Kappa coefficient, calculated according to the values obtained by two readers from the same device, became 0.96 (SE: 0.07). Furthermore, using the Rann scale cut-off of 2 without the GFD pa-

tients, sensitivity was 100% and specificity was 93.1% (95%CI: 83.3-98.1).

CONCLUSION: The new CD-LFIA rapid screening test shows good diagnostic accuracy, sensitivity and specificity, and may rule out CD in patients with CD-related symptoms.

Key words: Celiac disease; Deamidated gliadin; Total immunoglobulin A; Screening; Point-of-care assay

Core tip: The aim of the present study was to evaluate the clinical accuracy of a new point-of-care device that is based on deamidated gliadin peptides (DGP) for diagnosis of celiac disease (CD). One-hundred-and-twelve patients with clinical symptoms suggestive of CD and/or first-degree relatives of CD patients, and patients with confirmed CD on a gluten-free diet, were prospectively enrolled in the study. The actual CD diagnosis had been based on serum-positive immunoglobulin A anti-tissue transglutaminase results by enzyme-linked immunosorbent assay and on biopsy findings. Overall evaluation shows that the new DGP-based rapid point-of-care test is an excellent screening tool for high-risk populations.

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INTRODUCTION

Celiac disease (CD) is a common T cell-mediated gluten-sensitive enteropathy. CD diagnosis remains challenging since only a minority of celiac patients presents with specific gastrointestinal symptoms and the majority of patients manifests atypical extra-intestinal symptoms that may lead to missed diagnosis or misdiagnosis[1].

The initial diagnosis of CD is made by serological testing and confirmed either by histopathologic examination of small-bowel biopsy or further blood tests, depending on the serum concentration of anti-tissue transglutaminase (tTG) autoantibodies and the patient's age. Serology markers of CD have evolved over the years, as more specific antibodies have been identified. Currently, endomysial and anti-tTG autoantibodies are considered among the most reliable CD diagnostic markers[2,3]. Although both of these markers exhibit high sensitivity and specificity, their accuracy remains controversial in patients of a very young age or with a minor degree of mucosal damage; moreover, their accuracy for monitoring CD status in patients following a gluten-free diet (GFD) remains controversial[4,5]. Very recently, a new generation of assays based on the detection of antibodies against deamidated gliadin peptides (DGP) has demonstrated very high sensitivity, as well as a diagnostic accuracy that is at least equivalent to the established serological assays[6-9].

Given the high prevalence of the disease and likelihood of missed diagnosis, several simple immunoassays have been developed as a first step toward reducing the turnaround time for result delivery and initiating patient counseling and treatment[10]. Unfortunately, these new assays feature several drawbacks, including the reliance on serum samples, requirement for some basic laboratory equipment, their lack of sensitivity to identify celiac disease and to identify patients suffering from an immunoglobulin (Ig)A deficiency[11].

To overcome these issues, a multi-analytic lateral-flow immunochromato-graphic assay (LFIA) device, the Simtomax® Blood Drop system, has been developed that is based upon the detection of both IgA and IgG anti-DGP and total IgA. In this study, this new CD-LFIA test was evaluated in a ward setting to determine its accuracy, sensitivity, and specificity as compared to the established laboratory serology assay.

MATERIALS AND METHODS

Patients

Patients visiting the gastroenterology adult outpatient clinic and the gastroenterology consultation ward in the pediatric department of the University Hospital of Geneva from April 2008 to December 2009 were prospectively enrolled in this study. Criteria for study inclusion were clinical symptoms suggestive of CD and/or first-degree relatives (FDR) of CD-confirmed patients (n = 66), and CD-confirmed patients on a gluten-free diet (n = 46). Written informed consent was obtained from all subjects prior to study participation. The study was carried out with approval from the local ethics committee board (University Hospital of Geneva application 07-217).

Diagnostic methods

The diagnosis of CD was based on results of serologic enzyme-linked immunosorbent assay (ELISA) tests (described below) and small intestine mucosal biopsy examination.

The IgA and IgG anti-tTG QuantaLite™ ELISA tests from Inova Diagnostics (San Diego, CA, United States) were used to detect serum samples from all study participants. For both tests, concentrations > 30 U/mL were considered moderate to strongly positive for CD.

Total IgA was measured by the BN II nephelometer (Dade Behring Ltd., Milton Keynes, United Kingdom) according to the manufacturer's protocol. Results were evaluated by referring to a standard curve and by using < 0.05 g/L as the cut-off point to identify IgA deficiency. For the study population, normal values ranged between

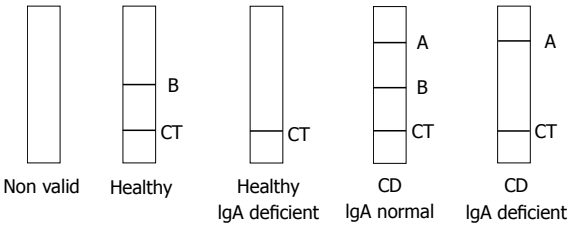

Figure 1 Celiac disease lateral-flow immunochromatographic assay visual result interpretation. CT: Control line; A: Position for detection of IgA and IgG anti-DGP; B: Position for detection of total IgA; CD: Celiac disease; IgA: Immunoglobulin A.

0.05 and 4.07 g/L, depending on the patient's age.

Small-bowel biopsies were obtained from all patients who tested positive by serology tests. The mucosal biopsy sections were analyzed by an experienced histopathologist, who assessed the following pathologic features of CD: villus atrophy, crypt hyperplasia, increased intraepithelial lymphocytes, and chronic inflammation in the lamina propria. The diagnosis of CD was subsequently confirmed according to the modified Oberhuber-Marsh classification system[12].

CD-LFIA test

Serum samples were collected from all study participants, stored at -20 ℃, and tested in duplicate on the Simtomax® Blood Drop system (Augurix SA, BioArk, Monthey, Switzerland). This CD-LFIA device was developed as an antigen direct sandwich assay capable of detecting both human IgA and IgG anti-DGP, as well as total IgA. A synthetic DGP conjugated to a carrier protein[7] was attached to the device's nitrocellulose membrane at the test line A position for detection of IgA and IgG anti-DGP. Mouse anti-human IgA was attached at the test line B position for detection of total IgA. In the test, secondary gold-conjugated antibodies bind to the patient's antibodies to form detectable complexes that are captured by the test in lines A and B. The control line, CT, is formed by the interaction of nitrocellulose-attached goat anti-mouse antibodies with the secondary gold-conjugated antibodies. All the lines are formed in 10-15 min. A CD-positive test result was indicated by detection of both the CT and A lines. IgA deficiency was indicated by absence of the B line. Figure 1 illustrates the device run with samples representative of the various diagnoses. Each sample was tested by two independent user-operators blinded to the subject's histories and laboratory findings and each of whom performed the CD-LFIA interpretations twice on two independent devices.

The CD-LFIA test lines were semi-quantitatively evaluated by using the Rann colorimetric scale (British Biocell International, Cardiff, United Kingdom). A series of five pink/red lines with a colloidal gold solution of decreasing optical density were sprayed on a card, and yielded line intensities ranging from 10 (maximum line intensity) to 2 (weakest visible line). Accordingly, the cut-off value for a positive result was set at 2. Spiked celiac serum equivalent to the ELISA QuantaLite™ cut-off value was used to set the visual limit of detection.

Statistical analysis

Statistical analyses were carried out by the STATA software (version 11; College Station, TX, United States). The StatXact-8 software (Cytel Inc., Cambridge, MA, United States) was used to calculate the 95%CIs. The diagnostic performance of the CD-LFIA test was evaluated by generating receiver operating characteristic (ROC) curves for each CD-LFIA device used and for each user-operator[13]. The areas under the ROC curves (AUCs) were provided with the corresponding 95%CIs. The "gold standard" diagnostic methods of laboratory ELISA and biopsy results were used for comparative analyses to evaluate the testing features of CD-LFIA. The cut-off of 2 Rann, which represented the delimitation between a "positive" and "negative" result (visible/invisible band) was used to calculate the CD-LFIA test' s sensitivity, specificity, and positive and negative likelihood ratios (LR+, LR-). Concordance between sample and device replicates was evaluated by calculating the Kappa coefficient and its SE.

RESULTS

Overall agreement between CD-LFIA and ELISA IgA-tTG laboratory test results

A total of 112 patients (71 females, 36 males; no sex information was available for five patients) with a mean age of 24.6 years old (median 13.9 years; range: 1.8-79.2 years) were analyzed.

Based on the laboratory values and biopsy results, a group of eight newly diagnosed celiac patients was found amongst the group of 66 patients composed of FDR and patients with clinical symptoms suggestive of CD. Thus, the CD prevalence in this study was 12.1%. All of the eight newly diagnosed CD patients were correctly identified by the CD-LFIA test (range of Rann values between 3-10). Among them, one did not undergo intestinal biopsy but had typical clinical presentation of CD and high positive titers of IgA-tTG (137 U/mL). The remaining seven had a positive intestinal biopsy (Marsh 3 and 4) and positive titers of IgA-tTG (119 -197 U/mL). Out of the 58 CD sero-negative patients, four were positive by the CD-LFIA test, however their Rann scores were just near cut-off: 2-3.

Of the 46 CD GFD patients, two patients showed selective IgA deficiency, and the CD-LFIA test detected this at 100%. Out of the 46 CD GFD patients, eleven of the CD GFD patients tested positive on the IgA-tTG ELISA, with three having high levels (116-170 U/mL) and the remaining eight having moderate levels (near the cut-off value; 30-55 U/mL). Among those 11 patients with positive IgA-tTG serology, four had negative results with the CD-LFIA test. These four patients had IgA-tTG ELISA levels near the cut-off values (36-55 U/mL for IgA-tTG for ELISA) and values of 0 Rann for CD-LFIA. The remaining 35 CD GFD patients were cor-

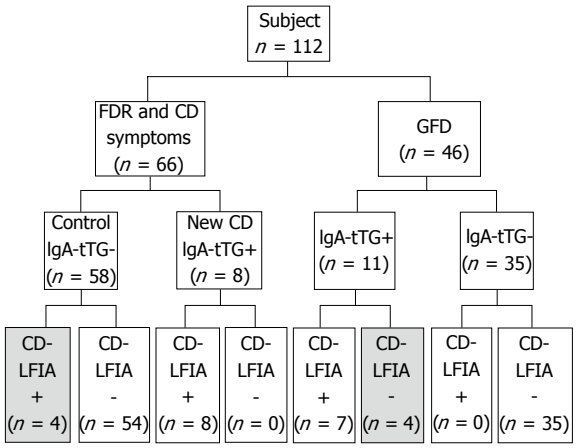

Figure 2 Histogram showing the immunoglobulin A-tissue transglutaminase enzyme-linked immunosorbent assay and celiac disease lateral-flow immunochromatographic assay test results. Text in gray indicates false-positive and false-negative results by celiac disease lateral-flow immunochromatographic assay (CD-LFIA). FDR: First-degree relatives; GFD: Gluten-free diet; Control: First-degree relatives and patients with celiac disease symptoms diagnosed as celiac disease (CD)-negative; IgA-tTG: Immunoglobulin A-tissue transglutaminase.

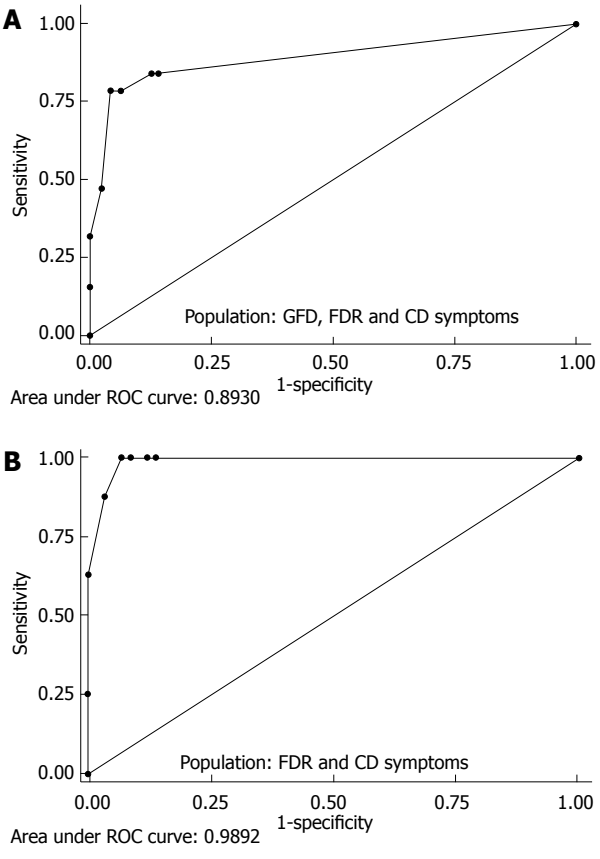

Figure 3 Diagnostic performance of the celiac disease lateral-flow immunochromatographic assay test determined by receiver operating characteristic curve analysis. A: GFD, FDR and CD symptoms; B: FDR and CD symptoms. FDR: First-degree relatives; CD: Celiac disease symptoms; GFD: Gluten-free diet; ROC: Receiver operating characteristic.

rectly identified as CD-negative by CD-LFIA. The overall agreement between the CD-LFIA test and the ELISA laboratory test results is shown in Figure 2.

Thus, CD-LFIA tests showed four false-positive results, all in the FDR and CD symptoms group. All of the ELISA laboratory test results were below the cut-off value and the Rann scores were between 2 and 3, just near the cut-off value. There were also four false-negative results obtained by the CD-LFIA device, all of which were from the CD GFD group. The serological IgA-tTG level of these patients was near the cut-off values.

Table 1 Celiac disease lateral-flow immunochromatographic assay result compared to diagnosis of celiac disease

		IgA-tTG ELISA		Total
		Positive	Negative	
GFD, FDR and CD symptoms				
CD-LFIA	Positive	15	4	19
	Negative	4	89	93
		19	93	112
FDR and CD symptoms				
CD-LFIA	Positive	8	4	12
	Negative	0	54	54
		8	58	66

Celiac disease lateral-flow immunochromatographic assay (CD-LFIA) result compared to diagnosis of celiac disease based on elevated titers of immunoglobulin A-tissue transglutaminase (IgA-tTG) in a population including gluten-free diet (GFD), first-degree relatives (FDR) and patients with celiac disease (CD)-related symptoms and FDR and patients with CD-related symptoms only. ELISA: Enzyme-linked immunosorbent assay.

Evaluation of the diagnostic performance of CD-LFIA on a population including patients monitored for compliance with GFD

The AUCs for each CD-LFIA device used and for each user-operator were 0.869 (95%CI: 0.764-0.975) and 0.893 (95%CI: 0.798-0.988), indicating excellent diagnostic performance of the test (Figure 3).

These results yield a sensitivity for the CD-LFIA device of 78.9% (95%CI: 54.4-93.9) and a specificity of 95.7% (95%CI: 89.4-98.8), as compared to the serological IgA-tTG levels detected by the ELISA laboratory tests. Considering the newly diagnosed CD patients (n = 8), the sensitivity was 100% (95%CI: 63.1-100) for both user-operators (Table 1).

Although the CD-LFIA is dependent upon the user-operator's semi-quantitative assessment of the colors of the reactive bands, the results were very reproducible between devices and user-operators. The concordance between user-operators and devices was indicated by the Kappa coefficients of 0.96 (SE = 0.06) and 0.92 (SE = 0.05), respectively.

In addition, an LR+ of 18.4 (95%CI: 7.0-51.8) and an LR- of 0.22 (95%CI: 0.08-0.46) were found for the CD-LFIA test when compared to the IgA-tTG ELISA (Table 1).

Evaluation of the diagnostic performance of CD-LFIA on a high-risk population

Exclusion of the CD GFD patients from the ROC anal-

ysis brought the AUC up to 0.989 (95%CI: 0.971-1.000), depending on the device and user-operator (Figure 3).

The kappa coefficient was 0.96 (SE = 0.07), indicating an excellent concordance between devices and user-operators.

In addition, when the CD GFD patients were excluded and the Rann cut-off of 2 was used, the sensitivity was of 100% (95%CI: 63.1-100) and the specificity remained nearly unchanged at 93.1% (95%CI: 83.3-98.1) (Table 1).

The LR+ became 14.5 (95%CI: 5.8-49.0) and the LR- became 0.00 (95%CI: 0.00-0.39), respectively (Table 1).

DISCUSSION

Diagnostic tests play a vital role in medicine, not only to confirm the presence of diseases but also to rule them out[14]. Diagnosis of CD has improved significantly in the past 20 years, as highly sensitive and specific biomarkers were identified[15]. Nevertheless, the prevalence of CD has dramatically increased over this same period (rising from a previously assumed 0.1% to up to 1.0%)[16,17]. This increase is probably largely due to identification of patients suffering from mild or atypical forms of CD. Moreover, large epidemiological screening studies have revealed that CD is a worldwide health concern[18]. Besides the improved detection methods, other etiological factors appear to have contributed to the increased prevalence[16], and, similar to other autoimmune conditions, these may include different environmental factors, such as gluten, antigens in breast milk, or from other pathogenic infections[19,20].

Unfortunately, CD remains one of the most common underdiagnosed medical conditions, with estimates of more than 90% of the patients being unrecognized[19,21]. Due to mild and atypical symptoms, the diagnosis of CD is often a challenge for many physicians, resulting in delays in diagnosis (up to 11 years[21]) and high rates of patient dissatisfaction and discomfort.

A large retrospective study of a managed-care population demonstrated that timely CD diagnosis was associated with a significant overall cost reduction that was attributable to reduced amounts of office visits, laboratory services, diagnostic and imaging support services, and endoscopy procedures[22]. Several simple, visual assays have been developed to promote the feasibility of CD screening programs[4,23-27]. However, while these assays have been demonstrated as reliable and easy-to-use, they are limited in sensitivity and lack the ability to concomitantly detect IgA deficiency[11].

Therefore, there is a clear unmet clinical need for a rapid and discriminative point-of-care test that could facilitate the management of patients consulting in primary care centers for CD-related symptoms. To this end, in this study, we compared the validity of the newly developed rapid point-of-care diagnostic device for detecting both human IgA and IgG anti-DGP to the measurements of serological IgA and IgG anti-tTG levels detected by routine laboratory ELISA. Sensitivity and specificity are two features of a diagnostic test that measure the validity of a new test as compared to a gold standard test, such as the ELISA. The ROC curves, as well as the corresponding AUCs, are effective measures of the inherent validity of a diagnostic test. Here, we found that the CD-LFIA test had a sensitivity of 100% for the detection of new CD cases, and result interpretation appeared unambiguous between multiple devices and multiple user-operators. The ROC curve indicated that, at a cut-off of 2 Rann, the device has a good discriminative ability between patients with CD and those without CD. The high values of the AUCs (up to 0.989) indicated an excellent accuracy of the CD-LFIA test. LR+ and LR- values represent measures of the performance of a diagnostic method, independent of disease prevalence[18,28]. The CD-LFIA test in this study achieved a LR+ of 14.5, indicating that patients having CD are 15 times more likely to have a positive test than those who are healthy. Moreover, the LR- of 0.0 indicated that the CD-LFIA test is very good at ruling out the disease.

The particular challenges to this test concerned interpretation of samples with weak reactivity that were exclusively representative of the CD GFD patients and would affect monitoring of CD status in this patient population. For this specific group, another approach may be required.

Here, we showed that CD-LFIA is highly accurate in detecting untreated celiac patients. It can be easily performed during the course of a consultation in primary care to test patients with symptoms suggestive of CD, and may represent a reliable alternative to the traditional laboratory assays. With specificity and sensitivity of 93.1% and 100%, respectively, and a LR-value of 0.0, CD-LFIA appears highly suitable for ruling out CD, representing an interesting tool in an exclusion diagnostic strategy. In case of positive serology, the physician can proceed to further investigations by the traditional laboratory assay. Therefore, CD-LFIA can be used as a rapid and accurate test to rule out CD in patients presenting with CD-related symptoms to primary care centers.

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COMMENTS

Background

Traditionally thought to be a rare childhood disease, celiac disease (CD) is currently recognized as a frequent condition both in adults and children and has become a widespread public health concern. CD diagnosis can be quite challenging for physicians since only a minority of celiac patients suffer from specific gastrointestinal symptoms. The majority of patients present with an atypical extra-intestinal manifestation that may not raise the physician's suspicion of CD. Laboratory-based methods, such as enzyme-linked immunosorbent assays (ELISA), remain the primary screening tool for CD. However, these tests are labor intensive and relatively high cost. Development and implementation of simple immunoassays will be a first step toward reducing the turnaround time for

result delivery and patient counseling and treatment.

Research frontiers

Serology markers of CD have evolved over the years with the identification of more disease-specific antibodies. Endomysial and anti-tissue transglutaminase (tTG) autoantibodies are currently considered among the most reliable of the CD-related markers. Although these markers exhibit a high sensitivity and specificity, their accuracy in very young children, in patients with a minor degree of mucosal damage, and for the follow-up of CD patients under a gluten-free diet remains controversial. Very recently, a new generation of assays based on the detection of antibodies against deamidated gliadin peptides (DGP) has demonstrated very high sensitivity for CD, as well as diagnostic accuracy that is at least equivalent to the traditional immunoassays.

Innovations and breakthroughs

A new rapid point-of-care serologic screening test based on detection of anti-DGP antibodies (immunoglobulin, IgA and IgG) and total IgA by a lateral flow immunochromatographic assay was evaluated in a pediatric and adult population and compared to ELISA reference laboratory serology assays. The new test was found to be rapid and highly accurate for ruling out CD in patients with CD-related symptoms.

Applications

The test can be easily performed during the course of a consultation visit and may represent a reliable alternative to the traditional laboratory assays, and appears to be highly suitable for ruling out CD in primary care centers in patients with CD-related symptoms.

Peer review

The manuscript evaluates the use of a new point-of-care assay for diagnosing CD in a clinical setting and compares its use to traditional tTG ELISA measurements. The test is based on simultaneous detection of IgA and IgG DGP antibodies and total IgA. The test shows a good accuracy in diagnosing CD. This is important as it suggests the test as a reliable alternative to laboratory assays for ruling out CD in patients with CD-related symptoms.

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BRIEF ARTICLE

Rikkunshito improves globus sensation in patients with proton-pump inhibitor-refractory laryngopharyngeal reflux

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Abstract

AIM: To investigate the effect of rikkunshito on laryngopharyngeal reflux (LPR) symptoms and gastric emptying in patients with proton-pump inhibitor (PPI)-refractory LPR.

METHODS: In total, 22 patients with LPR were enrolled. Following a 2-wk treatment with PPI monotherapy, PPI-refractory LPR patients were randomly divided into two treatment groups (rikkunshito alone or rikkunshito plus the PPI, lansoprazole). LPR symptoms were assessed using a visual analog scale (VAS) score, gastrointestinal symptoms were assessed using the gastrointestinal symptom rating scale (GSRS), and gastric emptying was assessed using the radio-opaque marker method prior to and 4 wk following treatments.

RESULTS: The 4-wk treatment with rikkunshito alone and with rikkunshito plus the PPI significantly decreased the globus sensation VAS scores. The VAS score for sore throat was significantly decreased following treatment with rikkunshito plus PPI but not by rikkunshito alone. Neither treatment significantly changed the GSRS scores. Rikkunshito improved delayed gastric emptying. We found a significant positive correlation between improvements in globus sensation and in gastric emptying (r^2 = 0.4582, P < 0.05).

CONCLUSION: Rikkunshito improved globus sensation in patients with PPI-refractory LPR, in part, because of stimulation of gastric emptying. Thus, rikkunshito is an effective treatment for PPI-refractory LPR.

Key words: Laryngopharyngeal reflux; Gastroesophageal reflux disease; Globus sensation; Gastric emptying; Rikkunshito

Core tip: Regarding the treatment of laryngopharyngeal reflux (LPR) symptoms such as globus sensation and a scratchy feeling, proton pump inhibitors (PPIs) are considered the mainstay. We investigated the effects of rikkunshito on globus sensation and gastric emptying in patients with PPI-refractory LPR.

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INTRODUCTION

Symptoms or complaints of globus sensation ("globus"), a "lump in the throat" feeling located between the upper edge of the sternum and the cricoid region, are common. Recently, gastroesophageal reflux disease (GERD) has been identified as a major cause of globus[1-3]. Stom-

ach acid reflux produces a number of extraesophageal symptoms in the laryngopharynx, commonly referred to as laryngopharyngeal reflux (LPR)[1,2], which include a hoarse voice, cough, a scratchy feeling in the throat, and globus[1-3]. However, the etiology of globus remains unclear. Recent studies have suggested the condition may be caused by hypertonicity in the upper esophageal sphincter (UES)[4,5]. We have demonstrated that elevated UES pressure resulting from gastroesophageal reflux without direct exposure of the hypopharynx to acid can cause the globus sensation[6].

Proton-pump inhibitors (PPIs) are considered the mainstay treatment for LPR[7]. However, LPR requires more aggressive and prolonged therapy than GERD, and PPIs do not improve extraesophageal symptoms in the laryngopharynx in all cases[7,8]. Furthermore, increasing evidence suggests that duodeno-gastroesophageal reflux may be related to several laryngeal disorders[9]. Thus, stimulation of gastric emptying or esophageal clearance in addition to inhibition of gastric acid secretion may be an effective treatment for LPR. Ezzat *et al*[10] reported that adding prokinetics, such as cisapride and itopride, to PPIs to treat LPR reduced the recurrence of symptoms. However, few studies have investigated the efficacy of prokinetics in the treatment of LPR.

Rikkunshito, a traditional Japanese medicine, is widely used to treat upper gastrointestinal symptoms such as gastroesophageal reflux[11,12] and dyspepsia[13,14]. Rikkunshito has been shown to accelerate gastric emptying in functional dyspeptic patients[13,14] and rats[15]. Furthermore, rikkunshito improved upper gastrointestinal symptoms in PPI-refractory GERD patients[12]. Thus, we investigated the effects of rikkunshito on globus sensation and gastric emptying in patients with PPI-refractory LPR.

MATERIALS AND METHODS

Subjects

In total, 22 patients with PPI-refractory LPR were enrolled at Tokyo Medical University Hospital, from March, 2007 to December, 2008. PPI-refractory LPR was defined as the presence of LPR symptoms (globus sensation, sore throat, excessive throat clearing) despite therapy using a standard dose of PPI for 2 or more weeks. Enrolled patients met the following inclusion criteria: (1) 20-76 years of age; (2) received standard-dose therapy with a PPI for at least 2 wk prior to commencement of the study; (3) a score of three or higher than the average gastrointestinal symptom rating scale (GSRS) score for acid reflux, abdominal pain, or indigestion; (4) had LPR symptoms (globus sensation, sore throat, or excessive throat clearing); and (5) provided written informed consent for study participation. Exclusion criteria were: (1) use of an antipsychotic drug, skeletal muscle relaxant, anti-ulcer drug (with the exception of a PPI), digestive drug, or antacid within 2 wk of the start of the present study; (2) patients who had globus sensation, laryngopharyngeal pain, or chronic cough due to an organic disease; (3) cervical spine disease; (4) sinusitis; (5) bronchial asthma; (6) patients with serious complications; (7) a history of drug hypersensitivity; (8) females who were pregnant or wished to become pregnant during the study or follow-up period, and lactating females; and (9) patients who were considered unsuitable by the chief investigator.

Study design

This prospective, randomized, comparative parallel group study examined the efficacy and safety of a therapeutic strategy using rikkunshito in patients with PPI-refractory LPR. The study was conducted according to ethical guidelines for clinical studies and with consideration of patients' human rights and privacy. The protocol was approved by the Institutional Review Board of Tokyo Medical University.

Study procedures

All patients were treated with a standard-dose PPI for at least 2 wk prior to obtaining written informed consent. After obtaining written informed consent, LPR symptoms and gastrointestinal symptoms were evaluated using a visual analog scale (VAS) score and the GSRS scores. Following treatment with the PPI, lansoprazole (30 mg/d, *qd*), for at least 2 wk, patients with PPI-refractory LPR who met the inclusion and none of the exclusion criteria were enrolled in the study. Enrolled patients were randomly divided into two groups using the envelope method: rikkunshito (7.5 g/d, *tid*) alone and rikkunshito (7.5 g/d, *tid*) plus a standard dose of lansoprazole (30 mg/d). We used a powdered extract of rikkunshito (Tsumura & Co., Tokyo, Japan) obtained by spray drying a hot water extract mixture of the following eight crude herbs: *Atractylodis lanceae Rhizoma* (4.0 g), *Ginseng radix* (4.0 g), *Pinelliae tuber* (4.0 g), *Hoelen* (4.0 g), *Zizyphi fructus* (2.0 g), *Aurantii nobilis pericarpium* (2.0 g), *Glycyrrhizae radix* (1.0 g), and *Zingiberis rhizoma* (0.5 g). LPR symptoms, gastrointestinal symptoms, and gastric emptying were evaluated before and after a 4-wk treatment regimen using rikkunshito or rikkunshito plus PPI.

Assessment of LPR symptoms and gastrointestinal symptoms

LPR symptoms of globus sensation, sore throat, and excessive throat clearing were assessed using a VAS scale. Gastrointestinal symptoms were assessed using the GSRS, a 15-item questionnaire used to assess general gastrointestinal symptoms[16]. Each GSRS item is rated on a seven-point Likert scale, from no discomfort (1) to very severe discomfort (7). According to a factor analysis, the 15 GSRS items are divided into five domains: abdominal pain (abdominal pain, hunger pain, and nausea), reflux syndrome (heartburn and acid regurgitation), diarrhea syndrome (diarrhea, loose stools, urgent need for defecation), indigestion syndrome (borborygmus, abdominal distension, eructation, increased flatus), and constipation syndrome (constipation, hard stools, feeling of incomplete evacuation).

Table 1 Subjects' characteristics

	Rikkunshito	Rikkunshito + PPI
Number of patients	11	11
Mean age (range)	55.9 (39-76)	56.6 (25-76)
Sex (male/female)	4/7	4/7
Smoking (yes/no)	5/6	3/8

There is no significant difference between the rikkunshito and rikkunshito + proton-pump inhibitor (PPI) groups (Fisher's exact test or Wilcoxon's rank sum test).

Measurement of gastric emptying using radio-opaque markers

Radio-opaque markers were used to evaluate gastric emptying according to the method proposed by Cremonini *et al*[17]. Briefly, 18 subjects swallowed a capsule containing 40 radio-opaque markers (Sitzmarks, Konsyl Pharmaceuticals, Fort Worth, TX, United States) before and after 4 wk treatment with rikkunshito or rikkunshito plus PPI. A plain abdominal radiograph was obtained 3 h after intake of the capsule, and the number of markers in the stomach was counted.

Adverse events, safety and tolerability

Safety and tolerability were assessed by recording all adverse events, and changes in hematological and clinical laboratory variables were measured at the screening visit. An adverse event was defined as any unfavorable or unintended sign, whether or not it was considered to be causally related to the drugs used in this study.

Compliance

Treatment compliance was defined as the percentage of the test drug used. A treatment compliance of at least 66.6% was considered acceptable.

Statistical analysis

Within-group treatment responses in were evaluated according to pre- and post-treatment VAS and GSRS scores using a paired *t* test or the Wilcoxon signed-rank test. Mean the pre- and post-treatment scores were compared between groups using the Wilcoxon rank-sum test. Between-group age and demographic factors were compared using the Wilcoxon rank-sum test, and the distributions of sex and smoking status were compared using Fisher's exact test. We calculated the correlation between change in globus sensation and change in gastric emptying values using the non-parametric Spearman's *r* correlation. *P* values < 0.05 were considered to indicate statistical significance. All data are expressed as mean ± SD.

RESULTS

Patient characteristics

We found no marked differences in age, sex, or smoking status between the groups (Table 1). No difference was found between pre- and post-PPI monotherapy for globus sensation (VAS score, 58.7 ± 25.2 and 56.7 ± 20.1, respectively) or gastrointestinal symptoms (overall GSRS score, 2.2 ± 0.9 and 2.0 ± 0.7, respectively) in the enrolled patients.

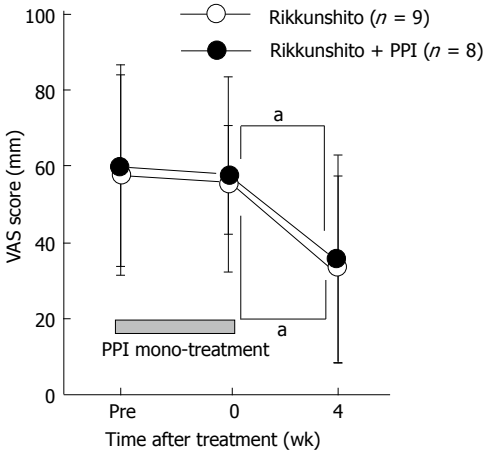

Figure 1 Effects of proton-pump inhibitor monotherapy and subsequent treatment with rikkunshito alone or rikkunshito plus proton-pump inhibitor on visual analog scale scores for globus sensation in patients with proton-pump inhibitor-refractory laryngopharyngeal reflux. Proton-pump inhibitor (PPI) monotherapy was delivered for at least 2 wk prior to the experiment. Each value represents the mean ± SD. $^{a}P < 0.05$, significantly different from the visual analog scale (VAS) score at week 0 in each group (paired *t* test). No significant between-group differences were found at any time point.

Changes in LPR and gastrointestinal symptoms after rikkunshito or rikkunshito plus PPI treatment

The 4-wk treatment regimen significantly decreased the globus sensation VAS scores in both treatment groups (Figure 1). Furthermore, the post-treatment VAS scores were not significantly different between treatment groups.

The effects of rikkunshito alone or rikkunshito plus PPI treatments on sore throat and excessive throat clearing in patients with PPI-refractory LPR are shown in Table 2. The VAS scores for sore throat and excessive throat clearing did not decrease following the 2-wk PPI monotherapy. The VAS score for sore throat decreased after treatment with rikkunshito plus PPI but not after rikkunshito alone. The VAS score for excessive throat clearing did not change in either treatment group.

Neither the rikkunshito alone nor rikkunshito plus PPI treatment group showed a significant change in the overall GSRS or five subscale scores following the 4-wk treatment period (Table 3).

Changes in gastric emptying following rikkunshito alone or rikkunshito plus PPI treatment

Changes in gastric emptying following rikkunshito or rikkunshito plus PPI treatment are shown in Figure 2. The number of markers in the stomach tended to decrease after treatment with rikkunshito alone, but the difference was not statistically significant. However, the number of markers in the stomach was significantly decreased following treatment with rikkunshito plus PPI. We found no between-group difference in the number of markers in

Table 2 Effects of rikkunshito and rikkunshito plus proton-pump inhibitor treatments on sore throat and excessive throat clearing in patients with proton-pump inhibitor-refractory laryngopharyngeal reflux

	Week	Visual analog scale score (mean ± SD)		
		A: Rikkunshito	B: Rikkunshito + PPI	*P* (A *vs* B)
Sore throat		(*n* = 4)	(*n* = 5)	
	-2	35.4 ± 21.6	44.3 ± 30.5	0.730
	0	24.0 ± 28.1	45.2 ± 28.4	0.234
	4	24.8 ± 32.8	31.8 ± 30.2[a]	0.538
Excessive throat clearing		(*n* = 4)	(*n* = 8)	
	-2	48.0 ± 12.8	40.8 ± 32.5	1.000
	0	37.2 ± 21.5	45.7 ± 25.0	0.514
	4	39.8 ± 34.9	25.7 ± 24.2	0.569

Each value represents the mean ± SD. [a]*P* < 0.05, significantly different from the visual analog scale score at week 0 in each group (paired *t* test). No significant differences were found between the rikkunshito and rikkunshito plus proton-pump inhibitor (PPI) treatments at any time point.

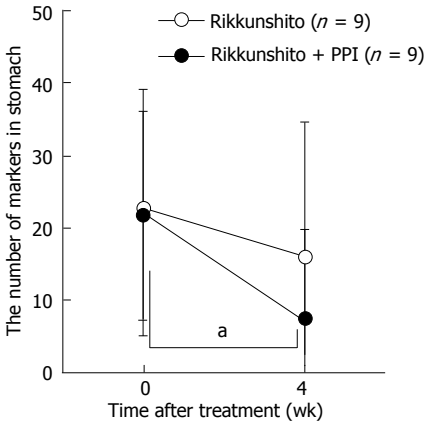

Figure 2 Effects of rikkunshito alone and rikkunshito plus proton-pump inhibitor on gastric emptying in patients with proton-pump inhibitor-refractory laryngopharyngeal reflux. Each value represents the mean ± SD. [a]*P* < 0.05, significantly different from the number of markers at week 0 in the rikkunshito + proton-pump inhibitor (PPI) group (Wilcoxon signed-rank test). We found no significant difference between treatment groups after the 4-wk treatment period (Wilcoxon rank-sum test).

the stomach following the 4-wk treatment period.

Correlation between improvement in globus sensation and improvement in gastric emptying

The correlation between improvement in globus sensation and improvement in gastric emptying is shown in Figure 3. A marked improvement in globus sensation was observed in patients with PPI-refractory LPR as gastric emptying improved. The correlation analysis revealed a significant positive correlation between the improvement in globus sensation and the improvement in gastric emptying ($r^2 = 0.4582$, $P < 0.05$).

Safety of rikkunshito

No adverse event/reaction requiring treatment occurred in any patient during the study period.

DISCUSSION

As no diagnostic gold standard is available for LPR, few studies have investigated this condition. However, previous reports indicate that 74.4% of GERD patients experience extraesophageal or atypical manifestations with prevalences of globus sensation and laryngitis/pharyngitis in GERD patients of 38.7% and 19.9%, respectively[8]. LPR requires more aggressive and prolonged therapy than GERD, and several cases in which PPIs did not improve extraesophageal symptoms in the laryngopharynx have been reported[7,8]. We examined PPI-refractory patients whose LPR symptoms of globus sensation, sore throat, or excessive throat clearing did not improve after at least 2 wk of PPI treatment. Rikkunshito has been shown to improve upper gastrointestinal symptoms in PPI-refractory GERD patients[12]; thus, we investigated the efficacy of rikkunshito in improving extraesophageal symptoms in patients with PPI-refractory LPR. Our findings indicate that a 4-wk treatment regimen of rikkunshito alone or rikkunshito plus PPI improved globus sensation in patients with PPI-refractory LPR. Two theories of LPR pathogenesis have been proposed. According to the direct impairment theory, LPR occurs when stomach acid acts directly on the hypopharynx, whereas the reflex theory holds that acid reflux in the lower esophagus causes coughing or other symptoms through a vagal reflex[1-3]. Moreover, we demonstrated previously that globus sensation can be caused by elevated upper esophageal sphincter pressure resulting from gastroesophageal reflux without direct exposure of the hypopharynx to acid[6]. Thus, acid secretion control alone is not sufficient for the treatment of LPR, which is caused by several factors. Unlike the PPIs, rikkunshito does not have an antisecretory effect[18], and, thus, may improve the globus sensation via a different mechanism. Kawahara *et al*[11] reported that rikkunshito reduced esophageal acid exposure through improved esophageal acid clearance in GERD patients. The hesperidine and atractylodin, components of rikkunshito, have been shown to improve delayed gastric emptying in L-NNA-administered rats[15,19], and rikkunshito improved upper GI symptoms *via* stimulation of gastric emptying in functional dyspeptic patients[13,14] and in patients who had undergone pylorus-preserving gastrectomy[20]. A recent study showed that rikkunshito stimulated secretion of a ghrelin, which has stimulatory effects on appetite and gastrointestinal motor activity[21,22]. Furthermore, rikkunshito and atractylodin enhance reactivity of its receptor[23]. Nahata *et al*[24] found an association between impaired ghrelin signaling and gastrointestinal motility dysfunction and demonstrated that rikkunshito restored gastrointestinal motility by improving the ghrelin response in rat GERD models. If rikkunshito reduces gastric contents, it seems reasonable that a subsequent reduction in the reflux volume may reduce acid exposure in the esophagus, pharynx, and larynx. We calculated the correlation between improved globus sensation and improved gastric emptying to investigate the association between rikkunshito-induced stimulation of gastric emptying improved globus sensation. We found

Table 3 Gastrointestinal symptom rating scale scores after 4 wk treatments of rikkunshito with or without proton-pump inhibitor

	Week	Rikkunshito (mean ± SD)	Test[1] *P* value	Rikkunshito + PPI (mean ± SD)	Test[1] *P* value	Test[2] *P* value
Overall scores	-2	2.25 ± 1.06	0.232	2.19 ± 0.73	0.375	1.000
	0	2.12 ± 0.85	-	1.96 ± 0.50	-	0.778
	4	1.83 ± 0.84	0.148	1.73 ± 0.37	0.195	0.736
Subscale scores						
Reflux syndrome	-2	2.25 ± 1.06	0.055	2.79 ± 0.91	0.170	0.369
	0	2.23 ± 1.60	-	2.45 ± 1.42	-	0.540
	4	1.94 ± 1.16	1.000	1.94 ± 0.86	0.106	0.801
Abdominal pain	-2	2.27 ± 1.29	0.168	2.33 ± 1.12	0.058	0.658
	0	1.87 ± 0.86	-	1.77 ± 0.85	-	0.914
	4	1.59 ± 0.78	0.250	1.50 ± 0.40	0.223	0.805
Indigestion syndrome	-2	2.40 ± 1.04	0.615	2.54 ± 1.29	0.551	0.844
	0	2.30 ± 1.03	-	2.20 ± 0.86	-	1.000
	4	2.17 ± 1.22	0.201	1.94 ± 0.75	0.139	0.961
Diarrhea syndrome	-2	1.77 ± 1.05	0.750	1.71 ± 0.71	1.000	0.878
	0	1.61 ± 0.68	-	1.77 ± 0.75	-	0.661
	4	1.41 ± 0.49	0.098	1.71 ± 0.68	0.866	0.345
Constipation syndrome	-2	1.77 ± 1.05	0.341	1.71 ± 0.71	0.784	0.138
	0	1.61 ± 0.68	-	1.77 ± 0.75	-	0.254
	4	1.41 ± 0.49	0.134	1.71 ± 0.68	1.000	0.883

0 week: Baseline. Test[1]: There is also no significantly different compared from gastrointestinal symptom rating scale score at week 0 (Wilcoxon's signed rank test); Test[2]: There is no significant difference between the rikkunshito with or without plus proton-pump inhibitor (PPI) treatment at each period (Wilcoxon's rank sum test).

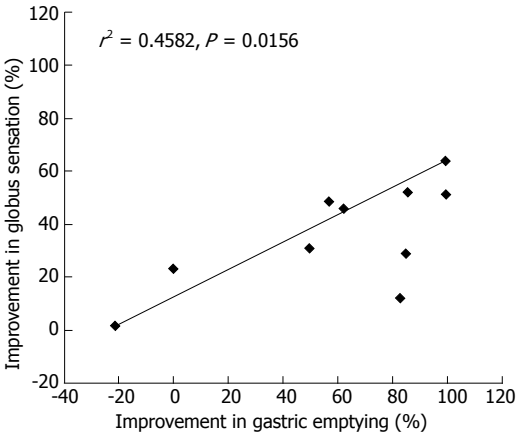

Figure 3 Correlation between improvement in globus sensation and improvement in gastric emptying. The improvement in globus sensation calculation based on pre- and post-treatment visual analog scale (VAS) scores using the following formula: Improvement (%) = [(pre-score) - (post-score)]/(post-score) × 100. Pre-score: VAS score before the start of rikkunshito or rikkunshito + proton-pump inhibitor treatment; Post-score: VAS score after the 4-wk treatment period. Improvement in gastric emptying was calculated based on the number of markers in the stomach before and after treatment.

a significant positive correlation between improved globus sensation and improved gastric emptying. Thus, the improvement in globus sensation following treatment with rikkunshito may be the result, at least in part, of improved gastric emptying. In addition to the globus sensation, patients with LPR typically experience sore throat or excessive throat clearing. Treatment with rikkunshito plus PPI, but not with rikkunshito alone, improved the tingling sensation in patients with PPI-refractory LPR in the present study, suggesting that acid may play a greater role in causing a sore throat than in globus sensation. Moreover, the LPR symptoms of globus sensation, sore throat, and excessive throat clearing may be induced by different mechanism. Johnston *et al*[25] reported absence or decreased expression of mucosal-protective proteins in laryngeal epithelial cells in 64% of patients with LPR. Thus, reducing the gastric content that passes into the laryngopharyngeal tissue *via* mucosal defenses may be an effective treatment for LPR. Rikkunshito has an effect on mucosal defenses in the gastroesophageal region, although the effect in the laryngopharynx is unclear[26,27]. In addition to the inhibitory effects of PPIs on acid, rikkunshito-induced stimulation of gastric emptying and effects on mucosal defense may contribute to the improvement in sore throat in the laryngopharynx.

The present study demonstrated that rikkunshito did not improve gastrointestinal symptoms in patients with PPI-refractory LPR assessed using the GSRS. In contrast, rikkunshito has been shown to improve upper gastrointestinal symptoms in PPI-refractory GERD patients assessed using the frequency scale for the symptoms of GERD score[12]. This discrepancy may be related to differences in the pathology and/or assessment tools used in the two studies.

In conclusion, rikkunshito treatment improved the globus sensation in patients with PPI-refractory LPR. The effect may be the result, at least in part, of the stimulation of gastric emptying. Rikkunshito plus PPI therapy may be an effective novel therapeutic strategy for PPI-refractory LPR symptoms, including globus sensation and sore throat.

COMMENTS

Background

Regarding the treatment of laryngopharyngeal reflux (LPR) symptoms such as globus sensation and a scratchy feeling, proton pump inhibitors (PPIs) are considered the mainstay. However, cases exist in which extraesophageal symp-

toms in the laryngopharynx are not improved by PPI.

Research frontiers

Recently, gastroesophageal reflux disease (GERD) has been considered a major cause of globus. However, the etiology of globus remains unclear. The authors have demonstrated that the cause of the globus sensation is elevated upper esophageal sphincter pressure, resulting from gastroesophageal reflux without direct exposure of the hypopharynx to acid.

Innovation and breakthroughs

Stimulation of gastric emptying or esophageal clearance in addition to inhibition of gastric acid secretion may also be efficacious in the treatment of LPR. It has been reported that addition of prokinetics, such as cisapride and itopride, to PPIs in the treatment of LPR reduced the recurrence of symptoms. However, there are few reports of the efficacy of prokinetics in the treatment of LPR.

Applications

Rikkunshito, a traditional Japanese medicine, has a dual action on the stomach: relaxation of the proximal stomach and contraction of the distal stomach. Recently, it was reported that rikkunshito improved upper gastrointestinal symptoms in PPI-refractory GERD patients. This was a prospective, randomized, parallel comparative study performed to examine the efficacy and safety of a therapeutic strategy using rikkunshito in patients with PPI-refractory LPR.

Peer review

The authors examined the effect of an herbal medicine "rikkunshito" on symptoms and gastric emptying in patients with LPR. The outcome of the study is interesting and important for the care of patients with PPI-refractory LPR.

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BRIEF ARTICLE

Emergency balloon-occluded retrograde transvenous obliteration of ruptured gastric varices

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Abstract

AIM: To evaluate the effectiveness and safety of emergency balloon-occluded retrograde transvenous obliteration (BRTO) for ruptured gastric varices.

METHODS: Emergency BRTO was performed in 17 patients with gastric varices and gastrorenal or gastrocaval shunts within 24 h of hematemesis and/or tarry stool. The gastric varices were confirmed by endoscopy, and the gastrorenal or gastrocaval shunts were identified by contrast-enhanced computed tomography (CE-CT). A 6-Fr balloon catheter (Cobra type) was inserted into the gastrorenal shunt *via* the right internal jugular vein, or into the gastrocaval shunt *via* the right femoral vein, depending on the varices drainage route. The sclerosant, 5% ethanolamine oleate iopamidol, was injected into the gastric varices through the catheter during balloon occlusion. In patients with incomplete thrombosis of the varices after the first BRTO, a second BRTO was performed the following day. Patients were followed up by endoscopy and CE-CT at 1 d, 1 wk, and 1, 3 and 6 mo after the procedure, and every 6 mo thereafter.

RESULTS: Complete thrombosis of the gastric varices was not achieved with the first BRTO in 7/17 patients because of large gastric varices. These patients underwent a second BRTO on the next day, and additional sclerosant was injected through the catheter. Complete thrombosis which led to disappearance of the varices was achieved in 16/17 patients, while the remaining patient had incomplete thrombosis of the varices. None of the patients experienced rebleeding or recurrence of the gastric varices after a median follow-up of 1130 d (range 8-2739 d). No major complications occurred after the procedure. However, esophageal varices worsened in 5/17 patients after a mean follow-up of 8.6 mo.

CONCLUSION: Emergency BRTO is an effective and safe treatment for ruptured gastric varices.

Key words: Emergency balloon-occluded retrograde transvenous obliteration; Gastric varices; Bleeding; Portal hypertension; Ethanolamine oleate

Core tip: As ruptured gastric varices are associated with high rates of recurrent bleeding and mortality, quick treatment is essential. Balloon-occluded retrograde transvenous obliteration (BRTO) is a minimally invasive treatment for gastric varices with a high success rate and a low recurrence rate. Emergency BRTO is an effective and safe treatment, providing temporary hemostasis of ruptured gastric varices can be achieved, allowing the sclerosant to accumulate in the varices.

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INTRODUCTION

As gastric varices have greater blood flow compared with esophageal varices, ruptured gastric varices can cause massive hemorrhage, and are associated with high rates of recurrent bleeding and mortality (45%-55%)[1,2]. Therefore, ruptured gastric varices must be treated as quickly as possible. Balloon-occluded retrograde transvenous obliteration (BRTO) is a minimally invasive treatment for gastric varices, with a high success rate and a low recurrence rate[3-7]. However, few reports have evaluated emergency BRTO for ruptured gastric varices[8,9]. In this study, we report the long-term outcomes of emergency BRTO performed within 24 h of hematemesis and/or tarry stool.

MATERIALS AND METHODS

The effectiveness and safety of emergency BRTO for ruptured gastric varices were evaluated retrospectively. Between March 1998 and December 2008, BRTO was performed for gastric varices with gastrorenal or gastrocaval shunts in 79 patients. Of these patients, emergency BRTO was performed for ruptured gastric varices within 24 h of hematemesis and/or tarry stool in 17 patients. The patients' ages ranged from 33 to 79 years, and the mean age was 58.8 years. All patients had liver cirrhosis corresponding to Child-Pugh class A in 2 patients, class B in 12 patients, and class C in 3 patients. The etiologies of liver cirrhosis were hepatitis C in seven patients, hepatitis B in three patients, alcoholic liver disease in four patients, primary biliary cirrhosis in one patient, and unknown in two patients. Mean creatinine value before BRTO was 0.82 mg/dL (normal range: 0.64-1.11 mg/dL), and we had no patients with renal dysfunction. Informed consent for BRTO was obtained from all patients.

The gastric varices were confirmed by endoscopy. According to Sarin classification[2], isolated varices in the fundus of the stomach were found in 13 of 17 patients, and gastroesophageal varices in the remaining 4 patients. Also, white plugs which indicated bleeding sites were found in 7 patients, and oozing in 3 patients. Temporary hemostasis was achieved spontaneously in 9/17 patients and by balloon compression in 8/17 patients. The presence and diameter of the gastrorenal or gastrocaval shunts were evaluated by contrast-enhanced computed tomography (CE-CT). The gastric varices drained *via* the gastrorenal shunt in 16 patients, and by the gastrocaval shunt in 1 patient. An 8-Fr sheath (Cobra type; Medikit, Tokyo, Japan) was inserted into the left renal vein through the right internal jugular vein with ultrasound-guided puncture while an 8-Fr sheath (Straight type; Medikit) was inserted into the inferior vena cava through the right femoral vein. A 6-Fr balloon catheter (Cobra type; Clinical Supply, Gifu, Japan) was inserted into the gastrorenal or gastrocaval shunt. The balloon diameter was 13 or 20 mm. In patients with a shunt diameter $\geqslant$ 13 mm, a 20 mm diameter balloon was used. A sclerosing agent, 5% ethanolamine oleate iopamidol (EOI), was infused through a balloon catheter or a microcatheter placed close to the gastric varices during balloon occlusion. In the 13 most recent cases, microcatheters were used to decrease the sclerosant dose. We prepared 5% EOI by mixing 10 mL of contrast material with 10 mL of 10% ethanolamine oleate (Oldamin; Glelan Pharmaceutical, Tokyo, Japan). The infusion of 5% EOI was continued until the entire gastric varices and feeding veins were rendered opaque. The mean dose of 5% EOI per procedure was 21.3 mL (range 2-40 mL). The balloon occlusion time ranged from 12 to 48 h. The catheters were fixed in place using sterilized tape (Hogy Medical, Tokyo, Japan). The morning after BRTO, thrombosis of the gastric varices was evaluated by CE-CT. In patients with incomplete thrombosis after the first BRTO, a second BRTO was performed the following day[7]. After complete thrombosis of gastric varices was confirmed by CE-CT, the catheters were removed. To prevent renal damage caused by EOI-related hemolysis, 4000 units of haptoglobin (Mitsubishi Pharma, Osaka, Japan) was intravenously administered to all patients[10,11]. Patients underwent endoscopy and CE-CT at 1 d, 1 wk, and 1, 3 and 6 mo after the procedure, and every 6 mo thereafter.

RESULTS

Complete thrombosis of the gastric varices was not achieved with the first BRTO in 7/17 patients because of large gastric varices. These patients underwent a second BRTO on the next day, and additional sclerosant was injected through the catheter[7]. Complete thrombosis which led to disappearance of the varices was achieved in 16/17 patients (Figures 1 and 2), while the other patient had incomplete thrombosis of the varices. None of the patients experienced rebleeding or recurrence of the gastric varices during a median follow-up of 1130 d (range 8-2739 d). However, esophageal varices worsened in 5/17 patients during a mean follow-up of 8.6 mo[12-14] (Table 1). In two of these five patients, red-colored esophageal varices were treated by endoscopic sclerotherapy. Reddening of the variceal mucosa is associated with a high risk of variceal bleeding[15].

All of the complications were transient[16], and included sclerosant-induced hematuria (7/17 patients), abdominal pain (8/17), high fever (6/17), sclerosant-induced blood pressure elevation (1/17), headache (2/17), pleural effusion (15/17), and ascites (12/17). Although extravasation of the sclerosant occurred in one patient

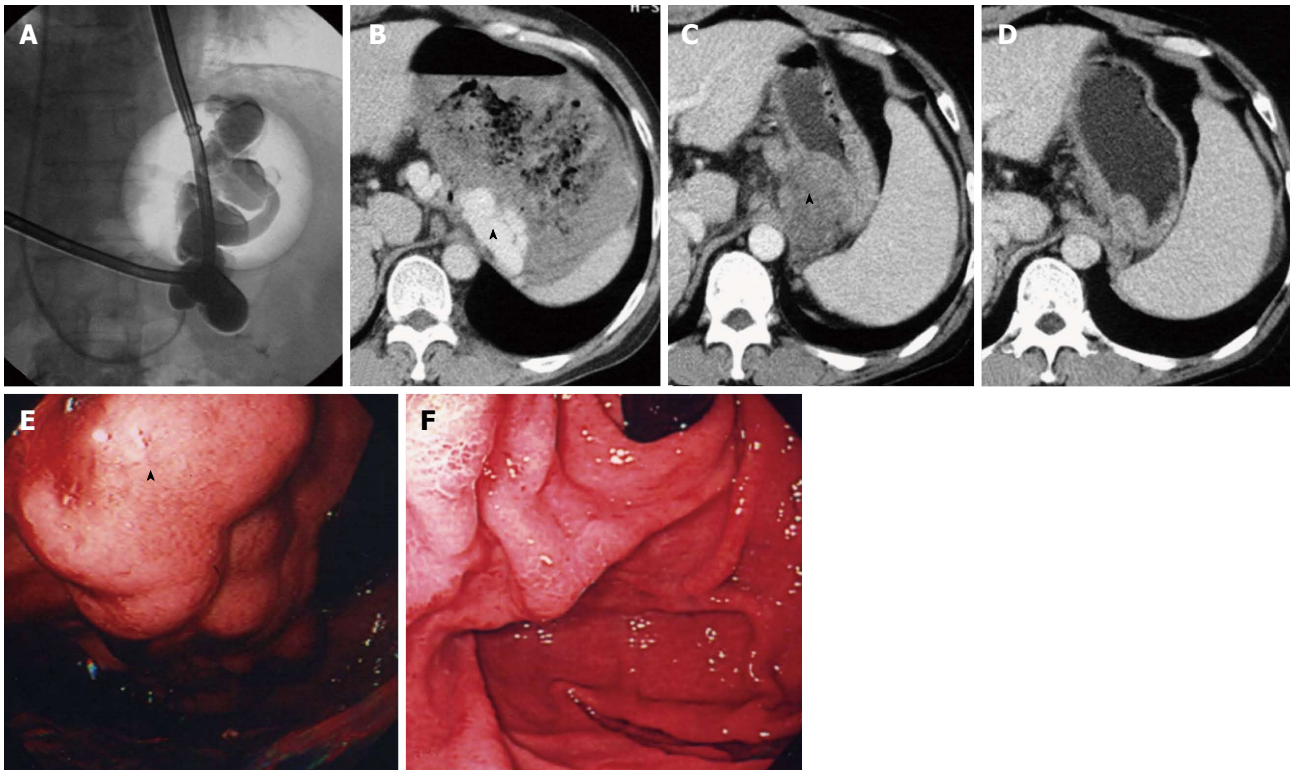

Figure 1 Gastric varices with a gastrorenal shunt (case 15). A: Balloon-occluded retrograde transvenous obliteration (BRTO) was performed 11 h after hematemesis. The gastric varices and a gastrorenal shunt were filled with 36 mL of 5% ethanolamine oleate iopamidol. A Sengstaken–Blakemore tube was inserted into the stomach for temporary hemostasis; B: Contrast-enhanced computed tomography (CE-CT) image taken before BRTO shows gastric varices (arrowhead) with a massive hematoma; C: CE-CT image taken 1 wk after BRTO shows complete thrombosis of the varices (arrowhead); D: CE-CT image taken 6 mo after BRTO shows complete disappearance of the varices; E: Endoscopy performed before BRTO shows gastric varices (arrowhead) with oozing; F: Endoscopy performed 6 mo after BRTO shows complete disappearance of the varices.

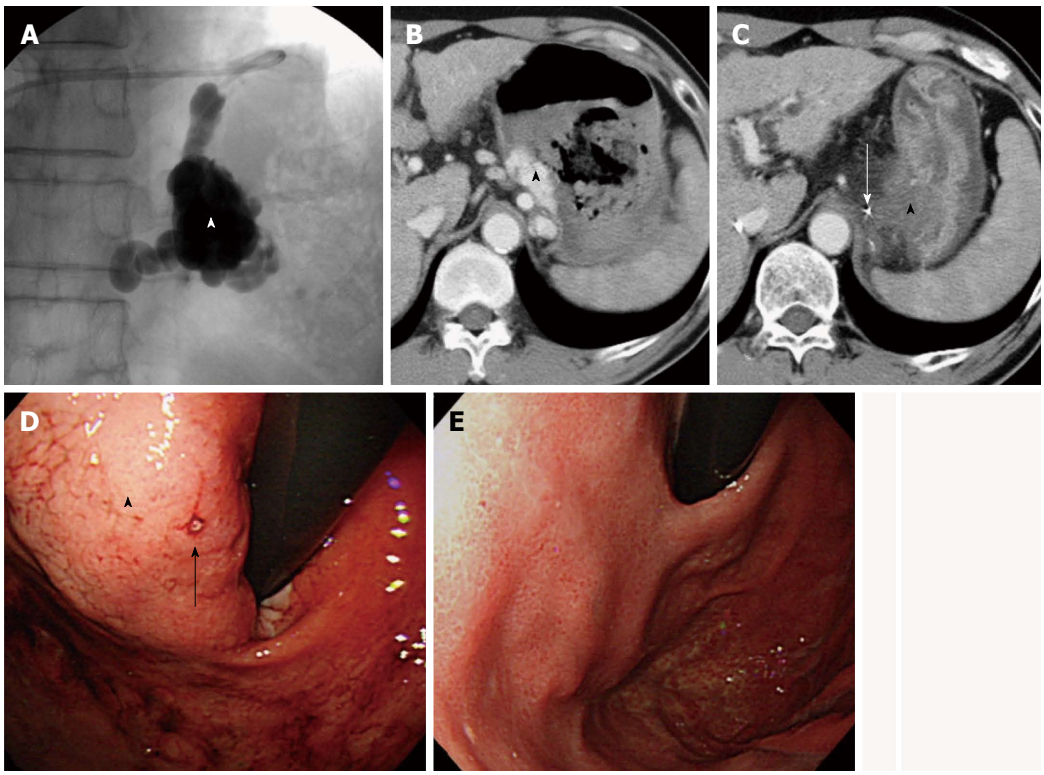

Figure 2 Gastric varices with a gastrocaval shunt (case 8). A: A balloon catheter was inserted into a gastrocaval shunt and 18 mL of 5% EOI was injected through the microcatheter that had been advanced close to the gastric varices; B: Contrast-enhanced computed tomography (CE-CT) image taken before balloon-occluded retrograde transvenous obliteration (BRTO) shows gastric varices (arrowhead) with a massive hematoma; C: CE-CT image taken the day after BRTO shows complete thrombosis of the varices (arrowhead) and that the tip of the microcatheter (arrow) is close to the varices; D: Endoscopy performed before BRTO shows bleeding site (arrow) of the gastric varices (arrowhead); E: Endoscopy performed 3 mo after BRTO shows complete disappearance of the varices.

Table 1 Patient characteristics and clinical outcomes

Case	Age (yr)	Sex	Cause of LC	Child-Pugh class	Temporary hemostasis	Drainage route	Dose of 5% EOI (mL)	Eradication of GV	Worsening of EV	Follow-up time (d)	Clinical outcome
1	63	M	HCV	B	Spontaneous	GR	30	Complete	–	205	Alive
2	59	M	HBV	B	Spontaneous	GR	40	Complete	+	2229	Alive
3	58	F	PBC	A	Spontaneous	GR	18	Complete	–	2197	HF[1]
4	67	M	HCV	A	Spontaneous	GR	12	Complete	–	2529	HCC[2]
5	79	F	Unknown	C	Spontaneous	GR	35	Complete	–	135	HF[1]
6	46	M	HCV	B	Balloon	GR	36	Complete	–	388	Alive
7	66	F	Alcohol	B	Spontaneous	GR	10 + 17	Complete	+	2739	HF[1]
8	59	M	Alcohol	B	Spontaneous	GC	18	Complete	–	1501	Alive
9	70	M	HCV	B	Spontaneous	GR	17	Complete	–	8	Alive
10	33	F	Unknown	B	Balloon	GR	30 + 10	Complete	–	1293	Alive
11	46	M	HBV	C	Balloon	GR	36 + 23	Complete	–	41	Alive
12	57	M	HBV	B	Balloon	GR	30 + 28	Complete	+	164	HF[1]
13	66	M	Alcohol	B	Balloon	GR	18 + 13	Partial	–	14	HF[1]
14	65	F	HCV	B	Balloon	GR	10 + 8	Complete	–	1130	Alive
15	51	M	HCV	B	Balloon	GR	36	Complete	+	2466	HF[1]
16	53	M	Alcohol	C	Balloon	GR	15	Complete	+	545	HF[1]
17	62	F	HCV	B	Spontaneous	GR	20 + 2	Complete	–	1835	Alive

[1]Died of hepatic failure (HF); [2]Died of hepatocellular carcinoma (HCC). LC: Liver cirrhosis; PBC: Primary biliary cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; GR: Gastrorenal shunt; GC: Gastrocaval shunt; EOI: Ethanolamine oleate iopamidol; GV: Gastric varices; EV: Esophageal varices.

during BRTO, the procedure was continued and achieved complete thrombosis of the varices. No major complications, such as renal failure, pulmonary embolism, or liver failure, occurred after the procedure.

DISCUSSION

The cumulative risk for hemorrhage from gastric fundal varices has been reported to be 16%, 36% and 44% at 1, 3 and 5 years, respectively[17]. Ruptured gastric varices are also associated with high rates of rebleeding and mortality (45%-55%)[1,2]. Therefore, ruptured gastric varices must be treated as quickly as possible.

As most patients with ruptured gastric varices are in a critical state because of hypovolemic shock, surgical treatment and transjugular intrahepatic portosystemic shunts (TIPS) are too invasive and risky. The mortality of patients with esophageal varices undergoing emergency surgery was reported to be 38.4%[18]. Although TIPS is a treatment for portal hypertension to decrease the portal pressure, Miller-Catchpole[19] reported some of the problems of TIPS, which included technical failure, restenosis or occlusion of the shunt, dislocation of the stent, and hepatic encephalopathy. Overall, 21% of patients (86/416) died because of bleeding, liver failure, or multiple organ failure. Only 50% of patients had improvements in gastric fundal varices after TIPS[20]. Furthermore, the cumulative gastric variceal bleeding rate at 1 year was 20% in patients who underwent TIPS compared with 2% in patients who underwent transcatheter sclerotherapy ($P < 0.01$) (Kaplan-Meier method and Log-rank test)[21]. Although percutaneous transhepatic obliteration (PTO) may achieve temporary embolization of gastric varices, the varices recur very quickly[22]. Because gastric varices usually have many feeding veins, it is difficult to embolize all of them by PTO. Arai *et al*[23] reported that PTO achieved a success rate of 44% (8/18) but the recurrence rate of gastric varices was 38% (3/8). By contrast, BRTO was found to have a success rate of 81% (75/93) and the recurrence rate of gastric varices was just 4% (3/75). Although the Baveno Consensus[24] suggests endoscopic cyanoacrylate injection for bleeding from isolated gastric varices, it is also difficult to apply endoscopic methods, such as endoscopic injection sclerotherapy (EIS), endoscopic variceal ligation (EVL), and sclerotherapy using cyanoacrylate, to ruptured gastric varices because of their extensive blood supply. Additionally, the mortality rate of EIS in patients with bleeding gastric varices was 55%[1]. EVL using a rubber band[25] has also been associated with a high risk of adverse outcomes for treating ruptured gastric varices, as it often causes re-rupture during the procedure, and has a high incidence of rebleeding. The rebleeding rate of EVL was significantly higher than that of endoscopic obturation using cyanoacrylate (54% *vs* 31%; $P = 0.0005$)[26]. Endoscopic-guided injection of cyanoacrylate into the varices may induce multiple organ embolisms, such as cerebral infarction[27] and pulmonary embolisms[28,29], which are caused by leakage of the sclerosant into the systemic circulation. Furthermore, cyanoacrylate treatment of gastric variceal bleeding has a high rate of early bleeding (15.5%-20.5%)[30,31]. By contrast, BRTO is a minimally invasive treatment of gastric varices that is associated with a high success rate and a low recurrence rate[3-7]. Therefore, it may be much more effective than surgery, TIPS, PTO or endoscopic treatment for critical patients.

Ethanolamine oleate is a sclerosant that damages endothelial cells and induces thrombus formation in the vessel. EOI was prepared by mixing ethanolamine oleate with contrast medium to monitor the movement of EOI under fluoroscopy. To prevent EOI-related complications caused by excess sclerosant, we believe that < 40 mL of

5% EOI should be used during individual BRTO procedures. If complete thrombosis of large gastric varices is not achieved, a second BRTO can be performed the following day, and additional sclerosant can be injected through the catheter that was left in place overnight[7]. To decrease the sclerosant dose, 50% glucose solution[32] or polidocanol foam[33] may be used during BRTO procedures. Haptoglobin has also been intravenously administered to prevent renal failure[10,11].

In our study, the BRTO procedure achieved temporary hemostasis in all of the patients. If active bleeding from the gastric varices continues during the procedure, then 5% EOI may be unable to control the bleeding, because it will leak into the gastric lumen. This displaces the sclerosant and prevents it from accumulating in the gastric varices. In such situations, transportal or transesophageal sclerotherapy with cyanoacrylate and coils may be necessary. The coils serve as a scaffold to trap the cyanoacrylate preventing pulmonary embolism[34-36]. If temporary hemostasis by balloon compression is achieved, we perform BRTO. On the other hand, if temporary hemostasis by balloon compression is not achieved and the gastric varices continue to spurt blood, we perform transportal or transesophageal sclerotherapy with cyanoacrylate and coils.

Esophageal varices worsened in 5/17 patients in this study. The occlusion of a gastrorenal shunt probably induced the esophageal varices through another collateral route. BRTO was reported to significantly increase the portal systemic pressure gradient[13] and increase the bleeding rates of coexisting esophageal varices[12]. Other major risk factors identified for worsening of esophageal varices after BRTO were the presence of esophageal varices, higher Child-Pugh class, and higher resistance index assessed by endoscopic color Doppler ultrasonography before BRTO[14]. Therefore, esophageal varices should be endoscopically checked every 6 mo after BRTO.

As ruptured gastric varices are associated with high rates of recurrent bleeding and mortality, quick treatment is essential. BRTO is a minimally invasive treatment for gastric varices with a high success rate and a low recurrence rate. Emergency BRTO is an effective and safe treatment, providing temporary hemostasis of ruptured gastric varices can be achieved, allowing the sclerosant to accumulate in the varices.

COMMENTS

Background

As gastric varices have greater blood flow compared with esophageal varices, ruptured gastric varices can cause massive hemorrhage, and are associated with high rates of recurrent bleeding and mortality. Therefore, ruptured gastric varices must be treated as quickly as possible.

Research frontiers

In this study, the authors demonstrate the long-term results of emergency balloon-occluded retrograde transvenous obliteration (BRTO) for ruptured gastric varices.

Innovations and breakthroughs

Sixteen of 17 patients had complete thrombosis leading to disappearance of gastric varices. One patient had incomplete thrombosis leading to reduction of varices.

Applications

Patients who have ruptured gastric varices and gastrorenal or gastrocaval shunts can be treated with emergency BRTO within 24 h of hematemesis and/or tarry stool.

Terminology

Emergency BRTO is a procedure where a balloon catheter is inserted into a draining vein of gastric varices, and the sclerosant can be injected into the varices through the catheter during balloon occlusion. The sclerosant damages endothelial cells of the varices resulting in thrombosis and disappearance of the varices.

Peer review

The authors reported the results of a retrospective study on patients who underwent emergency BRTO for ruptured gastric varices. Emergency BRTO is an effective and safe treatment, providing temporary hemostasis of ruptured gastric varices can be achieved, allowing the sclerosant to accumulate in the varices.

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BRIEF ARTICLE

Connective tissue diseases in primary biliary cirrhosis: A population-based cohort study

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Abstract

AIM: To establish the frequency and clinical features of connective tissue diseases (CTDs) in a cohort of Chinese patients with primary biliary cirrhosis (PBC).

METHODS: Three-hundred and twenty-two Chinese PBC patients were screened for the presence of CTD, and the systemic involvement was assessed. The differences in clinical features and laboratory findings between PBC patients with and without CTD were documented. The diversity of incidence of CTDs in PBC of different countries and areas was discussed. For the comparison of normally distributed data, Student's t test was used, while non-parametric test (Wilcoxon test) for the non-normally distributed data and 2×2 χ^2 or Fisher's exact tests for the ratio.

RESULTS: One-hundred and fifty (46.6%) PBC patients had one or more CTDs. The most common CTD was Sjögren's syndrome (SS, 121 cases, 36.2%). There were nine cases of systemic sclerosis (SSc, 2.8%), 12 of systemic lupus erythematosus (SLE, 3.7%), nine of rheumatoid arthritis (RA, 2.8%), and 10 of polymyositis (PM, 3.1%) in this cohort. Compared to patients with PBC only, the PBC + SS patients were more likely to have fever and elevated erythrocyte sedimentation rate (ESR), higher serum immunoglobulin G (IgG) levels and more frequent rheumatoid factor (RF) and interstitial lung disease (ILD) incidences; PBC + SSc patients had higher frequency of ILD; PBC + SLE patients had lower white blood cell (WBC) count, hemoglobin (Hb), platelet count, γ-glutamyl transpeptidase and immunoglobulin M levels, but higher frequency of renal involvement; PBC + RA patients had lower Hb, higher serum IgG, alkaline phosphatase, faster ESR and a higher ratio of RF positivity; PBC + PM patients had higher WBC count and a tendency towards myocardial involvement.

CONCLUSION: Besides the common liver manifestation of PBC, systemic involvement and overlaps with other CTDs are not infrequent in Chinese patients. When overlapping with other CTDs, PBC patients manifested some special clinical and laboratory features which may have effect on the prognosis.

Key words: Cirrhosis; Biliary; Connective tissue disease; Sjögren's syndrome; Systemic sclerosis; Raynaud phenomenon

Core tip: This study demonstrated that primary biliary cirrhosis (PBC) is a complicated disease that not only involves the liver but also often coexists with other connective tissue diseases (CTDs). Evaluation of our cohort of 322 Chinese PBC patients showed that Sjögren's syndrome was the CTD that most frequently coexisted with PBC. In addition, it was also shown that when CTDs coexist with PBC, the clinical features and the disease course are different from those in patients with PBC alone. Our collective results suggest that Chinese patients with PBC may benefit from assessment of systemic involvement and screening for CTDs through detection of autoantibodies.

Wang L, Zhang FC, Chen H, Zhang X, Xu D, Li YZ, Wang Q, Gao LX, Yang YJ, Kong F, Wang K. Connective tissue diseases in primary biliary cirrhosis: A population-based cohort study. *World J Gastroenterol* 2013; 19(31): 5131-5137 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5131.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5131

INTRODUCTION

Primary biliary cirrhosis (PBC), which predominantly affects middle-aged women, is histologically characterized by chronic non-suppurative destructive cholangitis. Although the liver is the chief target, PBC may involve multiple systems, such as interstitial lung disease (ILD)[1,2], pulmonary artery hypertension (PAH)[3], and nephritis[4]. PBC is also associated with other connective tissue diseases (CTDs) and autoimmune disorders, such as Sjögren's syndrome (SS), systemic sclerosis (SSc), and systemic lupus erythematosus (SLE).

These co-existing conditions frequently increase the difficulty in making a diagnosis and treating the disease. They may also change the natural course and prognosis of PBC. We have established a database of PBC patients admitted to our hospital during the past ten years, to serve as a resource of data for studies of PBC features and outcomes. In this report, we describe our analysis of these patients' data to determine the frequencies of extrahepatic lesions and association of CTDs.

MATERIALS AND METHODS

Patients

Chinese patients with PBC (294 women, 28 men; age mean: 53 years, range: 20-81 years old), who attended our hospital during 2002-2012, were prospectively entered into our collective database and retrospectively analyzed in this study. PBC diagnosis was made according to the criteria published in the guidelines of the American Association for the Study of Liver Diseases[5,6]. The majority (91.9%) of the patients resided in northern China, with 23.6% of those individuals being from Beijing.

Diagnostic criteria for CTDs and definition of organ involvement

Diagnosis of SS was made if the patient fulfilled the 2002 European diagnostic criteria[7]. Diagnosis of SSc (including scleroderma) was made according to the 1980 American College of Rheumatology (ACR) criteria[8]. Diagnosis of SLE was made according to the 1997 revised ACR criteria[9] and the 2009 Systemic Lupus International Collaborating Clinic revision of the ACR classification criteria for SLE[10]. Diagnosis and classification of rheumatoid arthritis (RA) were made according to the 1987 revised ACR[11] and 2010 ACR/European League Against Rheumatism criteria[12]. Diagnoses of polymyositis (PM) or dermatomyositis (DM) were made according to the criteria reported by Bohan and Peter[13], and diagnosis of mixed connective tissue disease (MCTD) was made according to the 1987 Alarcon-Segovia criteria[14].

Renal involvement was defined by persistent protein urea of > 0.5 g/d, and/or glomerular haematuria, and/or cellular casts[15]. Cardiac involvement was defined by the presence of cardiomyopathy, pericarditis, or arrhythmia.

Statistical analysis

SPSS version 11.5 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis of the data. Main results were presented as mean ± SD. According to the type and distribution of the data, the statistical significance was estimated by Student's *t* test, Wilcoxon test, or $2 \times 2\ \chi^2$ or Fisher's exact tests. *P* values < 0.05 were considered to be statistically significant.

RESULTS

General characteristics of PBC patients

Of the 322 PBC patients enrolled in the study, the mean time from onset of symptoms to diagnosis was 5.8 years. Anti-nuclear antibody (ANA) was present in 87.0% of the patients, while anti-mitochondrial antibody (AMA) was present in 90.9% of the patients, among which 90.3% were also positive for the M_2 subtype of AMA (AMA-M_2). Seventy-two (22.4%) of the total patients underwent liver biopsy (Table 1).

CTDs in PBC patients and inter-study comparison with other countries

One-hundred and fifty (46.6%) of the patients had CTDs, 11 (3.4%) of which had two or more CTDs (Figure 1). SS (121 cases, 36.2%) was the most frequent CTD represented. There were nine cases of SSc (2.8%), 12 of SLE (3.7%), 9 of RA (2.8%), and 10 of PM (3.1%) in this cohort. no DM or MCTD coexisted with PBC.

The incidence of PBC + SS in the current study was significantly higher than that reported in either the United

Table 1 Baseline characteristics of the study population

Characteristic	Cohort representation
Sex, female/male	294/28
Age (yr)	53 ± 12
Duration of disease (yr)	5.8 ± 3.5
Mayo risk score	4.5 ± 1.1
Positive ANA	275/316 (87.0)
Positive AMA	290/319 (90.9)
Positive AMA-M2	262/290 (90.3)
Titers of AMA-M2 (IU/mL)	139.6 ± 107.9
Liver biopsy	72 (22.4)

Data are presented as mean ± SD, ratio or *n* (%). ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; AMA-M2: M2 subtype of anti-mitochondrial antibody.

Table 2 Inter-study comparison of patterns of connective tissue diseases in primary biliary cirrhosis patients *n* (%)

	Wang *et al* China	Watt *et al*[16] United Kingdom	Marasini *et al*[17] Italy
PBC	322	160	170
CTDs in PBC	150 (46.6)	84 (53.0)	62 (36.5)
PBC + SS	121 (37.6)	40 (25.0)[a]	6 (3.5)[a]
PBC + SSc	9 (2.8)	12 (8.0)[a]	21 (12.3)[a]
PBC + SLE	12 (3.7)	2 (1.3)	3 (1.8)
PBC + RA	9 (2.8)	27 (17.0)[a]	3 (1.8)
PBC + PM	10 (3.1)	0 (0.0)	1 (0.6)
PBC + MCTD	0 (0.0)	0 (0.0)	1 (0.6)

[a]$P < 0.05$ *vs* Wang *et al* (China, the current study). PBC: Primary biliary cirrhosis; CTDs: Connective tissue diseases; SS: Sjögren's syndrome; SSc: Systemic sclerosis; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; PM: Polymyositis; MCTD: Mixed connective tissue disease.

Kingdom study[16] (37.6% *vs* 25.0%, $P = 0.006$) or the Italy study[17] (37.6% *vs* 3.5%, $P = 0.000$), while the frequency of PBC + SSc was much lower (2.8% *vs* 8.0%, $P = 0.017$; 2.8% *vs* 12.3%, $P = 0.000$). The frequency of RA in the current study was less than that in the UK study (2.8% *vs* 17.0%, $P = 0.000$) but about the same as in the Italy study. The frequencies of PBC + SLE, + PM and + MCTD were not different between the three studies (Table 2).

Primary biliary cirrhosis patients with and without connective tissue diseases

There were no significant differences between PBC patients with and without CTDs in terms of sex, age, incidences of Raynaud's phenomenon (RP) or PAH, or levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), ANA, AMA, or AMA-M2 (Table 3).

Compared with PBC patients, the PBC + SS patients had significantly higher incidence of fever (6.4% *vs* 15.7%, $P = 0.010$) and ILD (7.6% *vs* 22.3%, $P = 0.000$), while the PBC + RA patients had significantly higher incidence of arthralgia (21.5% *vs* 100%, $P = 0.000$) and the PBC + SSc patients also had significantly higher incidence of ILD (7.6% *vs* 33.3%, $P = 0.035$). Patients with PBC + PM were likely to have cardiac involvement, most frequently cardiomyopathy (40% *vs* PBC patients: 2.9%, $P = 0.001$), and renal involvement was more common in patients with

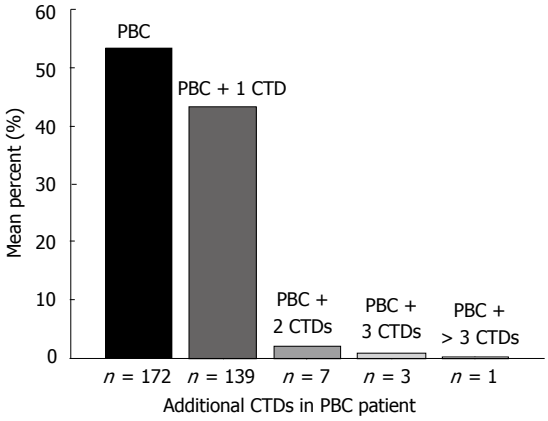

Figure 1 Percentage of primary biliary cirrhosis patients with varying numbers of connective tissue diseases. CTDs: Connective tissue diseases; PBC: Primary biliary cirrhosis.

PBC + SLE (33.3% *vs* PBC patients: 5.2%, $P = 0.006$).

The most common disease coexisting with PBC was SS. Compared to PBC patients without SS, the PBC + SS patients had higher serum level of immunoglobulin G (IgG; 17.1 ± 6.2 g/L *vs* 21.0 ± 12.4 g/L, $P = 0.004$), faster erythrocyte sedimentation rate (ESR; 41 ± 28 mm/h *vs* 57 ± 38 mm/h, $P = 0.032$), and higher rates of positivity for rheumatoid factor (RF; 19.9% *vs* 76.7%, $P = 0.000$). There were no significant differences in the clinical characteristics of patients who had PBC + SSc and those with PBC alone.

Compared to PBC patients, the PBC + SLE patients had lower white blood cell count (WBC; 5.7 ± 2.6 × 10^9/L *vs* 4.0 ± 1.1 × 10^9/L, $P = 0.005$), level of hemoglobin (Hb; 126 ± 20 g/L *vs* 102 ± 20 g/L, $P = 0.003$), platelet count (PLT; 189 ± 82 × 10^9/L *vs* 96 ± 75 × 10^9/L, $P = 0.001$), serum levels of γ-glutamyl transpeptidase (γ-GT; 320 ± 340 U/L *vs* 207 ± 153 U/L, $P = 0.048$), and IgM (4.5 ± 4.7 g/L *vs* 1.9 ± 1.2 g/L, $P = 0.001$). Compared to PBC patients without RA, patients with PBC + RA had lower Hb (126 ± 20 g/L *vs* 110 ± 11 g/L, $P = 0.001$), but higher levels of serum alkaline phosphatase (ALP, 250 ± 221 U/L *vs* 487 ± 411 U/L, $P = 0.047$) and IgG (17.1 ± 6.2 g/L *vs* 22.2 ± 5.1 g/L, $P = 0.004$), ESR (41 ± 28 mm/h *vs* 76 ± 30 mm/h, $P = 0.004$), and ratio of positive RF (19.9% *vs* 100.0%, $P = 0.009$). Compared to PBC patients, patients with PBC + PM had higher WBC count (5.7 ± 2.6 × 10^9/L *vs* 7.9 ± 3.4 × 10^9/L, $P = 0.048$).

DISCUSSION

Autoimmune diseases exhibit an increased immune response to self-antigens, occur predominantly in females, and share some similar pathogenic pathways or genetic etiologies[18,19]. Consequently, it is common for more than one autoimmune condition to occur in a single patient. For instance, the classic model of SS shows its secondary nature to SLE[20] and SSc overlapping with PM[21]. Similarly PBC often overlaps with other autoimmune diseases and conditions, thereby causing not only liver damage but

Table 3 Clinical features and laboratory results of patients with primary biliary cirrhosis alone and patients with primary biliary cirrhosis plus one other connective tissue diseases

	PBC (n = 172)	PBC + SS (n = 121)	PBC + SSc (n = 9)	PBC + SLE (n = 12)	PBC + RA (n = 9)	PBC + PM (n = 10)
Female/male	153/19	112/9	9/0	12/0	7/2	7/3
Age (yr)	53 ± 11	53 ± 12	51 ± 6[1]	50 ± 9[1]	59 ± 121	53 ± 8[1]
Fever	11 (6.4)	19 (15.7)[a]	0 (0)	3 (25.0)	1 (11.1)	1 (10.0)
RP	32 (18.6)	23 (19.0)	4 (44.4)	5 (41.7)	0 (0)	0 (0)
Arthralgia	37 (21.5)	31 (25.6)	2 (22.2)	4 (33.3)	9 (100)[a]	1 (10)
ILD	13 (7.6)	27 (22.3)[a]	3 (33.3)[a]	0 (0)	1 (11.1)	2 (20)
PAH	11 (6.4)	13 (10.7)	2 (22.2)	1 (8.3)	0 (0)	0 (0)
Cardiac	5 (2.9)	3 (2.5)	0 (0)	0 (0)	0 (0)	4 (40.0)[a]
Renal	9 (5.2)	8 (6.6)	1 (11.1)	4 (33.3)[a]	0 (0)	0 (0)
WBC (10^9/L)	5.7 ± 2.6	5.0 ± 2.9	5.7 ± 2.8[1]	4.0 ± 1.1[1,a]	5.7 ± 2.4[1]	7.9 ± 3.4[1,a]
Hb (g/L)	126 ± 20	118 ± 18	120 ± 23[1]	102 ± 20[1,a]	110 ± 11[1,a]	127 ± 16[1]
PLT (10^9/L)	189 ± 82	135 ± 69	190 ± 103	96 ± 75[a]	247 ± 142	206 ± 9[1]
ALT (U/L)	79 ± 76	91± 75	76 ± 62[1]	69 ± 72[1]	56 ± 46[1]	73 ± 56[1]
AST (U/L)	76± 62	90 ± 64	96 ± 41[1]	69 ± 55[1]	79 ± 52[1]	73 ± 33[1]
ALP (U/L)	250 ± 221	287 ± 224	291 ± 166[1]	173 ± 98[1]	487 ± 411[1,a]	153 ± 98[1]
γ-GT (U/L)	320 ± 340	309 ± 290	344 ± 346[1]	207 ± 153[1,a]	239 ± 166[1]	264 ± 275[1]
IgG (g/L)	17.1 ± 6.2	21.0 ± 12.4[a]	17.3 ± 5.8[1]	15.8 ± 5.2[1]	22.2 ± 5.1[1,a]	16.4 ± 4.4[1]
IgM (g/L)	4.5 ± 4.7	4.3 ± 3.9	3.4 ± 1.8[1]	1.9 ± 1.2[1,a]	4.2 ± 3.6[1]	5.4 ± 3.1[1]
ESR (mm/1h)	41 ± 28	57 ± 38[a]	47 ± 34[1]	47 ± 25[1]	76 ± 30[1,a]	48 ± 20[1]
RF+	31/156 (19.9)	92/120 (76.7)[a]	4/8 (50.0)	5/11 (45.5)	9/9 (100)[a]	4/10 (40.0)
ANA	142/169 (84.0)	111/119 (93.3)	9/9 (100)	12/12 (100)	8/9 (88.9)	10/10 (100)
AMA	153/171 (89.5)	109/120 (90.8)	8/9 (88.9)	11/12 (91.7)	8/9 (88.9)	10/10 (100)
AMA-M_2 (IU/mL)	147± 125	130 ± 105	119 ± 115[1]	160 ± 116[1]	139 ± 118[1]	172 ± 138[1]

Data are presented as mean ± SD, ratio or n (%).[1]Non-normally distributed data compared with PBC patients by the Wilcoxon test. [a]$P < 0.05$ *vs* PBC patients. PBC: Primary biliary cirrhosis; SS: Sjögren's syndrome; SSc: Systemic sclerosis; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; PM: Polymyositis; WBC: White blood cell count; Hb: Hemoglobin; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GT: γ-glutamyl transpeptidase; IgG: Immunoglobulin G; IgM: Immunoglobulin M; ESR: Erythrocyte sedimentation rate; RF: Rheumatoid factor; ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; AMA-M_2: M_2 subtype of anti-mitochondrial antibody.

also extrahepatic injury.

An epidemiological study from United States showed that one-third of 1032 patients with PBC were affected by another autoimmune disease, most commonly SS, RP, autoimmune thyroid disease, scleroderma, or SLE[22]. Yet another United States-based study reported that about 72% of the PBC patients also had SS, and 20% of PBC patients had joint disease[23]. Similarly, a previous study of the United Kingdom showed that 53% of the PBC patients had at least one additional autoimmune condition, with the most common being SS, autoimmune thyroid disease, RA, and SSc[16]. None of these patients had concomitant PM or DM. An Italian-based study of 170 PBC cases showed that the highest-frequency CTD was SSc (21 cases, 12.3%)[17].

The data from the current study showed that SS was the most common CTD that coexisted with PBC. The frequency was higher compared to rates reported in Europe[16,17]. SS and PBC are both characterized by immune-mediated progressive destruction of the epithelial tissues, with SS mainly affecting the salivary and lacrimal glands and PBC mainly affecting the small bile ducts[24]. Clinically, many PBC patients also present with dry eyes or mouth (47%-73%), and focal lymphocytic infiltration of labial glands (26%-93%)[25-27]. Nevertheless, in these PBC patients, anti-SSA or anti-SSB antibodies were rarely detected, and sequelae were milder than in the primary SS patients. They were also found to express lower levels of human leukocyte antigen-B8, DR3, and DRW52 compared to the primary SS patients[24]. Perhaps, only PBC patients who met the criteria of SS and also had exact anti-SSA or anti-SSB antibodies had really overlapping SS. In the current study, all of the PBC patients who met the criteria of SS were included[7], regardless of whether specific antibodies were present or not, which likely explains the particularly high number of PBC + SS patients in the current study's cohort. Compared to patients with PBC only, the PBC + SS patients were more likely to have fever and elevated ESR, suggesting that the inflammatory reaction may have been more severe in the concomitant cases. The PBC + SS cases also showed higher serum IgG levels and more frequent RF and ILD incidences; thus, treatment with glucocorticoids or immunosuppressive agents, in addition to ursodesoxycholic acid, might be beneficial for these cases.

SSc was the first reported CTD to coexist with PBC[28]. Although the known molecular targets of SSc and PBC are distinct, the two diseases share similar outcomes: sclerosis in the case of SSc and cirrhosis in the case of PBC. As both conditions result in fibrogenesis, there may be some similar epitopes or sequences in the target antigens of the two diseases that are involved in the effects on the fibrogenic pathway. According to the data from the Italian-based study[17], SSc was the most frequent comorbidity in PBC; moreover, a future study suggested that this rate might be underestimated[29]. In the Chinese-based study, the frequency of PBC + SSc was much lower than that of PBC + SS. In China, SSc cases

with only skin involvement usually consult a dermatologist for diagnosis and treatment, instead of a rheumatologist. It is likely that many cases of PBC with SSc remain undiagnosed. On the other hand, prevalence of SSc has been reported to be much higher in North America and Australia than in Japan, another Asian country[30]. The exact epidemiologic data for SSc in China is not available, but considering the similarity in genetic backgrounds of Asian ethnicities it is possible that the incidence and prevalence of SSc in China may be close to that in Japan, and lower than that in Europe. Such a situation may partially explain the observed low frequency of PBC + SSc in our Chinese cohort. Recent study from United Kingdom have demonstrated that patients affected by both PBC and SSc manifested a less aggressive form of liver disease, suggesting an active interaction between the two conditions[31]. Such characteristics were not observed in the current study's Chinese cohort. Specifically, there were no significant differences in the results of laboratory tests from the PBC patients and the PBC + SSc patients; however, the latter had higher frequency of ILD due to the existence of SSc.

PBC mostly affects middle-aged women, while the majority of SLE cases occur in women of childbearing age[32]. Therefore, the likelihood of co-existence of PBC and SLE is theoretically low. In fact, the reported frequencies of PBC + SLE in PBC are 1.25%-1.80%[16,17] and in SLE are 1.4%-7.5%[33-35]. In the current Chinese cohort, the frequency of PBC + SLE was 3.7%, which was higher than that of PBC + SSc (2.8%). Compared to patients with PBC alone, the PBC + SLE patients had lower WBC count, Hb, and PLT, and higher frequency of renal involvement, all of which are distinctive features of SLE. The coexistence of SLE in PBC patients appeared to be associated with much less extensive liver damage, as reflected by lower γ-GT and IgM levels. These finding suggest that SLE may protect against progression of PBC by inducing a slower progression to cirrhosis and delaying the need for liver transplantation[36,37].

Arthralgia is a non-specific symptom, which is very common in CTD, and inflammation of multiple joints with arthralgia is characteristic of RA. A study from the United States indicated that the rate of prevalence of RA in PBC patients did not differ from that in healthy controls[22]. However, the incidence of RA in PBC patients in the current study (2.8%) was higher than the incidence of 0.5%-1.0%[38] reported worldwide, but less than that reported in the United Kingdom study[16]. In the current cohort, the PBC + RA patients had lower Hb levels but higher serum levels of IgG, faster ESR and a higher ratio of RF positivity. They also had elevated serum ALP level, from which we conclude that coexistence of RA maybe a negative-prognosis factor for PBC[36,37].

Regarding the overlap of PBC and PM/DM, the current data did not confirm that it was as rare as reported in the previous studies in the literature. Interestingly, no cases of PBC + DM were detected. In contrast, there have been several case reports of PBC complicated by PM[39-41], and many of these cases have been asymptomatic or showing mild (early) histological changes. Higher WBC count meant more severe inflammation in PBC + PM. The PBC + PM patients in our Chinese cohort showed a tendency towards myocardial involvement, and that rate was much higher than that in the PM/DM patients[42]. It is unclear why the heart is particularly involved in PBC complicated with PM. Treatment with high-dose steroids or even pulse therapy is a particularly effective strategy[43] and has been shown to decrease mortality[44]. It is intriguing to consider that the pathogenesis of this syndrome might be related to the presence of various subtypes of AMA[45]; however, further studies are necessary to investigate whether the preferential myocardial involvement is a diagnostic finding in patients with PBC and PM.

There are several limitations inherent to the current study's design, which may have affected the results. Less than one-fourth of the patients underwent liver biopsy, which precluded our ability to perform statistical analyses of the differential pathologic features in patients with CTDs and those without CTDs. In addition, the retrospective and descriptive nature of the study restricted our investigations to only the fundamental relationship between PBC and CTDs. Future studies should be designed to investigate the relation with genetics and immune regulator factors to help identify common and distinct pathways involved in pathogeneses of the various CTDs. Finally, the follow-up was relatively short, and longer-term follow-up will help to determine the differential prognosis and mortality profiles of the various CTDs.

In conclusion, many CTDs coexist with PBC, which suggests that PBC and CTDs may share similar pathogenic mechanisms. When various CTDs coexist with PBC, different manifestations and some specific organ involvement may appear. PBC is a systemic autoimmune disease and not organ-specific. Clinicians should screen for CTDs in PBC patients, especially those who have RP, renal manifestation, or signs of involvement of other organ systems. Detailed medical history should be obtained, and laboratory examination of autoantibodies, such as ANA, should be performed to screen for co-existing CTDs and PBC.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is often thought of as an organ-specific autoimmune disease which mainly targets the liver. However, accumulating evidence has indicated that PBC may involve multiple systems and may be associated with other connective tissue diseases (CTDs). It remains unknown whether these complicated PBC cases have distinctive clinical features and/or prognoses, especially in ethnic Chinese.

Research frontiers

The current study assessed the frequency of extrahepatic lesions and the association of CTDs in a cohort of 322 Chinese patients with PBC. In addition, the clinical and laboratory features were compared between the subsets of PBC patients with and without various CTDs.

Innovations and breakthroughs

According to some studies from Europe, systemic sclerosis is the CTD that

most frequently coexists with PBC. However, in the current study of a Chinese cohort, Sjögren's syndrome was the CTD that most frequently coexisted with PBC. This report is the first retrospective cohort study to investigate the differences in the clinical features and extrahepatic involvement between PBC patients with and without CTDs.

Applications

PBC is a systemic autoimmune disease and without organ-specificity. It is necessary to evaluate CTDs in PBC patients, especially those with Raynaud phenomenon, renal manifestation, and signs of involvement of multiple organ systems. Detailed collection of medical history and laboratory examination of related autoantibodies should be performed to help diagnose cases of coexisting CTDs and PBC.

Terminology

PBC is characterized by chronic non-suppurative destructive cholangitis and presence of anti-mitochondrial antibody. It ultimately progresses to cirrhosis and hepatic failure. PBC is an autoimmune liver disease that may involve multiple systems and may be associated with other CTDs.

Peer review

This is a descriptive study in which the authors analyzed the frequency and clinical features of CTDs in a cohort of 322 Chinese patients with PBC. The results showed that there were some interesting manifestations in the PBC patients with other CTDs and suggest that assessment of systemic involvements and examination of associated autoantibodies may be beneficial for patients with PBC.

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BRIEF ARTICLE

Preclinical evaluation of herpes simplex virus armed with granulocyte-macrophage colony-stimulating factor in pancreatic carcinoma

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Abstract

AIM: To investigate the therapeutic efficacy and mechanisms of action of oncolytic-herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) in pancreatic carcinoma.

METHODS: Tumor blocks were homogenized in a sterile grinder in saline. The homogenate was injected into the right armpit of each mouse. After vaccination, the mice were randomly assigned into four groups: a control group, a high dose $HSV^{GM\text{-}CSF}$ group [1×10^7 plaque forming units (pfu)/tumor], a medium dose $HSV^{GM\text{-}CSF}$ group (5×10^6 pfu/tumor) and a low dose $HSV^{GM\text{-}CSF}$ group (5×10^5 pfu/tumor). After initiation of drug administration, body weights and tumor diameters were measured every 3 d. Fifteen days later, after decapitation of the animal by cervical dislocation, each tumor was isolated, weighed and stored in 10% formaldehyde solution. The drug effectiveness was evaluated according to the weight, volume and relative volume change of each tumor. Furthermore, GM-CSF protein levels in serum were assayed by enzyme-linked immunosorbent assays at 1, 2, 3 and 4 d after injection of $HSV^{GM\text{-}CSF}$.

RESULTS: Injection of the recombinant mouse HSV encoding GM-CSF resulted in a significant reduction in tumor growth compared to the control group, and dose-dependent effects were observed: the relative tumor proliferation rates of the low dose, medium dose and high dose groups on 15 d after injection were 45.5%, 55.2% and 65.5%, respectively. The inhibition rates of the tumor weights of the low, middle, and high dose groups were 41.4%, 46.7% and 50.5%, respectively. Furthermore, the production of GM-CSF was significantly increased in the mice infected with $HSV^{GM\text{-}CSF}$. The increase in the GM-CSF level was more pronounced in the high dose group compared to the other two dose groups.

CONCLUSION: Our study provides the first evidence that $HSV^{GM\text{-}CSF}$ could inhibit the growth of pancreatic cancer. The enhanced GM-CSF expression might be responsible for the phenomenon.

Key words: Pancreatic carcinoma; Gene therapy; Animal test; Herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor

Core tip: Herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) is an engineered oncolytic virus. The key features of $HSV^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and the

ICP47 gene as well as interruption of the *ICP6* gene and insertion of the therapeutic gene GM-CSF. Our study provides the first evidence that HSV^{GM-CSF} could inhibit the growth of pancreatic cancer in a dose-dependent manner. Enhanced GM-CSF expression might be responsible for the phenomenon.

Liu H, Yuan SJ, Chen YT, Xie YB, Cui L, Yang WZ, Yang DX, Tian YT. Preclinical evaluation of herpes simplex virus armed with granulocyte-macrophage colony-stimulating factor in pancreatic carcinoma. *World J Gastroenterol* 2013; 19(31): 5138-5143 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5138.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5138

INTRODUCTION

Pancreatic cancer is a rapidly fatal malignancy with one-year relative survival rates less than 30% and nearly all patients die from their disease within 7 years of surgery[1,2]. More than 80% patients are unsuitable for radical resection. Further more, it is insensitive to current chemotherapy, radiotherapy and immunotherapy.

Gene therapy of pancreatic carcinoma is considered a novel model, and has become an emerging research area in recent years. Successful drugs for gene therapy may result in prolonged survival. Oncolytic herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor (HSV^{GM-CSF}) is an attenuated, replication-competent oncolytic virus. It can activate the host's own immune system against infected tumor cells. Some clinical trials of HSV for the treatment of various cancers have been completed, providing preliminary data about its safety and effectiveness[3-7]. However, there is little data for pancreatic cancer.

Therefore, we conducted a preclinical evaluation of effects of HSV^{GM-CSF} on pancreatic cancer and explored the mechanisms that may be involved in any antitumor response.

MATERIALS AND METHODS

Experimental chemical

The OrienGene Biotechnology Ltd. (Beijing, China) provided the mouse recombinant GM-CSF herpes simplex virus (HSV^{GM-CSF}) (OrienX010).

Experimental cell and animals

Panc-2 cells: All cells used in this study represent mouse pancreatic carcinoma cell lines. Panc-2 cells were grown in Dulbecco's modification of Eagle's medium.

Animals: Female C-57B mice (4-6 wk, 16-18 g) were provided by the Experimental Animal Center, Peking Union Medical College. The Committee of Animal Care and Use of the university approved the experimental protocol, which met the regulatory requirements of Tumor Hospital, Chinese Academy of Medical Science for the use of experimental animals. All mice were bred in a standard environment and were provided with free access to food and water.

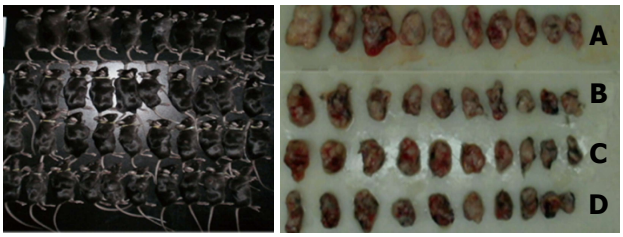

Figure 1 Inhibition of herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor on the proliferation of PANC-2 pancreatic carcinoma xenografts in mice. A: Control group; B: High dosage; C: Middle dosage; D: Low dosage. Fifteen days later, after decapitation of the animals by cervical dislocation, each tumor was isolated.

Experimental procedure

Injection of transplanted tumors and drug administration in mice followed standard methods used internationally. The Discussion Draft of Guidance Principles of Pharmacodynamics of Antitumor Drugs[8] and Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval[9] were used for guidance. Panc-2 cells were first recovered and amplified for collection of tumor cells, of which a total of 1×10^7-1×10^8 plaque forming units (pfu) virus were subcutaneously injected into each mouse. When the resulting tumor had grown to 2-3 cm in diameter, the tumor tissue was dissected under sterile conditions and cut into blocks of 2 mm^3 with sterile scissors. The tumor blocks were homogenized in a sterile grinder with normal saline. The homogenate was injected into the right armpit of each mouse. After vaccination, the mice were randomly grouped for intratumoral administration of drugs. After initiation of drug administration, body weights and tumor diameters were measured every 3 d. Fifteen days later, after decapitation of the animals by cervical dislocation, each tumor was isolated, weighed and stored in 10% formaldehyde solution (Figure 1). The drug effectiveness was evaluated according to the weight, volume and relative volume change of each tumor.

GM-CSF quantification by enzyme-linked immunosorbent assay

In vivo blood collected by tail vein bleed was centrifuged, and serum was collected and stored at -20 ℃. Mouse GM-CSF concentration was determined by an enzyme-linked immunosorbent assay (ELISA) (Abcam Inc, MA, United States), according to manufacturer's protocol, for cells infected with 1×10^7, 5×10^6 and 5×10^5 pfu/mL.

Animal grouping and drug administration

Three days after vaccination of tumors, the mice were randomly divided into groups with the weights of the

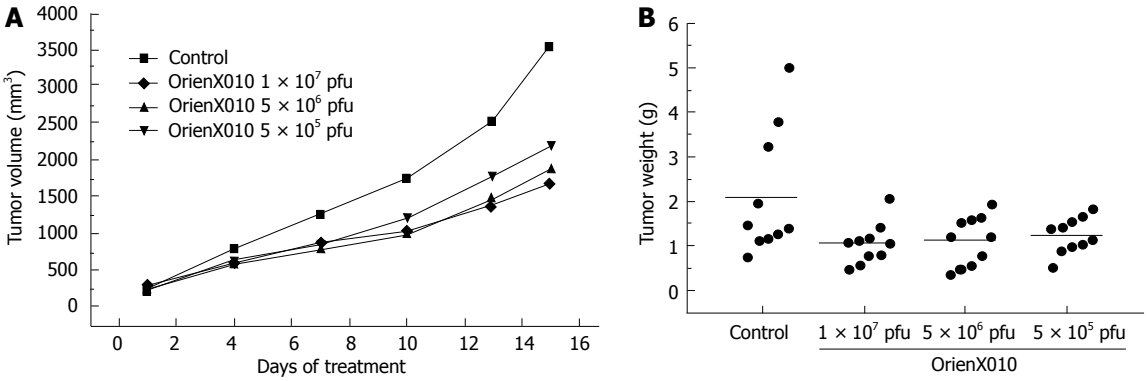

Figure 2 Effect of OrienX010 on the tumor volume (A) and tumor weight (B) of PANC-2 pancreatic carcinoma xenografts in mice. pfu: Plaque forming units.

Table 1 Inhibition of OrienX010 on the growth of PANC-2 pancreatic carcinoma xenografts in mice

Administration	Host (mice) reaction		Tumor reaction					
	Animal number beginning/end	Body weight, g (mean ± SD) beginning/end	Tumor weight, g (mean ± SD)	*Z*	Tumor volume (mm^3)	*J*	RTV	T/C (%)
	10/10	16.5 ± 1.2/19.8 ± 1.9	2.10 ± 1.41		3555.8 ± 1849.8		15.4 ± 5.7	
Tumor injection × 4	10/10	16.4 ± 0.9/18.2 ± 2.4	1.04 ± 0.45	50.50%	1668.3 ± 661.9	53.10%	7.0 ± 2.5[b]	45.5
Tumor injection × 4	10/10	16.0 ± 0.6/18.6 ± 1.3	1.12 ± 0.55	46.70%	1869.8 ± 846.6	47.40%	8.5 ± 4.4[b]	55.2
Tumor injection × 4	10/10	16.2 ± 0.8/18.5 ± 0.8	1.23 ± 0.39	41.40%	2200.3 ± 826.5	38.10%	10.1 ± 4.2[a]	65.6

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; *Z*: Inhibition rate of tumor weight; *J*: Inhibition rate of tumor volume; RTV: Relative tumor volume.

animals being similar in each group: control group, high dose group (1×10^7 pfu/tumor), middle dose group (5×10^6 pfu/tumor) and low dose group (5×10^5 pfu/tumor). The drug was administered via intratumoral injections of 0.1 mL/tumor on the first day.

Statistical analysis

Data were expressed as mean ± SD. The inhibition rate of tumor proliferation = (tumor weight of control group - tumor weight of drug group)/tumor weight of control group × 100%. Tumor volume (V) = $1/2ab^2$ (a = tumor major diameter; b = tumor minor diameter). The inhibition rate of tumor volume proliferation = (tumor volume of control group - tumor volume of drug group)/tumor volume of control group × 100%. Relative tumor volume (RTV) = Vt/Vo (Vo = tumor volume pre-drug, Vt = tumor volume measured each time after drug administration). The relative tumor proliferation rates (T/C) = RTV of drug group/RTV of control group × 100%. SPSS13 was used for statistical analysis of inter-group difference using t tests and for plotting of the tumor volume, relative growth curve of volume-time, tumor weight and related tables and figures.

RESULTS

The tumor volume on day 15 post-treatment in the control group was 3555.8 ± 1849.8 mm^3. The tumor volume of the group treated with a single intratumoral injection of low dose virus was 2200.3 ± 826.5 mm^3 ($P < 0.05$ *vs* control). For the middle dose virus group, the tumor volume on day 15 post-treatment was 1869.8 ± 846.6 mm^3 ($P < 0.05$ *vs* control). The tumor volume on day 15 post-treatment of the high dose virus group was 1668.3 ± 661.9 mm^3 ($P < 0.01$ *vs* control) (Figure 2A). The inhibition rates of the tumor volumes of dose of the low, middle, and high dose groups were 38.1%, 47.4% and 54.3%, respectively. Thus, $HSV^{GM\text{-}CSF}$ could inhibit pancreatic cancer in a dose-dependent manner. The relative tumor proliferation rates of the low, middle, and high dose groups were 45.5%, 55.2% and 65.5%, respectively (Table 1).

The present study showed that the tumor weight of the control group was 2.10 ± 1.41 g, 1.23 ± 0.39 g in the low dose group ($P > 0.05$ *vs* control), 1.12 ± 0.55 g in the middle dose group ($P > 0.05$ *vs* control), and 1.04 ± 0.45 g in the high dose group ($P < 0.05$ *vs* control). The inhibition rates of the tumor weights of the low, middle, and high dose groups were 41.4%, 46.7% and 50.5%, respectively (Figure 2B). Only the high dose group showed a significant difference compared with the control group ($P < 0.05$). There was no significant difference in mouse body weight among these four groups ($P > 0.05$) (Figure 3). Also, none of the mice died or showed skin ulceration/necrosis at the tumor location during the experiment.

The results of serum GM-CSF protein level showed that $HSV^{GM\text{-}CSF}$ significantly increased GM-CSF production, peaking at day 3 after treatment (Figure 4). There may be a correlation between the dose of $HSV^{GM\text{-}CSF}$ and the GM-CSF protein level.

Dissection of the mice at 15th day after administration of the drug showed no adhesions around the tumors, and there were no ascites or metastasis of the tumors in the peritoneal cavity; the tumors appeared as gray in color, had uniform textures and showed no necrosis.

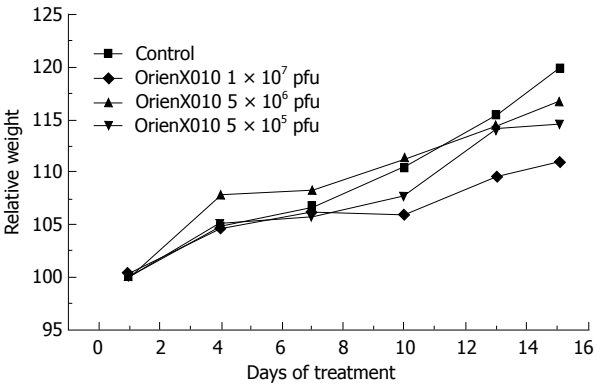

Figure 3 Changes in relative mouse body weights. pfu: Plaque forming units.

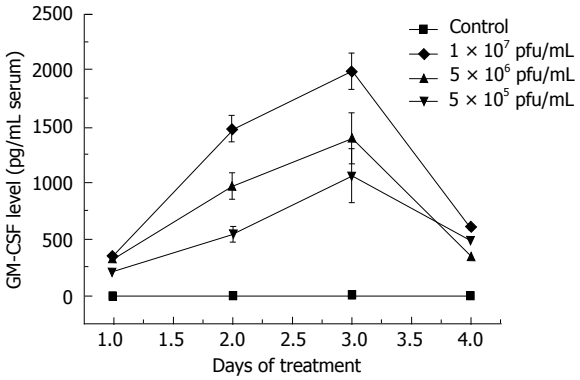

Figure 4 Quantification of expressed granulocyte-macrophage colony-stimulating factor. pfu: Plaque forming units; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

DISCUSSION

Compared to the traditional therapeutic methods, gene therapy is a recent and active research field. Since 1999, Germany, the United Kingdom and the United States have approved gene therapy projects for pancreatic carcinoma to enter clinical stage Ⅰ/Ⅱ trials, some of which are complete, providing preliminary data about its safety and effectiveness. The data suggested that the gene drugs were well tolerated in cancer patients and could suppress tumor growth[3-7]. The aim of the present study was to evaluate the efficacy of HSV$^{GM\text{-}CSF}$, an attenuated, replication-competent oncolytic virus, for treating mouse pancreatic carcinoma.

HSV$^{GM\text{-}CSF}$ is an engineered oncolytic virus. It belongs to a conditional replication HSV-1 mutant that uses the differences in cellular structure and metabolic pathways between tumors and normal tissues and retains the genes related to virus replication. The key features of HSV$^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and *ICP47* genes, as well as interruption of the *ICP6* gene and insertion of the therapeutic gene *GM-CSF*. GM-CSF is a pleiotropic cytokine secreted by many kinds of cells, including activated lymphocytes, macrophages and endothelial cells. Several previous studies demonstrated that GM-CSF was one of the most potent cytokines[5] that could influence the immune response in several ways, including recruitment and stimulation of antigen-presenting cells, such as dendritic cells, and induction of myeloid precursor cells to proliferate and differentiate into monocytes, macrophages, neutrophils and eosinophils[10]. Viral lysis and the mechanism mediated by the transgene protein, represent two parallel mechanisms of tumor destruction that can be achieved using HSV$^{GM\text{-}CSF}$.

The encouraging results of the present study suggested that the proliferation speed of tumors in the mouse experimental groups was reduced after 15 d of administration of HSV$^{GM\text{-}CSF}$ compared with the control group ($P < 0.05$). The reduction of tumor growth was dose-dependent. However, there was no obvious difference in the host response between the different dosages, which may be related to the small differences in drug dosages and could be resolved with the promotion of pharmacological techniques for high-concentration drugs.

The sera of cells infected with the three doses of the virus showed high expression of GM-CSF. There was no GM-CSF secretion in the control group. The results suggested that the HSV$^{GM\text{-}CSF}$ enhanced *GM-CSF* gene expression. Additionally, the increase was more pronounced in the group injected by the high dose virus than in the middle and low dose groups. There may be a correlation between the dose of HSV$^{GM\text{-}CSF}$ and the GM-CSF protein level. These results indicated that HSV$^{GM\text{-}CSF}$ could regulate immunity in cancer-bearing mice. The increased GM-CSF levels might be responsible for the dose-dependent relationship between the drug and the tumor response.

Currently, HSV vectors alone, and HSV vectors armed with GM-CSF or other recombinant genes have been successfully tested for safety in humans and have exhibited efficacy in preclinical animal models against various human cancers. And HSV mutant has been shown to be an effective strategy for lysing tumor cells *in vitro* and in multiple experimental animal models[10-16]. Geevarghese *et al*[17] evaluated the anti-tumor effects of NV1020 (another HSV-1 mutant), which showed that the NV1020 stabilized liver metastases in patients, and extended survival by resensitizing the cancer cells to chemotherapy. Both Yang *et al*[18] and Malhotra *et al*[19] suggested that the HSV vectors armed with GM-CSF had a significantly better antitumor effect compared to treatment with HSV vectors alone in mouse colon cancer. Furthermore, Derubertis *et al*[20] declared that mouse colorectal cancer hepatic metastases could be suppressed by HSV vectors armed with GM-CSF. HSV$^{GM\text{-}CSF}$ combined with cisplatin-based chemoradiotherapy was well tolerated in patients with stage Ⅲ/Ⅳ head and neck cancer. The present study showed that HSV$^{GM\text{-}CSF}$ enhanced the inhibition rate of mouse pancreatic cancer by regulating the expression of GM-CSF. The results were similar to those provided in previous studies[19,20].

Although the agent was highly attenuated and replication restricted, the use of a virus still raises concerns about viral proliferation and dissemination. During the experimental period, the body weights of the mice in the

experimental groups were similar to the control group at the beginning, and gradually and stably increased. At the later stages, the body weights slowly increased, particularly in the high-dosage group, compared to the control group, but there was no statistical difference. In addition, there was no occurrence of treatment-related death or ulceration/necrosis of the skin, suggesting that the drug is safe and effective, with low toxicity and side effects, and is tolerated by mice. The existence of antiviral drugs, such as ganciclovir, provides us with a further margin of safety.

In the present study, the transplanted tumor cell was injected into the armpits of mice and the drug was administrated by intratumoral injection. If applied in a clinic, the drug could be administered through a fine needle puncture technique with the guidance of CT/endoscopic ultrasonography or through vascular intervention, which several research centers have proved to be effective. Mulvihill *et al*[21] performed a clinical trial of intratumoral injection of the *ONYX-015* gene with the guidance of CT, while Löhr *et al*[22] reported their experimental results of clinical stage I and II trials of drug administration via vascular intervention.

Previous studies indicated that the HSV vector had a significant effect on multiple solid tumors[23-26], and could enhance the effect of other combined common therapies, such as radiotherapy and chemotherapy. Most studies that combined viral gene therapy with other therapies observed a synergistic effect in preclinical models[27-29]. Recently a stage I / II clinical trial of combined HSV with radiotherapy in head and neck tumors ended and showed no obvious side effects[7]. We are performing experimental research using an injection solution of recombined mouse $HSV^{GM\text{-}CSF}$ combined with radiotherapy to find a new approach in treating pancreatic carcinoma.

During the last two decades, gene therapy has made great progress. Simultaneous use of basic research and clinical experiments may become one of the fastest-developing areas in the field of medicine in the next 10 years. The development of gene therapy has proved difficult, and application in the clinic is still a long way off. The immunity, safety, transduction rate and tissue specificity of current vectors require further study and improvement, which is a common problem in gene therapy. The vectors used in the clinic in the future should have the advantage of combining non-viral vectors and alternative viral vectors that can be customized according to different requirements to express the target gene in specific tissues, and effectively modulate their expression level and duration.

COMMENTS

Background

Pancreatic cancer is a rapidly fatal malignancy with one-year relative survival rates less than 30%; nearly all patients die from their disease within 7 years of surgery. Gene therapy of pancreatic carcinoma is considered a novel model, and has become an emerging research area in recent years.

Research frontiers

The gene therapy model for pancreatic carcinoma has emerged recently. Successful drugs for gene therapy may result in prolonged survival. Oncolytic herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) is an attenuated, replication-competent oncolytic virus. It can activate the host's own immune system against infected tumor cells. Some clinical trials of HSV for the treatment of various cancers had been completed, providing preliminary data about its safety and effectiveness. However, there is little data for pancreatic cancer.

Innovations and breakthroughs

$HSV^{GM\text{-}CSF}$ is an engineered oncolytic virus. It is a conditional replication HSV-1 mutant that utilizes differences in cellular structure and metabolic pathways between tumor and normal tissues, and retains the genes related with virus replication. The key features of $HSV^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and *ICP47* gene as well as interruption of the *ICP6* gene and insertion of the therapeutic gene GM-CSF.

Peer review

The study is very interesting. Liu *et al* investigated the therapeutic efficacy of oncolytic $HSV^{GM\text{-}CSF}$ in a mouse model of pancreatic carcinoma and explored mechanisms that may be involved in the antitumor response. The authors provide evidence that $HSV^{GM\text{-}CSF}$ could inhibit the growth of pancreatic cancer.

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BRIEF ARTICLE

HMGB1 gene polymorphisms in patients with chronic hepatitis B virus infection

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Abstract

AIM: To characterize high mobility group box chromosomal protein 1 (*HMGB1*) polymorphisms in patients infected with hepatitis B virus (HBV) and determine the different patterns in patient subgroups.

METHODS: A total of 1495 unrelated Han Chinese HBV carriers were recruited in this hospital-based case-control study. The *HMGB1 1176 G/C* polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism assay.

RESULTS: A significant association was observed between *HMGB1 1176 G/C* polymorphism and outcome of HBV infection. The subjects bearing 1176G/G genotype had an increased risk of susceptibility to chronic hepatitis B, liver cirrhosis and severe hepatitis B when compared with those bearing at least one 1176C allele.

CONCLUSION: Patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection.

Key words: High mobility group box chromosomal protein 1; Hepatitis B virus; Polymorphism; Intron

Core tip: We analyzed the relationship between the high mobility group box chromosomal protein 1 (*HMGB1*) *1176 G/C* polymorphism and the susceptibility and outcome to hepatitis B virus (HBV) infection in a large hospital-based case-control study. Our results indicated that patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection. Our study emphasizes the importance of HMGB1 in the pathophysiology of HBV-related diseases on the population level and will provide researchers new clue for the further basic research in pathogenesis of chronic HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection is associated with a variety of diseases, including asymptomatic carrier (AsC), fulminant hepatitis, chronic hepatitis (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). Persistent

HBV infection has been considered as a multifactorial and polygenic disorder with viral, environmental and genetic components. HBV genomic variability and a number of conventional risk factors, including age, gender, concurrent infection with hepatitis C virus, hepatitis D virus and human immune deficiency virus, are clearly the important factors contributing to the incidence of persistent HBV infection[1-4]. However, segregation analysis and twin studies strongly support the role of host genetic components in determining the chronicity of HBV infection[5,6]. A known and unknown number of identified or unidentified genes are likely to modify the susceptibility to persistent HBV infection[7-10]. Single nucleotide polymorphism (SNP) is currently believed to be a powerful tool for identifying genetic susceptibilities to common complex diseases[11,12].

The intranuclear architectural protein termed high mobility group box chromosomal protein 1 (HMGB1) has recently been identified as a potent proinflammatory mediator when passively released to extracellular by necrotic cells, as opposed to apoptotic cells that will induce inflammation[13,14]. Furthermore, HMGB1 can also be actively secreted by stimulated macrophages or monocytes[15-17]. Active secretion from living inflammatory cells and passive release from necrotic cells implicate that HMGB1 may play a central role in proinflammatory reactions. It is well known that HBV infection is closely related with cytokines. Polymorphisms of cytokine gene, such as human leukocyte antigen, estrogen receptor alpha (*ESR1*), have been reported to be associated with HBV infection[18-21]. However, so far there has been no report on the association between *HMGB1* gene and HBV infection. We conducted a hospital-based case-control study including more than one thousand subjects with HBV infection to characterize the relationship between *HMGB1* gene polymorphism and HBV infection.

MATERIALS AND METHODS

Patients

Patients with HBV infection were randomly selected from the outpatient and inpatient referral center affiliated to the Institute for Infectious Diseases of Southwest Hospital treated between February 2002 and February 2012. Informed consent was obtained from all the patients to participate in the study. Participants finally included in the current study were from a subset of unrelated individuals from the referral center. The diagnostic criteria for chronic HBV infection were as follows: persistent presence of hepatitis B surface antigen (HBsAg), absence of anti-hepatitis B surface antibodies (anti-HBs), presence of anti-core IgG antibodies (anti-HBc), and presence of hepatitis B early antigen (HBeAg) or anti-hepatitis B e antibodies (anti-HBe) for 6 mo or longer despite of virus replication. Asymptomatic carriers had no fluctuation of serum alanine aminotransferase (ALT) levels and no obvious clinical symptoms. Chronic hepatitis B had a serum ALT fluctuation, 1 × the upper limit of normal (ULN) < ALT < 5 × ULN, with or without other abnormal hepatic functions. Severe hepatitis B (SHB), which is currently equal to acute-on-chronic liver failure, presents the following symptoms: (1) fatigue with striking gastrointestinal tract symptoms; (2) rapidly worsening jaundice, with serum total bilirubin (TBIL) 10 times higher than ULN, or with a daily increase ≥ 17.1 μmol/L; (3) hemorrhagic tendency with international normalised ratio ≥ 1.5 or prothrombin activity ≤ 40% where other causes have been excluded; (4) progressive reduction in liver size; and (5) occurrence of hepatic encephalopathy. Liver cirrhosis and HCC were confirmed by liver biopsy, ultrasound, and/or computerized tomography scan. Healthy control individuals were recruited from Red Cross blood donor centers with or without anti-HBs, but HBsAg, anti-HBc, HBeAg, and anti-HBe were negative.

DNA extraction

The leukocytes genomic DNA from 5 mL whole blood was isolated using Miller's method[22]. DNA samples were diluted to 8 ng/μL and distributed into 96-well plates (DNA panels), with 94 samples and 2 controls (DNA-free water) in each plate.

Gene polymorphism

We used the current recommendations of human genome SNP described at http://www.ncbi.nlm.gov/SNP under accession number NT024524. The higher allele variation frequency selected in position 1176 G/C, the intron 4 of *HMGB1* gene, was studied to determine whether any association identified was specific to HBV infection. The SNP was named in a same way to *HMGB1* (1176G/C).

Genotype

The genotyping was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Appropriate primer pairs (sense 5'-3' GTCTCCTTTGCCCAGTGTATCTC and anti-sense 5'-3'GTACACAGCCTTTGTCTGAGTCTG) were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, United States). PCR condition was as follows: one cycle of predenature 3 min at 95 ℃, 30 cycles of denature 30 s at 94 ℃, hybridization for 30 s at 54 ℃, an extension cycle of 50 s at 72 ℃, and a last cycle of delay 5 min at 72 ℃. Restriction enzyme BcLI (recognition site T/GATCA) was obtained from NEB; the fragments were separated by electrophoresis on 3% agarose gel and stained with ethidium bromide for visualization under ultraviolet light. The observed genotypes were also identified by direct sequencing before large-scale test was started.

Statistical analysis

An allele frequency was directly calculated by its genotype. The observed genotype frequencies and allele frequency were compared using χ^2 test between the variables to determine if they were in Hardy-Weinberg equilibrium.

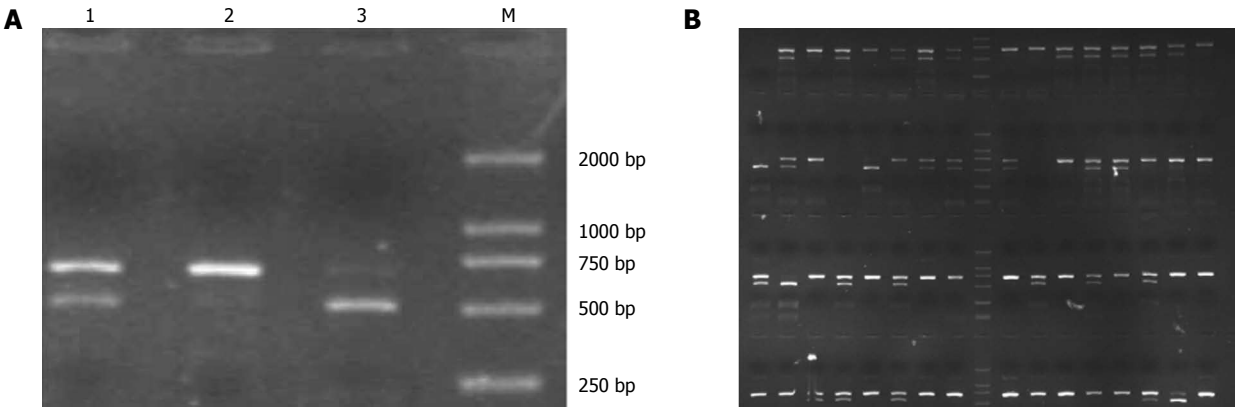

Figure 1 High mobility group box chromosomal protein 1 (1176G/C) restriction fragment length polymorphism genotyping. A: The typical pattern of three genotypes; B: Panel genotyping. 1: GC genotype; 2: GG genotype; 3: CC genotype; M: Marker DL2000.

Table 1 High mobility group box chromosomal protein 1 polymorphism (1176G/C) between various clinical subgroups infected with hepatitis B virus

	Sex	Age (yr)	Genotype			Allele frequency	
	(M/F)	(mean ± SD)	GG	CC	GC	G	C
AsC (n = 199)	116/83	34.762 ± 11.282	107	9	83	0.7462	0.2538
AHB (n = 15)	11/4	30.201 ± 10.221	9	1	5		
CHB (n = 929)	730/199	34.312 ± 11.549	572	33	324	0.6530	0.3470
SHB (n = 157)	129/28	39.989 ± 11.792	91	6	60	0.7707	0.2293
LC (n = 175)	142/33	41.950 ± 11.437	104	13	58	0.7600	0.2300
HCC (n = 20)	14/6	49.256 ± 12.232	10	1	9		
LC + CHB (n = 1104)	872/232	35.461 ± 11.642	676	46	382	0.7853	0.2147
LC + CHB + SHB (n = 1261)	1001/260	36.001 ± 11.852	767	52	442	0.7835	0.2165

There was age difference in any two subgroups except between AsC and CHB, $P < 0.05$; There was sex difference between AsC and any other subgroups, $P < 0.05$; AsC: Asymptomatic carrier; AHB: Acute hepatitis B; CHB: Chronic hepatitis B; SHB: Severe hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

An observed $P > 0.01$ was considered in Hardy-Weinberg equilibrium, and $P < 0.05$ was considered significantly different between the variables. All the analyses were performed with SPSS11.0 statistical software (SPSS Inc., Chicago, IL, United States).

RESULTS

HMGB1 1176G/C polymorphism genotyping

HMGB1 1176G/C polymorphism was genotyped by PCR-RFLP assay (Figure 1). A total of 1495 clearly diagnosed and genotyped patients were enrolled. The clinical characteristics, such as age and sex, are listed in Table 1. Apparently, age or sex difference existed in the studied subgroups. Hardy-Weinberg equilibrium by χ^2 test showed $P = 0.494 > 0.01$ (Table 1), which confirmed that the studied group was in Hardy-Weinberg equilibrium.

Case-control association study

Because age or sex difference existed in the studied subgroups, it is essential to detect the association between observed SNP and HBV infected subgroups, and age and sex factors were considered by logistic regression (Table 2). A statistically significant difference in the distribution of *HMGB1* polymorphism (1176G/C) was observed between subgroups of AsC and LC (OR = 1.571, 95%CI: 1.108-2.227, $P = 0.011$, codominant model); AsC and CHB (OR = 1.354, 95%CI: 1.085-1.689, $P = 0.007$, codominant model); AsC and CHB + SHB + LC (OR = 1.401, 95%CI: 1.010-1.944, $P = 0.044$, recessive model); OR = 1.329, 95%CI: 1.070-1.651, $P = 0.010$, codominant model, AsC and CHB + LC (OR = 1.406, 95%CI: 1.011-1.956, $P = 0.043$, recessive model; OR = 1.355, 95%CI: 1.088-1.687, $P = 0.007$, codominant model).

DISCUSSION

HMGB1 is a nuclear DNA-binding protein, which also functions as a pleiotropic cytokine, implicated in the pathology of several different immune-mediated diseases. The human *HMGB1* gene is located on chromosome 13. Kornblit *et al*[23] firstly elaborated six polymorphisms and four mutations identified in the *HMGB1* gene, located in -1615A/G, 982C/T, 3814C/G, 1779T/G, -196C/A, 1808C/G, 4519_4521delGAT, -1377delA,1747delT, 1888insT, respectively. In other studies, several associations have been observed, revealing the importance of the genetic variation in the *HMGB1* gene. In their report, the -1377delA$^{A/-}$ genotype or the -1377delA$^{-/-}$ genotype

Table 2 Association between high mobility group box chromosomal protein 1 (1176G/C) single nucleotide polymorphism and hepatitis B virus infected subgroups

Subgroup	Dominant model		Recessive model		Codominant model	
	P	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)
LC/CHB	0.075	0.533 (0.267-1.065)	0.945	0.988 (0.701-1.392)	0.120	0.844 (0.682-1.045)
LC/SHB	0.183	0.508 (0.188-1.376)	0.747	1.075 (0.693-1.669)	0.326	0.872 (0.664-1.146)
LC/AsC	0.168	2.320 (0.702-7.672)	0.108	1.572 (0.906-2.728)	0.011	1.571 (1.108-2.227)
AsC/SHB	0.473	1.616 (0.436-5.999)	0.489	1.204 (0.711-2.040)	0.206	1.254 (0.883-1.782)
AsC/CHB	0.238	1.660 (0.716-3.851)	0.052	1.389 (0.997-1.935)	0.007	1.354 (1.085-1.689)
SHB/CHB	0.916	1.050 (0.425-2.592)	0.474	1.137 (0.800-1.614)	0.462	1.090 (0.866-1.371)
SHB + LC/CHB + AsC	0.362	0.760 (0.421-1.371)	0.824	0.971 (0.747-1.262)	0.316	0.918 (0.777-1.085)
AsC/CHB + SHB + LC	0.330	1.505 (0.662-3.421)	0.044	1.401 (1.010-1.944)	0.010	1.329 (1.070-1.651)
AsC/CHB + LC	0.256	1.619 (0.704-3.721)	0.043	1.406 (1.011-1.956)	0.007	1.355 (1.088-1.687)

The association was analyzed by logistic regression analysis with adjustment for covariates, including age, sex, and alcohol consumption. Dominant model: GG + GC/CC, recessive model: GG/GC + CC, codominant model: GG/GC/CC; *P* and OR values were calculated by logistic regression. AsC: Asymptomatic carrier; AHB: Acute hepatitis B; CHB: Chronic hepatitis B; SHB: Severe hepatitis B; LC: Liver cirrhosis; OR: Odds ratio.

showed a significant association with delayed mortality, independent of age and number of the systemic inflammatory response syndrome (SIRS) criteria[24]. Subsequent estimation revealed that several polymorphisms have a potential regulatory impact on HMGB1 transcription. Genetically determined risk factors associated with early and late mortality and death due to infection have been identified, explaining some of the inherited risks in this heterogeneous patient population. Associations between genetic variation and disease severity parameters are also established. Studies of association between *HMGB1* polymorphisms and disease have been also reported with allogeneic hematopoietic cell transplantation (HCT)[25]. Patient homozygosity or heterozygosity for the-1377delA minor allele is associated with increased risk of relapse and increased relapse-related mortality. Furthermore, patient homozygosity for the 3814C/G minor allele is associated with increased overall survival and progression-free survival. Patient carriage of the 2351insT minor allele can reduce the risk from grade II to IV acute graft-versus-host disease whereas donor homozygosity is associated with chronic acute graft-versus-host disease. These findings suggest that the inherited variation in HMGB1 is associated with outcome after allogeneic HCT following myeloablative conditioning regimens. Zeng *et al*[26] found that three SNPs act as tag SNPs for the entire *HMGB1* gene in multiple organ dysfunction syndromes. The rs2249825 and the haplotype TCG can be used as relevant risk estimate for the development of sepsis in patients with major trauma.

As is well known, the susceptibility of HBV infection is closely related to the variation of some important genes. Deng *et al*[27] have demonstrated that polymorphisms at the *ESR1* gene locus are associated with persistent HBV infection. Subsequently, Yan *et al*[18] have also demonstrated an association between cis-acting regulatory variation of the *ESR1* gene and hepatitis B virus-related liver cirrhosis. Some important variations of cytokine gene also influenced the susceptibility to HBV infection. Deng *et al*[28] have found that the novel regulatory polymorphism G-201A in the promoter of interferon gamma-inducible protein of 10 kilodaltons (*IP-10*, *CXCL10*) gene might be a part of the genetic variation underlying the susceptibility of individuals to disease progression of chronic HBV infection. In another study, Yan *et al*[29] demonstrated that the -592C allele and the -1082A-819C-592C haplotype in the *IL-10* gene promoter were associated with an increased susceptibility to acute liver failure in HBV carriers.

Nevertheless, there are few reports about the association between *HMGB1* gene and HBV infection, especially reports about the association between *HMGB1* polymorphisms and HBV infection. In this study, we used the current recommendations of human genome SNPs described at http://www.ncbi.nlm.gov/SNP under accession number NT024524. The higher allele variation frequency was selected in position 1176 G/C, the intron 4 of *HMGB1* gene. There has been no report about this SNP up to date. We genotyped the polymorphisms of 1495 cases, including AsC, CHB, SHB, LC and HCC. The distribution of *HMGB1* 1176G/C genotypes in studied sample of unrelated men and women from the referral center were in Hardy-Weinberg equilibrium ($P > 0.01$), so it is important to consider whether our studied sample could be representative. Yan *et al*[18] and Deng *et al*[28] had scanned the polymorphisms on the same cohort. Because differences in age or sex existed in the studied subgroups, we detected the association by logistic regression between observed SNP and HBV infected subgroups, and the age and sex factors were considered. As a result, there was statistically significant evidence of association. The fraction calculated by relative risk indicated that *HMGB1* 1176G/G genotype was more susceptible to CHB, LC and SHB than 1176C/C and 1176G/C genotype. In other words, the patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection. The results suggest that allele 1176G is closely related to the ponderance of disease. These findings underscore a potentially important role of *HMGB1* in influencing the development of HBV infection.

In another study, we had successfully cloned and analyzed 154 bp nucleotides in intron 4 near the fourth

exon-intron boundary, and found that the region contained sequences *1176 G/C* polymorphism characteristic of an enhancer using PGL3 reporter gene systems. We demonstrated that the SNP 1176 G/C could affect the function. Furthermore, this activity was enhanced by the SNP: G→C change in position 1176, providing the basis for molecular investigations of the *HMGB1* gene in HBV infection. Subsequent reports would focus on this investigation.

In conclusion, our results showed that the *HMGB1* 1176G/G genotype was related to the outcomes of hepatitis B infection, and patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection. The subjects bearing 1176G/G genotype have an increased risk of susceptibility to CHB, LC and SHB compared with those bearing at least one 1176C allele. However, further work is needed to validate our results, and clarify more potential functions of human *HMGB1* gene.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is a serious public health problem worldwide. Host genetic factors play a role in determining to the outcome and progression of the infection. A large number of studies on the association between cytokine gene polymorphisms and the risk of chronic hepatitis B (CHB) have been conducted. High mobility group box 1 (HMGB1) functioned as a pleiotropic cytokine and implicated in the pathology of several different immune-mediated diseases. However, there has been no report about the association between *HMGB1* gene and HBV infection.

Research frontiers

HMGB1 has recently been identified as a potent proinflammatory mediator when passively released extracellularly by necrotic cells, as opposed to apoptotic cells that will induce inflammation. Furthermore, HMGB1 can also be actively secreted by stimulated macrophages or monocytes. Active secretion from living inflammatory cells and passive release from necrotic cells implicate that HMGB1 may play a central role in proinflammatory reactions.

Innovations and breakthroughs

This study characterize the relationship between *HMGB1* gene polymorphism and HBV infection, and concluded that the *HMGB1 1176G/G* genotype was related to the outcomes of hepatitis B infection, and patients with 1176G/G genotype of *HMGB1* gene are more likely to have a progressive status in HBV infection.

Applications

The study results suggest that the subjects bearing *HMGB1 1176G/G* genotype have an increased risk of susceptibility to CHB, liver cirrhosis and severe hepatitis B compared with those bearing at least one 1176C allele, which will provide new clue for the further basic research in pathogenesis of chronic HBV infection.

Peer review

The authors have done a good job and found an association between the 1176G/C polymorphism of *HMGB1*, a proinflammatory mediator, and hepatitis B virus infection. The results are interesting and suggest that HMGB1 is a mediator of the immune response to HBV infection.

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BRIEF ARTICLE

Radical lymph node dissection and assessment: Impact on gallbladder cancer prognosis

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Abstract

AIM: To investigate the lymph node metastasis patterns of gallbladder cancer (GBC) and evaluate the optimal categorization of nodal status as a critical prognostic factor.

METHODS: From May 1995 to December 2010, a total of 78 consecutive patients with GBC underwent a radical resection at Liaocheng People's Hospital. A radical resection was defined as removing both the primary tumor and the regional lymph nodes of the gallbladder. Demographic, operative and pathologic data were recorded. The lymph nodes retrieved were examined histologically for metastases routinely from each node. The positive lymph node count (PLNC) as well as the total lymph node count (TLNC) was recorded for each patient. Then the metastatic to examined lymph nodes ratio (LNR) was calculated. Disease-specific survival (DSS) and predictors of outcome were analyzed.

RESULTS: With a median follow-up time of 26.50 mo (range, 2-132 mo), median DSS was 29.00 ± 3.92 mo (5-year survival rate, 20.51%). Nodal disease was found in 37 patients (47.44%). DSS of node-negative patients was significantly better than that of node-positive patients (median DSS, 40 mo *vs* 17 mo, χ^2 = 14.814, P < 0.001), while there was no significant difference between N1 patients and N2 patients (median DSS, 18 mo *vs* 13 mo, χ^2 = 0.741, P = 0.389). Optimal TLNC was determined to be four. When node-negative patients were divided according to TLNC, there was no difference in DSS between TLNC < 4 subgroup and TLNC ⩾ 4 subgroup (median DSS, 37 mo *vs* 54 mo, χ^2 = 0.715, P = 0.398). For node-positive patients, DSS of TLNC < 4 subgroup was worse than that of TLNC ⩾ 4 subgroup (median DSS, 13 mo *vs* 21 mo, χ^2 = 11.035, P < 0.001). Moreover, for node-positive patients, a new cut-off value of six nodes was identified for the number of TLNC that clearly stratified them into 2 separate survival groups (< 6 or ⩾ 6, respectively; median DSS, 15 mo *vs* 33 mo, χ^2 = 11.820, P < 0.001). DSS progressively worsened with increasing PLNC and LNR, but no definite cut-off value could be identified. Multivariate analysis revealed histological grade, tumor node metastasis staging, TNLC and LNR to be independent predictors of DSS. Neither location of positive lymph nodes nor PNLC were identified as an independent variable by multivariate analysis.

CONCLUSION: Both TLNC and LNR are strong predictors of outcome after curative resection for GBC. The retrieval and examination of at least 6 nodes can influence staging quality and DSS, especially in node-positive patients.

Key words: Gallbladder neoplasms; Lymphatic metastasis; Lymph node excision; Lymph node ratio; Prognosis

Core tip: The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected gallbladder cancer (GBC). The present study evaluates the prognostic impact of number, location and ratio of involved lymph nodes, in addition to well described prognostic parameters, in patients with curatively resected GBC. The results demonstrate that total lymph node count and lymph node ratio are more appropriate to stratify GBC patients with regards to prognosis; removal and pathological examination of at least six lymph nodes can influence staging quality and disease-specific survival especially in node-positive patients.

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INTRODUCTION

Gallbladder cancer (GBC) is one of the most common malignancies of the biliary tract with poor prognosis, because it is usually detected at an advanced stage due to no specific symptoms. Treatment options for GBC have evolved over the last decade, as it has become well accepted that patients benefit from radical resection[1-4]. The spread modes of GBC are direct, lymphatic, vascular, neural, intraperitoneal and intraductal. Lymph node is one of the most common sites of metastasis of GBC. The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC[5-8]. However, the method of optimally categorizing lymph nodal involvement in GBC remains controversial[9,10]. It is increasingly being recognized that an inadequate number of lymph nodes examined may adversely influence survival and lead to understaging of GBC[11]. Some investigators have highlighted the importance of metastatic lymph node count as a means of stratification, while others rely on the location of involved nodes[12]. Some investigators have emphasized the total number of lymph nodes resected during operation[13,14]. Recent studies have also demonstrated the influence of involved lymph node count and metastatic to examined lymph nodes ratio (LNR) on survival of patients with GBC[15,16]. The present study evaluates the prognostic impact of number, location and ratio of involved lymph nodes, in addition to well described prognostic parameters, in patients with curatively resected gallbladder cancer.

MATERIALS AND METHODS

Patient population

From May 1995 to December 2010, a total of 78 consecutive patients with GBC underwent a radical resection at Liaocheng People's Hospital. A radical resection was defined as removing both the primary tumor and the regional lymph nodes of the gallbladder. Cancer arising in the cystic duct was also included as gallbladder cancer. Eight patients with early pT stages (Tis or T1) were excluded due to their resection of only simple cholecystectomy without lymphadenectomy. Eleven patients were excluded due to incomplete clinicopathologic data or follow-up loss. As a result, 78 patients were retrospectively reviewed; these included 46 women and 32 men ranging in age from 33 years to 82 years (median, 59 years).

Table 1 Number of radical resection procedures and their relationship with tumor node metastasis stages

TNM stage procedure	0	I	II	IIIA	IIIB	IVA	IVB	Sum
C + N	1	2	1[1]		3			7
C + WR + N		2	4	6	5		2	19
C + S4aS5 + N				8	9		1	18
C + ELH + N							1	1
C + ERH + N						2		2
C + BD + N		1[2]						1
C + WR + BD + N		1	3	1	3	1	1	10
C + S4As5 + BD + N				1	3	1	1	6
C + CH + BD + N						1		1
C + S4aS5 + other + N				3	1	1		5
C + S4As5 + BD + other + N						1		1
C + ERH + BD + other + N						1		1
HPD + N						2	4	6
Sum	1	6	8	19	24	10	10	78

[1]Tumor of the patient infiltrated the serosa at the visceral surface of the gallbladder bottom; [2]This patient was an incidental gallbladder cancer with a diagnosis confirmed during the initial operation by frozen section with a preoperative diagnosis of choledochal cyst. C: Cholecystectomy; N: Lymphadenectomy; WR: Wedge resection of the gallbladder fossae; S4aS5: Liver resection of segments IVa and V; ELH: Extended left hepatectomy; ERH: Extended right hepatectomy; CH: Central hepatectomy; BD: Resection of the bile duct; HPD: Hepatopancreaticoduodenectomy; Other: Other organ tissue resection; TNM: Tumor node metastasis.

Radical resection procedures

Radical resection procedures consisted of cholecystectomy, *en bloc* hepatic resection, and lymphadenectomy with or without bile duct excision. Lymphadenectomy included *en bloc* clearance of cystic duct, pericholedochal, hepatic artery, portal vein, periduodenal and peripancreatic lymph nodes. Celiac artery, perigastric, superior mesenteric artery and para-aortic nodal clearances were not performed routinely in every patient, but if there was any evidence of tumor infiltration or metastasis to the near organ or tissues, these nodes would be cleared by an extended radical operation such as pancreaticoduodenectomy. The extent of liver resection was guided by the extent of the tumor's liver infiltration, and the guiding principle is acquiring a negative surgical margin while at the same time preserving the maximal amount of liver parenchyma. A 2-cm non-anatomical wedge of gallbladder fossa was performed if the tumor was confined to gallbladder, and formal resection of segments V and IV a was performed if there was gross liver involvement.

The operative procedures are shown in Table 1. All patients underwent lymphadenectomy. The operative pro-

cedures included cholecystectomy (n = 8), wedge resection (n = 29), resection of segments Ⅳa and Ⅴ (n = 30), resection of the bile duct (n = 20), extended hepatectomy (n = 5), hepatopancreaticoduodenectomy (n = 6), with other organ tissue resection (n = 7), portal vein resection and reconstruction (n = 2), proper or right hepatic artery resection (n = 3).

Pathological examination

Immediately after resection, the operating surgeon separated the lymph nodes from the node-bearing adipose tissues of the fresh surgical specimen, which were then divided by the surgeon into individual node groups according to their locations. The specimen was then fixed in 10% buffered formaldehyde solution. Primary tumor was examined to determine the histologic type, tumor grade, depth of infiltration, tumor involvement of excised contiguous viscera and resection margins. Histologic grade was determined based on the areas of tumor with highest grade. Lymph node metastasis was defined as tumor cells detected on histopathologic examination using hematoxylin and eosin stain.

The lymph nodes retrieved were examined histologically for metastases routinely from each node. The positive lymph node count (PLNC) as well as the total lymph node count (TLNC) was recorded for each patient. Here, PLNC and TLNC represented the sum of regional, celiac artery, perigastric, superior mesenteric artery and paraaortic nodes evaluated in the patient. Then the metastatic to examined LNR was calculated.

Patient follow-up after resection

Of 78 patients, one died during the hospital stay because of liver failure after the definitive resection, giving an in-hospital mortality rate of 1.28%. Patients discharged to home were followed up regularly every 1-6 mo, with a median follow-up time of 26.50 mo (range, 2-132 mo). Adjuvant chemoradiation therapy was administered to 23 patients at the discretion of the individual surgeons. Only deaths from tumor recurrence were treated as failure cases in the analysis of disease-specific survival (DSS), whereas those from other causes were recorded as censored cases. The survival time in each patient was defined as the interval between the date of definitive resection and the date of last follow up or death.

Statistical analysis

Categorical variables were compared using the Pearson χ^2 test. Numerical variables were compared using paired samples t test. Survival curves were constructed using the Kaplan-Meier method, and differences in survival were evaluated with the log rank test. Cox regression analysis was used to identify independent predictors of disease-specific survival using factors found to be significant by univariate analysis. The IBM SPSS 16.0 software (SPSS Inc., Chicago, IL, United States) was used for all statistical evaluations. All tests were two-tailed and P values less than 0.05 were considered statistically significant.

RESULTS

Pathologic features

Pathological findings were documented using the American Joint Committee on Cancer (AJCC) cancer staging manual (7th edition)[17]. Resection margin status was judged as no residual tumor (R0) in all 78 patients. The primary tumor was pTis in 1 patient, pT_1 in 7 patients, pT_2 in 12 patients, pT_3 in 44 patients, and pT_4 in 14 patients. The lymph node stage was N0 in 41 patients, N1 in 31 patients and N2 in 6 patients. The M stage was M0 in 74 patients and M1 in 4 patients. Of the metastasis patients, 1 was a single metastasis lesion on the visceral peritoneum and the other 3 were liver metastases. Then the patients were classified according to tumor node metastasis (TNM) staging: stage 0 (n = 1), stage I (n = 6), stage Ⅱ (n = 8), stage ⅢA (n = 19), stage ⅢB (n = 24), stage ⅣA (n = 10) and stage ⅣB (n = 10).

Distribution of lymph nodes metastasis

A total of 465 lymph nodes taken from the 78 studied patients were evaluated. TLNC ranged from 1 to 24 (median, 4) per patient. According to the AJCC cancer staging manual (7th edition)[17], the topographical distribution of the analyzed lymph nodes included 361 first-station nodes and 104 second-station nodes (Table 2). There were significantly more first-station nodes per patient (median = 4; range: 1-12) than second-station nodes (median = 0; range: 0-12) (t = 10.46, P < 0.001).

Of the 78 studied patients, 37 (47.44%) had a total of 98 positive lymph nodes. The number of positive nodes per patient ranged from 1 to 9 (median = 2). There were 5 (25.00%) of 20 patients with pTis to pT_2 stage who had positive nodal disease, whereas 32 (55.17%) of 58 patients with pT_3 to pT_4 stage had positive nodal disease. The occurrence of lymph node metastasis was increased obviously with the advance of pT stage (χ^2 = 5.430, P = 0.020).

The topographical distribution of all positive lymph nodes is shown in Table 2. Among the 37 node-positive patients, the prevalence of nodal disease was highest in the pericholedochal (n = 20, 54.05%) or the cystic duct (n = 18, 48.65%) node group, followed by the periportal (n = 12, 32.43%), hepatic artery (n = 10, 27.03%), postero-superior pancreaticoduodenal (n = 6, 16.22%), hilar (n = 4, 10.81%), and right celiac (n = 1, 2.70%) node groups. The paraaortic, superior mesenteric artery and perigastric nodes were not involved in any of our patients.

Of 13 patients with a single positive node, 11 (84.62%) had nodal disease in either the pericholedochal (n = 6) or cystic duct (n = 5) node group, suggesting that initial nodal involvement occurred primarily in these node groups.

Analysis of the topographical distribution of positive lymph nodes may be helpful to derive the route of lymphatic spread from GBC (Table 2). In this study, GBC primarily spread to the first-station nodes, then to the second-station nodes.

Table 2 Topographical distribution of 465 lymph nodes evaluated in 78 patients with gallbladder cancer *n* (%)

Node group	Patients with node group evaluated	Lymph nodes evaluated	Patients with positive nodes	Positive nodes
Cystic duct[1]	41 (53.95)	46 (9.89)	18 (23.08)	19 (19.39)
Pericholedochal[1]	68 (81.18)	146 (31.40)	20 (25.64)	29 (29.59)
Periportal[1]	47 (60.26)	74 (15.91)	12 (15.38)	18 (18.37)
Hepatic artery[1]	48 (61.54)	69 (14.84)	10 (12.82)	12 (12.24)
Posterosuperior pancreaticoduodenal[2]	22 (28.21)	56 (12.04)	6 (7.69)	12 (12.24)
Hilar[1]	18 (23.08)	26 (5.59)	4 (5.13)	6 (6.12)
Right celiac[2]	8 (10.26)	21 (4.52)	1 (1.28)	2 (2.04)
Perigastric[2]	4 (5.13)	6 (1.29)	0 (0.00)	0 (0.00)
Superior mesenteric artery[2]	6 (7.69)	11 (2.37)	0 (0.00)	0 (0.00)
Paraaortic[2]	6 (7.69)	10 (2.15)	0 (0.00)	0 (0.00)
Sum	78 (100)	465 (100)	37 (47.44)	98 (100)

[1]First-station nodes; [2]Second-station nodes; according to the American Joint Committee on Cancer cancer staging manual (7th edition). Here, hilar lymph nodes classified as first-station nodes and perigastric lymph nodes classified as second-station nodes.

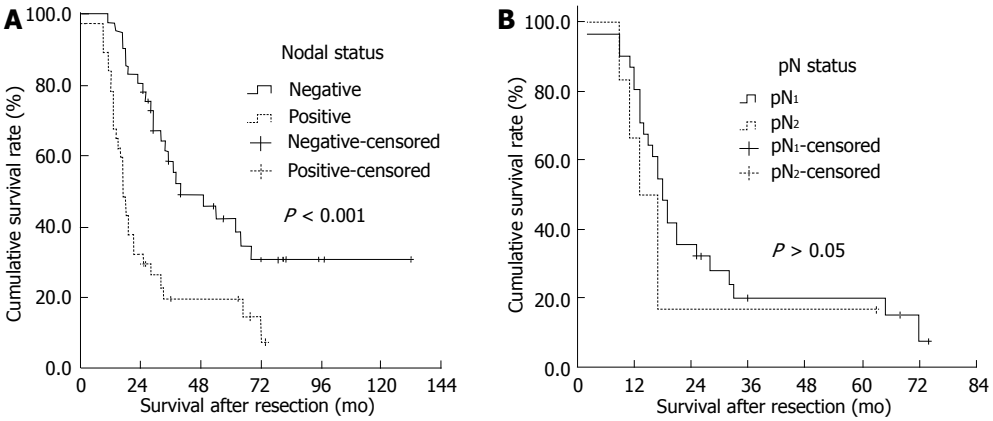

Figure 1 Kaplan-Meier survival estimates stratified. A: Lymph node status (negative *vs* positive; median disease-specific survival, 40 mo *vs* 17 mo); B: pN status in nodal positive patients (pN1 *vs* pN2; median disease-specific survival, 18 mo *vs* 13 mo).

Survival after regional lymphadenectomy

Of the overall patients, there were 22 patients who survived more than 3 years and 16 patients survived more than 5 years by the end of the follow-up; the median DSS was 29.00 ± 3.92 mo (5-year survival rate, 20.51%). The postoperative DSS of node-negative patients was significantly better than that of node-positive patients (median DSS, 40 mo *vs* 17 mo, $\chi^2 = 14.814$, $P < 0.001$, Figure 1A). Most node-negative patients achieved long-term survival after R0 resection (5-year survival rate, 26.83%). Of the 37 node-positive patients after an R0 resection, only 5 patients survived more than 5 years (5-year survival rate, 13.51%).

We then focused on a subgroup of 37 node-positive patients who had undergone an R0 resection for survival analysis; they comprised 31 N1 stage patients and 6 N2 stage patients. The postoperative DSS was not significantly different between N1 node-positive patients (median survival time, 18 mo; 5-year survival rate, 12.90%) and N2 node-positive patients (median survival time, 13 mo; 5-year survival rate, 16.67%) ($\chi^2 = 0.741$, $P = 0.389$, Figure 1B). Of the 5 patients with node-positive disease who survived for more than 5 years, there were two patients who underwent a pancreaticoduodenal lymph node dissection with hepatopancreaticoduodenectomy for suspected N2 nodal disease. These findings suggested that regional lymphadenectomy could achieve an acceptable rate of long-term survival even in patients with advanced stage of nodal metastasis, provided that an R0 resection is feasible.

Cut-off values for the TNLC, PNLC and LNR

Based on the magnitude of the Log-rank test χ^2 statistic, the optimal cut-off value was four nodes for the number of TLNC. Based on these results, the number of TLNC was placed into two categories in subsequent analyses (< 4 or ≥ 4, respectively). DSS of TLNC < 4 group was worse than that of TLNC ≥ 4 group (median DSS, 18 mo *vs* 33 mo, $\chi^2 = 5.606$, $P = 0.018$, Figure 2A). When node-negative patients were divided according to TLNC, there was no difference in DSS between TLNC < 4 subgroup ($n = 60$) and TLNC ≥ 4 subgroup ($n = 21$) (median DSS, 37 mo *vs* 54 mo, $\chi^2 = 0.715$, $P = 0.398$, Figure 2B). For node-positive patients, DSS of TLNC < 4 subgroup was worse than that of TLNC ≥ 4 subgroup (median DSS, 13 mo *vs* 21 mo, $\chi^2 = 11.035$, $P < 0.001$, Figure 3A). Moreover, for node-positive patients, a new cut-off value of six nodes for the number of TLNC clearly stratified

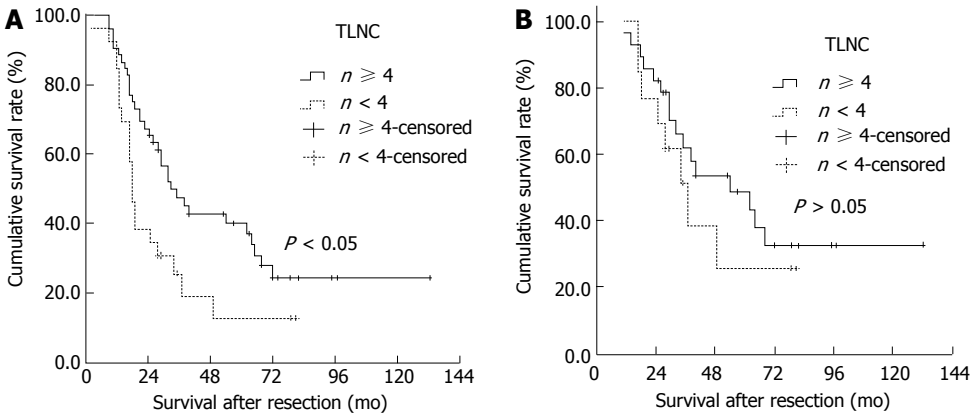

Figure 2 Kaplan-Meier survival estimates stratified for total lymph node count status (< 4 or ⩾ 4, respectively). A: In 78 patients who underwent an R0 resection (median disease-specific survival, 18 mo *vs* 33 mo); B: In 41 node-negative patients (median disease-specific survival, 37 mo *vs* 54 mo). TLNC: Total lymph node count.

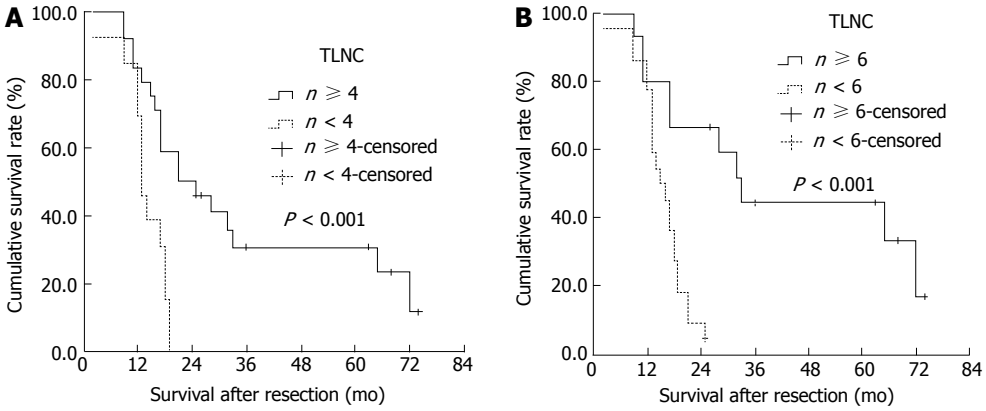

Figure 3 Kaplan-Meier survival estimates stratified for total lymph node count status in 37 node-positive patients. A: *n* < 4 or ⩾ 4, respectively; median disease-specific survival, 13 mo *vs* 21 mo; B: *n* < 6 or ⩾ 6, respectively; median disease-specific survival, 15 mo *vs* 33 mo. TLNC: Total lymph node count.

them into 2 separate survival groups (< 6 or ⩾ 6, respectively; median DSS, 15 mo *vs* 33 mo, $\chi^2 = 11.820$, $P < 0.001$, Figure 3B).

DSS progressively worsened with increasing PLNC and LNR, but no definite cut-off value could be identified. Based on the previous literature, we left the cut-off value as 3 nodes for PLNC and 50% for LNR separately[13,16].

Factors influencing disease-specific survival after resection

Univariate analyses identified liver invasion, venous invasion, pT classification, pN classification, pM classification, TNM staging, lymph node invasion, TLNC, PLNC, LNR and histological grade as significant prognostic factors (Table 3).

The univariately significant variables were then entered into multivariate analysis. Histological grade, TNM staging, TLNC and LNR remained as independently significant variables (Table 4). Neither location of positive lymph nodes nor PLNC were identified as an independent variable by multivariate analysis.

DISCUSSION

Studies have demonstrated that the presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC[5,13,18-20]. Patients with lymph node metastasis have significantly worse survival than those with negative nodes[1,21]. With the increasing safety of hepatic and pancreatic surgery, various radical procedures have been advocated to improve the curative outcome for advanced GBC[22-24]. Recent data also suggest that aggressive resection may improve long-term survival, even in patients with advanced stage disease[3,12,25].

It had been confirmed that the main lymphatic pathway of the gallbladder descends along the common bile duct and into the retroportal nodes, then to the posterosuperior of the head of the pancreas or around the hepatic artery, and finally to the paraaortic nodes near the left renal vein[26-28]. Based on these detailed anatomical studies, it has been suggested that lymphatic metastasis from GBC spreads widely through the hepatoduodenal ligament towards the peripancreatic region and beyond. In

Table 3 Univariate analysis of clinical and histopathologic variables

Variable	Number of patients	Survival rate		*P* value
		3-yr	5-yr	
Age (yr)				0.222
< 60	41	34.15%	17.07%	
≥ 60	37	24.32%	18.92%	
Sex				0.523
Female	46	28.26%	17.39%	
Male	32	31.25%	18.75%	
Cholelithiasis				0.374
Present	25	24.00%	16.00%	
Absent	53	32.08%	18.87%	
Type of radical resection				0.179
Extended cholecystectomy	7	42.86%	42.86%	
Partial hepatectomy[1]	37	32.43%	13.51%	
Partial hepatectomy and EBD resection	23	30.43%	26.09%	
Extended hepatectomy[2]	5	0.00%	0.00%	
Hepatopancreaticoduodenectomy	6	50.00%	33.33%	
Hepatic infiltration				0.005
Present	41	14.63%	4.88%	
Absent	37	51.35%	37.84%	
Bile duct infiltration				0.238
Present	17	29.41%	23.53%	
Absent	61	32.79%	19.67%	
Venous invasion				0.001
Present	10	0.00%	0.00%	
Absent	68	36.76%	23.53%	
Perineural invasion				0.539
Present	9	22.22%	22.22%	
Absent	69	33.33%	20.29%	
Lymph node involvement				< 0.001
Present	37	16.22%	13.51%	
Absent	41	46.34%	26.83%	
pT classification[3]				0.001
pT_0-pT_2	20	60.00%	45.00%	
pT_3-pT_4	58	22.41%	12.07%	
pN classification[3]				< 0.001
pN_0	41	46.34%	26.83%	
pN_1	31	16.13%	12.90%	
pN_2	6	16.67%	16.67%	
pM classification[3]				0.002
pM_0	74	33.78%	21.62%	
pM_1	4	0.00%	0.00%	
TNM staging[3]				< 0.001
0-Ⅱ	15	80.00%	60.00%	
Ⅲ	43	23.26%	11.63%	
Ⅳ	20	15.00%	10.00%	
TLNC				0.018
< 4	26	15.38%	7.69%	
≥ 4	52	40.38%	26.92%	
Number of positive lymph nodes				< 0.001
0	41	46.34%	26.83%	
< 3	24	16.67%	16.67%	
≥ 3	13	15.38%	7.69%	
LNR				< 0.001
0	41	46.34%	26.83%	
< 50	15	33.33%	33.33%	
≥ 50	22	4.55%	0.00%	
Histological type				0.706
Adenocarcinoma	69	33.33%	20.29%	
Others	9	22.22%	22.22%	
Histological grade				0.042
G1-G2	58	36.21%	24.14%	
G3-G4	19	15.79%	5.26%	

[1]Includes wedge resection and resection of segments Ⅳa and Ⅴ; [2]Includes extended right hepatectomy, extended left hepatectomy and central hepatectomy; [3]According to the American Joint Committee on Cancer cancer staging manual (7th edition). G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated; G4: Undifferentiated; EBD: Endoscopic balloon dilatation; TNM: Tumor node metastasis; TLNC: Total lymph node count; LNR: Lymph node ratio.

Table 4 Results of Cox multivariate regression analysis

Variable	Parameter estimate	Wald χ^2	*P*	Hazard ratio	95%CI
Tumor node metastasis staging		20.559	< 0.001		
0-Ⅱ				1.000	
Ⅲ	-3.112	19.846	< 0.001	0.045	0.011-0.175
Ⅳ	-1.044	9.341	0.002	0.352	0.180-0.688
Lymph node ratio	2.424	20.247	< 0.001	11.293	3.929-32.465
Total lymph node count	-0.147	14.273	< 0.001	0.864	0.800-0.932
Histological grade	-0.755	5.512	0.019	0.470	0.250-0.883

this study, initial nodal involvement occurred primarily in the cystic duct or pericholedochal nodes, followed by periportal and hepatic artery nodes. Posterosuperior pancreaticoduodenal and right celiac lymph nodes were involved in 16.22% of node-positive patients and were classified as N2 disease, according to the 7th edition of AJCC classification. However, we observed that the categorization of patients as having N2 disease did not adversely influence DSS as compared to those with N1 disease. Hence, we believe that even patients with N2 lymph node metastasis can achieve an ideal survival if radical lymphadenectomy is performed. An addition of pancreaticoduodenectomy could result in an R0 resection by removing extensive peripancreatic nodal disease in a select group of patients[22,23,29]. Furthermore, Murakami *et al*[30] suggested that it is lymph node metastasis but not para-aortic lymph node metastasis that is associated independently with longer survival by multivariate analysis. In this study, six patients were treated with pancreaticoduodenectomy and two patients survived more than five years.

The high propensity for lymphatic spread in GBC renders adequate lymphadenectomy indispensable for improving patient outcomes after resection[8,19]. However, because of the rarity of disease and low resectability rates, which limit the ability to perform large cohort studies or prospective randomized trials, the optimal extent of lymphadenectomy remains unresolved and there are no uniform evidence-based guidelines on the issue[9,10]. Accuracy of nodal staging depends on a critical number of lymph nodes analyzed; insufficient number of nodes retrieved during surgery or examined pathologically leads to underestimation of disease stage[14]. Although the 6th edition of AJCC suggests a minimum of three lymph nodes to be assessed for appropriate pathologic nodal staging of gallbladder cancer, the basis of recommendation is not clear, and there are no established standards. A large population-based study on the SEER database demonstrated that of the 2835 resected patients with T1-T3 M0 GBC, only 5.3% had a lymphadenectomy of three or more lymph nodes[31]. Also, Ito *et al*[14] independently suggested that retrieval and evaluation of at least six lymph nodes improves risk stratification after resection in node-negative patients. These observations indicate that retrieval of a larger number of lymph nodes than previously practiced is warranted not only for accurately staging the nodal status, but also for improving survival due to better clearance of nodal disease[13].

Although a greater number of examined nodes might improve the survival of the disease, the results of our study suggest that retrieval and evaluation of at least four nodes is perhaps optimal. Furthermore, TLNC significantly correlated with DSS in node-positive patients and allowed better prognostic substratification of these patients. For node-positive patients in this study, we can get a new cut-off value of six nodes for the number of TLNC that clearly stratifies them into 2 separate survival groups, which is more optimal than four nodes. But no definite cut-off value of TLNC could be identified for node-negative patients. Since the TLNC-survival relationship was observed only in node-positive patients and not in those node-negative patients, we believe that a higher count not only helps in stage purification but also helps improve therapeutic benefit, which is more serious in node-positive patients. These findings should heighten awareness about the importance of TLNC amongst surgeons performing lymphadenectomy for suspected node-positive patients. We believe that adequate lymphadenectomy is indispensable for improving the prognosis after radical resection in patients with GBC.

Endo *et al*[32] first suggested that the PLNC is more useful in assessing nodal status than the location of positive nodes in GBC. Sakata *et al*[12] additionally showed that the number, but not location, of positive nodes independently determined prognosis after resection. The burden of nodal disease (PLNC) also had an impact on prognosis; there was significantly reduced DSS observed in this study with involved nodes. The DSS progressively worsened with increasing PLNC; however, we were not able to identify any specific cut-off value. The use of PLNC as a prognostic factor might be limited by inherent bias of inadequate number of lymph nodes retrieved or histologically examined which leads to the phenomenon of "stage migration". However, many recent studies (including this study) have reported a number of long-term survivors after resection for GBC with multiple positive lymph nodes[11,29,30,33]. These observations indicate that regional lymph node dissection for GBC provides long term survival for selected patients with multiple positive lymph nodes, provided that R0 resection is feasible.

LNR has been shown to be an important predictor of survival for many gastrointestinal tract cancers after surgery because it is a better and reproducible method of stratifying nodal status which incorporates not only the burden and biology of disease (PLNC) but also the quality of lymphadenectomy and pathologic examination (TLNC)[34-36]. Negi *et al*[16] first found that LNR, and

not PLNC, was an independent prognostic factor in their study cohort comprising 57 patients with a relatively small TLNC. Our study suggests that, along with tumor TNM staging, LNR is an independent prognostic factor and another important lymph nodal variable in patients undergoing curative resection for GBC. The prognostic impact of LNR was observed in the entire group, including the subgroup of patients with positive nodes, even though we could not find an optimal cut-off value in this study. LNR is of particular value in patients who cannot adequately be staged because of the limited number of lymph nodes evaluated. In the case of insufficient lymph node evaluation, LNR will more accurately reflect the nodal status than the number of positive nodes in GBC. Patients with high LNR after radical resection might need adjuvant chemoradiation therapy to improve their prognosis.

The strengths of our study include the reasonably sized cohort of patients managed in a single institution using a standardized treatment approach. The current study has several limitations: the retrospective nature of the analysis, the relatively small number of patients spanning a long period of time, some variability in the degree of nodal dissection, and the short follow-up time for some patients. The observations need to be confirmed in larger, especially population-based, cohort. We believe, however, that these limitations did not greatly affect the results of the study as the differences between groups were too marked to have resulted from bias. In addition, the role of TLNC and LNR in assessing the nodal status for GBC is now more clearly defined than previously, based on the current study. Our results thus provide useful information for accurately staging nodal disease, predicting prognosis after resection, and selecting candidates for adjuvant chemoradiation therapy after resection.

The results of the present study demonstrate that, rather than categorizing GBC patients based on PLNC or location of involved nodes, TLNC and LNR are more appropriate tools to stratify patients with regards to prognosis. Our data also suggest that removal and pathological examination of at least six lymph nodes can influence staging quality and disease-specific survival especially in node-positive patients. This knowledge should heighten awareness amongst surgeons about the importance of performing lymphadenectomy for suspected node-positive patients, aiming to retrieve and examine an adequate number of lymph nodes.

COMMENTS

Background

Lymph node is one of the most common sites of metastasis of gallbladder cancer (GBC). The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC. However, the method of optimally categorizing lymph nodal involvement in GBC remains controversial.

Research frontiers

It is increasingly being recognized that an inadequate number of lymph nodes examined may adversely influence survival and lead to understaging of GBC. Some investigators have highlighted the importance of metastatic lymph node count as a means of stratification while others rely on the location of involved nodes. Some investigators emphasized the total number of lymph nodes resected during operation. Recent studies have also demonstrated the influence of involved lymph node count and metastatic to examined lymph nodes ratio (LNR) on survival of patients with GBC.

Innovations and breakthroughs

The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC. The present study evaluates the prognostic impact of number, location and ratio of involved lymph nodes, in addition to well described prognostic parameters, in patients with curatively resected GBC. The results demonstrate that total lymph node count (TLNC) and LNR are more appropriate to stratify GBC patients with regards to prognosis, and removal and pathological examination of at least six lymph nodes can influence staging quality and disease-specific survival especially in node-positive patients.

Applications

The study results suggest that TLNC and LNR are more appropriate to predict the prognosis of GBC patients, while surgeons need to achieve clearance and pathologically examine at least six lymph nodes to improve staging quality and disease-specific survival especially in node-positive patients.

Peer review

The lymph node is one of the most common sites of metastasis of GBC. The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected GBC.

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BRIEF ARTICLE

Effects of SAHA on proliferation and apoptosis of hepatocellular carcinoma cells and hepatitis B virus replication

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Abstract

AIM: To investigate the effects of suberoylanilide hydroxamic acid (SAHA) on proliferation and apoptosis of a human hepatocellular carcinoma cell line (HepG2.2.15) and hepatitis B virus (HBV) replication.

METHODS: HepG2.2.15 cells were treated with different concentrations of SAHA. Cell morphology was examined by confocal laser scanning microscopy, and cell proliferation was determined using a MTT colorimetric assay. Flow cytometry was used to detect apoptosis and determine cell cycle phase, while hepatitis B surface antigen and hepatitis B e antigen content were measured using chemiluminescence. Reverse transcription polymerase chain reaction was performed to measure HBV DNA in cell lysate.

RESULTS: Cell proliferation rates were significantly reduced by the addition of SAHA. The inhibitory effect of SAHA on cell proliferation was both time- and dose-dependent. After 24 h of treatment with SAHA, the early cell apoptotic rate increased from 3.25% to 21.02% (P = 0.041). The proportion of G_0/G_1 phase cells increased from 50.3% to 65.3% (P = 0.039), while that of S phase cells decreased from 34.9% to 20.6% (P = 0.049). After 48 h of treatment, hepatitis B surface antigen and hepatitis B e antigen content increased from 12.33 ± 0.62 to 25.42 ± 2.67 (P = 0.020) and 28.92 ± 1.24 to 50.48 ± 1.85 (P = 0.026), respectively. Furthermore, HBV DNA content increased from 4.54 ± 0.46 to 8.34 ± 0.59 (P = 0.029).

CONCLUSION: SAHA inhibits HepG2.2.15 cell proliferation, promotes apoptosis, and stimulates HBV replication. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

Key words: Human hepatocellular carcinoma; HepG2.2.15 cells; Suberoylanilide hydroxamic acid; Hepatitis B virus

Core tip: HepG2.2.15 cells were treated with different concentrations of suberoylanilide hydroxamic acid (SAHA). Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) content were measured using chemiluminescence. Reverse transcription polymerase chain reaction was performed to measure hepatitis B virus (HBV) DNA in cell lysate. Results found that, the inhibitory effect of SAHA on cell proliferation was both time- and dose-dependent. After 24 h of treatment, the early cell apoptotic rate increased. After 48 h of treatment, HBsAg and HBeAg content both increased. Furthermore, HBV DNA content increased. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

Wang YC, Yang X, Xing LH, Kong WZ. Effects of SAHA on proliferation and apoptosis of hepatocellular carcinoma cells and hepatitis B virus replication. *World J Gastroenterol* 2013; 19(31): 5159-5164 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5159.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5159

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. The worldwide incidence of HCC ranks fifth out of all malignant tumors, and the number of patients with HCC in China accounts for more than half of total cases in the world[1]. Etiological factors of HCC vary for different countries and areas. Histone deacetylase inhibitors (HDACIs) are a series of new anticancer drugs with a wide scope of application. In recent years, HDACIs have generated considerable interest due to their high efficiency to inhibit a variety of solid tumors with low toxicity[2-6]. In the current study, the effects of suberoylanilide hydroxamic acid (SAHA), a potent HDACI, on proliferation and apoptosis of human HCC cells HepG2.2.15 and hepatitis B virus (HBV) replication were investigated. The study objective was to characterize a potentially new treatment option for HCC.

MATERIALS AND METHODS

Cell culture and treatment

HepG2.2.15 cells (obtained from the Cell Center of the Chinese Academy of Medical Sciences; prepared by transfection of HepG2 cells with HBV genome) were maintained in DMEM (HyClone Laboratories, Inc., New England, United States) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin and 380 mg/L G418 in a thermostatic and sealed incubator (37 ℃, 5% CO_2). About 100 mmol/L SAHA (Sigma-Aldrich Corp, Missouri, United States) in dimethylsulfoxide (DMSO) was prepared and stored at -20 ℃ until further use. HepG2.2.15 cells were divided into a control group and several treatment groups to receive different concentrations of SAHA. The adherent cells were washed down with 0.25% trypsin, followed by passage.

Inhibition of cell proliferation

An MTT colorimetric assay was used to monitor inhibition of cell proliferation by the addition of different concentrations of SAHA to cell culture medium. For three 96-well plates, 100 μL HepG2.2.15 cells (1×10^5 cells/mL) was added to each well and incubated for 12 h at 37 ℃ in 5% CO_2. Once cells were adhered to the wells, SAHA was added to a final concentration of 2.5, 5, 7.5 or 10 μmol/L. Wells without SAHA were used as negative controls. After the addition of SAHA, a culture plate was incubated for 24, 48 or 72 h. Cell morphology was examined by confocal laser scanning microscopy (CLSM). Then, 20 μL of MTT (5 mg/mL) was added to each well. After incubation for 4 h, 150 μL of DMSO was added, followed by mixing for 10 min. Lastly, absorbance (A) at 490 nm was measured using a microplate reader. The inhibition rate of cell proliferation was calculated as follows: Cell proliferation inhibition rate (%) = $(1 - A_{\text{SAHA group}}/A_{\text{Negative control group}}) \times 100\%$.

Detection of apoptosis and determination of cell cycle phase

Control group and SAHA groups (2.5 and 5 μmol/L) were cultured for 24 and 48 h respectively. The single cell suspension was then prepared. After centrifugation at 2000 *g* for 5 min, the cell pellet was resuspended in 0.5 mL PBS (final concentration, $1\text{-}5 \times 10^5$ cells/mL). For detection of apoptosis, binding buffer (500 μL) and 5 μL annexin V-fluorescein isothiocyanate (Annexin V-FITC) were added to the cell resuspension, followed by 5 μL propidium iodide (PI) (Nanjing KGI Biological Technology Co., Ltd., Nanjing, China). After incubation for 5-15 min (room temperature, avoiding light), samples were subjected to flow cytometry (FCM). For determination of cell cycle phase, 5 mL of obtained cell resuspension was infused into 70% cold ethanol, followed by fixation at 4 ℃ overnight. During the next day, the cell solution was centrifuged at 800 r/min for 15 min, followed by two phosphate buffer saline (PBS) washes and resuspension in 0.4 mL PBS. RNaseA was added to a final concentration of 50 μg/mL, followed by digestion for 30 min in a 37 ℃ water bath. Lastly, PI was added to a final concentration of 65 μg/mL, followed by incubation for 30 min. After filtration through a nylon mesh, FCM was conducted.

Determination of hepatitis B surface antigen and hepatitis B e antigen content

HepG2.2.15 cells (2.5×10^5 cells/mL) were plated onto a 6-well plate. In triplicate, 1 μL SAHA (7.5 μmol/L) or an equivalent volume of DMSO was added to an individual well. After incubation for 72 h, cells were centrifuged at 800 r/min for 5 min. The supernatant was collected, and the hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) content were quantitated using quantitative chemiluminescence detection kits in i4000sR automatic chemiluminescence immunoassay analyser (R.D. Abbott Company, Inc., California, United States).

Determination of HBV DNA content

A 6-well cell culture plate was prepared as previously described above. HBV negative and positive controls were prepared as follows: 100 μL of cell supernatant was mixed with an equal amount of DNA extraction liquid (shaking for 15 s), followed by centrifugation at 12000 *g* for 10 min to remove supernatant. Then, 20 μL of DNA extraction liquid was added to the sediment, followed by incubation for 10 min in a 100 ℃ water bath.

HBV-polymerase chain reaction (PCR) reaction liquid (35.6 μL) and Taq enzyme (0.4 μL) were mixed in a 0.2 mL Eppendorf tube. Two μL of treated sample supernatant was then added to each tube and centrifuged at 8000 *g* for several seconds. Quantitative fluorescent PCR was performed under the following conditions: 95 ℃ for 3 min; 94 ℃ for 15 s (40 cycles); 60 ℃ for 30 s (40 cycles).

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis

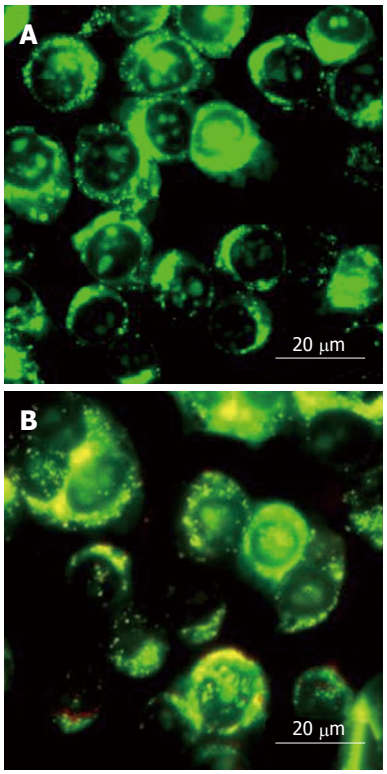

Figure 1 Cell morphology changes in confocal laser scanning microscopy. A: Control group; B: Suberoylanilide hydroxamic acid group.

was performed using SPSS 16.0 statistical software. Single factor analysis of variance and *t* tests were conducted for comparison among multiple groups. $P < 0.05$ was considered as statistically significant.

RESULTS

Effect of SAHA on cell morphology

CLSM showed that proliferation of untreated HepG2.2.15 cells was rapid, and the cells were compactly arranged. The adjacent cells fused into pieces, with clear edge. The cytoplasm was small, with a large nucleus. In SAHA-treated groups, cell proliferation rate was significantly slower. There were sparse adherent cells, with blurred configuration. The cytoplasm increased, presenting morphological changes similar to normal cells (Figure 1).

Effect of SAHA on cell proliferation

Multiple concentrations of SAHA could inhibit proliferation of HepG2.2.15 cells, and the inhibitory rate increased with increasing concentrations of SAHA ($P < 0.05$). With each SAHA concentration, the inhibition rate gradually increased with prolonged treatment time ($P < 0.05$). Taken together, the inhibitory effect of SAHA on cell proliferation was time- and dose-dependent (Table 1).

Effect of SAHA on cell apoptosis and cell cycle

After 24 h of treatment with 2.5 μmol/L SAHA, early apoptosis rate of HepG2.2.15 cells increased from 3.25% to 16.28% ($P = 0.032$), and the middle-late apoptosis rate increased from 1.08% to 5.16% ($P = 0.035$). In the 5 μmol/L SAHA group, early and middle-late apoptosis rate increased from 3.25% to 21.02% ($P = 0.041$) and 1.08% to 10.70% ($P = 0.045$), respectively (Table 2 and Figure 2). After 24 h of treatment with 2.5 and 5 μmoL/L SAHA, the proportion of G_0/G_1 phase cells increased from 50.3% to 69.9% and 65.3%, respectively, and the proportion of S phase cells decreased from 34.9% to 22.3% and 20.6%, respectively. Most cells were arrested in the G_0/G_1 phase (Table 3).

Table 1 Inhibition rate of suberoylanilide hydroxamic acid on HepG2.2.15 cells

SAHA (μmol/L)	24 h	48 h	72 h
2.5	8% ± 0.54%	15% ± 1.52%	23% ± 1.39%
5.0	13% ± 0.63%	22% ± 1.68%	34% ± 1.61%
7.5	28% ± 1.56%	39% ± 1.67%	50% ± 1.70%
10.0	42% ± 1.72%	51% ± 1.82%	66% ± 1.76%

$P < 0.05$ for comparison among different concentration and different treatment time, respectively. SAHA: Suberoylanilide hydroxamic acid.

Table 2 Effect of suberoylanilide hydroxamic acid on apoptosis rate of HepG2.2.15 cells

Group	24 h		48 h	
	Early apoptosis rate	Middle-late apoptosis rate	Early apoptosis rate	Middle-late apoptosis rate
Control	3.25%	1.08%	3.58%	1.26%
2.5 μmol/L SAHA	16.28%	5.16%	23.06%	8.42%
5.0 μmol/L SAHA	21.02%	10.70%	26.44%	17.55%

SAHA: Suberoylanilide hydroxamic acid.

Table 3 Effect of suberoylanilide hydroxamic acid on proportion of HepG2.2.15 cells with different phases

Group	24 h			48 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	50.3%	34.9%	14.8%	46.3%	38.2%	15.5%
2.5 μmol/L SAHA	69.9%	22.3%	7.8%	70.9%	26.1%	3.0%
5.0 μmol/L SAHA	65.3%	20.6%	14.1%	68.9%	25.8%	5.3%

SAHA: Suberoylanilide hydroxamic acid.

HBsAg and HBeAg content and HBV DNA content

Positive expression of HBsAg and HBeAg in the SAHA group and control group, respectively, was observed. After 48 h of treatment with SAHA, HBsAg and HBeAg content increased from 12.33 ± 0.62 to 25.42 ± 2.67 ($P = 0.020$) and 28.92 ± 1.24 to 50.48 ± 1.85 ($P = 0.026$), respectively, and HBV DNA content increased from 4.54 ± 0.46 to 8.34 ± 0.59 ($P = 0.029$).

DISCUSSION

Abnormality of any step of epigenetics can affect gene

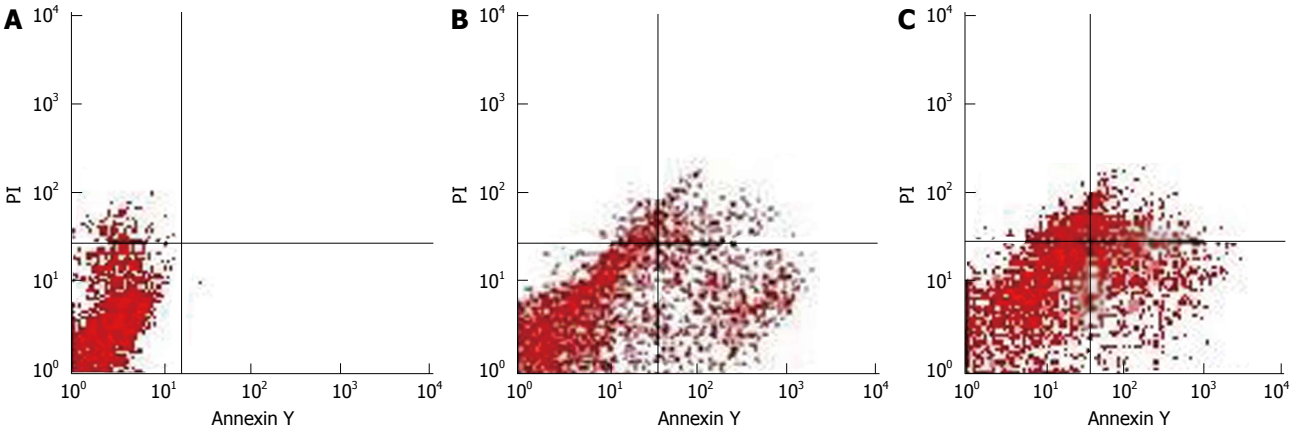

Figure 2 Effect of suberoylanilide hydroxamic acid on apoptosis of HepG2.2.15 cells. A: Control group; B: 2.5 μmol/L suberoylanilide hydroxamic acid (SAHA) group; C: 5.0 μmol/L SAHA group. PI: Propidium iodide.

expression or function, leading to the occurrence of disease, such as cancer. As a main epigenetic pattern, histone acetylation is closely related with tumor occurrence. HDACIs are often used to alter histone acetylation for treatment of cancer[7-9]. SAHA is a broad-spectrum HDACI, and was approved for treating T-cell lymphoma in 2006 (in phase I and II clinical trial). It has obvious inhibitory effects on histone deacetylase, and can inhibit the growth of HCC cells by arresting cell cycle progression and inducing cell differentiation and apoptosis. HDACIs have been shown to exhibit a broad-spectrum inhibitory activity on blood and solid tumors[10,11].

Unrestricted division and proliferation is an important feature of tumor cells. Detection of an inhibitory effect on tumor cell proliferation is a basic index for screening of anti-tumor drugs. In this study, CLSM and a MTT colorimetric assay were used to show that cell proliferation rates was significantly decreased by treatment with SAHA. Specifically, the number of cells was reduced, and adherent cells became sparse. Time- and dose-dependencies of SAHA inhibition on cell proliferation were evident. The cytoplasm increases, presenting morphological changes similar to normal cells, which is consistent with results from a previous study[12].

Apoptosis is programmed cell death. The process of apoptosis and the clearance of apoptotic cells is one of the most important factors for maintaining liver health. In this study, after 24 h of treatment with 5 μmol/L SAHA, early apoptosis rate and middle-late apoptosis rate of HepG2.2.15 cells increased from 3.25% to 21.02% (P = 0.029) and 1.08% to 10.70% (P = 0.045), respectively, indicating that SAHA may interfere with the balance between apoptosis and anti-apoptosis, induce the expressions of pro-apoptotic genes (*Bmf*, *Bim*, *TRAIL* and *DR5*)[13], and activate the expression of transcription factor E2F1. Furthermore, SAHA can induce the expression of apoptosis signal-regulating kinase 1 (ASK1), which promotes apoptosis of tumor cells through the death receptor and intracellular apoptotic pathways[14,15]. In addition, SAHA can activate the expression of pro-apoptotic proteins, including Bax and Bak, and inhibit expression of anti-apoptosis proteins, including Bcl-2 and Bcl-xL, thus inducing apoptosis of tumor cells[16]. In the extracellular apoptotic pathway, activated caspase-8 can cleave Bid to truncated Bid (tBid), as well as cause Cyt C release and Bax expression, leading to activation of caspase-9 and caspase-3. Caspase-3 can promote activation and cleavage of PARP to subsequently activate the intracellular apoptotic pathway[17]. SAHA has been shown to induce transcription of CDK inhibitor p21/wafl in T24 bladder cancer cells, reducing proliferation and increasing apoptosis[14].

Abnormality of cell cycle regulation is one of intrinsic factors for tumor occurrence. HDACIs can arrest tumor cell cycle, inhibiting growth. Results of this study show that, after 24 h of treatment with 5 μmoL/L SAHA, the proportion of G_0/G_1 phase cells increases from 50.3% to 65.3%, and the proportion of S phase cells decreases from 34.9% to 20.6%. Most cells were arrested in the G_0/G_1 phase and induced to undergo apoptosis. This may be related to increased expression of CDK inhibitor p21/waf1, which is induced by SAHA treatment. Nearly all HDACIs can induce expression of p21/waf1 to inhibit the activities of cyclin and CDK, resulting in cell cycle arrest and inhibition of differentiation. In addition, SAHA has been shown to influence expression of p27. After SAHA treatment, the degree of histone acetylation is elevated, stabilizing the activity of p53 (an important intracellular tumor suppressor protein) and leading to cell cycle arrest[18-20]. The Ras-Raf-MEK-ERK pathway is closely related with tumor cell proliferation. ERK can be activated by various growth factors, leading to interaction with transcription factors (mitogen, c-Jun, c-fos, c-Myc, cERK1) and nuclear proteins to promote the transcription and expression of a variety of oncogenes and genes related to cell cycle regulation, thus promoting cell proliferation and inhibiting apoptosis[21-23].

HBV is a risk factor for development of HCC. An epidemiological survey demonstrated that the carrying rate of HBsAg in China is 7.18%. HBV can be actively

replicated in patients with HCC, causing further liver damage[24-27]. HepG2.2.15 cells can continuously excrete intact HBV Dane particles into culture media. Upon treatment with SAHA, HBsAg and HBeAg content were 2.06 and 1.75 times greater than the control group, respectively, and HBV DNA content was 1.83 times greater than the control group. Taken together, SAHA stimulated replication of HBV. Histone acetylation is involved in regulation of gene transcription. After treatment with SAHA, the level of histone acetylation in HBV DNA is increased, and chromosome structure became incompact. This facilitates the combination of transcription factor with HBV DNA polymerase, thus stimulating HBV replication. However, this mechanism needs further validation. SAHA is an effective drug for HBV-negative HCC patients, but should be cautiously used in HBV-positive HCC patients in combination with anti-HBV drugs.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. Its occurrence is related to the multiple-step development process of different genetic alterations. At present, there is no effective treatment method. Suberoylanilide hydroxamic acid (SAHA) is a newly discovered anti-tumor drug which has broad application prospect. It exhibits inhibitory effect of tumor growth, which is been further confirmed in clinical trials.

Research frontiers

Histone deacetylase inhibitors (HDACIs) are a class of new anticancer drugs emerging in recent years, which has attracted widespread attention. Previous clinical trials find that, SAHA has broad-spectrum anti-hematological and solid tumor activities, with good tolerance. However, the effect of SAHA on hepatitis B virus (HBV) replication has not been reported.

Innovations and breakthroughs

SAHA inhibits HepG2.2.15 cell proliferation, promotes apoptosis, and stimulates HBV replication. In combination with anti-HBV drugs, SAHA may potentially be used cautiously for treatment of hepatocellular carcinoma.

Applications

SAHA has been applied in previous clinical trials. Results show that, it has broad-spectrum anti-hematological and solid tumor activities. SAHA can inhibit HepG2.2.15 cell proliferation, deduce the differentiation, and promote the apoptosis. At the same time, it can stimulate the replication of HBV. Therefore, SAHA should be cautiously used for treatment of HCC, and be combined with anti-HBV drugs if necessary. It can be used in the treatment of HBV-negative HCC patients.

Peer review

HCC is one of the most common malignant tumors, and HDACIs are a series of new anticancer drugs with a wide scope of application. In this manuscript, the authors investigated the effects of suberoylanilide hydroxamic acid, a potent HDACI on proliferation and apoptosis of a human hepatocellular carcinoma cell line (HepG2.2.15) and HBV replication. The manuscript is very well written.

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META-ANALYSIS

Single-incision laparoscopic appendectomy *vs* conventional laparoscopic appendectomy: Systematic review and meta-analysis

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Abstract

AIM: To assess the differences in clinical benefits and disadvantages of single-incision laparoscopic appendectomy (SILA) and conventional laparoscopic appendectomy (CLA).

METHODS: The Cochrane Library, MEDLINE, Embase, Science Citation Index Expanded, and Chinese Biomedical Literature Database were electronically searched up through January 2013 to identify randomized controlled trails (RCTs) comparing SILA with CLA. Data was extracted from eligible studies to evaluate the pooled outcome effects for the total of 1068 patients. The meta-analysis was performed using Review Manager 5.2.0. For dichotomous data and continuous data, the risk ratio (RR) and the mean difference (MD) were calculated, respectively, with 95%CI for both. For continuous outcomes with different measurement scales in different RCTs, the standardized mean difference (SMD) was calculated with 95%CI. Sensitivity and subgroup analyses were performed when necessary.

RESULTS: Six RCTs were identified that compared SILA (n = 535) with CLA (n = 533). Five RCTs had a high risk of bias and one RCT had a low risk of bias. SILA was associated with longer operative time (MD = 5.68, 95%CI: 3.91-7.46, P < 0.00001), higher conversion rate (RR = 5.14, 95%CI: 1.25-21.10, P = 0.03) and better cosmetic satisfaction score (MD = 0.52, 95%CI: 0.30-0.73, P < 0.00001) compared with CLA. No significant differences were found for total complications (RR = 1.15, 95%CI: 0.76-1.75, P = 0.51), drain insertion (RR = 0.72, 95%CI: 0.41-1.25, P = 0.24), or length of hospital stay (SMD = 0.04, 95%CI: -0.08-0.16, P = 0.57). Because there was not enough data among the analyzed RCTs, postoperative pain was not calculated.

CONCLUSION: The benefit of SILA is cosmetic satisfaction, while the disadvantages of SILA are longer operative time and higher conversion rate.

Key words: Single incision; Laparoscopic; Appendectomy; Meta-analysis; Systematic review

Core tip: The clinical benefit of single-incision laparoscopic appendectomy (SILA), compared to the conventional three-port laparoscopic appendectomy, has been a controversial issue in recent years. We performed the first systematic review and meta-analysis of randomized controlled trails (RCTs) that have assessed the clinical benefits and disadvantages between SILA and conventional laparoscopic appendectomy (CLA). Six RCTs conducted between 2011 and 2013 were identified and pooled to determine outcomes using meta-analytic methods. From this analysis, we conclude that SILA is as safe as CLA. Although patients receiving SILA had longer operative times and a higher conversion rate, one benefit of SILA is cosmetic satisfaction.

Cai YL, Xiong XZ, Wu SJ, Cheng Y, Lu J, Zhang J, Lin YX, Cheng NS. Single-incision laparoscopic appendectomy *vs* conventional laparoscopic appendectomy: Systematic review and meta-analysis. *World J Gastroenterol* 2013; 19(31): 5165-5173 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5165.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5165

INTRODUCTION

Appendectomy is one of the most commonly performed surgical procedures of the abdomen in the world. This surgical procedure has been performed for over 100 years, after first being described by McBurney[1]. With rapidly developing, minimally invasive surgery, the laparoscopic appendectomy has become a selectable method for appendectomy. Previous studies have reported that laparoscopic appendectomy has many advantages in comparison to open appendectomy, such as shorter hospital stays, reduced risks of complications, and better cosmetic satisfaction[2,3]. Therefore, the laparoscopic appendectomy, like laparoscopic cholecystectomy, is considered to be a favorable procedure for appendectomy in the future.

In addition, the use of single-incision laparoscopic techniques, which have been described with promising results in multiple studies[4-11], has increased over the past few years. Under such circumstances, surgical appendectomy may be undergoing a transition from the conventional three-port laparoscopic surgery toward the less-invasive, single-incision laparoscopic surgery. With the number of incisions reduced to just one umbilical incision, the potential advantages of single-incision surgery include better cosmetic outcome, less postoperative pain, and faster postoperative recovery. At the same time, this new technique may present potential disadvantages, such as increased operative time, higher conversion rates, and more complications.

Although a number of studies in the last few years have compared the single-incision laparoscopic appendectomy (SILA) with conventional laparoscopic appendectomy (CLA), most only demonstrated the feasibility and safety of SILA. Well-described benefits and disadvantages are still lacking in the literature. To our knowledge, there are no published meta-analyses describing randomized controlled trails (RCTs) comparing SILA with CLA. Therefore, we conducted a systematic review and meta-analysis of RCTs to assess the clinical benefits and disadvantages associated with SILA and CLA.

MATERIALS AND METHODS

Searching strategy

We searched the following databases up through January 2013 to identify RCTs: The Cochrane Library, MEDLINE, EMBASE, Science Citation Index Expanded, and the Chinese Biomedical Literature Database. The search strategies are shown in Table 1. Language was not used as a criterion for selection of studies, and both English and non-English studies were included. Moreover, the citations within the reference lists of the articles were searched manually to identify additional eligible studies. After all searches were completed, the search results were merged using the software package Endnote X6 to remove duplicate records. The title and abstract of every identified record was scanned by two independent authors (Wu SJ and Cheng Y) for the inclusion criteria. If compliance was not clear from the abstract, full-texts were retrieved for further assessment.

Inclusion and exclusion criteria

The aim of this meta-analysis was to specifically compare the benefits and disadvantages of SILA and CLA methods. Therefore, only those studies which provided comparison between those two methods mentioned above were included. Reliability was the most important point considered in this meta-analysis, so only RCTs were included. Prospective non-randomized, retrospective, and improperly performed RCTs were excluded from the analysis.

The definition of SILA was surgery through a single intra-umbilical incision. The included studies used various multiport devices or multiple conventional ports through a single skin incision but with multiple fascial incisions. CLA was defined as surgery with the standard three-port technique *via* a supra-umbilical or infra-umbilical port, a left lower quadrant port, and a right lower quadrant or supra-pubic region port.

Data extraction and validity assessment

Two independent authors (Lu J and Zhang J) extracted and confirmed the data and entered them into an electronic data collection form. Any disagreement in the two reviewers' data collection and quality assessment was discussed until a consensus was reached. For the validity assessment, another two authors (Cai YL and Lin YX) independently assessed the methodological quality of the included trials using the quality checklist recommended by the Cochrane Handbook. The assessment contained six dimensions: (1) random sequence generation; (2) allocation concealment; (3) blinding; (4) addressing of incomplete outcome data; (5) selective reporting; and (6) other bias. Following the evaluation of the above domains, an included trial was judged as having low risk of bias if it was evaluated as "low" in all of the above domains. If the risk of bias was judged as "unclear" or "high", then the trial was listed under the group of trials with "high risk of bias." Otherwise, all disagreements were resolved by discussion and referral to a third author (Xiong XZ) for resolution.

Outcomes

Data for the following outcomes were extracted: total operative time, total complications (wound infection, abscess, ileus, stump leakage, *etc.*), drain insertion, conversion rate, length of hospital stay, postoperative pain as

Table 1 Search strategies

Databases	Period of search	Search strategies
The Cochrane Library	Through January 30, 2013	(1) MeSH descriptor Appendectomy, Laparoscopic explode all trees (2) (laparoscop* or coelioscop* or celioscop* or peritoneoscop*) and appendectom* (3) 1 or 2 (4) "single incision" or "single port" or "single site" or "one port" or "one incision" or "one site" (5) 3 and 4
MEDLINE (Pubmed)	Through January 30, 2013	(1) Appendectomy, laparoscopic [MeSH] (2) (laparoscop* or coelioscop* or celioscop* or peritoneoscop*) and appendectom* (3) 1 or 2 (4) "single incision" or "single port" or "single site" or "one port" or "one incision" or "one site" (5) (randomised controlled trial [pt] or controlled clinical trial [pt] or randomised [tiab] or placebo [tiab] or drug therapy [sh] or randomly [tiab] or trial [tiab] or groups [tiab]) not (animals [mh] not humans [mh]) (6) 3 and 4 and 5
EMBASE (OvidSP)	Through January 30, 2013	(1) (appendectomy.af.) or (exp appendectomy/) (2) ((laparoscop* or coelioscop* or celioscop* or peritoneoscop*).af.) or (exp Laparoscopy/) (3) (single incision or single port or single site or one port or one incision or one site).af. (4) (random* or factorial* or crossover* or placebo*).af. (5) expcrossoverprocedure/or exp double-blind procedure/or exp randomized controlled trial/or single-blind procedure/ (6) 4 or 5 (7) 1 and 2 and 3 and 6
Science Citation Index Expanded	Through January 30, 2013	(1) TS = (appendectom*) (2) TS = (laparoscop* or coelioscop* or celioscop* or peritoneoscop*) (3) TS = ("single incision" or "single port" or "single site" or "one port" or "one incision" or "one site") (4) TS = (random* or blind* or placebo* or meta-analysis) (5) 1 and 2 and 3 and 4
CBM	Through January 30, 2013	Search strategy in was performed in Chinese. Includes search terms similar to the terms used in MEDLINE

MEDLINE: Medical Literature Analysis and Retrieval System Online; EMBASE: Excerpta Medica Database; CBM: Chinese Biomedical Literature Database; MeSH: Medical Subject Heading.

assessed using the visual analogue scale (VAS), and cosmetic satisfaction.

Statistical analysis

We performed all the statistical analyses of the extracted data with Review Manager 5.2.0. For dichotomous data and continuous data, we calculated the risk ratio (RR) and the mean difference (MD) with 95%CIs for both. For continuous outcomes with different measurement scales in different RCTs, we calculated the standardized mean difference (SMD) with 95%CI. Heterogeneity was described with the χ^2 test. A P value less than 0.10 was considered to be significant heterogeneity and the I^2 statistic was used to measure the quantity of heterogeneity. If significant heterogeneity existed, a random-effect model was used. In the absence of significant heterogeneity, a fixed-effect model was adopted.

In the case of missing data, we contacted the original investigators to request further information. If there was no reply, we performed the analysis on an "intention-to-treat" principle, if applicable. Otherwise, we adopted the available-case analysis, also known as the per-protocol analysis. A few published clinical trials reported a median and a range instead of a mean and SD. To adjust this difference, we assumed that the median was equal to the mean, and we estimated the SD as a quarter of the reported range. Funnel plots were used to determine reporting biases. We conducted the meta-analysis and systematic review according to the Cochrane Handbook for Systematic Reviews of Interventions and Preferred Reporting Item for Systematic Reviews and Meta-Analysis.

RESULTS

Search results

We identified a total of 111 records through electronic searches of The Cochrane Library (n = 12), MEDLINE (n = 21), EMBASE (n = 32), Science Citation Index Expanded (n = 44), Chinese Biomedical Literature Database (n = 0), and a manual search of the references in the included RCTs (n = 2). We excluded 39 duplicates and 72 clearly irrelevant records by reading titles and abstracts. Fourteen full-text articles were retrieved for further assessment. We excluded seven articles for the reasons listed in Figure 1.

Description of included trials and risk of bias

Six RCTs published between 2011 and 2013 were identified that fulfilled the inclusion criteria[12-17]. A total of 1068 patients were included. There were 535 patients who received SILA and 533 who received CLA. Two included trials were of pediatric patients[14,15], and the remaining four trials were of adult patients[12,13,16,17]. Details on the included studies are shown in Table 2. The risk of bias is summarized in Table 3. Five RCTs had a high risk of bias[13-17], and one RCT had a low risk of bias[12].

Effect of interventions

Total operative time: All six RCTs reported the operative time to complete appendectomy[12-17]. The operative time

Table 2 Study characteristics

Study	Area	Study design	Participants (SILA/CLA)	Mean age, yr (SILA/CLA)	Male:female ratio (SILA/CLA)
Teoh *et al*[12]	Hong Kong	Multi-center	195 (98/97)	39.2/40.7	58:40/59:38
Lee *et al*[13]	South Korea	Single-center	229 (116/113)	28.4/28.5	64:52/68:45
Perez *et al*[14]	United States	Single-center	50 (25/25)	8.7/8.9	10:15/15:10
St Peter *et al*[15]	United States	Single-center	360 (180/180)	11.1/11.1	99:81/92:88
Sozutek *et al*[16]	Turkey	Single-center	50 (25/25)	30.6/30.0	12:13/7:18
Frutos *et al*[17]	Spain	Single-center	184 (91/93)	28.0/31.0	42:49/47:46

SILA: Single-incision laparoscopic appendectomy; CLA: Conventional laparoscopic appendectomy.

Table 3 Risk of bias assessment

Study	Sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting
Teoh *et al*[12]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Lee *et al*[13]	Low risk	Uncertain	High risk	High risk	Uncertain	Low risk
Perez *et al*[14]	Low risk	Low risk	Low risk	Low risk	Uncertain	High risk
St Peter *et al*[15]	Low risk	Low risk	Low risk	High risk	Low risk	Low risk
Sozutek *et al*[16]	Low risk	Uncertain	High risk	High risk	Uncertain	High risk
Frutos *et al*[17]	Low risk	Uncertain	High risk	High risk	Low risk	High risk

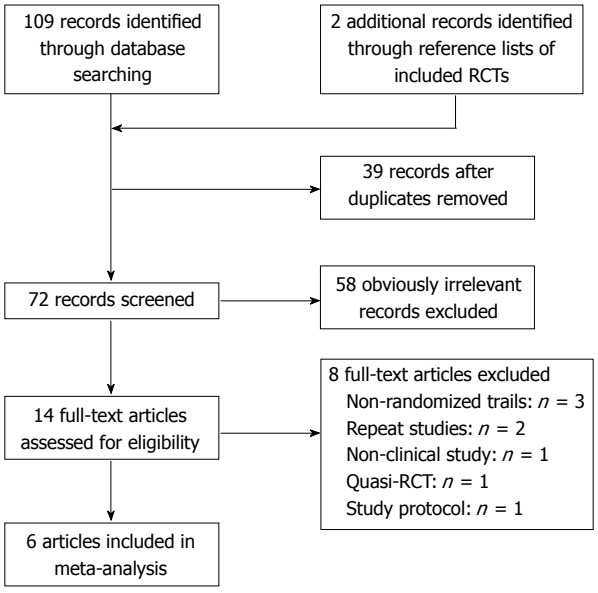


Figure 1 Flow diagram demonstrating the study selection process. RCT: Randomized controlled trial.

was significantly longer in the SILA group than in the CLA group (Figure 2A; MD = 5.68, 95%CI: 3.91-7.46, $P < 0.00001$). There was no evidence of statistical heterogeneity ($\chi^2 = 4.61$, $P = 0.47$, $I^2 = 0\%$).

Total complications: All six RCTs reported the total complications after appendectomy[12-17]. There was no significant difference in the overall incidence of postoperative complications between the two groups (Figure 2B; RR = 1.15, 95%CI: 0.76-1.75, $P = 0.51$). There was no evidence of statistical heterogeneity ($\chi^2 = 2.25$, $P = 0.81$, $I^2 = 0\%$).

Conversion rate: All six RCTs reported the conversion rates during appendectomy[12-17]. This included placement of additional laparoscopic ports for SILA and conversion to open appendectomy from both SILA and CLA .The conversion rate was 7.48% (40 of 535 patients) and 0.75% (4 of 533 patients) in the SILA and CLA groups, respectively. The rate was significantly higher in patients who received SILA than CLA (Figure 2C; RR = 5.14, 95%CI: 1.25-21.10, $P = 0.02$). Significant heterogeneity was present in the trials ($\chi^2 = 6.82$, $P = 0.15$, $I^2 = 41\%$).

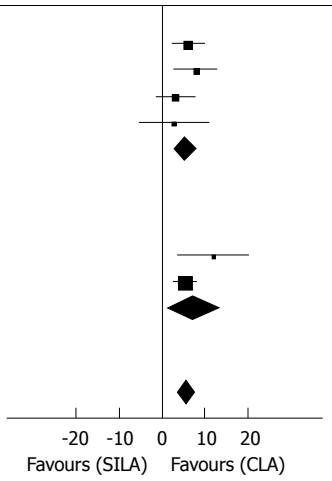
Drain insertion: Only two RCTs reported drain insertion during appendectomy[12,13]. There was no significant difference in the incidence of drain insertion between the two groups (Figure 2D; RR = 0.72, 95%CI: 0.41-1.25, $P = 0.24$). There was no evidence of statistical heterogeneity ($\chi^2 = 0.13$, $P = 0.71$, $I^2 = 0\%$).

Length of hospital stay: The length of hospital stay was evaluated in all studies[12-17], but only three studies reported this data in the form of mean and SD[12,16,17]. By contacting the authors personally by email, we were able to retrieve the mean and SD data for the other two studies[14,15]. Another study provided the mean and range[13]. According to our predefined plan, we equated the SD with a quarter of the reported range. There was no significant difference between the two groups (Figure 2E; SMD = 0.04, 95%CI: -0.08-0.16, $P = 0.57$). There was no evidence of statistical heterogeneity ($\chi^2 = 5.31$, $P = 0.38$, $I^2 = 6\%$).

Postoperative pain: Four of the included trials reported postoperative pain scores using the VAS (10-point or 100-mm) after appendectomy[12,13,16,17]. Teoh *et al*[12] indicated that there were no significant differences in the overall pain scores and the pain scores at rest ($P = 0.109$ and 0.154, respectively), while significantly worse pain was experienced in the SILA group after coughing 10 times and on standing ($P = 0.001$ and 0.038, respectively). Lee *et al*[13] stated that postoperative pain scores were not statistically different between the two groups at 12 h, 24 h, 36 h and 14 d postoperatively ($P = 0.651$, 0.555, 0.570

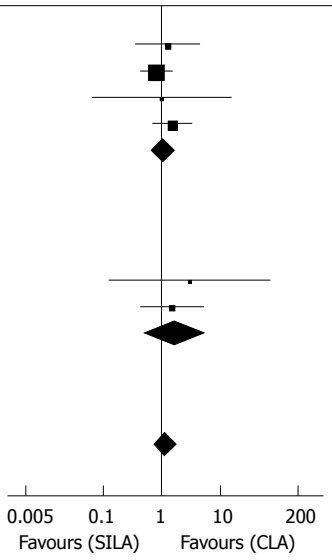
A

Study or subgroup	SILA Mean	SILA SD	SILA Total	CLA Mean	CLA SD	CLA Total	Weight	Mean difference IV, random, 95%CI
1.1.1 Adult								
Frutos *et al*[17]	38.13	13.49	91	32.12	12.44	93	22.4%	6.01 [2.26, 9.76]
Lee *et al*[13]	43.8	21.3	116	35.8	18.9	113	11.6%	8.00 [2.79, 13.21]
Sozutek *et al*[16]	32.6	9.9	25	29.5	6.8	25	14.2%	3.10 [-1.61, 7.81]
Teoh *et al*[12]	63	27.2	98	60.2	31.7	97	4.6%	2.80 [-5.49, 11.09]
Subtotal (95%CI)			330			328	52.8%	5.38 [2.94, 7.83]
Heterogeneity: Tau^2 = 0.00; χ^2 = 2.35, df = 3 (P = 0.50); I^2 = 0%								
Test for overall effect: Z = 4.32 (P < 0.0001)								
1.1.2 Children								
Perez *et al*[14]	46.8	17.5	25	34.8	12.5	25	4.4%	12.00 [3.57, 20.43]
St Peter *et al*[15]	35.2	14.5	180	29.8	11.6	180	42.8%	5.40 [2.69, 8.11]
Subtotal (95%CI)			205			205	47.2%	7.44 [1.46, 13.42]
Heterogeneity: Tau^2 = 11.57; χ^2 = 2.13, df = 1 (P = 0.14); I^2 = 53%								
Test for overall effect: Z = 2.44 (P = 0.01)								
Total (95%CI)			535			533	100.0%	5.68 [3.91, 7.46]
Heterogeneity: Tau^2 = 0.00; χ^2 = 4.61, df = 5 (P = 0.47); I^2 = 0%								
Test for overall effect: Z = 2.44 (P = 0.01)								
Test for subgroup differences: χ^2 = 0.39, df = 1 (P = 0.53); I^2 = 0%								



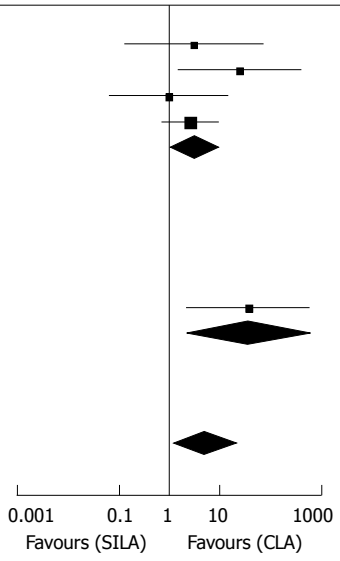
B

Study or subgroup	SILA Events	SILA Total	CLA Events	CLA Total	Weight	Risk ratio M-H, fixed, 95%CI
3.1.1 Adult						
Frutos *et al*[17]	5	91	4	93	10.8%	1.28 [0.35, 4.61]
Lee *et al*[13]	15	116	18	113	49.6%	0.81 [0.43, 1.53]
Sozutek *et al*[16]	1	25	1	25	2.7%	1.00 [0.07, 15.12]
Teoh *et al*[12]	14	98	9	97	24.6%	1.54 [0.70, 3.39]
Subtotal (95%CI)		330		328	87.8%	1.08 [0.69, 1.69]
Total events	35		32			
Heterogeneity: χ^2 = 1.62, df = 3 (P = 0.65); I^2 = 0%						
Test for overall effect: Z = 0.33 (P = 0.74)						
3.1.2 Children						
Perez *et al*[14]	1	25	0	25	1.4%	3.00 [0.13, 70.30]
St Peter *et al*[15]	6	180	4	180	10.9%	1.50 [0.43, 5.23]
Subtotal (95%CI)		205		205	12.2%	1.67 [0.53, 5.28]
Total events	7		4			
Heterogeneity: χ^2 = 0.16, df = 1 (P = 0.69); I^2 = 0%						
Test for overall effect: Z = 0.87 (P = 0.39)						
Total (95%CI)		535		533	100.0%	1.15 [0.76, 1.75]
Total events	42		36			
Heterogeneity: χ^2 = 2.25, df = 5 (P = 0.81); I^2 = 0%						
Test for overall effect: Z = 0.66 (P = 0.51)						
Test for subgroup differences: χ^2 = 0.47, df = 1 (P = 0.49); I^2 = 0%						



C

Study or subgroup	SILA Events	SILA Total	CLA Events	CLA Total	Weight	Risk ratio M-H, random, 95%CI
5.1.1 Adult						
Frutos *et al*[17]	1	91	0	93	14.1%	3.07 [0.13, 74.28]
Lee *et al*[13]	12	116	0	113	16.7%	24.36 [1.46, 406.59]
Sozutek *et al*[16]	1	25	1	25	17.5%	1.00 [0.07, 15.12]
Teoh *et al*[12]	8	98	3	97	34.9%	2.64 [0.72, 9.65]
Subtotal (95%CI)		330		328	83.2%	3.21 [1.04, 9.92]
Total events	22		4			
Heterogeneity: Tau^2 = 0.14; χ^2 = 3.30, df = 3 (P = 0.35); I^2 = 9%						
Test for overall effect: Z = 2.03 (P = 0.04)						
5.1.2 Children						
Perez *et al*[14]	0	25	0	25		Not estimable
St Peter *et al*[15]	18	180	0	180	16.8%	37.00 [2.25, 609.33]
Subtotal (95%CI)		205		205	16.8%	37.00 [2.25, 609.33]
Total events	18		0			
Heterogeneity: Not applicable						
Test for overall effect: Z = 2.53 (P = 0.01)						
Total (95%CI)		535		533	100.0%	5.14 [1.25, 21.10]
Total events	40		4			
Heterogeneity: Tau^2 = 1.05; χ^2 = 6.82, df = 4 (P = 0.15); I^2 = 41%						
Test for overall effect: Z = 2.27 (P = 0.02)						
Test for subgroup differences: χ^2 = 2.52, df = 1 (P = 0.11); I^2 = 60.3%						



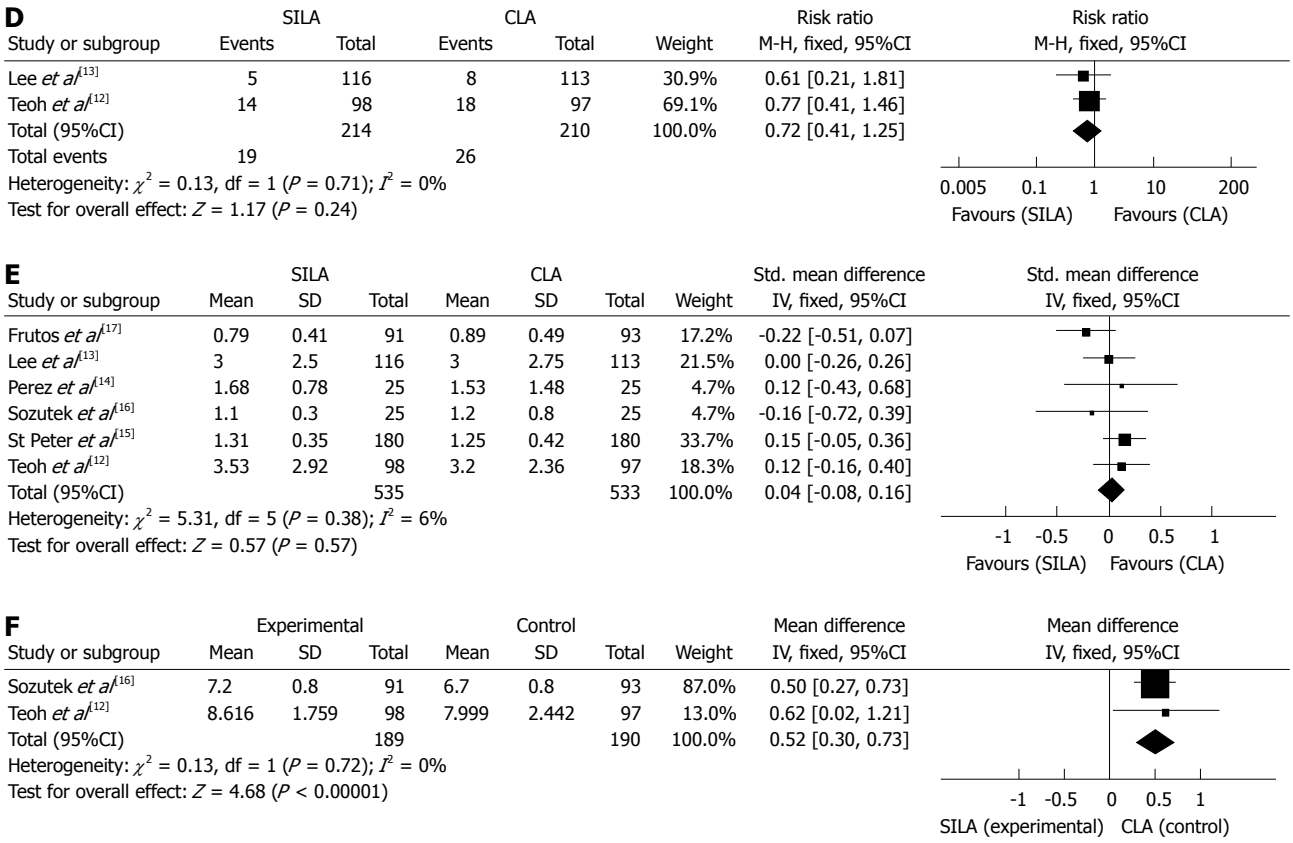


Figure 2 Forest plots of the meta-analysis. A: Comparisons of single-incision laparoscopic appendectomy (SILA) *vs* conventional laparoscopic appendectomy (CLA) in total operative time; B: Total complications; C: Conversion rate; D: Drain insertion; E: Length of hospital stay; F: Cosmetic satisfaction.

and 0.631, respectively). Likewise, Sozutek *et al*[16] stated that no difference was detected in terms of postoperative pain ($P = 0.991$). However, in Frutos' trial, less pain was found in SILA group (SILA/CLA: 2.76 ± 1.64/3.78 ± 1.76, $P < 0.001$). Only one study provided the mean and SD, so those values were not calculated in this analysis.

Cosmetic satisfaction: Three studies reported cosmetic satisfaction scores[12,13,16]. The cosmetic score was also measured by a 5-point VAS with a higher score indicating better satisfaction. The meta-analysis of two studies[12,16], which provided the mean and SD, reported that the cosmetic scores were significantly higher in the SILA group than in the CLA group (Figure 2F; MD = 0.52, 95%CI: 0.30-0.73, $P < 0.00001$). There was no evidence of statistical heterogeneity ($\chi^2 = 0.13$, $P = 0.72$, $I^2 = 0\%$). However, the remaining trial reported no significant difference between the two groups with VAS scores of 4.0 and 3.3 for SILA and CLA, respectively ($P = 0.128$)[13].

Subgroup analysis: Because the age of the patients may have influenced the eventual outcome, we performed a subgroup analysis for operative time, total complications, and conversion rate. In the subgroup analysis of age, the outcomes were also equivalent.

DISCUSSION

The single-incision method of laparoscopic appendectomy, compared to the conventional three-port method, has been a controversial issue in recent years. Numerous studies have been performed to evaluate the differences; however, most of them were non-RCT studies. Fortunately, six new RCTs published between 2011 and 2013[12-17] evaluated the benefits and disadvantages of SILA and CLA in a quantitative manner and provided the basis of this study. This meta-analysis and systematic review of those six RCTs indicated that although SILA was associated with a longer operative time and a higher conversion rate, patients had better cosmetic satisfaction compared with CLA. No significant differences were found in total complications, drain insertion, length of hospital stay, and postoperative pain between the two procedures.

Regarding operative time, a meta-analysis of non-RCTs concluded that there was no difference between the two groups[18]. Those results were inconsistent with the results of this analysis, which determined that the SILA operative time was longer by 5.68 min. This discrepancy may have been due to the lack of surgical experience using the new technique. Performing SILA requires experience in laparoscopic surgery, and a certain number of cases must be performed to overcome the learning curve. A retrospective study by Lee *et al*[19] reported that the operation time tended to shorten when the surgeon gained more experience and accumulated cases. This finding is in agreement with a separate report by Perez *et al*[14], which reported that in the first 25 patients enrolled, the differ-

ence in operative time was significantly greater (49.31 min *vs* 33.50 min, $P = 0.049$) and that this difference decreased in a subsequent group of 25 patients (44.08 min *vs* 36.00 min, $P = 0.123$). Although one disadvantage of SILA is a longer operative time, we believe that with increased experience and developed instrumentation SILA will reach equivalent effectiveness to conventional three-port methods.

Conversion rate is another major concern for surgeons. The high conversion rate is an important disadvantage and has considerably limited the widespread use of SILA. In our meta-analysis, we found that the heterogeneity was very high among the analyzed studies. Therefore, in order to assess the reliability and stability of this outcome, we conducted a sensitivity analysis; only two of the evaluated RCTs precisely described the conversion-fulfilled, predefined outcome[13,15]. After this analysis, a significantly higher rate was observed in the SILA group (RR = 30.64, 95%CI: 4.22-222.68, $P = 0.0007$) and no heterogeneity was found ($\chi^2 = 0.04$, $P = 0.84$, $I^2 = 0\%$). Thus, we confirmed that a higher conversion rate was consistent with SILA treatment. Technical difficulty could account for this. Complicated appendicitis exists in 30% of all appendicitis cases[20] and when the operation is difficult, such as with serious adhesion or significant inflammation, the single-incision approach can be somewhat cumbersome.

In such scenarios, extra incision sites or use of surgical instruments may become necessary. In a study by St Peter *et al*[15], surgeons rated the degree of technical difficulty for every case, excluding perforated appendicitis, on a subjective scale from 1 to 5 with 1 indicating an easy case and 5 indicating a difficult case. Higher surgical difficultly ratings were noted for SILA relative to the standard three-port laparoscopic appendectomy (2.3 ± 1.4 *vs* 1.7 ± 1.0, $P < 0.001$). Thus, not only in complicated appendicitis, but also in uncomplicated appendicitis, the decision to add an additional site or use additional instrumentation is dependent on a lower comfort level with single-site procedures. However, Crohn's disease can be performed with a single-site procedure in the presence of significant inflammation[21]. This indicates that if only to promote surgeon comfort level, pure SILA could become easier to complete. Further technical research and developments are needed to reduce the difficulty of SILA and to allow surgeons to comfortably perform this procedure. This may be the only way to reduce the conversion rate when implementing SILA.

Postoperative pain is another controversial topic to be discussed when a single-incision technique is applied. As a result of a reduced trocar use, less surgical pain was postulated in SILA[22]. A small case series and a retrospective analysis reported that reduced pain was found with SILA[23,24]. Conversely, the combined size of the fascial incision at the umbilicus required to accommodate the single-incision port may give rise to more potential pain compared with multiple, smaller fascial incisions in CLA. A 40-patient pilot trial in adults found significantly greater pain scores in the initial 24 h after SILA[25]. Moreover, from an anatomical point of view, the true pelvic peritoneum has less sensitivity to acute pain than the parietal peritoneum in the umbilicus[26]. Thus, the two ports in the lower abdomen in CLA may cause less pain than repositioning them to the umbilicus. Thus, by analyzing previous studies, whether there is less postoperative pain with SILA is uncertain.

In this analysis, three of the included RCTs indicated that the pain scores were comparable between the two groups[12,13,16]. Although, Teoh *et al*[12] concluded that more pain was identified in activity, the overall scores demonstrated no significant difference. This is in agreement with a previous non-RCT meta-analysis[18]. Moreover, the same comparison in cholecystectomy also showed no significant difference in pain scores at 6 and 24 h between single-incision and multiple-incision procedures[27]. Conversely, another RCT showed less pain was found with SILA, although this difference was very small[17]. Thus, we believe that the pain is not much different between SILA and CLA. However, the overall length of incision may be an important factor in this debate. As many discrepancies exist in the analyzed studies, data from future RCTs are anticipated to resolve these potential differences.

This meta-analysis highlighted cosmetic satisfaction as the significant benefit of SILA over CLA. This so-called "scarless" procedure meets the demand of expecting to conceal the surgical history of patients, especially in young females. Although SILA definitely reduces the number of incisions and often results in better cosmetic satisfaction among patients, there was not enough clinical data to support this claim previously. We recognize that some studies showed better scores without significant differences[13,28], possibly due to existing high cosmetic scores with CLA and leaving only slightly more room for improvement with SILA.

Some limitations exist in assessing cosmetic satisfaction. First, a standard tool to assess the appearance of the wound is still lacking. Second, patients rate the score by their own subjective feeling without a more quantitative reference. We speculate that after surgery, patients may be more focused on whether the disease had been cured rather than on a cosmetic score. Third, wound healing is a long-term process, and the cosmetic benefit should be assessed during both short-term and long-term follow-up examinations. Therefore, prospective RCTs with long-term follow-up are needed to confirm the cosmetic benefits of SILA. Establishing a validated scar assessment tool is also necessary for adequate quantitative analysis.

Six RCTs were included in this review. Most included patients with perforated appendicitis, while only one study excluded patients with perforated appendicitis. Thus, our results were relevant to all types of acute appendicitis. However, the quality of these newly analyzed RCTs was low as only one RCT had a low risk of bias[12].

Meta-analysis is an increasingly popular method of data analysis to examine discrepancies in the literature. Nevertheless, there were some limitations in our research.

First, the number of included RCTs was small, and, among those, two RCTs were also of small sample size. Funnel plots were not performed to assess the publication bias due to the small number of included RCTs. Second, the surgical techniques among the studies were varied; thus, there may be variances in operative time, conversion rate, and complications. Third, a cost analysis was not conducted in this research as cost is always higher with the development of a new technique and the instruments varied significantly with each study.

In conclusion, despite the limitations mentioned above, this review currently provides the best available evidence for comparison of single-incision laparoscopic appendectomy *vs* conventional laparoscopic appendectomy. From a curative perspective, SILA is comparable to CLA in terms of total complications, drain insertion, length of hospital stay, and postoperative pain. The disadvantages of SILA are a longer operative time and a higher conversion rate. One benefit of SILA is patient cosmetic satisfaction. Thus, the option of this new treatment alternative should be carefully discussed with patients. More RCTs are needed to clarify the benefits and disadvantages of SILA compared to CLA.

COMMENTS

Background

Appendectomy is one of the most commonly performed surgical procedures of the abdomen in the world. In recent years, minimally invasive surgery has rapidly developed and conventional laparoscopic appendectomy (CLA) has been widely used. In addition, single-incision laparoscopic appendectomy (SILA), as a new technique, has been introduced as an alternative to conventional three-port laparoscopic appendectomy.

Research frontiers

Both SILA and CLA are used for patients undergoing appendectomy. Many studies, including randomized controlled trials (RCTs), have compared SILA with CLA in the last few years. However, most have only demonstrated the feasibility and safety of SILA. The clinical benefits and disadvantages between SILA and CLA are still controversial.

Innovations and breakthroughs

The authors identified all RCTs comparing SILA with CLA. A meta-analysis and systematic review was conducted according to the Cochrane Handbook. From this study, the disadvantages of SILA were determined to be longer operative times and higher conversion rates, while the benefit of SILA was cosmetic satisfaction among patients. This has not been clearly identified in previous studies.

Applications

From a curative perspective, SILA is proven to be a safe and effective treatment that is comparable to CLA. Based on the benefits and disadvantages of SILA, surgeons should carefully assess each patient's situation and discuss surgical options that meet their needs.

Peer review

This article is a good meta-analysis about single-incision laparoscopic appendectomy *vs* conventional laparoscopic appendectomy. The conclusions are unbiased and give good clues to the readers.

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CASE REPORT

Green tea extract: A potential cause of acute liver failure

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Abstract

The use of herbal products has increased significantly in recent years. Because these products are not subject to regulation by the Food and Drug Administration and are often used without supervision by a healthcare provider, the indication for and consumption of these supplements is quite variable. Moreover, their use is generally regarded as safe and natural by the lay-public. Unfortunately, there has been an increase in the number of reported adverse events occurring with the use of herbal products. We present a case of acute impending liver failure in an adolescent male using a weight-loss product containing green tea extract. Our case adds to the growing concern surrounding the ingestion of green tea extract and serves to heighten healthcare provider awareness of a potential green tea extract hepatotoxicity. Despite the generally touted benefits of green tea as a whole, clinical concern regarding its use is emerging and has been linked to its concentration in multiple herbal supplements. Interestingly, the suspected harmful compounds are those previously proposed to be advantageous for weight-loss, cancer remedy, and anti-inflammatory purposes. Yet, we emphasize the need to be aware of not just green tea extract, but the importance of monitoring patient use of all dietary supplements and herbal products.

Key words: Green tea; Plant extract; Dietary supplements; Liver failure; Liver injury; Hepatotoxicity

Core tip: Green tea extract is one of the most common herbal supplements ingested worldwide and is manufactured into more than 100 different over-the-counter products. Although traditionally considered safe, it has been linked to hepatotoxicity and led to acute impending liver failure in our adolescent patient. Eliminating multiple etiologies and with tissue evidence, a weight-loss supplement containing green tea extract was likely to blame. Recovery was over a two-month course. The lack of regulation and provider guidance in the use of this product and dietary supplements in general is significant. We highlight the importance of monitoring patient use of dietary supplements.

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INTRODUCTION

In the United States, herbal products are classified as dietary supplements, and their use has been increasing over recent decades. In fact, the use of herbal medicine increased from 2.5% in the general population in 1990 to

Table 1 Laboratory studies

Days post-hospitalization	Admission day 1	Hospitalized day 15	Discharge day 24	Follow-up day 45	Follow-up day 94	Follow-up day 185
Aspartate aminotransferase (U/L)	2106	958	525	59	33	35
Alanine aminotransferase (U/L)	2984	1169	665	165	44	31
Alkaline phosphatase (U/L)	186	86	137	148	120	94
Gamma glutamyl transferase (U/L)	78	65	104	49	41	28
Conjugated bilirubin (mg/dL)	12.9	14.7	10.3	0.0	0.0	0.0
Unconjugated bilirubin (mg/dL)	1.9	2.0	2.1	0.8	0.2	0.2
Albumin (g/dL)	4.0	2.5	3.2	3.9	4.1	4.1
Protime (s)	15.9	18.2	14.9	-	-	-
International normalised ratio	1.3	1.5	1.2	1.0	1.0	1.0
Factor 7	-	42%	-	102%	-	-

12.1% in 1997, with $5.1 billion spent as out of pocket expenditures for herbal therapies in 1997[1]. Moreover, according to the 2007 National Health Interview Survey, 17.9% of adults reported use of an herbal supplement in the previous year[2]. Yet, as dietary supplements, these products are not subject to the same regulation as drugs approved by the Food and Drug Administration (FDA). Instead, in accordance with the Dietary Supplement Health and Education Act of 1994, dietary supplements do not need approval of their safety or efficacy by the FDA[3].

Green tea has been consumed worldwide for many years and is a popular herbal ingredient that has been manufactured into more than 100 over-the-counter supplements[4]. Green tea's most touted benefits are its antioxidant and weight-loss or thermogenic properties. Nonetheless, there has been increasing concern regarding the potential hepatotoxicity with the use of green tea extract[5-7]. Here, we present a case of acute impending liver failure in an adolescent male occurring with the use of a weight-loss product containing green tea extract.

CASE REPORT

Our patient is a 16 year-old Hispanic male, who presented to our emergency room with new onset jaundice. The patient noticed yellowing of his skin and darkening of his urine six to seven days prior to admission. He denied abdominal pain, changes in his stool, fever, changes in mental status, alcohol consumption, sick contacts or recent travel. He is currently a high school student.

He does have a history of obesity and was taking several dietary supplements as part of an unsupervised weight-loss plan. Specifically, he was taking Applied Nutrition® Green Tea Fat Burner beginning 60 d prior to admission and took 2 pills daily (or 400 mg epigallocatechin-3-gallate, EGCG, daily). He started whey protein 30 d prior to admission and mixed 1 scoop in 16.9 oz of water three times per week. In addition, he used GNC Mega Men® Sport, taken 2 pills three times per week, beginning 30 d prior to admission. And lastly, he was taking Nopal® (Cactus), 1 pill daily, beginning 60 d prior to admission. Over this time period, he lost 56 pounds.

On physical exam, the patient was jaundiced, most evident in the face and sclera, but also present on the chest and upper extremities. Mental status was intact. Abdominal exam was insignificant, with the liver difficult to appreciate given that the patient was still overweight at the time of exam. Initial labs included: aspartate aminotransferase (AST) 2106 U/L (normal range 15-40 U/L), alanine aminotransferase (ALT) 2984 U/L (normal range 10-45 U/L), alkaline phosphatase 186 U/L (normal range 116-483 U/L), gamma glutamyl transferase (GGT) 78 U/L (normal range 12-33 U/L), conjugated bilirubin (CB) 12.9 mg/dL (normal range < 0.3 mg/dL), unconjugated bilirubin (UB) 1.9 mg/dL (normal range < 0.1 mg/dL), albumin 4 g/dL (normal range 3.7-5.5 g/dL), partial thromboplastin time 33.9 s (normal range 25.4-34.9 s), protime 15.9 s (normal range 11.2-15.4 s), international normalised ratio (INR) 1.3 (normal range 0.8-1.2), and glucose 99 (Table 1). Thus, he was admitted for work-up of acute liver injury and possible impending liver failure. During his hospitalization his peak INR and CB were 1.5 and 17.5 mg/dL, respectively. His lowest albumin and factor 7 level was 2.5 g/dL and 39% (normal range 58%-150%), respectively, indicating a decline in liver synthetic function and impending liver failure. Radiological exam was done on admission and consisted of an abdominal ultrasound with Doppler, read as mild hepatomegaly with normal right upper quadrant Doppler evaluation.

Extensive lab work was ordered to determine the etiology of his impending liver failure. Serological markers of autoimmune hepatitis (filamentous actin and Liv/Kid antibodies), infectious hepatitis A, B and C (serologies for infectious hepatitis E were not performed given insignificant incidence in the United States), Wilson's disease (ceruloplasmin), and alpha-1-antitrypsin deficiency were negative. In addition, cytomegalovirus and Epstein-Barr virus immunoglobulin M/immunoglobulin G and adenovirus polymerase chain reaction were negative.

Given lab work as stated, the patient had an ultrasound-guided liver biopsy completed on hospital day 5 (Figure 1). Liver histology was notable for diffuse portal and lobular mixed inflammatory cell infiltrates with acute and chronic inflammation that included scattered eosinophils and interface hepatitis. There was hepatocyte unrest and ballooning degeneration with multifocal individual hepatocyte necrosis and cholestasis. Injury was most

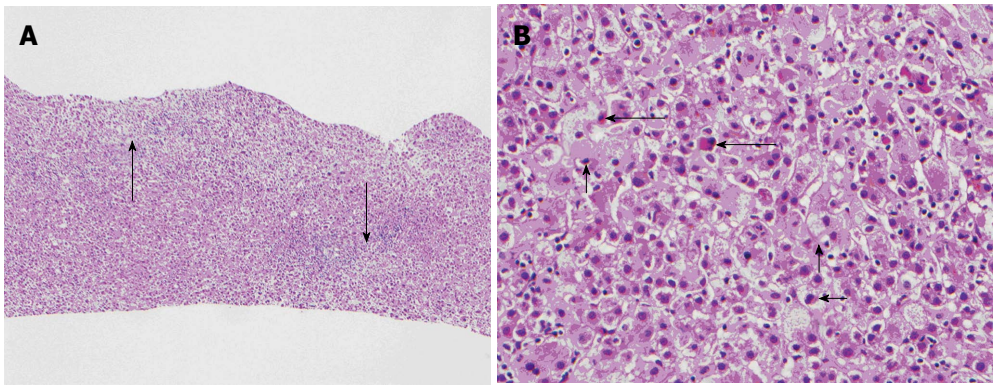

Figure 1 Pathological liver tissue. A: Diffuse portal and lobular inflammatory cell infiltrates (long arrows) [hematoxylin and eosin (HE), × 20]; B: Hepatocytes are reactive with prominent ballooning degeneration (short arrows) and individual cell necrosis (long arrows) (HE, × 200).

prominent in zone 1, but pan-lobular as depicted in Figure 1. He was observed in the hospital until his liver panel began to improve on hospital day twenty-four. Treatment during this admission included initiation of oral vitamin K 5 mg daily on hospital day 2 and ursodiol on hospital day 3. He also received intravenous fluids with a 5% dextrose content, initiated one week after admission and discontinued one week prior to discharge. He was seen again in our clinic at three weeks, ten weeks, and twenty-three weeks after discharge at which time labs (AST, ALT, alkaline phosphatase, GGT, CB, UB, INR and albumin) were repeated. All values continued to improve, along with normalization of both albumin and factor 7 levels, indicating resolution and recovery of his liver function (Table 1).

As several causes of acute liver injury were ruled out, and given his liver histology consistent with previously published reports of toxicity associated with green tea extract, his liver injury can most likely be attributed to his ingestion of this commercially available herbal supplement.

DISCUSSION

As with our patient, many patients using herbal supplements use a combination of products. Again the use is commonly unsupervised and a deviation from the products' user instructions. We are associating our patient's impending liver failure to his ingestion of green tea extract given the history taken, histological findings, and after literature review of all the products and ingredients ingested. A search of the United States National Library of Medicine Dietary Supplements Labels Database and United States National Library of Medicine Clinical and Research Information on Drug Induced Liver Injury for GNC Mega Men® Sport, Nopal® (Cactus), and Whey Protein returned no warnings[8,9]. A PubMed review was significant for a case of acute cholestatic liver injury following ingestion of Whey protein and Creatine supplements. Yet, there have been no other reports that demonstrate this relationship, and instead there have been studies that suggest the hepatoprotective effect of Whey protein in acute and chronic hepatitis[10]. Further investigation of the individual ingredients within the supplements taken, as listed on their respective supplement labels, was concerning for contribution of both Vitamin A and chromium (both contained in the GNC Mega Men® Sport) in development of the liver injury and failure. However, current evidence suggests that Vitamin A toxicity occurs with ingestion of greater than 40000 IU daily or about 12000 micrograms daily[11]. Our patient was taking 5000 IU three times per week, or only 15000 IU per week. In addition, there is no established upper limit of intake of chromium set forth by the Institute of Medicine as there have been few adverse side effects reported[12]. Although likely multi-factorial in nature, current evidence suggests that our patient's liver outcome is most likely secondary to the green tea extract-containing supplement.

Green tea is made from steaming of the tea plant, *Camellia sinensis*. Polyphenols, including catechins and flavanols make up 30%-40% of the extractable solid of dried green tea leaves. The main catechins consist of epicatechin, epicatechin-3-gallate, epigallocatechin, and EGCG. It is proposed that these compounds or extracts give green tea its anticarcinogenic, antioxidant, probiotic, and thermogenic properties[13].

Despite studies that show the benefits of green tea, there have been several recent reports that demonstrate hepatotoxicity following the consumption of concentrated green tea extract. Much interest in green tea hepatotoxicity came after the discontinuation of Exolise, a weight-loss product containing a hydroalcoholic extract of green tea, in France and Spain following the report of acute liver injury with the use of this product. The United States Pharmacopeia subsequently reviewed the safety information for green tea products. They found 34 reports of liver damage, ranging from acute hepatitis to fulminant liver failure requiring transplant, following the use of multiple green tea extract preparations[5]. As a result, the United States Pharmacopeia have suggested, but not mandated, a warning, stating symptoms of liver injury be placed on any green tea extract monograph produced[5]. The green tea product ingested by our patient was without such a warning of potential hepatotoxicity.

In reports of green tea extract-associated hepatotoxicity reviewed between 1999 and 2008, histological exam of the livers showed pathology characteristic of inflammatory infiltrates, cholestasis, steatosis, and necrosis[6]. The hepatotoxicity that follows may be attributed to

those same compounds within green tea extract that have previously been described as beneficial, and in particular to the catechins, of which EGCG is the most abundant and may be the most potent. The major cytotoxic mechanisms include destruction of mitochondrial membranes and the induction of reactive oxygen species formation[14]. Liver injury typically occurs within three months of ingestion[4].

Thus, although green tea has traditionally been considered safe, emerging reports linking liver injury, and in some cases liver failure, with the use of green tea extract should not be ignored. Several issues remain unresolved, including determination of the preparation types and amounts that can be considered safe versus harmful. This is a difficult task to achieve given the lack of FDA regulation of herbal products and other dietary supplements. There are many supplements that contain various formulations (hydroalcoholic *vs* aqueous *vs* powder, *etc*.) and concentrations of green tea extract in combination with other potentially harmful ingredients. Moreover, there is often inconsistent information regarding the complete list of ingredients contained within dietary supplements. Yet, investigations regarding safety and efficacy of these products are lacking. Resources including the United States National Library of Medicine and The Drug Induced Liver Injury Network account for these adverse events and have been established to help us better understand supplement-related hepatotoxicity[4,7]. Yet, until appropriate standards are established, it is imperative that physicians monitor the use of green tea extract, recognize that it may be contained in a variety of products, and be cognizant of its hepatotoxic potential.

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CASE REPORT

Total dysphagia after short course of systemic corticotherapy: Herpes simplex virus esophagitis

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Abstract

A 72 year-old female developed a herpetic esophagitis after 3 d of oral corticotherapy for an acute exacerbation of chronic obstructive pulmonary disease, presenting as odynophagia and total dysphagia. Biospies were taken during a first esophagogastroduodenoscopy (EGD) and the patient was referred to the thoracic surgery service with a presumptive diagnosis of esophageal cancer. A second EGD was planned for dilatation, but by that time the stenosis was completely resolved. The biopsies taken during the first EGD revealed multiple herpetic viral inclusions and ulcerations without any dysplasia or neoplasia. In front of a severe esophageal stenosis, one must still exclude the usual differential diagnosis peptic stenosis and cancer. Visualization of endoscopic lesions can suggest the diagnosis but must be promptly confirmed by biopsy, viral culture or polymerase chain reaction. Although immune systemic effects of corticotherapy are well known and herpetic esophagitis occurs most frequently in immunocompromised individuals, this case emphasizes the importance of clinical awareness concerning short courses of corticotherapy for immunocompetent individuals. This article discusses the reactivation process of herpetic infection in this context and addresses its diagnostic and therapeutic issues.

Key words: Herpes simplex; Esophagitis; Dysphagia; Corticosteroids; Immunocompetence

Core tip: This article reports the case of a 72 year-old female who developed a herpetic esophagitis after 3 d of oral corticotherapy for an acute exacerbation of chronic obstructive pulmonary disease, presenting as odynophagia and total dysphagia. Although immune systemic effects of corticotherapy are well known and herpetic esophagitis occurs most frequently in immunocompromised individuals, this case emphasizes the importance of clinical awareness concerning short courses of corticotherapy for immunocompetent individuals.

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INTRODUCTION

The most frequent causes of infectious esophagitis are candida, cytomegalovirus and herpes simplex virus type 1 (HSV-1). Although typically these esophagitis occurs in the immunocompromised patients, it has been seen also

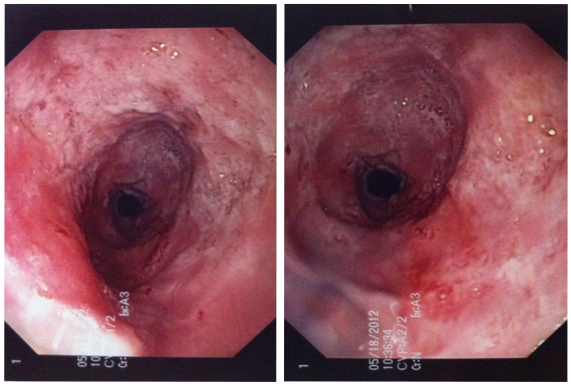

Figure 1 Stenosis of the esophagus, day 4.

in immunocompetent patients. We report a case of a 72 year-old female who developed a herpetic esophagitis presenting as odynophagia and total dysphagia after three days of oral corticotherapy for an acute exacerbation of chronic obstructive pulmonary disease (COPD).

CASE REPORT

A 72 year-old female was referred to the thoracic surgery department following the finding of a impassable stenosis of lower esophagus during an esophagogastroduodenoscopy (EGD). Her pertinent past medical history included repair of a hiatal hernia *via* right thoracotomy several years ago, gastroesophageal reflux and coronary artery disease (CAD) with a spastic component (Takutsubo). The patient also reported a history of caustic injury without subsequent dysphagia in her childhood. She does not recall having had oral or genital herpes, nor cutaneous lesions that would suggest a prior herpetic infection. Three months prior to this event, the patient had received a course of oral prednisone 15 mg daily for ten days, without any complication for her COPD.

The patient had been discharged nine days prior to this event for an acute exacerbation of COPD. During that hospitalization, she was discharged with a prescription of oral prednisone 30 mg daily with standard tapering doses. She decided to interrupt on her own the medication on the third day of this regimen since she presented sudden retrosternal pain, odynophagia and dysphagia to solids and liquids. She reported a slight tenderness at palpation in the epigastric region, but otherwise her complete physical examination was normal.

An EGD was performed by the gastroenterologist on day 4 of her present hospitalization and an impassable esophageal stenosis was discovered in the mid esophagus at 25 cm EGD (Figure 1). Biospies were taken and the patient was referred to the thoracic surgery service with a presumptive diagnosis of a mid esophageal cancer. We planned to repeat the EGD and proceed to a dilatation. But on day 8, the EGD was repeated by another gastroenterologist and by that time the patient reported a slight improvement of her dysphagic symptoms. Hyperhemia concordant with oesophagitis of the mid oesophagus was found, without any stenosis (Figure 2). At this moment, the patient still had some retrosternal pain but she reported improvement in swallowing liquids.

The biopsies taken during the first EGD revealed multiple herpetic viral inclusions and ulcerations without any dysplasia or neoplasia (Figure 3). A viral isolation on Vero cells and diploid human fibroblast cells demonstrated the presence of HSV-1. Acyclovir (5 mg/kg *iv* each 8 h for a total of 10 d) was started along with xylocaine 2% (15 mL *po qid prn*) for symptomatic relief. The patient rapidly improved and was discharged on day 6 with valacyclovir 1 g *po bid* for a total of 16 d.

DISCUSSION

Herpetic esophagitis occurs mostly in immunocompromised hosts such as organ transplant recipient (solid organ and bone marrow) or patients with human immunodeficiency virus (HIV) infection[1]. The main complaints in immunocompetent patients are fever and retrosternal chest pain[2]. In this case, there is occurrence of a complication not frequently described in the litterature: reversible esophageal stricture associated with herpes simplex esophagitis in immunocompetent host following short-term systemic corticotherapy.

Reflux disease and long-standing inflammation are well known causes of benign esophageal stricture. Malignancy must always be ruled out by endoscopy and biopsy. In the setting of herpetic oesophagitis, stenosis secondary to edema have been described in immunocompromised host with HIV[3]. Early endoscopic findings include vesicles, ulcerations and cobblestoning secondary to cluster lesions. The mucosa is friable and exudates are frequently present. Mucosal necrosis can also be seen in late stages[4].

Diagnosis is made by histopathology and virus isolation. The pathognomonic alterations include multinucleated cells with ground-glass, nuclear inclusions with chromatin margination. The cellular inclusions are surrounded by an inflammatory cell infiltrate. Pathologists use peroxidase-conjugated antibodies against HSV-1 and HSV-2. Virus isolation in tissue culture and nucleic acid amplification techniques are sensitive but may give false positive results from colonize oral mucosal surface.

Triggers known of HSV-1 recurrent labial infection are multiple, from immunosuppression to fatigue and stress[5]. In our case, the patient had an acute exacerbation of COPD and a subsequent short-term corticotherapy at low doses. Both of these events could have triggered HSV-1 reactivation, which will be discussed separately.

First, infections represent a major stress, especially in COPD disease. Viral infections have been identified as a causative factor of COPD exacerbation[6]. Although the role of bacterias during acute exacerbations is still under investigations, studies describe immunomodulatory effect of antibiotics as a mecanism leading to reduction of exacerbation rate and severity, addressing the bacterial chronic infection in COPD[7]. It is also important to mention abnormal Th1 responses among the various dis-

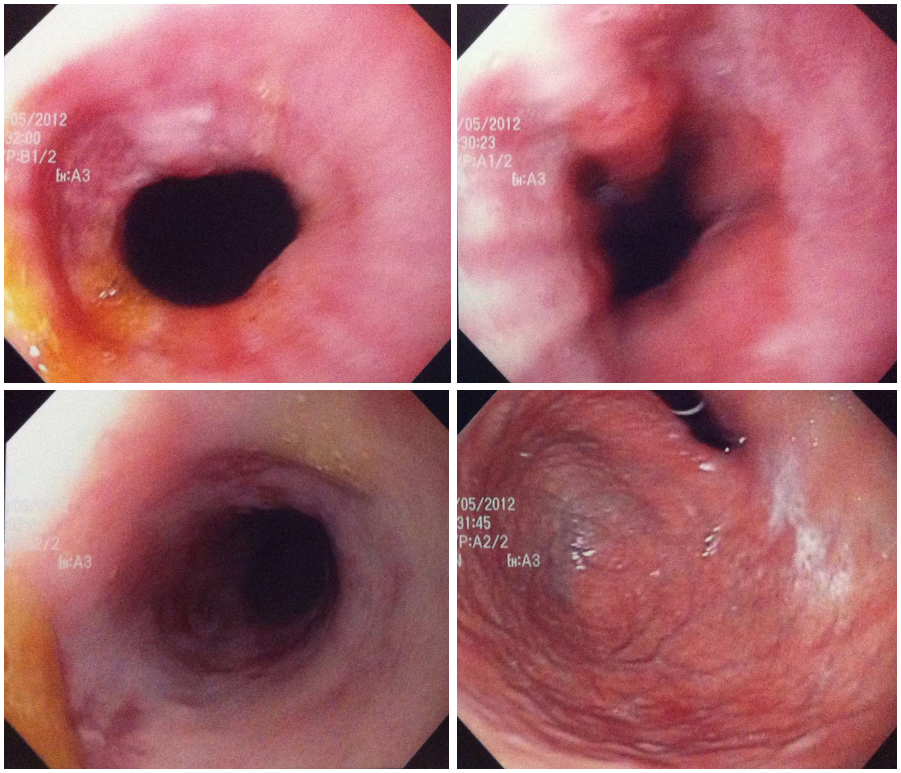

Figure 2 Hyperhemia consistent with esophagitis with complete resolution of the stenosis of the esophagus, day 8.

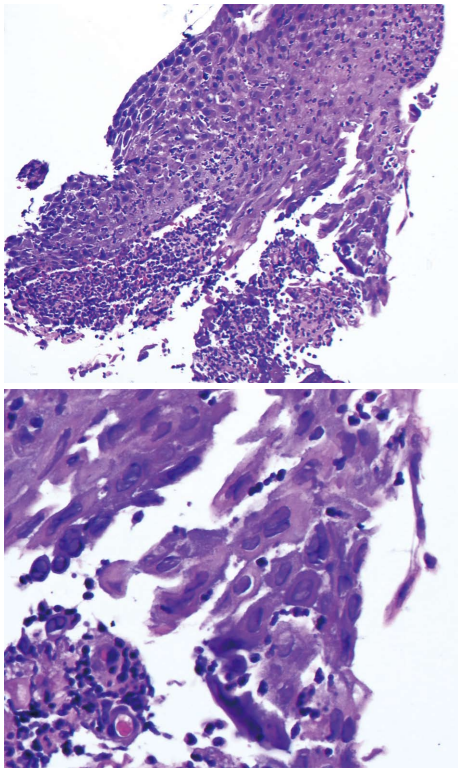

Figure 3 Ulcerated squamous epithelium with herpes virus inclusions easily visible on hematoxylin phloxine saffron coloration.

ruptions of immune system occuring in COPD[8]. Th-1, the main immune response to HSV infection, is then impaired. These evidences could partly explain HSV-1 reactivation in our case.

Second, a common side effect of corticotherapy is secondary infections. Topical corticosteroids have been a proven factor of bovine herpes virus reactivation in intranasal rabbit model[9]. No clinical evidence shows that systemic corticoids reactivate HSV, although topical application on active HSV infection can lead to expansion of lesions. However, glucocorticoids induced a polarization of Th2 over Th1 profile[10]. Th1, the main immune response to HSV infection, is then inhibited. It has not been proven however that reactivation of prior infection can be triggered by these mechanisms. Of interest, it is known that sudden cessation of glucorticoids can be a risk factor of HSV reactivation[11]. Two other case reports of HSV reactivation associated with corticotherapy have been published. One fatal HSV infection under corticotherapy in a patient with Darrier disease[12]. A case of fatal HSV hepatitis following eight days of prednisone 40 mg DIE given for ulcerative colitis has also been reported, in an otherwise immunocompetent patient[13]. In both cases, authors concluded that high clinical suspicion and prompt diagnosis are crucial.

Furthermore, it is interesting to remember that our patient also had a previous COPD exacerbation treated by corticotherapy three months before. HSV-1 esophagitis could have been triggered by the additional burden of the last exacerbation and corticotherapy.

Herpetic esophagitis is usually self-limited in immunocompetent patients, but Acyclovir therapy has been used successfully in many cases. Most authors suggest hasten symptomatic relief and subsequent shortening of clinical course with medical treatment[1,2,4,13-15]. For immunocompetent patients, suggested dosage is 5 mg/kg *iv*

every 8 h for 7-14 d that can be then completed orally as the patient swallowing returns. Oral viscous lidocaine solution (15 mL of 2% solution) can also be administered to lessen the odynophagia.

To conclude, clinicians must be aware that reactivation of herpetic infection can occur following a course of low dose corticotherapy and can cause herpetic esophagitis also in immunocompetent patient. In front of a severe esophageal stenosis, one must still exclude the usual differential diagnosis peptic stenosis and cancer. Visualization of endoscopic lesions can suggest the diagnosis but must be promptly confirmed by biopsy, viral culture or polymerase chain reaction. Acyclovir is considered to be the treatment of choice, with oral xylocaine for pain relief.

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CASE REPORT

Pancreatic duct drainage using EUS-guided rendezvous technique for stenotic pancreaticojejunostomy

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Abstract

The patient was a 30-year-old female who had undergone excision of the extrahepatic bile duct and Roux-en-Y hepaticojejunostomy for congenital biliary dilatation at the age of 7. Thereafter, she suffered from recurrent acute pancreatitis due to pancreaticobiliary maljunction and received subtotal stomach-preserving pancreaticoduodenectomy. She developed a pancreatic fistula and an intra-abdominal abscess after the operation. These complications were improved by percutaneous abscess drainage and antibiotic therapy. However, upper abdominal discomfort and the elevation of serum pancreatic enzymes persisted due to stenosis from the pancreaticojejunostomy. Because we could not accomplish dilation of the stenosis by endoscopic retrograde cholangiopancreatography, we tried an endoscopic ultrasonography (EUS) guided rendezvous technique for pancreatic duct drainage. After transgastric puncture of the pancreatic duct using an EUS-fine needle aspiration needle, the guidewire was inserted into the pancreatic duct and finally reached to the jejunum through the stenotic anastomosis. We changed the echoendoscope to an oblique-viewing endoscope, then grasped the guidewire and withdrew it through the scope. The stenosis of the pancreaticojejunostomy was dilated up to 4 mm, and a pancreatic stent was put in place. Though the pancreatic stent was removed after three months, the patient remained symptom-free. Pancreatic duct drainage using an EUS-guided rendezvous technique was useful for the treatment of a stenotic pancreaticojejunostomy after pancreaticoduodenectomy.

Key words: Balloon dilatation; Endoscopic ultrasound-guided fine needle aspiration; Pancreaticobiliary maljunction; Pancreaticoduodenectomy; Pancreatitis; Postoperative complication

Core tip: The usefulness of pancreatic duct drainage using endoscopic ultrasonography-guided rendezvous technique for stenotic pancreaticojejunostomy after pancreaticoduodenectomy. However, this procedure requires technically skill and the success rate is low. The main reason for failure is the inability to pass through the stenotic anastomosis due to its tightness. In our case, the stenosis was not so tight because the stenosis developed about a month after the operation. A

case of stenotic pancreaticojejunostomy that occurs at any early stage after pancreaticoduodenectomy with a dilated pancreatic duct is possibly a good indication.

Takikawa T, Kanno A, Masamune A, Hamada S, Nakano E, Miura S, Ariga H, Unno J, Kume K, Kikuta K, Hirota M, Yoshida H, Katayose Y, Unno M, Shimosegawa T. Pancreatic duct drainage using EUS-guided rendezvous technique for stenotic pancreaticojejunostomy. *World J Gastroenterol* 2013; 19(31): 5182-5186 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5182.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5182

INTRODUCTION

The surgical mortality rate after pancreaticoduodenectomy (PD) has decreased due to advances in surgical technique, but surgical morbidity has not yet decreased[1]. Stenotic pancreaticoenteric anastomosis is one of the complications after PD[2]. Endoscopic retrograde cholangiopancreatography (ERCP) is performed to treat this complication, but the success rate has been low[3]. Surgeons prefer to avoid re-operation for pancreaticoenteric stenosis due to the operative risks. Recently, interventional endoscopic ultrasonography (EUS) has greatly advanced in terms of the available devices and techniques[4-11]. Pancreaticobiliary cases treated by interventional EUS have been reported, but the usefulness of interventional EUS-fine needle aspiration (FNA) for the treatment of post-operative complications is not yet known. We here report a case with stenotic pancreaticojejunostomy that was efficiently treated by an EUS-guided rendezvous technique.

CASE REPORT

The patient was a 30-year-old female who had undergone excision of the extrahepatic bile duct and Roux-en-Y hepaticojejunostomy for congenital biliary dilatation at the age of 7 (Figure 1A). She was referred to our hospital for upper abdominal and dorsal pain. Pancreatic enzymes and inflammatory reactions (serum amylase: 210 IU/L, serum lipase: 390 IU/L, C-reactive protein: 2.4 mg/dL) were elevated, and abdominal computed tomography (CT) revealed peripancreatic fluid collection and pancreatic swelling. This patient was diagnosed as acute pancreatitis based on these findings and admitted to our hospital in 2007. Magnetic resonance imaging (MRI) and ERCP showed protein plugs in the main pancreatic duct (MPD) and pancreaticobiliary maljunction with a dilated common channel and dilated residual intra-pancreatic bile duct. The pancreaticobiliary maljunction was suspected to be the cause of the protein plugs in the MPD. We performed endoscopic pancreatic sphincterotomy and removed the protein plugs, but she suffered from acute pancreatitis due to recurrent protein plugs until

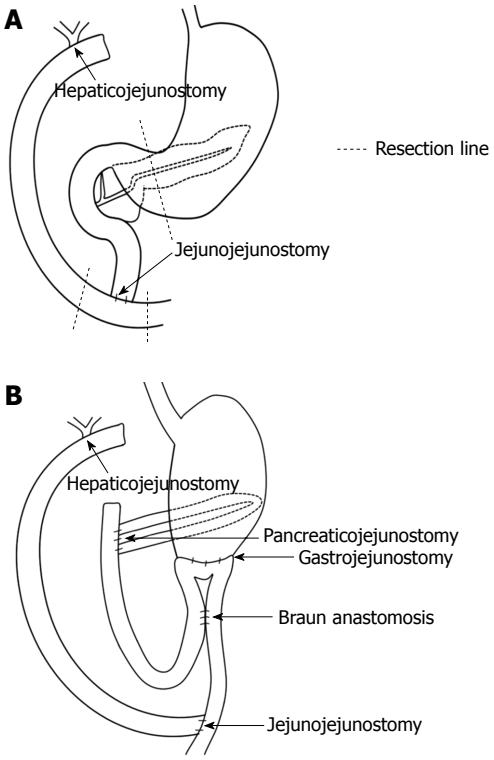

Figure 1 Re-construction after subtotal stomach-preserving pancreaticoduodenectomy. A: Before the operation (after excision of extrahepatic bile duct and Roux-en-Y hepaticojejunostomy; B: After the operation, pancreaticojejunostomy and gastrojejunostomy were performed. Roux-en-Y hepaticojejunostomy was re-established.

2011. PD was thought to be an adequate operation for preventing recurrent acute pancreatitis by protein plugs. After obtaining informed consent, the patient received subtotal stomach-preserving PD. Regarding reconstruction, pancreaticojejunostomy and gastrojejunostomy were performed, and Roux-en-Y hepaticojejunostomy was re-established (Figure 1B).

On the 16th post-operative day, the patient developed a high fever after accidental removal of an external drainage tube placed in the pancreatic duct. Abdominal CT revealed fluid collection and an intra-abdominal abscess near the anastomotic site of the pancreaticojejunostomy. After percutaneous drainage of the abscess and antibiotic therapy, her condition improved. However, upper abdominal discomfort and the elevation of serum pancreatic enzymes persisted. CT and EUS revealed a pancreatic duct dilatation (6 mm) (Figure 2). These symptoms were due to the stenosis of the pancreaticojejunostomy, and we tried ERCP using an oblique-viewing endoscope (GIF-XK240; Olympus, Tokyo, Japan). The anastomotic site of the pancreaticojejunostomy was identified, but we could not perform either pancreatography or a guidewire insertion into the pancreatic duct. We then tried EUS-guided rendezvous technique for drainage of the pancreatic duct on the 53rd post-operative day. We used a convex array echoendoscope (GF-UCT240-AL5; Olympus, Japan) and identified the echo image of the dilated pancreatic duct from the stomach. A vascular structure was confirmed by color

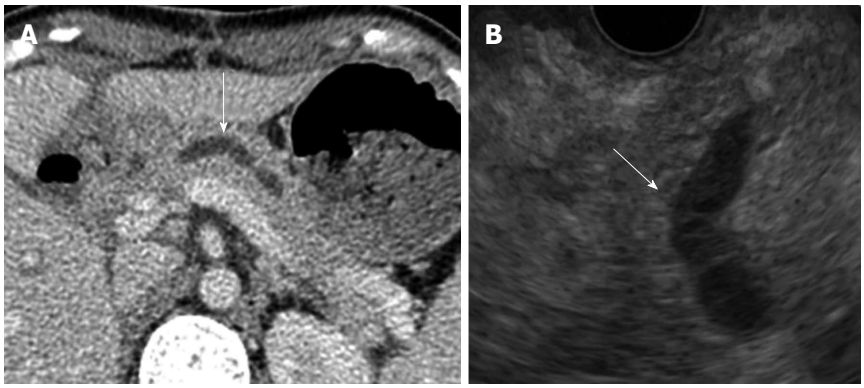

Figure 2 Computed tomography (A) and and endoscopic ultrasonography (B) revealed a dilated pancreatic duct (white arrows).

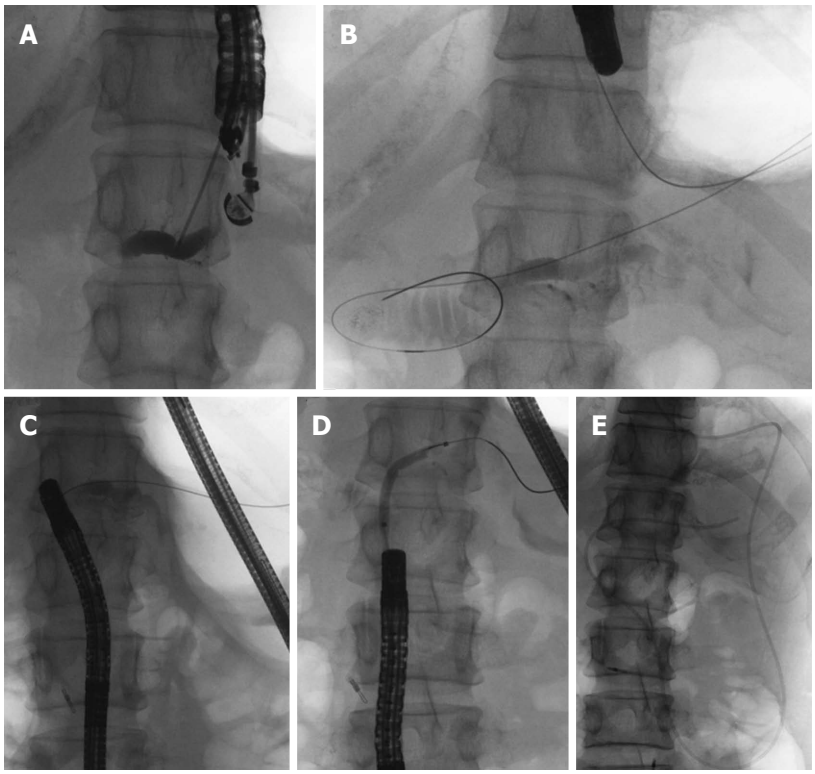

Figure 3 Pancreatic duct drainage procedures using endoscopic ultrasonograph-guided rendezvous technique. A: Pancreatic duct puncture and pancreatography using endoscopic ultrasonography; B: Introducing the guidewire into the jejunum through the pancreatic duct and the stenotic anastomosis; C: After exchanging echoendoscope for oblique-viewing endoscope, the guidewire was withdrawn into the working channel; D: Balloon dilatation of the stenotic anastomosis; E: Placement of an endoscopic naso-pancreatic drainage tube.

Doppler imaging and successfully avoided. The MPD was punctured using a 19-gauge needle (Echo Tip; Cook, Wilston-Salem, NC, United States). Pancreatography was obtained by the injection of contrast medium (Figure 3A), and a 0.025-inch guidewire (VisiGlide; Olympus, Japan) was inserted into the MPD and finally reached to the jejunum through the stenotic anastomosis (Figure 3B). The echoendoscope was removed, leaving the guidewire. After introducing an oblique-viewing endoscope (GIF-XK240; Olympus, Japan) up to the pancreaticojejunostomy, we grasped the guidewire by a snare and withdrew it through the working channel (Figure 3C). The stenosis of the pancreaticojejunostomy was dilated up to 4 mm by a wire-guided balloon catheter (MaxForce; Boston Scientific, Natric, MA, United States) (Figure 3D), and replaced with a 5-Fr endoscopic nasopancreatic drainage (ENPD) tube (GADELIUS, Tokyo, Japan) (Figure 3E). Eight days later, we replaced the ENPD tube with a 7-Fr pancreatic stent (GADELIUS). Her symptom was improved and the serum amylase and lipase values returned to the normal range. When we performed ERCP after 3 mo, we removed the pancreatic stent and confirmed good pancreatic juice drainage. At 1 year after the last endoscopic treatment, she was symptom-free and the blood chemistry test results were normal.

DISCUSSION

Pancreatoenteric anastomotic site stenosis can be a problematic complication after PD. Reid-Lombardo et al. reported that stenotic pancreaticojejunostomy requiring intervention was observed in 4.6% of PD patients[2]. The Patients with stenotic pancreaticojejunostomy after PD tended to be treated with ERCP-related procedures. However, the success rates were not often high due to the inability to reach or to identify the pancreaticojejunostomy through the afferent loop[3]. A long afferent loop and postoperative adhesions might hamper endoscopic

Table 1 Reported cases of pancreatic duct drainage using endoscopic ultrasonography-guided rendezvous technique for stenosis of after pancreticojejunostomy

Ref.	*n*	Age (yr)/sex	Indication	Pancreatic duct	Success	Reasons for failure	Postoperative period (yr)
Kikuyama *et al*[4]	19	72/M	ARP	N/A			N/A
	20	66/M	ARP				
	21	51/M	ARP				
Mallery *et al*[7]	1	35/F	ARP	Non dilated	Yes		4
	2	55/M	Chronic pancreaticocutaneous fistula	Non dilated	No	Failed guidewire passage	1
Kinney *et al*[8]	3		ARP		No		
	4		ARP		No		
	5		ARP, pancreatic stone		Yes	Failed pancreatic	
	6		ARP, pancreatic stone		Yes	duct puncture	
	7	N/A	ARP, chronic pain	N/A	Yes	(in two patients)	N/A
	8		ARP, chronic pain		Yes		
	9		ARP, chronic pain		No	Failed guidewire passage	
	10		Chronic pancreaticocutaneous fistula		No	(in three patients)	
	11		Inwardly migrated surgical stent with recurrent pancreatitis		No		
Barkay *et al*[9]	12	36/F	IPMN	Dilated	No	Failed guidewire passage	
	13	60/M	ARP	Dilated	Yes		
	14	22/F	CP, pancreatic divisum	Dilated	No	Failed guidewire passage	
	15	54/F	CP	Non dilated	No	Failed guidewire passage	N/A
	16	53/M	IPMN	Dilated	No	Failed guidewire passage	
	17	67/M	IPMN	Dilated	No	Failed guidewire passage	
	18	59/F	CP	Dilated	Yes		
DeWitt *et al*[10]	22	25/F	Chronic pain	Dilated	No	Failed guidewire passage	N/A
Itoi *et al*[11]	23	66/M	ARP	Dilated	Yes		12
	24	51/M	ARP	Dilated	Yes		8

F: Female; M: Male; ARP: Acute recurrent pancreatitis; N/A: Not available; IPMN: Intraductal papillary mucinous neoplasm; CP: Chronic pancreatitis.

treatment. Whether lateral-viewing, forward-viewing or oblique-viewing endoscope was chosen, it has been unclear which type is the appropriate choice. Kikuyama *et al*[4] suggested that oblique-viewing endoscope was a good option for ERCP in operated patients, since it was useful for deep cannulation and therapeutic procedures due to the instrument elevator and good angle of view for advancing into the afferent loop. If a conventional endoscope was unable to reach the pancreaticojejunostomy, single or double balloon enteroscopes should be used as an alternative[5]. However, these endoscopes were not appropriate for interventional endoscopic treatment due to the small forceps' channel. In our case, we performed ERCP using an oblique-viewing endoscope and we could reach and identify the pancreaticojejunostomy because the afferent loop was not long (Figure 1B). However, pancreatography and guidewire insertion into the pancreatic duct were not possible.

Recently, interventional EUS has greatly advanced in terms of available devices and techniques. Bataille *et al*[6] first reported pancreatic duct drainage with EUS-guided rendezvous technique in 2002. Thereafter, several reports have described this procedure in post-PD patients (Table 1)[4,7-11]. This procedure is technically challenging and has an approximately 50% success rate. The reasons for failure include the impossibility of puncturing the pancreatic duct without dilatation, and the inability to pass through the stenotic anastomosis due to its tightness and less than ideal orientation of the puncture. Although the early course of this patient was favorable, it is important to follow up this patient carefully due to the risk of restenosis of pancreaticojejunostomy.

Kikuyama *et al*[4] have described difficulty in passing the stenotic anastomosis due to a tight stenosis, since stenosis of the pancreaticoenteric anastomosis usually happens as a late complication[2]. In our case, we expected that the stenosis was not tight since the stenosis developed about a month after PD due to a pancreatic fistula and an intra-abdominal abscess. We supposed that the length of time after the operation and the severity of other complications such as a pancreatic fistula might affect the outcomes, in terms of fibrosis, edema and compression occupying the space around the anastomotic site.

The diameter of the pancreatic duct is an important factor in avoiding complications as well as success. We could achieve successful pancreatic duct drainage in this case since the MPD was dilated enough to puncture (6 mm). Fatal complications have never been reported with this procedure, while a few complications such as abscess, mild pancreatitis or transient fever were reported, and these complications mostly happened to patients with pancreatic ducts of normal diameter[7,9]. Accordingly, for the EUS-guided rendezvous techniques we should select patients who satisfy these conditions.

EUS-guided pancreaticogastrostomy was an option for the treatment of this case. EUS-guided pancreaticogastrostomy has the risk of stent dysfunctions such as obstruction and migration[12], whereas dilatation of the stenotic anastomosis by a balloon catheter has a small risk of restenosis[4]. We therefore selected balloon dilatation for stenotic pan-

creaticojejunostomy using the rendezvous technique. Our case suggests that stenotic pancreaticojejunostomy ocurring at any early stage after PD with a dilated pancreatic duct might be a good indication for this technique.

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CASE REPORT

Complete response to multidisciplinary therapy in a patient with primary gastric choriocarcinoma

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Abstract

Primary gastric choriocarcinoma is a rapidly growing neoplasm with an average survival of several months in untreated patients. Gastrectomy with lymph node dissection followed by chemotherapy is the treatment of choice. Regimens used for gastric adenocarcinoma are usually selected. However, median survival remains less than six months. In this case report, we describe a case of primary gastric choriocarcinoma with a clinical complete response to multidisciplinary treatment including surgery, chemotherapy, and radiofrequency ablation (RFA). The patient was originally referred for general malaise. Esophagogastroduodenoscopy demonstrated a large tumor occupying the fornix, and total gastrectomy with lymph node dissection was performed. Seven days later, multiple liver metastatic recurrences with high serum levels of beta-human chorionic gonadotropin (β-hCG) were recognized. Chemotherapy with a gonadal choriocarcinoma regimen consisting of etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine (EMA/CO), was initiated. After three cycles, serum β-hCG decreased markedly and the tumors disappeared. Six months later, multiple lung metastatic recurrences were found. After one cycle of EMA/CO, only one nodule remained. Computed tomography-guided RFA was performed for this oligometastatic tumor. The patient has been alive with no evidence of disease for 10 years after the initial diagnosis. To the best of our knowledge, this patient with recurrent primary gastric choriocarcinoma has achieved the longest survival. The present case is the first report of choriocarcinoma metastatic to the lung successfully treated with RFA. From our retrospective analysis of recurrent or unresectable primary gastric choriocarcinoma, we propose that gonadal choriocarcinoma regimens can be considered as first-line for primary gastric choriocarcinoma.

Key words: Primary gastric choriocarcinoma; Beta-human chorionic gonadotropin; Etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine; Oligometastatic; Radiofrequency ablation

Core tip: We described a case of primary gastric choriocarcinoma with a complete response to multidisciplinary treatment including surgery, chemotherapy, and radiofrequency ablation (RFA). The patient has been alive with no evidence of disease for 10 years. To the best of our knowledge, this patient with recurrent primary gastric choriocarcinoma has achieved the longest survival. The present case is the first report of choriocarcinoma metastatic to the lung successfully treated with RFA. From our retrospective analysis of recurrent or unresectable primary gastric choriocarcinoma, we propose that gonadal choriocarcinoma regimens can be considered as first-line for primary gastric choriocarcinoma.

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INTRODUCTION

Choriocarcinoma typically occurs in females at the origin of the chorionic epithelium of the placenta, and is commonly related to gestation. The tumor is rapidly growing, widely metastasizing, and highly invasive of surrounding tissues. Gonadal choriocarcinomas are usually highly sensitive to various types of anti-cancer agents[1].

Primary gastric choriocarcinoma is a type of non-gonadal choriocarcinoma that constitutes less than 1% of all gastric cancers[2]. It was first described by Davidson in 1905, and there are currently approximately 140 reported cases worldwide[3]. Most patients with primary gastric choriocarcinoma do not survive for even one year after surgery[4]. Chemotherapy regimens used successfully for gonadal choriocarcinoma are not as effective for primary gastric choriocarcinoma[5]. The prognosis is considerably worse than gastric adenocarcinoma[4]. Primary gastric choriocarcinoma with liver metastases has the worst prognosis[6].

We report a case of primary gastric choriocarcinoma successfully controlled by multidisciplinary therapy including surgery, chemotherapy, and radiofrequency ablation (RFA). The patient survived for approximately 10 years after initial diagnosis. To the best of our knowledge, the present case has the longest survival of recurrent primary gastric choriocarcinoma in the world.

CASE REPORT

A 65-year-old woman was referred to our clinic for general malaise and dizziness. She had no significant past medical history, and had never been hospitalized. On physical examination, the patient had pale skin due to anemia. Initial laboratory results were normal except for hemoglobin of 7.4 g/dL. Serum carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19 concentrations were within normal limits. Esophagogastroduodenoscopy demonstrated a large tumor from the fornix to the posterior wall of the upper body of the stomach, with a mixture of protruding and ulcerative lesions, as well as areas of hemorrhage. Biopsied specimens were interpreted as tubular adenocarcinoma with moderate differentiation. Abdominal computed tomography (CT) demonstrated wall thickening at the fornix with disappearance of adipose tissue at the gastrosplenic ligament, suggestive of penetration of the gastric serosa. There was no obvious evidence of metastasis to the lymph nodes or liver, or of peritoneal dissemination. Total gastrectomy with D2 lymphadenectomy was planned.

During laparotomy, the tumor showed invasion to the body of the pancreas, and metastasis to several adjacent lymph nodes was suspected. A 1 cm × 1 cm nodule was detected in the liver. Total gastrectomy with D3 lymph node dissection, distal pancreatectomy, splenectomy, and enucleation of the liver was performed, along with Roux-en-Y reconstruction.

Pathological findings

An elevated 10 cm × 8 cm tumor with surface ulceration and hemorrhage was located at the fornix (Figure 1A). On the cut surface of the specimen, there was a very large lobulated tumor, white to gray in color, with large areas of hemorrhage and necrosis (Figure 1B).

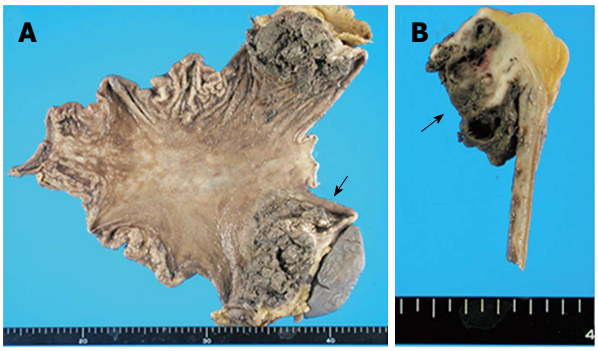

Figure 1 Gross appearance of the resected specimen. A: An elevated tumor with surface ulceration and hemorrhage was located in the fornix (arrow); B: On the cut surface of the specimen, there was a very large lobulated tumor, white to gray in color, with large areas of hemorrhage and necrosis (arrow).

Microscopically, the tumor had two components (Figure 2A). The first component, with histological features suggestive of choriocarcinoma, consisted of unusually-shaped multinucleated giant cells, similar to syncytiotrophoblasts in a characteristic dimorphic plexiform pattern, associated with hemorrhage and necrosis (Figure 2B). The second component consisted of atypical mononucleated cells similar to intermediate trophoblasts in a solid and sheet growth pattern (Figure 2C). Immunohistochemically, the tumor cells in the first component were diffusely positive for β-human chorionic gonadotropin (hCG) (Figure 2D). Immunoreactivity was also seen for human placental lactogen (Figure 2E). The tumor cells in the second component were diffusely positive for β-hCG and placental alkaline phosphatase (Figure 2F and G). Immunoreactivity was also seen for CEA (Figure 2H). These findings are identical to the World Health Organization (WHO) classification of primary gastric choriocarcinoma based on clinicopathological criteria[7]. Histologically, it showed an INF β growth pattern with invasion to the subserosa. Metastasis was detected in the lymph nodes along the short gastric vessels (no. 4SA) and at the right splenic hilum (no. 10). The liver nodule was also identified as a metastasis. The proximal and distal resection margins were clear. Peritoneal cytology was negative. The final classification was T3N1M1, stage Ⅳ, according to the Union for International Cancer Control guidelines.

Postoperative course and follow-up

The postoperative course was unremarkable. Serum β-hCG measured immediately after surgery was 12000 mIU/mL (normal range < 0.7 mIU/mL) (Figure 3). Seven days after surgery, multiple low-density lesions were detected in the liver on abdominal CT (Figure 4A and B). Recurrence was suspected and so systemic chemotherapy consisting of etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine (EMA/CO) was initiated. After one cycle, the serum β-hCG concentration

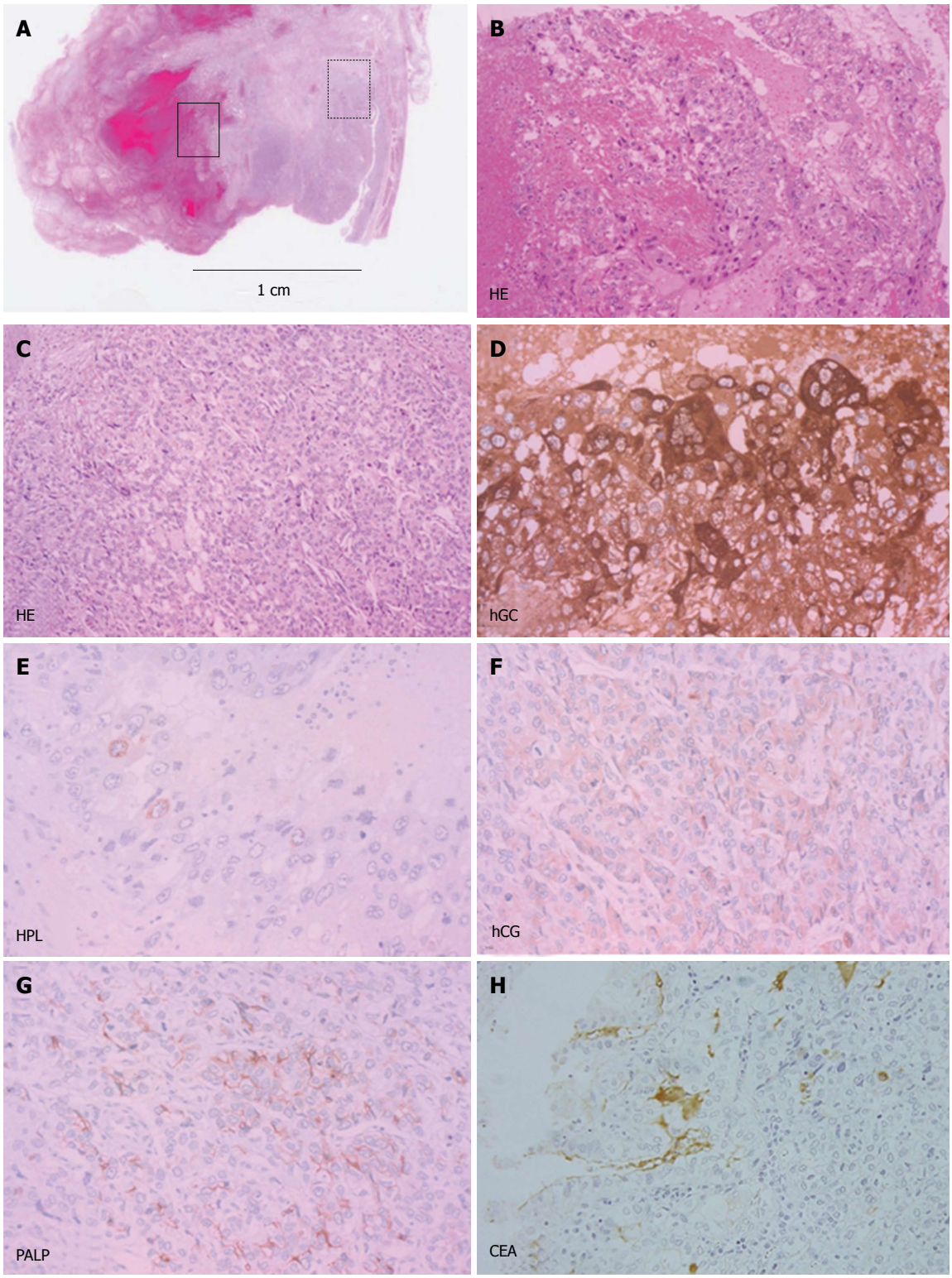

Figure 2 Pathological findings (hematoxylin/eosin staining and immunohistochemical staining). A: The tumor had two components, as indicated by small boxes with solid and dashed lines. Hematoxylin/eosin (HE) × 40; B: In the area marked by a solid line in panel A, unusual multinucleated giant cells in a characteristic dimorphic plexiform pattern associated with hemorrhage and necrosis were observed. HE × 100; C: Atypical mononucleated cells demonstrated a solid and sheet growth pattern in the area marked by a dashed line in panel A. HE × 100; D, E: Tumor cells were diffusely positive for β-human chorionic gonadotropin (hCG) and focally positive for human placental lactogen (HPL) in the area marked by a solid line in panel A. HE × 200; F, G: The tumor cells were positive for beta-human chorionic gonadotropin and placental alkaline phosphatase (PALP) in the area marked by a dashed line in panel A. HE × 200; H: Immunoreactivity was focally positive for carcinoembryonic antigen (CEA). HE × 100.

started to decrease and there was reduction in the size of the tumors on CT. After three cycles, the serum β-hCG level was almost within normal limits and the tumors disappeared with a clinical complete response and no major

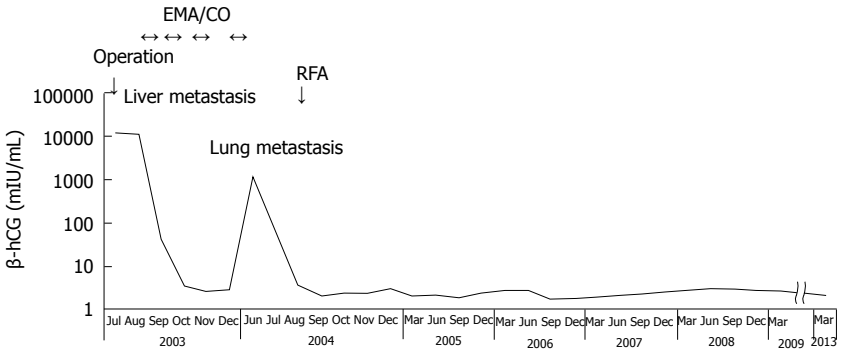

Figure 3 Tumor markers and chemotherapy. Seven days after surgery, metastatic recurrence in the liver was diagnosed. After starting etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine (EMA/CO), serum beta-human chorionic gonadotropin (β-hCG) concentrations decreased. After three cycles, serum β-hCG levels decreased markedly to almost within normal limits and clinically the tumors showed a complete response. Six months later, there was a sudden elevation in serum β-hCG levels with the emergence of multiple nodules in both lung fields. Metastatic recurrence in the lung was diagnosed and EMA/CO was restarted. After one cycle, most tumors, except for one nodule in the left lower lobe, disappeared concomitantly with declines in serum β-hCG levels. Computed tomography-guided radiofrequency ablation (RFA) was performed for the oligometastatic tumor. The patient has been alive with no evidence of disease for nine years after RFA.

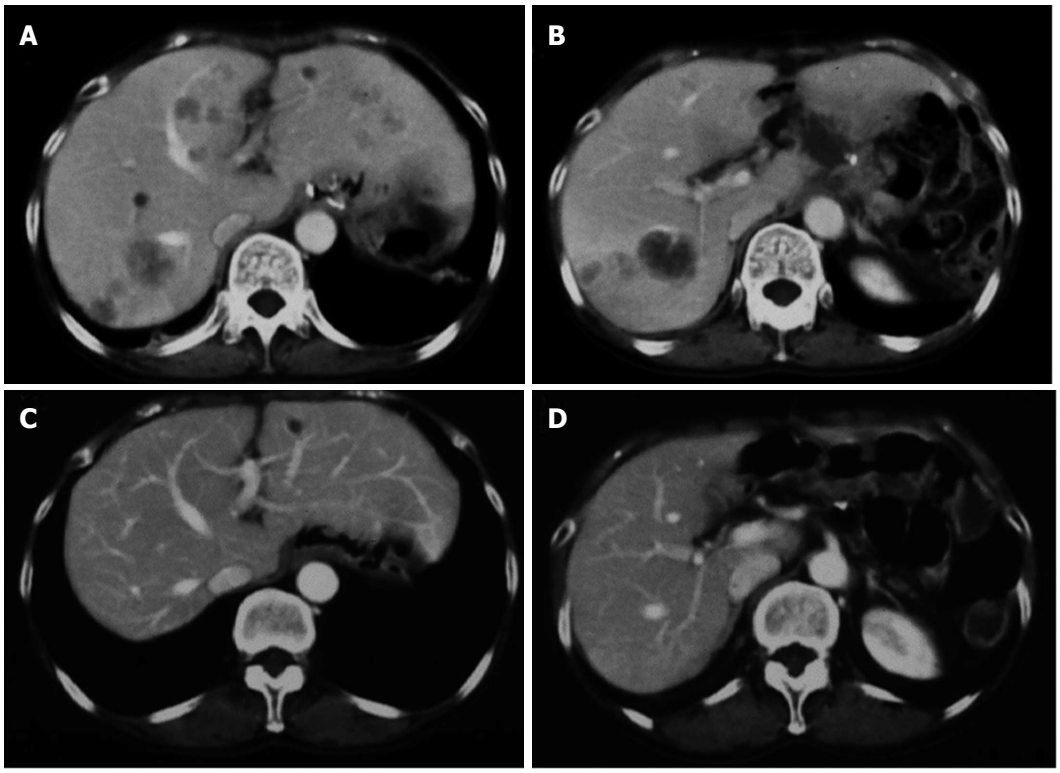

Figure 4 Computed tomography images before and after chemotherapy. A, B: Seven days after surgery, multiple low-density lesions were detected in the liver on abdominal computed tomography; C, D: After three cycles of etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine, the tumors disappeared with a clinical complete response.

side effects (Figure 4C and D). Six months after surgery, there were a sudden elevation in the serum β-hCG level (1100 mIU/mL) and the emergence of multiple nodules in both lung fields on CT. Lung metastasis was diagnosed and EMA/CO was restarted. After one cycle, most tumors, except one nodule in the left lower lobe, disappeared along with decreases of serum β-hCG. CT-guided RFA was performed for oligo-recurrence. The patient remains alive with no evidence of disease for nine years after RFA treatment.

Pooled analysis of reported cases of recurrent or unresectable primary gastric choriocarcinoma treated with chemotherapy

We retrospectively collected all reported cases of recurrent or unresectable (including initially unresectable tumors treated with neoadjuvant therapy) primary gastric choriocarcinoma treated with chemotherapy with a clear postoperative prognosis in the English and Japanese literature after 1990 (Table 1)[5,8-31]. Measurement of the overall survival (OS) period began at the time of initial

Table 1 Review of the English and Japanese literature for cases of recurrent or unresectable primary gastric choriocarcinoma treated with chemotherapy after 1990

Cases	Authors	Age (yr)/sex	Type	Site of metastasis	Chemotherapy regimen	Response	Prognosis (mo)
1	Present case	65/F	Recurrent	Liver	EMA/CO	CR	115 NED
2	Waseda *et al*[5]	68/M	Unresectable	Liver	EP	CR	24 NED
3	Shastri *et al*[8]	44/M	Unresectable	Liver	BEP	Size reduction	12 DOD
4	Shimuzu *et al*[9]	43/F	Unresectable	Distant lymph nodes	TS-1/CDDP	Size reduction	7 DOD
5	Yoon *et al*[10]	62/M	Unresectable	Liver	5-FU/USAN/L-OHP	Progression	16 DOD
					PTX/CDDP	Progression	
					5-FU/USAN/CPT-11	Progression	
					BEP	Progression	
					EMA/CO	Progression	
					VIP	Progression	
6	Yoon *et al*[10]	45/M	Unresectable	Liver	BEP	Progression	12 DOD
					VIP	Progression	
					EMA/CO	Progression	
					5-FU/USAN/CPT-11	Progression	
7	Kanemura *et al*[11]	79/M	Recurrent	No data	TS-1/PTX	Progression	5 DOD
8	Yasumoto *et al*[12]	76/F	Unresectable	Liver	5-FU	Progression	1 DOD
9	Mori *et al*[13]	36/F	Recurrent	Brain and lung	EMA/CO	CR	54 NED
10	Enokido *et al*[14]	54/M	Unresectable	Liver	TS-1	Progression	3 DOD
11	Adachi *et al*[15]	78/M	Unresectable	Liver	TS-1	CR	12 NED
12	Kishimoto *et al*[16]	69/M	Recurrent	Liver	Epi-ADM/MMC (TACE)	Progression	17 DOD
					5-FU (HAI)	Progression	
					UFT	Progression	
13	Kawaguchi *et al*[17]	60 M	Recurrent	Liver	MTX/BLM/CDDP/CPA	Progression	5 DOD
14	Liu *et al*[18]	36/F	Unresectable	Colon (infiltration)	BEP	Size reduction	6 DOD
					VBL/IFM/CDDP	Progression	
15	Inaki *et al*[19]	56/M	Recurrent	Liver	MAC (second-line after UFT as adjuvant)	Progression	3 DOD
16	Bayhan *et al*[20]	26/F	Recurrent	Lung	MAC	Size reduction	18 NED
17	Satoh *et al*[21]	58/M	Recurrent	Paraaortic lymph nodes	VP-16/CDDP	Progression	6 DOD
18	Kinoshita *et al*[22]	74/M	Unresectable	Liver	MTX	Progression	3 AWD
19	Imai *et al*[23]	63/F	Recurrent	Liver	MA	Progression	3 DOD
20	Fujimoto *et al*[24]	57/M	Recurrent	Liver	MAC	Progression	6 DOD
21	Ogura *et al*[25]	45/F	Recurrent	Liver	5-FU/MMC/Epi-ADM (HAI)	Size reduction	10 DOD
					VP-16/CDDP	Progression	
22	Kan *et al*[26]	67/M	Unresectable	Liver	5'DFUR/CDDP	Size reduction	10 DOD
					5'DFUR/CDDP/VP-16	Progression	
					MTX	Progression	
23	Saito *et al*[27]	57/M	Recurrent	CEA elevation	5-FU/CDDP (second-line after Tegafur as adjuvant)	SD	12 AWD
24	Imatake *et al*[28]	50/M	Unresectable	Liver	MMC/5-FU/lentinan	Progression	2 DOD
25	Okada *et al*[29]	57/F	Recurrent	hCG elevation	VAC	Progression	7 DOD
26	Kobayashi *et al*[30]	60/M	Unresectable	Liver	MTX/ADM (HAI)	Progression	5 DOD
27	Masuda *et al*[31]	79/M	Unresectable	Liver	UFT	Progression	1.5 DOD

M: Male; F: Female; USAN: Leucovorin; CDDP: Cisplatin; 5-FU: Fluorouracil; L-OHP: Oxaliplatin; CPT-11: Irinotecan; PTX: Paclitaxel; BLM: Bleomycin; CPA: Cyclophosphamide; VBL: Vinblastine; Epi-ADM: Epirubicin; IFM: Ifosfamide; VP-16: Etoposide; 5'DFUR: Doxifluridine; ACT-D: Actinomycin D; ADM: Adriamycin; VCR: Oncovin (vincristine); MMC; Mitomycin C; UFT: Tegafur-uracil; CEA: Carcinoembryonic antigen; β-hCG: β-human chorionic gonadotropin; NED: No evidence of disease; DOD: Died of disease; AWD: Alive with disease; CR: Complete response; SD: Stable disease; TACE: Transcatheter arterial chemoembolization; HAI: Hepatic arterial infusion; EMA/CO: Etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine.

diagnosis. Death due to primary gastric choriocarcinoma was the only endpoint considered for the purpose of this study. OS curves were obtained using the Kaplan-Meier method, and differences were compared using the log-rank test. P values < 0.05 were considered significant.

Our search revealed 12 previous cases treated using gonadal choriocarcinoma regimens. 11 patients received first-line chemotherapy, of whom two had a complete response with etoposide and cisplatin (EP)[5] and EMA/CO[13], respectively (Table 1). The median survival of the patients treated with gonadal choriocarcinoma regimens used as the first-line was 9.5 mo compared to 5.0 mo in patients treated with gastric adenocarcinoma regimens. Although the difference was not significant, treatment results showed a favorable prognosis with the gonadal choriocarcinoma regimen ($P = 0.1$) (Figure 5).

DISCUSSION

We report a rare case of primary gastric choriocarcinoma that showed a clinical complete response to multidisciplinary treatment, including surgery, chemotherapy, and RFA. The patient obtained nine years of disease-free survival. The present case represents the first report of cho-

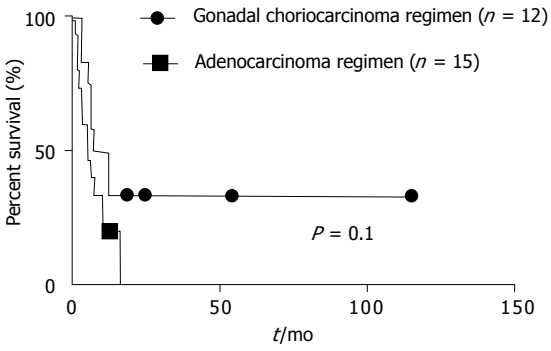

Figure 5 Overall survival with gonadal choriocarcinoma regimen and adenocarcinoma regimen. The median survival of patients treated with gonadal choriocarcinoma regimens used as the first-line was 9.5 mo compared to 5.0 mo in patients treated with gastric carcinoma regimens. Although this difference was not statistically significant, treatment results showed a favorable prognosis with gonadal choriocarcinoma regimens (P = 0.1).

riocarcinoma metastatic to the lung successfully treated with RFA. We propose that EMA/CO is useful as a first-line regimen for primary gastric choriocarcinoma.

Choriocarcinoma has been reported in extragonadal sites such as the lung, liver, breast, prostate, urinary bladder, nose, and gastrointestinal tract[32]. Primary gastric choriocarcinoma is extremely rare. There are several theories on the histopathogenesis of primary gastric choriocarcinoma (*i.e.*, histological resemblance to choriocarcinoma, arising from a gonadal anlage displaced in the abdomen, a long delayed metastasis from a genital primary lesion, arising from gastric teratoma, and retro-differentiation of gastric carcinoma cells to embryonal ectodermal status with the ability to form trophoblasts)[33,34]. In some cases, there is a combination of malignant cytotrophoblasts and syncytiotrophoblasts admixed with areas of typical glandular differentiation, which supports the retro-differentiation hypothesis[35]. In recent years, Okada *et al*[33] described the possibility of normal gastric cells with the ability to produce hCG, which can directly develop into gastric choriocarcinoma. However, most authors favor the concept of retro-differentiation within an area of adenocarcinoma over primary gastric cells developing into choriocarcinoma due to the fact that less than 25% of cases are pure choriocarcinoma[35]. In such cases, the more rapidly growing choriocarcinoma component seems to have replaced the adenomatous elements. In the present case, there was a component of adenocarcinoma, which was indicated by positive CEA immunohistological staining. This finding supports the hypothesis that primary gastric choriocarcinoma originates from pre-existing gastric adenocarcinoma.

Primary gastric choriocarcinoma is a rapidly growing neoplasm that has an average survival of only a few months in untreated patients[6]. Gastrectomy with lymph node dissection followed by chemotherapy is the treatment of choice. Although some case reports and small studies have reported benefits from chemotherapy, a standard treatment has not been established due to the rarity of this tumor. Chemotherapy regimens usually used successfully for gonadal choriocarcinoma, including MAC (methotrexate, actinomycin-D, cyclophosphamide), CHAMOCA (cyclophosphamide, hydroxycarbamide, doxorubicin, actinomycin D, methotrexate, melphalan, and vincristine), and EMA/CO are generally considered to have a lower success rate in the treatment of primary gastric choriocarcinoma[5]. Several studies employed regimens used for gastric adenocarcinoma, such as a combination of fluorouracil and cisplatin or TS-1-based therapy, based on the concept that primary gastric choriocarcinoma develops from the retro-differentiation of gastric adenocarcinoma[4,10,14,36]. However, despite recent advances in combination chemotherapy, median survival is still less than six months with these regimens. In our retrospective analysis, the median survival with gastric carcinoma regimens was 5.0 mo compared to 9.5 mo with gonadal carcinoma regimens. In the present case, we chose EMA/CO because it is the first-line regimen for high-risk gestational trophoblastic neoplasia due to its favorable effectiveness-to-toxicity ratio. EP, BEP (bleomycin, etoposide, cisplatin), or VIP (etoposide, ifosfamide, cisplatin) is used in refractory cases[1]. In fact, the recurrent tumor showed a dramatic response to EMA/CO even with metachronous tumors in the lung. From our analysis and experience, EMA/CO can be considered a candidate for first-line treatment of recurrent or unresectable primary gastric choriocarcinoma.

RFA has been gaining popularity rapidly as a treatment for lung cancer[37]. In recent years, RFA has been used to treat oligometastases and oligo-recurrences of metastatic lung cancer such as colorectal carcinoma, hepatobiliary carcinoma, renal cell carcinoma, and sarcoma[37]. Oligometastasis and oligo-recurrence refer to the presence of one or a few metastatic or recurrent lesions with a controlled primary tumor[38]. The International Registry of Lung Metastasis reported that the five-year OS of patients with complete resection of metastatic lung tumors was 36%, compared with 13% for patients without resection[39]. Furthermore, for patients whose lung metastases were completely resected, survival depended on the number of tumors, *i.e.*, fewer metastatic lesions indicated better survival. Such data may provide the rationale for using local therapy including RFA for oligometastases and oligo-recurrences. In the present case, since the recurrent tumors in the liver and lungs were well-controlled by EMA/CO, we treated the oligometastatic tumor in the lung with RFA. The patient was disease-free for nine years without additional chemotherapy after RFA. The present case is the first successful case report of metastatic choriocarcinoma of the lung treated by RFA in the world.

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CASE REPORT

Radical excision of Barrett's esophagus and complete recovery of normal squamous epithelium

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Abstract

To treat Barrett's esophagus (BE), radiofrequency ablation or cryotherapy are effective treatments for eradicating BE with dysplasia and intestinal metaplasia, and reduce the rates of Barrett's esophageal adenocarcinoma (BAC). However, patients with BE and dysplasia or early cancer who achieved complete eradication of intestinal metaplasia, BE recurred in 5% within a year, requiring expensive endoscopic surveillances. We performed endoscopic submucosal dissection as complete radically curable treatment procedure for BE with dysplasia, intestinal metaplasia and BAC.

Key words: Barrett's esophagus; Radiofrequency ablation; Cryotherapy; Endoscopic submucosal dissection; Radically curable treatment

Core tip: Radiofrequency ablation or cryotherapy is effective for eradicating Barrett's esophagus (BE) with dysplasia; however, it recurs in 5% in a year. Endoscopic submucosal dissection is a complete radically curable treatment procedure for BE with dysplasia and Barrett's esophageal adenocarcinoma.

Mori H, Kobara H, Rafiq K, Nishiyama N, Fujihara S, Ayagi M, Yachida T, Kato K, Masaki T. Radical excision of Barrett's esophagus and complete recovery of normal squamous epithelium. *World J Gastroenterol* 2013; 19(31): 5195-5198 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5195.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5195

INTRODUCTION

Barrett's esophagus (BE) is a condition of the esophageal mucosa where the esophageal squamous epithelium is replaced with columnar epithelium because of prolonged reflux of gastric acid and bile acid into the esophagus. However, the definition of the esophageal gastric junction (EGJ) is different between Japan and other countries and remains controversial. In Japan, EGJ is defined as the distal limit of the lower esophageal palisade vessels, but as the proximal margin of the gastric folds (Prague criteria). The japanese criteria may be more suitable for the definition of EGJ and for the diagnosis of endoscopic BE than other criteria[1]. Moreover, pathologically, the need to identify goblet cells in esophageal biopsies of BE is also controversial. Morphological evaluation of EGJ biopsies cannot distinguish whether the columnar epithelium comes from the distal esophagus or the proximal stomach[2,3]. There is also some controversy with regard to the definition, classification and histological findings and grading of dysplasia on BE[4]. The consensus indication for the treatment of BE are histological findings of

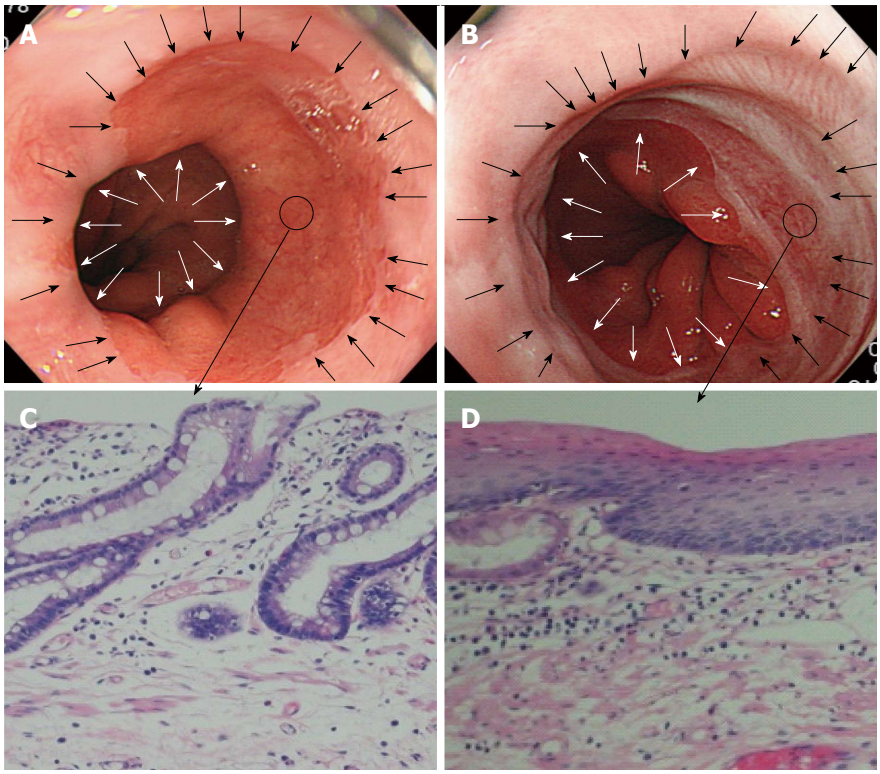

Figure 1 Short segment Barrett's esophagus undergoing endoscopic submucosal dissection. A: Short segment Barrett's esophagus (SSBE) predominantly of the right sidewall. The white arrows indicate the distal side, the black arrows indicate the proximal side, and the black encircled area indicates the preoperative biopsy site; B: After circumferential resection of SSBE with endoscopic submucosal dissection: regenerating squamous epithelium is seen between the distal side (white arrows) and the proximal side (black arrows). The black encircled area indicates the postoperative biopsy site; C: Specialized columnar epithelium with the preoperative intestinal metaplasia (hematoxylin and eosin stain, × 200); D: Postoperative regenerating squamous epithelium. Specialized columnar epithelium is not seen (hematoxylin and eosin stain, × 200).

high-grade dysplasia (HGD) by biopsies. After diagnosis of intestinal metaplasia of specific columnar epithelium and HGD, radio frequency ablation (RFA) or cryotherapy is performed because of the increased risk of Barrett's adenocarcinoma (BAC) associated with HGD[5]; however, the recurrence rate of BE is 5% within a year[6-9]. This indicates that even patients who underwent RFA require closer endoscopic surveillance. As a complete radically curable treatment, we performed entire circumference resection by endoscopic submucosal dissection (ESD) and subsequent steroid treatment. The application and permeation with balloon dilatation were performed to prevent stenosis[10]. We report two cases of en bloc resection of BE and BAC with ESD, in which the patients showed a complete recovery of normal squamous epithelium without recurrence.

CASE REPORT

Case 1

A 40-year-old man, whose mother died of BAC associated with BE, underwent screening with esophagogastroduodenoscopy and was diagnosed with circumferential short segment BE (SSBE), which was the same condition that caused his mother's death (Figure 1A and C). We could not detect severe dysplasia by biopsies before ESD. However, the patient insisted upon radical resection of SSBE. Moreover, narrow band imaging magnified endoscopy revealed irregular microvascular and microsurface patterns, which prompted us to recommend ESD. The patient underwent *en bloc* resection with ESD because he chose to undergo excision of SSBE with ESD to prevent BAC. For the resection line, the distal side was the border of the palisade vessel and the adoral fold, and the proximal side was 10 mm proximal to the columnar epithelium. Circumferential resection was performed with ESD. On days 5, 8, 12, 15 and 20 after ESD, steroid application and permeation with balloon dilatation were performed to prevent stenosis. We used triamcinolone acetonide (TA) gel as the steroid application. The TA gel was made and applied as follows: total of 10 mL TA (100 mg) was mixed with 7.5 mL Endolubri jelly (Olympus Medical Systems, Tokyo, Japan) to make 17.5 mL of gel. Beginning at the distal side of the artificial ulcer, steroid gel was applied to the ulcer floor while pulling the scope out spirally to the proximal side of the ulcer, using a spraying tube to apply the steroid gel precisely. Subsequently, 5 min of balloon dilatation (12-15 mm in diameter) was performed immediately to allow the steroid gel to permeate into the artificial ulcer. Three months after ESD and steroid treatment, squamous epithelium without stenosis was recovered at the excision site. The site returned to normal stratified squamous epithelium, which was confirmed by a biopsy of the regenerating squamous epithelium after resection

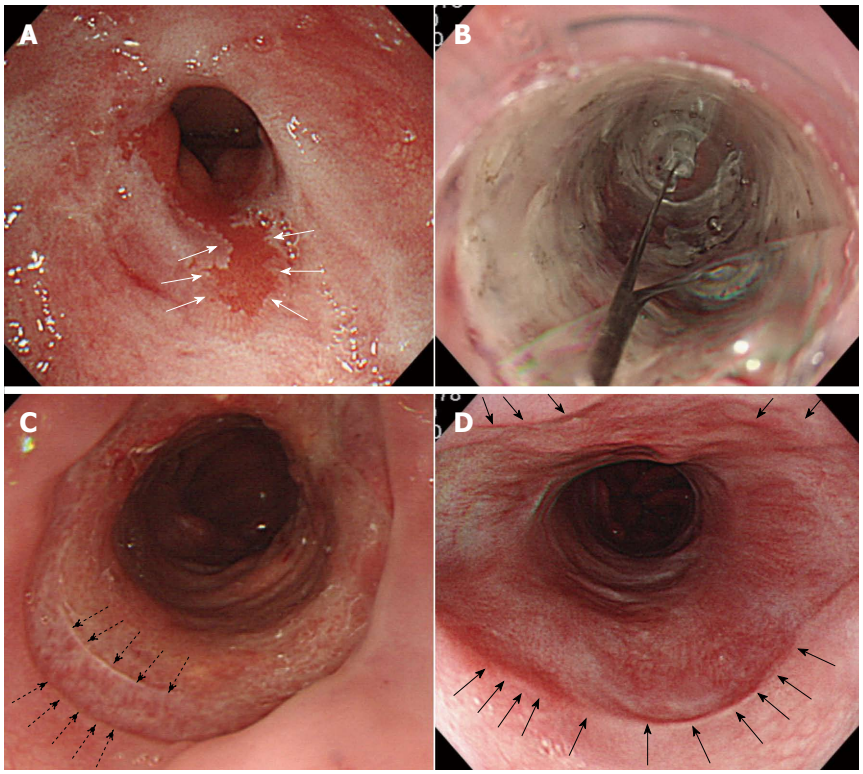

Figure 2 Barrett's adenocarcinoma with high-grade dysplasia and severe stenosis. A: Barrett's adenocarcinoma (white arrows) with high-grade dysplasia and severe stenosis of the lower esophagus; B: On days 5, 8, 12, 15 and 20 after endoscopic submucosal dissection, steroid application and balloon dilatation were performed to prevent stenosis; C: The base of the artificial ulcer on day 12 after surgery. Regenerating squamous epithelium can be seen from the resection margins of the proximal side indicated by the dotted arrows; D: On day 60, the base of the artificial ulcer is thoroughly covered with regenerating squamous epithelium (black arrows). No recurrence has been observed for 5 years.

(Figure 1B and D). This patient received only a proton pump inhibitor and has not relapsed for 4 years.

Case 2

A 65-year-old woman presented with a 20-year history of dysphagia associated with reflux esophagitis. BAC with HGD and severe stenosis of the lower esophagus was diagnosed (Figure 2A). The patient underwent ESD resection of BE and BAC and circumferential resection of the site of the stenosis. After ESD, steroid application and balloon dilatation were performed to prevent stenosis (Figure 2B). BAC was excised and BE was replaced with squamous epithelium (Figure 2C). The normal stratified squamous epithelium was observed at the excision site 6 mo after ESD, which was confirmed by a biopsy (Figure 2D). Narrow band imaging magnified endoscopy also showed a normal intraepithelial papillary capillary loop (IPCL type I). The patient has had no recurrence for 5 years. In addition, high-grade stenosis of the lower esophagus almost disappeared.

DISCUSSION

The cancerization rate and therapy of BE are still controversial[6,7]. Some researchers have reported lower incidences of dysplasia and BAC among patients with non-dysplasic BE, and most patients were cancer free after a long-term follow-up. Therefore, endoscopic surveillance intervals should be lengthened[8]. Others reported that the incidence of BAC in patients with BE was 30 times more frequent than in the general population[9]. Although closed endoscopic random biopsy is recommended for BE including HGD to detect BAC at an early stage, cancer detection is impossible unless BAC is at the site of biopsy[10,11]. RFA is an established treatment for BE with dysplasia. Although the short-term results of RFA have been determined, there have been concerns about recurrence of BE after RFA. It is reported that for BE treated by RFA, 56% were in complete remission after 24 mo. However, 33% of these patients had disease recurrence within the next 2 years[12,13], which is a very high recurrence rate. Therefore, we hypothesized that radical excision by ESD without recurrence and stenosis is best and most complete radically curative treatment procedure for BE with dysplasia, intestinal metaplasia and BAC. In Japan, ESD is recommend for Barrett's esophageal cancer after accurate diagnosis using narrow band imaging with magnifying endoscopy because of its high curative rate. However, the esophagus is a narrow organ and healing of an artificial ulcer that occupies two thirds or more of the circumference of the esophagus may result in the formation of a significant stricture. Recently, several studies have demonstrated the effectiveness of local injection or oral administration of steroids for preventing strictures[14,15]. We developed and reported a new method for preventing post-ESD stricture by steroid application and

permeation with balloon dilatation[7]. The proximal side of the lower esophageal sphincter has a lumen with a relatively high expansion ability, and is resectable circumferentially without stenosis by steroid application and permeation with balloon dilatation, even if BE with HGD is subjected to circumferential resection with ESD. In the ESD procedure, recurrence does not occur because the procedure involves en bloc resection and the regenerating epithelium returns to normal squamous epithelium. Thus, ESD seems to be effective in the treatment of BE associated with HGD.

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CASE REPORT

Isolated splenic metastases from gastric carcinoma: A case report and literature review

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Abstract

Isolated metastases to the spleen from gastric carcinoma is very rare. Only a few cases have been reported in the literature. We herein present a case of isolated splenic metastases in a 62-year-old man, occurring 12 mo after total gastrectomy for gastric carcinoma. The patient underwent a laparoscopic exploration, during which two lesions were found at the upper pole of the spleen, without involvement of other organs. A laparoscopic splenectomy was performed. Histological examination confirmed that the splenic tumor was a poorly differentiated adenocarcinoma similar to the primary gastric lesion. The postoperative course was uneventful and the patient has been well for 9 mo, with no tumor recurrence. The clinical data of 18 cases of isolated splenic metastases from gastric carcinoma treated by splenectomy were summarized after a literature review. To our knowledge, this is the first reported case of isolated splenic metastases undergoing laparoscopic splenectomy.

Key words: Metastasis; Splenic neoplasms; Stomach neoplasms; Laparoscopy; Splenectomy

Core tip: Isolated metastases to the spleen from gastric carcinoma is very rare. We report a case of isolated splenic metastases in a 62-year-old man, occurring 12 mo after total gastrectomy for gastric carcinoma who underwent laparoscopic splenectomy. The patient has been well for 9 mo after surgery with no tumor recurrence. The clinical data of 18 cases of isolated splenic metastases from gastric carcinoma treated by splenectomy were summarized after a literature review. To our knowledge, this is the first reported case of isolated splenic metastases undergoing laparoscopic splenectomy.

Zhu YP, Mou YP, Ni JJ, Zhou YC, Jiang JW, Jiang ZN, Wang GY. Isolated splenic metastases from gastric carcinoma: A case report and literature review. *World J Gastroenterol* 2013; 19(31): 5199-5203 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i31/5199.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i31.5199

INTRODUCTION

Splenic metastases from gastric carcinoma are uncommon and are generally detected as part of multi-organ metastases. Isolated splenic metastases from gastric carcinoma are exceedingly rare with only a few cases having been documented in the literature. Here, we report a case of isolated splenic metastases, occurring 12 mo after eradication of gastric carcinoma, which was successfully

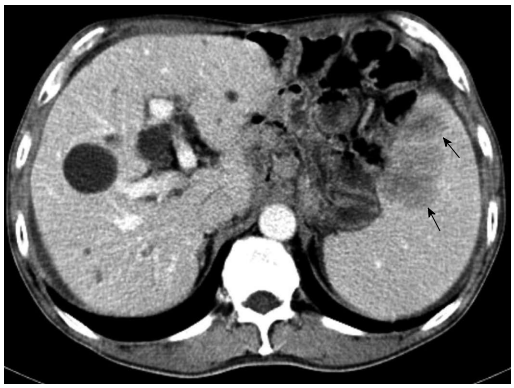

Figure 1 Computed tomography showed two low-density lesions (arrows), 4.5 cm × 3.5 cm and 2.5 cm × 2.0 cm in size respectively, at the upper pole of the spleen.

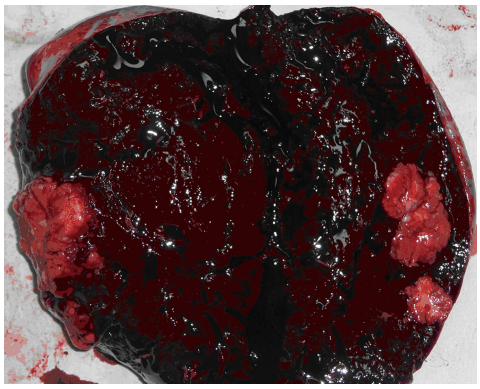

Figure 2 Cross section of the spleen showing two yellowish-white lesions at the upper pole.

treated by laparoscopic splenectomy. To the best of our knowledge, this is the first report documenting metachronous splenic metastases treated by laparoscopic surgery. In order to better understand the clinical behavior of isolated splenic metastases with gastric carcinoma origin, we reviewed a total of 18 such cases from the literature. The detailed features and prognoses of these cases were summarized in this study.

CASE REPORT

A 62-year-old man was diagnosed with gastric carcinoma located in the M and L portion of the stomach. The tumor was of ulcerative type in gross appearance (Borrmann Ⅲ type) and was 9 cm × 8 cm in size. The patient underwent a total gastrectomy with a standard D2 lymph node dissection in February 2011. Histology of the resected specimen revealed a poorly differentiated adenocarcinoma infiltrating the serosa with nodal involvement (12 of 25 nodes were positive for metastases), fulfilling the criteria of stage ⅢB according to the American Joint Committee on Cancer TNM staging classification for carcinoma of the stomach (7th ed, 2012)[1]. The patient received six cycles of intravenous chemotherapy consisting of 5-fluorouracil, leucovorin and oxaliplatin after surgery. Ultrasonography and abdominal computed tomography

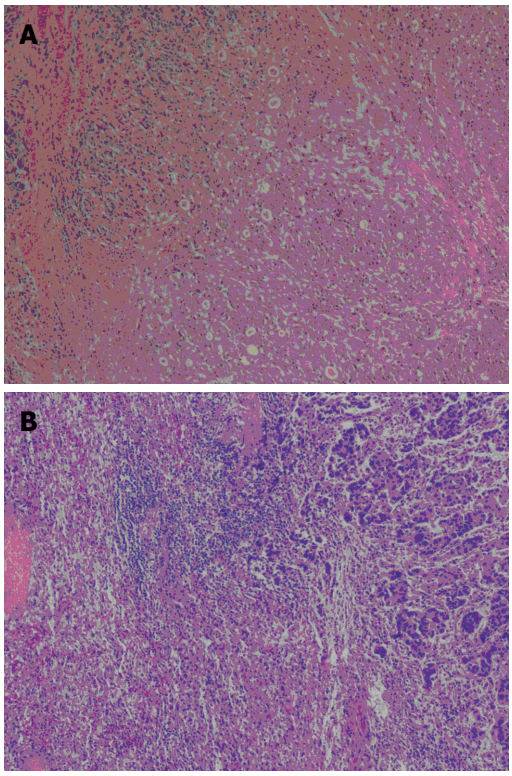

Figure 3 Histological findings of the primary gastric carcinoma (A) and the splenic metastatic tumor (B) (hematoxylin-eosin stain, × 40).

(CT) scan did not reveal any remarkable metastatic lesions during the postoperative follow-up.

In February 2012, an abdominal CT scan showed two low-density lesions, 4.5 cm × 3.5 cm and 2.5 cm × 2.0 cm, respectively, at the upper pole of the spleen without obvious contrast enhancement (Figure 1). Two hypoechoic lesions in the corresponding location of the spleen were revealed by ultrasonography. The previous history of gastric carcinoma contributed to a presumptive diagnosis of metachronous splenic metastases. The patient was given two cycles of chemotherapy consisting of intravenous 5-fluorouracil, leucovorin and oxaliplatin. A thorough diagnostic workup, including gastroscopy, CT scan of chest and abdomen, and ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography scan, was negative for extra-splenic tumor dissemination. However, ultrasound as well as CT scan revealed enlargement of the splenic lesions which indicated their poor responsiveness to the chemotherapeutics. The patient underwent a laparoscopic exploration since his splenic metastases were revealed to be isolated and resectable. The splenic lesions were confirmed during the procedure, while no other intra-abdominal organ metastasis or peritoneal dissemination was seen. A laparoscopic splenectomy was performed in April 2012. The specimen showed two lesions, measuring 4.5 cm × 4.0 cm and 3.0 cm × 2.0 cm respectively, occupying the upper pole of the spleen. The tumors were yellowish-white in color, which demarcated them quite clearly from the adjacent splenic parenchyma, and showed no bleeding or necrosis (Figure 2). Histological examination showed that the lesions were metastatic adenocarcinoma consistent with the features of the primary gastric carcinoma (Figure 3) with no lymph

Table 1 Summary of patients with isolated splenic metastasis from gastric carcinoma treated by splenectomy

No.	Source	Gender /age (yr)	Primary gastric carcinoma		Splenic metastasis			CT appearance	Survival time (mo)/status/ tumor dissemination
			Location[1]	Histology[2]	Interval[3] (mo)	Other involvement	Suggested route		
1	Takebayashi *et al*[10]	F/64	U, M	Por, T3, n1, M1	0	LNs	Lymphatic	NS	3/dead/lung
2	Fujita *et al*[11]	F/75	R	Tub, T4, n3, M1	0	LNs	Hematogenous	NS	NS
3	Mori *et al*[12]	M/49	R	Tub, T3, n3, M1	0	LNs	Hematogenous	NS	12/alive/NS
4	Okuno *et al*[13]	M/56	U, M	Por, T3, n2, M1	0	LNs and peritoneum	Lymphatic	NS	1/dead/peritoneum, bone, pancreas, adrenal, liver
5	Sakamoto *et al*[14]	M/67	U, M	Tub, T4, n2, M1	0	LNs	Hematogenous	NS	8/dead/liver
6	Ishida *et al*[15]	M/65	U	Por, T2, n2, M1	0	LNs	NS	NS	5/dead/tumor embolism of portal vein
7	Lu *et al*[16]	M/59	U	Hepatoid AC, T4	0	LNs	NS	Heterogenous low-density lesions	18/alive/liver
8	Ikeda *et al*[17]	M/57	U	Por, T3, n1, M0	17	None	NS	NS	15/alive/ NS
9	Shirai *et al*[18]	M/63	M	Pap, T1, n0, M0	33	None	Hematogenous	NS	20/alive/ NS
10	Tatsusawa *et al*[19]	M/54	M	Tub, T2, n2, M0	102	None	Hematogenous	NS	NS
11	Takahashi *et al*[20]	M/64	L	Tub, T2, n3, M0	16	None	Hematogenous	NS	7/dead/liver, lung
12	Opocher *et al*[4]	F/76	L	Tub, T2, N0, M0	57	None	Hematogenous	Round cystic area with fluid content	13/alive/none
13	Opocher *et al*[4]	M/66	NS	Por, T2, N1, M0	36	None	Hematogenous	NS	14/alive/none
14	Williams *et al*[5]	M/69	U	AC	43	None	NS	Soft tissue mass with cacification	NS
15	Yamanouchi *et al*[8]	M/69	L	Tub, T2, N1, M0	48	None	Hematogenous?	Low-density area	40/dead/liver, peritoneum
16	Sunitsch *et al*[21]	F/80	L, R	Por, T1, N0, M0; tp, T1, N0, M0	37	None	NS	NS	NS
17	Kawasaki *et al*[22]	M/76	U	Pap, T1, N1, M0	12	None	Hematogenous?	Low-density lesion	24/alive/none
18	Zhou *et al*[23]	M/76	U	Tub	36	None	NS	NS	30/alive/none

[1]U, M, and L: Indicate the upper, middle, and lower thirds of the stomach, respectively; R: Residual stomach; [2]Por: Poorly differentiated adenocarcinoma; tub: Tubular adenocarcinoma; pap: Papillary adenocarcinoma; tp: Tubulopapillary adenocarcinoma; AC: Adenocarcinoma; T1: Tumor invades lamina propria, muscularis mucosae or submucosa; T2: Tumor invades muscularis propria; T3: Tumor penetrates subserosal connective tissue without invasion of visceral peritoneum or adjacent structures; T4: Tumor invades serosa (visceral peritoneum) or adjacent structures[1]; n: n0 indicates no evidence of lymph node metastasis; n1, n2, and n3 indicate metastasis to the groups 1, 2, and 3 lymph nodes, respectively, according to the Japanese Classification of Gastric Carcinoma[24]; N: N0: No regional lymph node metastasis; N1, N2 and N3 indicate metastasis in 1-2, 3-6, 7 or more regional lymph nodes; M: M0: No distant metastasis; M1: Distant metastasis; [3]Interval: Interval from the detection of primary gastric carcinoma to the detection of the splenic secondary tumor. 0 indicates the the splenic secondary tumor detected at the same time as the primary tumor. LNs: Lymph nodes; CT: Computed tomography; NS: Not specified.

node involvement at the splenic hilum.

The patient recovered uneventfully and was discharged eight days after surgery. He recovered well and showed no evidence of tumor recurrence at the last follow-up in December 2012.

DISCUSSION

Splenic metastases from non-hematologic malignancies are infrequent, with an incidence of 0.6%-1.1% in populations with carcinoma according to a large clinicopathologic study[2]. The rarity of splenic metastases might be explained by the following reasons: (1) the poorly developed lymphoid system of the spleen, especially the lack of afferent lymphatic vessels, prevents the spleen from receiving metastatic tumor cells *via* the lymphatic route; (2) the sharp angle of splenic artery branching from the celiac trunk inhibits large clumps of tumor cells from passing through; and (3) the microenvironment of the spleen may hinder the growth of micrometastatic foci[3].

Most splenic metastases are accompanied by multivisceral tumor dissemination. Skin melanoma and carcinomas of the breast, lung, ovary, colorectum and stomach are the major primary sources of splenic metastases, and gastric carcinoma accounts for 6.9%[2]. Very few splenic metastases occur as isolated splenic lesions, synchronous or metachronous to the primary tumor. To our knowledge, only seven cases of synchronous splenic metastases and 11 cases of metachronous splenic metastases from gastric carcinoma have been treated by splenectomy to date. A summary of these cases is shown in Table 1.

Isolated splenic metastases are often first identified by ultrasonography or CT scan as most of them are asymptomatic. However, some patients harboring splenic me-

tastases complain of fatigue, weight loss, fever, abdominal pain, splenomegaly, anemia, or thrombocytopenia[3]. Generally, when an isolated splenic lesion is found during the oncologic follow-up, a metastatic origin should be suspected. Serum levels of carcinoembryonic antigen and carbohydrate antigen 19-9 have been reported to be of predictive value in detecting the appearance of isolated splenic metastases in advance of imaging identification[4]. In our review, the splenic metastases presented variously on CT scan, and ranged from a cystic lesion, low-density occupying lesion to a solid mass, and showed different patterns of enhancement. A calcified splenic mass, which is a common feature of metastases from primary mucinous adenocarcinoma, was also described[5]. In this regard, it is sometimes difficult to distinguish the suspected splenic metastases from primary splenic lesions such as lymphoma, vascular tumor, or infectious disorder. It has been reported that ^{18}F-FDG positron emission tomography was of value in distinguishing benign from malignant masses of the spleen[6]. As a highly vascular organ, the spleen is often considered an inappropriate target for fine-needle aspiration (FNA) due to the potential risk of bleeding. Nevertheless, Cavanna *et al*[7] reported a series of 160 patients who underwent biopsy of the splenic mass by FNA with an overall accuracy rate of 98.1% and with no complications, which demonstrated that the technique is safe and effective.

In the present case, two masses were detected in the spleen by imaging techniques 12 mo after a radical gastrectomy. The previous history of gastric carcinoma indicated the diagnosis of a splenic metastasis which was subsequently supported by an ^{18}F-FDG positron emission tomography scan. Ultimately, the pathological study of the surgical specimen, which showed a papillary adenocarcinoma with a high morphological resemblance to the primary gastric carcinoma, confirmed our clinical diagnosis.

Gastric carcinoma in the U or M portion is likely to invade the spleen directly because of their anatomical contiguity. In such cases, the metastatic lesions are always detected synchronously or very shortly after a radical gastrectomy. In contrast, splenic metastases are often detected later when they are caused by the hematogenous route. In our literature review, the mean time from the diagnosis of primary stomach carcinoma to the development of secondary splenic metastases was 39.7 mo with the longest time being 102 mo[8]. The late occurrence of blood-borne isolated splenic metastases could be explained by some recent advances in the knowledge of the metastatic mechanism: it may develop from an early micro-metastasis within the spleen and progress to an observable lesion after a period of clinical latency[9]. We also noted that the mean duration from the detection of the primary gastric tumor to the detection of the splenic secondary tumor was shorter in Lam and Tang's review (3-36 mo, mean 8 mo)[2]. We speculate that there might have been a proportion of the splenic metastases in their analysis which were caused by direct invasion from primary gastric tumors, whereas there was not a single case in our review which was reported to be caused by direct invasion. This might explain the time difference between the two reviews.

As isolated splenic metastases from gastric carcinoma are rarely documented in the literature, it is still difficult to predict the clinical behavior of this disease. However, the existing preliminary records show a tendency that long-term remission could be expected after splenectomy in a metachronous splenic metastasis, while a synchronous splenic metastasis always indicates early tumor progression and a worse outcome. In our review, patients with gastric carcinoma and synchronous splenic metastases had a mean post-operative survival time of seven months, apparently shorter than that of the patients with metachronous splenic metastases (mean 20.3 mo after splenectomy).

Although splenectomy provides a possible means of radical treatment in patients with isolated splenic metastases, it should be decided with caution as a splenic metastatic lesion which is supposed to be "isolated" sometimes may represent an initial clinical manifestation of systemic metastases at multiple sites. Under such circumstances, surgical stress from splenectomy might cause adverse clinical effects in the patient. With this in mind, following the discovery of the splenic lesions in the present patient, rather than performing splenectomy immediately, he was given two cycles of chemotherapy and this time span was used for observation as well. As no new metastatic lesions emerged, a laparoscopic exploration and splenectomy was performed.

As a result of previous surgery, there was severe adhesion in the upper quadrant of the abdomen, especially between the transverse colon and the spleen, which needed careful separation. Conversely, the isolation of the splenic pedicle was not as difficult, as the connective tissue in this region had been removed and all the short gastric vessels had been severed during the previous operation of total gastrectomy. To the best of our knowledge, this is the first report documenting metachronous splenic metastases treated by laparoscopic surgery. The patient's postoperative course was uneventful and he has been well with no evidence of tumor recurrence for nine months.

According to our experience, laparoscopic splenectomy seems to be a promising approach in achieving long-term survival in patients with metachronous isolated splenic metastases after eradication of gastric carcinoma.

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Chronic pancreatitis as presentation of Crohn's disease in a child

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Abstract

It is reported that a pancreatic disease may precede the diagnosis of inflammatory bowel disease (IBD) both in children and in adults. Idiopathic chronic pancreatitis, however, occasionally co-exists with the IBD, mainly at pediatric age. We report a case of a patient who progressively developed the features of a chronic pancreatitis, before the diagnosis of Crohn's Disease (CD). Ten months after the onset of the first episode of pancreatitis the patient developed bloody diarrhea, mucus stools and biochemical findings of inflammation. The colonoscopy revealed a diffuse colitis without involvement of the last loop and the gastroscopy showed inflammation of the iuxta-papillary area. The histological findings confirmed the diagnosis of CD that involved the colon and the duodenum. In conclusion, in children the idiopathic chronic pancreatitis may be an unusual presentation of CD. Thus, if other known cause of chronic pancreatitis are not found, a not invasive work up to exclude the IBD should be warranted. An early coincidental diagnosis of the IBD may delay the progression of the pancreatic disease.

Key words: Inflammatory bowel disease; Crohn's Disease; Pediatric age; Bloody diarrhea; Pancreatic disease

Core tip: We report a cases of chronic pancreatitis associated with Crohn's Disease (CD). We have not been able to find reports of this association in the pediatric medical literature. The present case suggests that in children the idiopathic chronic pancreatitis may be an unusual presentation of CD. Thus, if other known cause of chronic pancreatitis are not found, a not invasive work up to exclude the inflammatory bowel disease should be warranted. The early recognition of the CD, indeed, may help in delay the progression of the pancreatic disease.

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TO THE EDITOR

It is well known that pancreas can be involved in the course of inflammatory bowel diseases (IBD)[1]. The pancreatic disease can occur in cases of biliary lithiasis or of the administration of 5-aminosalicylates (5-ASA), corticosteroids, and azathioprine (AZA) or 6-mercaptopurine[1,2]. Most cases of pancreatitis are clinically silent

and the frequency of clinical pancreatitis is markedly lower than that of asymptomatic hyperamylasemia or of evidence of exocrine pancreas insufficiency[1]. The potential association of the IBD with the pancreatic diseases makes therefore a periodic assessment of the pancreatic function in all patients affected by IBD advisable. A pancreatic disease may also precede the onset of the IBD. It is shown that an acute pancreatitis may represent the picture of presentation of the IBD in children such as in adults[3]. As recently reported by Broide *et al*[3] the prevalence of acute pancreatitis as symptom of the onset of the IBD is 2.17% in children and 0.06 in adults[3]. Two previous reports found a prevalence of acute pancreatitis preceding IBD in 27%[4] and in about 5%[5] of the cases. Therefore it was suggested[3] that in children, after an episode of acute pancreatitis, specific attention should be paid to other IBD susceptibility factors, that could indicate investigations by colonoscopy and gastroscopy. Idiopathic chronic pancreatitis, on the other hand, was occasionally reported in association with the IBD, mainly at pediatric age[6-8]. We report a case of a patient who progressively developed the features of a chronic pancreatitis, before the diagnosis of Crohn's disease (CD). When she was 4 years old, she was admitted with abdominal pain, slightly raised C-reactive protein and of erithro-sedimentation rate and high serum level of amylase and lipase. No gallstones were found, but only edema and enlargement of the pancreatic gland were reported on abdominal ultrasound. She received the treatment of the acute pancreatitis (intra-venous fluids, bowel rest, antibiotic, protease and gastric acid inhibitors), with beneficial effects on the pain and of the biochemical indices. Thereafter, she developed numerous episodes of acute pancreatitis. Therefore we planned the imaging and blood examinations to exclude all the causes of the chronic and recurrent pancreatitis. Serological tests for Cytomegalovirus, Epstein-Barr virus, Echovirus, Rubella, Adenovirus, Coxsackie virus, *Legionella* and *Mycoplasma* resulted negative. Immunoglobulin G serum level was into the normal range, so to exclude an autoimmune pancreatic process. The sweet test was negative. Genetic analysis for the mutations of cystic fibrosis transmembrane conductor regulator gene, for the cationic trypsinogen (*PRSS1*) gene, and serine protease inhibitor Kazal type 1 (*SPINK1*) were all negative. The magnetic resonance cholangiopancreatography showed a picture of chronic pancreatitis. This result along with the persistence of severe abdominal pain led us to schedule an endoscopic retrograde cholangiopancreatography to have a clear pancreatogram and therefore to define the chronic process but also to perform the pancreatic sphinterectomy[9]. The sphinterectomy was followed by partial extraction of the pancreatic concretions and by the placement of a plastic stent into the main pancreatic duct for transpapillary drainage. The pancreatogram showed pancreatic calcifications and the distortion of the main pancreatic duct, both findings consistent with established chronic pancreatitis. The sphinterectomy and the placement of the stent reduced the abdominal pain but did not impede the course of the pancreatic failure that required the enzymes replacement. Ten months after the onset of the first episode of pancreatitis the patient developed bloody diarrhea, mucus stools and relevant increase of inflammation indices. The following not invasive work up to define the suspected IBD showed positive anti-saccaromyces cerevisiae antibodies and fecal calprotectin. Stool cultures and stool test for *Clostridium difficile* toxins A and B were all negative. Upper and lower endoscopies were therefore scheduled. Colonoscopy revealed a diffuse colitis without involvement of the last loop. The gastroscopy revealed a duodenal involvement, with inflammation of the iuxta-papillary area. Then the histological examination confirmed the diagnosis of CD localized in the colon and in the duodenum. Furthermore the nutritional treatment with an amino-acid based formula by naso-gastric tube induced a partial regression of the intestinal symptoms that relapsed when she restarted a diversified oral nutrition. The use of 5-ASA and of AZA determined an immediate increase of the pancreatic enzymes; thus the patient received corticoid alone, that improved the intestinal symptoms, without influencing the pancreatic function. The clinical phenotype of the CD in this patient was very severe, with several relapses requiring repeated courses of steroid treatment and therefore we began the biological drugs. We started with infliximab but, after the second administration that induced a severe anaphylactic reaction, we temporary suspended this treatment and we started a period of bowel rest by home parenteral nutrition. The following re-exacerbations were treated by short courses of steroids that also showed beneficial effects on the pancreatic exacerbations, with immediate regression of the pancreatic pain and of the inflammatory indices. When she was 13 years old she begun the adalimumab that determined a prolonged period of remission. The girl is now 16 years old, she reached the pubertal development and she is treated by adalimumab, without severe re-exacerbations of the intestinal disease. The pancreatic function is supported by the pancreatic enzymes. To our knowledge in literature are reported 16 cases of chronic pancreatitis associated with CD[5-8] and none of them occurred at pediatric age. Therefore we report the first pediatric case of chronic pancreatitis as picture of presentation CD. In our experience this is the first case of CD presenting as chronic pancreatitis and therefore we may consider this association very rare according with the literature data. It' s not clear if the pancreatic inflammation may be a metastatic presentation of CD or the complication of the duodenal involvement[1]. In our patients the CD-associated pancreatitis was due to the duodenal and to the iuxta-papillary area involvement, causing duodenal reflux and papilla obstruction. In this case the diagnosis of IBD was made only 10 mo from the onset of the pancreatic complaints, when the patient had already developed the intestinal signs suggestive of intestinal inflammation. When the CD was recognized the pancreatic disease had already progressed towards a chronic relapsing process

with intractable pain and exocrine pancreas insufficiency. We may speculate that in this case the earlier diagnosis of CD might reduce the severity of the pancreatic disease, delaying the course of the pancreatic failure. Both pancreatic pain and biochemical inflammation recovered indeed after short treatments with steroids, so confirming that the progression of the pancreatic disease might be influenced by a timely diagnosis of CD. In conclusion in children the idiopathic chronic pancreatitis may be an unusual presentation of CD. Thus, if other known cause of chronic pancreatitis are not found, a not invasive work up to exclude the IBD should be warranted. An early coincidental diagnosis of the IBD may delay the progression of the pancreatic disease.

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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

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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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

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Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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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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