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EDITORIAL

Gastrointestinal and hepatic complications of hematopoietic stem cell transplantation

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Abstract

Recognition and management of gastrointestinal and hepatic complications of hematopoietic stem cell transplantation has gained increasing importance as indications and techniques of transplantation have expanded in the last few years. The transplant recipient is at risk for several complications including conditioning chemotherapy related toxicities, infections, bleeding, sinusoidal obstruction syndrome, acute and chronic graft-versus-host disease (GVHD) as well as other long-term problems. The severity and the incidence of many complications have improved in the past several years as the intensity of conditioning regimens has diminished and better supportive care and GVHD prevention strategies have been implemented. Transplant clinicians, however, continue to be challenged with problems arising from human leukocyte antigen-mismatched and unrelated donor transplants, expanding transplant indications and age-limit. This review describes the most commonly seen transplant related complications, focusing on their pathogenesis, differential diagnosis and management.

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Key words: Stem cell transplantation; Graft-versus-host disease; Sinusoidal obstruction syndrome; Complications

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INTRODUCTION

The indications of both autologous and allogeneic hematopoietic stem cell transplantation have expanded over the past decade including for malignant and nonmalignant disorders[1,2]. Transplant clinicians routinely encounter gastrointestinal and hepatic disorders and complications before, during and after the transplant. The outcome of transplant is often closely related to how well these complications are managed. This review provides a current overview of these disorders including pathogenesis, clinical diagnosis and management.

Table 1 Emetogenic potential of chemotherapeutic agents used in stem cell transplantation

	Chemotherapeutic agent
High (90%) emetogenic risk	Carmustine > 250 mg/m^2
	Cyclophosphamide > 1500 mg/m^2
Moderate (30%-90%) emetogenic risk	Busulfan
	Cytarabine > 200 mg/m^2
	Melphalan
Low (10%-30%) emetogenic risk	Etoposide
Minimal (< 10%) emetogenic risk	Fludarabine
	Rituximab

EVALUATION OF TRANSPLANT CANDIDATE

Patients referred for hematopoietic stem cell transplantation undergo a detailed evaluation including history and physical examination which encompasses the history of disease requiring transplant as well as pre-existing conditions including dental problems, vaccination, travel, blood transfusion and infectious disease exposure history[3,4]. Imaging studies including computerized tomography (CT) with/without positron emission tomography scan or magnetic resonance imaging (MRI) can be performed to localize and restage the malignancy. Infectious disease markers including human immunodeficiency virus (HIV)-1 and (HIV)-2, human T-cell leukemia virus (HTLV)-1 and (HTLV)-2, hepatitis B virus (HBV) and hepatitis C virus serologies, cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV)-1 and (HSV)-2, varicella zoster virus (VZV) and rubella titers are checked within 30 d prior to transplant admission. In addition to routine blood count, liver function and coagulation studies are obtained. Donors for allogeneic transplant also undergo the same serologic testing within 30 d of stem cell collection. Donors with viral hepatitis B and hepatitis C pose the risk of disease transmission to the recipient up to 30% and 100% respectively[5,6]. The risk of fatal HBV infection in recipients who become HBsAg positive is about 12%[5] By contrast, HCV transmission during transplant does not usually pose an increased short or mid-term clinical risk to the recipient yet does increase the long-term risk of cirrhosis[7]. Therefore donors should be treated with anti-viral agents; pegylated interferon α (IFN-α), famciclovir or lamivudine for hepatitis B and IFN-α plus ribavirin for hepatitis C before stem cell collection if time permits[8,9]. Of note, IFN-α should be stopped at least 1 wk before stem cell collection to avoid engraftment problems.

Patients with existing nausea, heartburn, dysphagia, abdominal pain, diarrhea, Crohn's disease or ulcerative colitis should be investigated with endoscopy prior to transplant to rule out mucosal ulcers and infections as the risk of bleeding is increased during the periods of thrombocytopenia.

Abnormal liver enzymes and organomegaly should be investigated with ultrasound, CT or MRI. Liver biopsy is indicated in patients with positive hepatitis B surface antigen or hepatitis C antibody to rule out hepatic fibrosis or cirrhosis which increases the risk of fatal sinusoidal obstruction syndrome (SOS)[10]. Evidence of advanced liver fibrosis is a contraindication to proceed to stem cell transplantation because of excess transplant-related mortality. Pre-existing liver dysfunction can be secondary to viral hepatitis as well as alcoholic or non-alcoholic steatohepatitis, iron overload, fungal infection, chemotherapy-induced cholestatic injury or hepatocyte damage, fibrosis, extramedullary hematopoiesis or prior liver irradiation[11]. Myeloablative conditioning regimens, recent exposure to alkylating agents (especially cyclophosphamide) and exposure to newer drugs, such as imatinib and gemtuzumab ozogamicin, are known associations with increased risk of SOS[12-14].

Hepatitis B infected transplant candidates are at risk for hepatitis flare and fulminant hepatitis can occur in up to 50% of transplant recipients in the absence of antiviral prophylaxis[15,16]. In the presence of isolated HBV core antibody, observation or prophylaxis are both acceptable approaches. If HBV surface antigen is detected, it is usually recommended that prophylaxis with oral nucleoside therapy is initiated prior to transplant and that HBV DNA levels are monitored frequently during the post-transplant period[17]. Even in the absence of cirrhosis, HCV infected patients have an increased risk of SOS especially if pre-transplant aspartate aminotransferase is elevated[18]. There is no effective prophylaxis or treatment of hepatitis C for transplant recipients as pegylated IFN-α is contraindicated due to myelosuppression and has the potential to exacerbate graft-versus-host disease (GVHD). Ribavirin alone can be tried while patient is on immunosuppressive therapy.

Patients with conditions causing transfusion dependency such as myelodysplastic syndrome, leukemia, lymphoma and aplastic anemia should be screened for iron overload as excess iron can impair Kupffer cell function and increase the risk for mold infections[19]. The excess iron can be demonstrated either by quantification of iron in liver biopsy tissue or MRI of the liver. Patients with severe iron overload can benefit from chelation pre-transplant. However the urgent need for the transplant may preclude this option. The relationship between iron overload and transplant-related toxicity has not been well established and in most cases chelation can be postponed after the transplant.

NAUSEA AND VOMITING

Many chemotherapeutic agents, with or without total body irradiation used in the conditioning regimens, have significant emetogenic potential (Table 1). The pathogenesis includes stimulation of the chemotherapy trigger zone in the brainstem which activates the vomiting center by increasing efferent output to target organs in the gastrointestinal tract, resulting in subsequent emesis. Chemotherapy also causes cell damage in the gastroin-

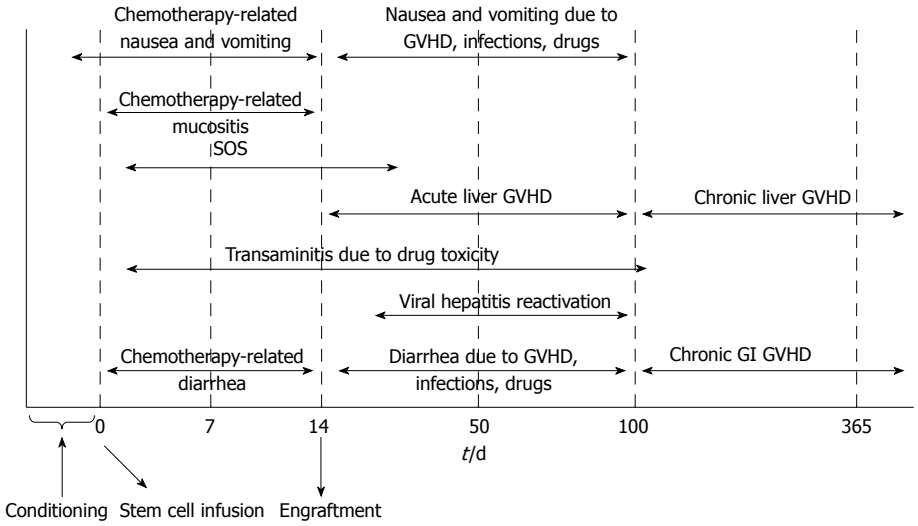

Figure 1 Timeline summary of hepatic and gastrointestinal complications of stem cell transplantation. GVHD: Graft-versus-host disease; SOS: Sinusoidal obstruction syndrome; GI: Gastrointestinal.

testinal (GI) tract, resulting in the release of neuroactive agents and vagal stimulation, increasing afferent input to the chemotherapy trigger zone and the vomiting center in the brainstem.

Patients usually experience the chemotherapy related side effects during the early post-transplant (15 d) period before engraftment (Figure 1). Nausea and vomiting in later phases may be due to other potential etiologies including upper GI acute GVHD and infections such as HSV, VZV, CMV, adenovirus, fungus and *Helicobacter pylori*. Persistent or recurrent nausea not responsive to routine anti-emetic regimens should be investigated further for GVHD with upper GI endoscopy which may show mucosal edema and erythema and biopsy findings consistent with local lymphocytic infiltrates and epithelial apoptosis[20,21]. Specimens should also be studied for bacterial or fungal cultures, HSV and CMV infections as viral infections can be detected in the GI tract without the presence of virus DNA in serum. Other common medical etiologies such as medication intolerance, gastroparesis, intestinal obstruction, intraabdominal infections, neurologic and metabolic causes should also be considered.

Prevention is the key to success in managing nausea and vomiting during the peri-transplant period[22]. Acute emesis prevention (up to 24 h after chemotherapy) can be achieved with a combination of corticosteroids (dexamethasone 10-20 mg iv/po daily or methylprednisolone 40-125 mg iv/po daily) and 5-hydroxytryptamine-3 receptor antagonists (ondansetron 16-24 mg iv/po daily or granisetron 1-2 mg daily). Delayed emesis (up to 5 d after treatment) can usually be prevented with corticosteroids. Aprepitant neurokinin-1 antagonist is an effective agent for this purpose; however it may interact with several post-transplant immunosuppressive agents and therefore is sparingly and cautiously used, especially in the allogeneic stem cell transplant setting.

Treatment options for breakthrough nausea and vomiting include phenothiazines (prochlorperazine, promethazine), metoclopramide, lorazepam, haloperidol, dronabinol and corticosteroids[23].

OROPHARYNGEAL MUCOSITIS AND DYSPHAGIA

Breakdown of mucosal barrier presenting as mucositis is a common complication during the early post-transplant period. It affects up to 80% of transplant recipients, especially with radiation-based myeloablative regimens[24]. Chemotherapeutic agents commonly causing mucositis include busulfan, etoposide, melphalan and methotrexate. Pre-existing periodontal disease and prior radiation to the head and neck area increase the risk of post-transplant complications. Mucositis can result in significant oral pain and dysphagia, decreased oral caloric intake as well as bleeding, infection, upper airway edema and obstruction. Clinically apparent mucositis usually starts 5-10 d after initiation of the conditioning regimen (Figure 1). Initial erythema and atrophy is followed by ulceration and healing phases. It may take up to two weeks for healing of chemotherapy-induced mucositis.

Infectious causes of mucositis include CMV, HSV, VZV, varicella zoster virus, Candida species and bacterial pathogens. The incidence of viral and fungal infections has been significantly lower since the standardization of antiviral and antifungal prophylaxis regimens. Other noninfectious causes of dysphagia should be included in the differential diagnosis such as acid-reflux disease, pill esophagitis and acute and chronic GVHD with esophageal strictures.

Prevention and early treatment is critical to minimize the duration and severity of symptoms. Frequent mouth rinsing with topical agents and oral cryotherapy with ice chips are started with chemotherapy and continued until

Table 2 Differential diagnosis of post-transplant diarrhea

Conditioning regimen-related
Acute GVHD
Drug toxicity
Antibiotic-related
Opioid withdrawal
Mycophenolate mofetil toxicity
Tacrolimus (thrombotic microangiopathy)
Proton pump inhibitors
Promotility agents
Magnesium salts
Metoclopramide
Infectious
Clostridium difficile
CMV
Rotavirus
Adenovirus
EBV
HSV
Astrovirus
Norovirus
Bacterial infections including ESBL
Fungal infections
Parasitic infections (*Cryptosporidium, Microsporidia, Giardia*)
Mycobacterial infections
Others
Lactose intolerance
Malabsorption
Pancreatic insufficiency

CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HSV: Herpes simplex virus; GVHD: Graft-versus-host disease; ESBL: Extended spectrum β lactamase.

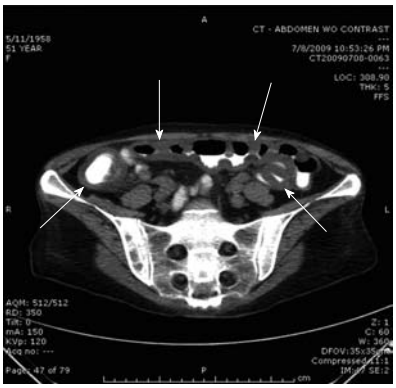

Figure 2 Bowel wall edema in a patient with gastrointestinal graft-versus-host disease.

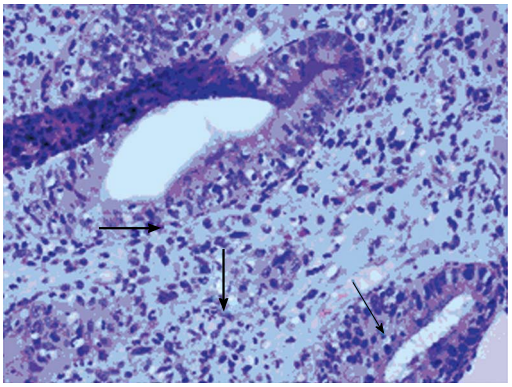

Figure 3 Histologic findings of acute graft-versus-host disease of the colon (hematoxylin and eosin stain, x 400). Thin arrow marks apoptotic bodies; thick arrow marks pericryptal acute inflammation.

engraftment. Keratinocyte growth factor (palifermin) has been shown to decrease the incidence of mucositis by 40% in patients receiving autologous stem cell transplant with aggressive total body irradiation (TBI)-based regimens[25,26]. It is administered iv for 3 d before and after cytotoxic therapy. Other supportive measures include saline and bicarbonate rinses, mucosal coating agents (such as aluminum hydroxide), topical anesthetics such as lidocaine rinse and/or narcotic analgesia, topical nystatin for signs of candidiasis and proton-pump inhibitor prophylaxis. Total parenteral nutrition should be considered for patients who are unable to tolerate oral supplementation for more than 7 d.

DIARRHEA

Diarrhea occurs in almost half of patients receiving high-dose chemotherapy conditioning and radiotherapy. It is most commonly associated with toxicity of conditioning regimens within the first 2 wk after transplant (Figure 1). Alkylating agents, busulfan and combination regimens are frequent etiologies and cause diarrhea due to mucosal inflammation. Several other etiologies should be considered in patients having diarrhea in the post-transplant period (Table 2). Acute GVHD is the most common reason for diarrhea after engraftment (> 15 d) in allogeneic transplants[27]; persistent or new diarrhea beyond 3 wk of transplant should be investigated for GVHD. The diarrhea with GVHD can be watery, mucoid and in large volumes; can be accompanied by vomiting, gastrointestinal bleeding and severe abdominal pain[28]. Infectious etiologies account for only 10%-15% of cases, yet diarrhea at any time after transplant should still prompt obtaining stool studies for *Clostridium difficile* toxin[29,30] as well as bacterial, viral and parasitic cultures if indicated. Abdominal imaging with CT may show bowel wall edema and/or pneumatosis intestinalis which may be associated with either GVHD or CMV infection (Figure 2). If cultures are negative, patients are usually treated with loperamide 4 mg po once followed by 2 mg/24 h as needed up to 24 mg/24 h. If diarrhea persists, strategies include scheduling loperamide every 4-6 h, adding atropine and diphenoxylate or tincture of opium. Octreotide starting at 150 mg iv every 8 h can be considered for protracted cases and can be titrated to response[31]. Other critical measures include maintaining adequate hydration and electrolyte supplementation, treating infections, discontinuation of medications causing diarrhea and assessment of nutritional status. Persistent symptoms despite the above measures and/or new diarrhea presenting after engraftment should be investigated with endoscopy and biopsy.

Visual findings of acute GVHD may include mucosal

Table 3 Staging of acute graft-versus-host disease (modified Keystone criteria)

Stage	Intestinal tract	Liver	Skin
0	Diarrhea ≤ 500 mL/d	Bilirubin < 2.0 mg/dL	No rash
1	Diarrhea 501-1000 mL/d or nausea (± vomiting)	Bilirubin 2.0-3.0 mg/dL	Maculopapular rash < 25% of body surface
2	Diarrhea 1001-1500 mL/d	Bilirubin 3.1–6.0 mg/dL	Maculopapular rash 25%-50% of body surface
3	Diarrhea > 1501 mL/d	Bilirubin 6.1–15 mg/dL	Generalized erythroderma
4	Severe abdominal pain +/- ileus	Bilirubin > 15 mg/dL	Generalized erythroderma with blister/bullous formation and desquamation

Table 4 Grading of acute graft-versus-host disease (modified Keystone criteria)

Grade	Gut	Liver	Skin
0 (none)	0	0	0
Ⅰ (mild)	0	0	1-2
Ⅱ (moderate)	1	1 or	3 or
Ⅲ (severe)	2-4	2-3 or	0-3
Ⅳ (life-threatening)		4	4 or

Table 5 Differential diagnosis for liver function abnormalities after hematopoietic stem cell transplantation

First 3 wk post-transplant
Drug toxicity
Conditioning regimens (cyclophosphamide, total body irradiation, bis-chloroethylnitrosourea, busulfan)
Calcineurin inhibitors
Azole antifungals
SOS
Sepsis, candidiasis
Ischemic liver disease
From 3 wk to 3 mo post-transplant
Acute GVHD
Drug toxicity
SOS
Hepatitis (fulminant, acute or chronic):
Viral (HBV, HCV, HSV, VZV, adenovirus) reactivation
Bacterial or fungal Infection
Fungal abscess
Gall bladder disease/cholecystitis
Hyperalimentation
Post-transplant lymphoproliferative disorder (EBV-related)
After 3 mo post-transplant
Chronic GVHD
Iron overload
Chronic viral hepatitis
Drug toxicity
Liver fibrosis or cirrhosis:
SOS
Viral infections
Hemosiderosis
Disease recurrence or new malignancy including hepatocellular carcinoma, lymphoproliferative disorder
Nodular regenerative hyperplasia
Gallbladder disease

GVHD: Graft-versus-host disease; SOS: Sinusoidal obstruction syndrome; EBV: Epstein-Barr virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

edema/erythema and ulceration/bleeding. The diagnostic yield is the best when biopsies are obtained either from the stomach and distal colon or from the colon and ileum[32]. Histologic findings include crypt epithelial cell apoptosis and dropout, crypt destruction (Figure 3), pericapillary hemorrhage and variable lymphocytic and eosinophilic infiltration of the epithelium and lamina propria[33]. It is also important to study the biopsy specimens for CMV involvement which is the only common infectious cause of enteritis after transplant that requires biopsy for diagnosis. The negative predictive value of other infectious studies in stools is high and therefore usually does not necessitate endoscopy.

The severity of acute GVHD is clinically determined by the amount of diarrhea which helps defining the staging and grading (Tables 3 and 4). In addition to the general management measures described above, patients with high clinical suspicion for ≥ grade Ⅱ acute GVHD or biopsy proven acute GVHD should be promptly started on high dose steroids[34]; methylprednisolone 0.5-2.0 mg/kg per day iv in addition to their existing GVHD prophylaxis regimens. Half of the patients respond to steroid treatment[35] which can be tapered starting after approximately 1 wk. Patients who do not respond to steroids tend to have a poor prognosis; several second line immunosuppressive agents have been tried with variable success[34]. Other supportive measures consist of addition of oral non-absorbable steroids (budesonide)[36] and cholestyramine as well as dietary adjustments (bowel rest and starting total parenteral nutrition) particularly for moderate to severe diarrhea.

GASTROINTESTINAL BLEEDING

The incidence of bleeding has been significantly reduced (1%-2%) as post-transplant care has improved with routine anti-viral, anti-fungal and GVHD prophylaxis, yet it remains one of the major causes of transplant related mortality, particularly among patients with GVHD[37,38]. Common infectious etiologies include CMV, VZV, adenovirus, fungal and clostridial infections. Mucosal necrosis from conditioning therapy, acute and chronic GVHD, peptic ulcer disease, mycophenolate-related ulcerations and gastric antral vascular ectasia (GAVE) are the common known etiologies of noninfectious causes of bleeding. Treatment is mainly focused on supportive care (platelet transfusion and continuous octreotide infusion) as well as implementing prophylaxis for viral infections and GVHD. Treating the underlying cause is important as the benefit of endoscopic methods alone is limited to focal lesions.

Table 6 Risk factors for sinusoidal obstruction syndrome

Existing liver disease:
Chronic viral hepatitis
Alcohol related hepatitis
Steatohepatitis
Cirrhosis, lobular fibrosis
Cholestatic disorders
Extramedullary hematopoiesis with sinusoidal fibrosis
Prior history of:
SOS
Extensive chemotherapy and stem cell transplantation
Hepatic radiation
Drugs
Recent gemtuzumab ozogamicin use
Conditioning agents:
High dose TBI (> 14 Gy)
Cyclophosphamide metabolite: acrolein
Busulfan
Melphalan
Concomitant use of sirolimus during conditioning

SOS: Sinusoidal obstruction syndrome; TBI: Total body irradiation.

LIVER FUNCTION ABNORMALITIES AND JAUNDICE

Severe liver dysfunction after transplant (total serum bilirubin > 4 mg/dL) may be an indicator of poor outcome as there is often no curative treatment[39]. Therefore, efforts are usually geared towards identifying the transplant candidates at risk as well as routine implementation of prophylactic measures such as ursodiol[40] (through 80 d post transplant), viral and fungal prophylaxis and careful selection of conditioning regimens to minimize hepatotoxicity[41]. The incidence of liver-related complications has declined significantly over the past decade with the preventive measures integrated into standard care. Some common etiologies for liver function abnormalities after transplant are summarized in Table 5. Drug toxicity, sepsis, GVHD and SOS are the most common etiologies for liver dysfunction. Calcineurin inhibitors (cyclosporine and less commonly tacrolimus, sirolimus), azole antifungal agents, trimethoprim-sulfamethoxazole, ribavirin, busulfan and bis-chloroethylnitrosourea are commonly associated with cholestasis. A declining incidence of acute GVHD is observed in 20%-25% of allogeneic transplant recipients due to widespread use of prophylactic immunosuppressive drugs, with peak incidence after engraftment (day 15) until the first 100 d of transplant[42]. It typically follows skin and/or GI GVHD and manifests by progressive parallel elevations of serum bilirubin and alkaline phosphatase; serum aminotransferase enzymes are elevated up to 10 times the upper limit of normal (Tables 3 and 4). Hepatitic-variant of GVHD has also been described where serum aminotransferase enzymes are elevated more than 10 times the upper limit of normal and clinical presentation resembles acute viral hepatitis[43]. The diagnosis is usually made by transjugular liver biopsy which typically reveals lymphocytic infiltration of small bile ducts with epithelial cell apoptosis[44]. Routine ursodiol prophylaxis is usually continued through day 80 of allogeneic transplants and reduces the incidence of GVHD[45]. The initial treatment of acute hepatic GVHD is similar to cutaneous and GI GVHD with high dose steroids as described above. However, only 30%-50% of patients respond to initial treatment and half of patients develop chronic GVHD.

SINUSOIDAL OBSTRUCTION SYNDROME

SOS (AKA veno-occlusive disease or VOD) is a clinical entity characterized by tender hepatomegaly, elevated serum bilirubin levels and weight gain which typically complicates myeloablative hematopoietic stem cell transplantation (HSCT) and was first described in 1979[46]. SOS is a well-recognized conditioning-related toxicity. The incidence is quite variable, ranging from less than 5% to as high as 70% in different reports[47,48].

Endothelial injury appears to be the initiating event triggering the hepatic changes and clinical manifestation of SOS. The prevailing hypothesis centers on damage to the hepatic venular and sinusoidal endothelium as an initial trigger inducing a hypercoagulable state by activation of the coagulation cascade, favoring clot formation over natural anticoagulation[49,50]. As a result, the venular and sinusoidal lumen is reduced due to an edematous concentric subendothelial zone containing fragmented red cells and fibrillar material, inducing partial to complete fibrotic obliteration of the venular lamina.

There are several risk factors for SOS and patients may present with elevated transaminase levels prior to the conditioning regimen due to various pre-existing conditions (Table 6). Previous cumulative exposure to high doses of cytotoxic agents may contribute to these risks including a second HSCT. Infection with hepatitis B is not considered a risk factor alone for SOS unless it is complicated with cirrhosis. The relationship between HCV infection and SOS is somewhat controversial. One report supports the increased risk even in the absence of cirrhosis[18] while another cohort did not confirm the association although there was an increased long term risk of non-relapse mortality for transplant patients who had chronic hepatitis C[51]. Other risk factors include certain conditioning regimens such as cyclophosphamide, busulfan, and/or total body irradiation[52-54].

Cyclophosphamide is a common conditioning agent with the highest incidence of SOS, which becomes a particular concern in regimens combined with TBI and busulfan. The hepatotoxicity is usually dependent on the toxic metabolite acrolein and the exposure to toxic metabolites can be minimized by metabolism-based dosing. The incidence of SOS seems to be higher in transplant recipients who receive TBI doses over 14 Gy[53]. Various fractionated schedules of TBI have been associated with decreased incidence. Increasing the interval between TBI and cytotoxic therapy also may decrease the risk. Busulfan exposure, on the other hand, is not proven to be directly related to SOS although it potentiates the toxicity of cyclophosphamide especially when it is administered

after this drug[54]. Oral busulfan has a variable and unpredictable absorption and studies have shown that the risk of SOS increases when the area under the curve for busulfan is greater than 1500 μmol/min. When busulfan is adjusted to normal drug levels by close monitoring, a decreased incidence has been reported[54].

Gemtuzumab ozogamicin may induce sinusoidal injury[55] (15%-40% risk) especially if it is administered preceding a cyclophosphamide-based conditioning regimen.

Most cases are observed within the first 3 wk after transplantation. Usually, an unexplained weight gain is the first symptom. This weight gain, attributable to water and sodium retention by the kidney, appears within 6 d to 8 d following the transplant in 95% of patients. This is often followed by varying degrees of hyperbilirubinemia and elevation in aspartate aminotransferase and alkaline phosphatase levels. Most patients develop ascites and pain in the upper right quadrant, and clinical examination usually reveals a firm and painful hepatomegaly. Platelet refractoriness is a common occurrence[56]. Renal insufficiency in the form of hepatorenal syndrome is also present in 50% of patients developing SOS (mainly patients with severe form) and 25% of them will require hemodialysis. Finally, patients with advanced disease can display severe encephalopathy and/or multiorgan failure.

Doppler ultrasound of the liver usually shows reversal of portal and/or hepatic venous flow in severe cases. Most patients are diagnosed on clinical basis, given the risk of liver biopsy in the setting of coagulopathy and platelet refractoriness. Nevertheless where feasible, the clinical suspicion should be confirmed by transjugular liver biopsy which is the gold standard for diagnosis. Hepatic venous pressure gradients are often measured at the time of biopsy; a gradient greater than 10 mmHg is highly specific for SOS[57] and correlates with worse prognosis.

Given the very high mortality rate in patients with severe SOS, it is critical to implement preventive measures such as ursodeoxycholic acid which has been shown to reduce the incidence of SOS[40]. The efficacy of low-dose heparin has not been confirmed and is usually not part of standard management.

Up to 70% of patients with SOS will recover spontaneously and the focus of treatment is supportive care such as maintaining intravascular volume and renal perfusion without causing fluid overload by optimizing sodium restriction and diuretics and transfusions to keep hematocrit levels higher than 40%. The role of albumin or other colloids is unclear but could be considered in patients with severe hypoalbuminemia and large third space fluid accumulations. Low-dose dopamine has been used in patients with renal insufficiency because the mechanism of renal dysfunction appears to be hepatorenal in origin. Avoidance of other hepatotoxic drugs is important in these patients, and infections should be identified and treated promptly. Therapeutic paracentesis can help relieve symptoms in patients with large, tense ascites and may help improve renal function. Also, use of hemodialysis or continuous venous hemofiltration is reported to help with fluid overload in patients with a poor response to diuretics.

There are no effective established treatments for patients with severe SOS characterized by rapidly increasing serum bilirubin and transaminase levels, portal vein thrombosis and multiorgan failure. Several antithrombotic agents have been tested with mixed results. Defibrotide which is an antithrombotic agent without significant systemic effects and with a manageable side effect profile, has been reported to improve signs and symptoms of SOS in 42% of patients[58]. Its mechanism of action is poorly understood. Other tested agents include prostaglandin E1 and tissue plasminogen activator with or without concurrent heparin, intravenous N-acetylcysteine, human antithrombin Ⅲ concentrate, activated protein C and prednisone. None of these approaches are considered a part of standard management.

LONG-TERM COMPLICATIONS

Chronic GVHD

Long-term survivors of stem cell transplantation are at increased risk of several serious complications related to chronic GVHD which may affect liver, GI tract, skin, mucosal surfaces, lungs, joints, eyes and bone marrow; these patients should be followed regularly. Complications may occur in up to 50% of transplant recipients. Liver GVHD usually manifests with progressive or sudden elevation of alkaline phosphatase and gamma glutamyl transpeptidase. Hyperbilirubinemia is usually a late manifestation that coincides with development of cirrhosis and findings of small bile destruction in biopsy[44]. Viral etiologies should be excluded and liver biopsy should be performed to establish the diagnosis, followed by initiation of immunosuppressive therapy which usually includes steroids with or without a calcineurin inhibitor. An improvement in liver function studies is usually observed within four weeks of treatment and 50%-80% of patients respond to the initial therapy with improvement in histopathologic findings. Addition of ursodeoxycholic acid should also be considered and this is usually well tolerated. The prognosis of patients who do not respond to immunosuppressive regimens is poor and correlates with shortened survival[59].

Chronic GVHD may affect several parts of the GI tract, causing esophageal webs and strictures leading to dysphagia, failure to thrive and chronic aspiration. Early lesions can be reversible with immunosuppression, proton pump inhibitors and dilatation. Patients may also experience chronic intermittent diarrhea, clinically and histologically similar to acute GVHD, which may respond to non-absorbable steroids. Chronic malabsorption is rare and is usually a result of long term inadequate treatment.

Chronic viral hepatitis and cirrhosis

Longstanding viral hepatitis C and B can lead to end-stage liver disease in transplant survivors. Rate of progression to cirrhosis in patients with chronic hepatitis

B is comparable to non-transplant patients whereas the incidence of cirrhosis in transplant patients with chronic hepatitis C infection seems to be higher than controls and it can be as high as 24% after 20 years[60]. Otherwise these patients are also at risk of developing hepatocellular carcinoma (HCC) (2%-8% per year) and lymphoproliferative disorders. Patients with chronic hepatitis C should therefore be routinely monitored for viral load and considered for combination therapy with ribavirin and pegylated IFN-α. They should also be screened for HCC with α-feto-protein and ultrasound every 6 mo. Close monitoring is essential for treatment complications such as neutropenia and thrombocytopenia secondary to pegylated IFN-α or exacerbation of coexisting chronic GVHD. Screening and treatment of iron overload may augment the success of anti-viral therapy. Liver transplantation for end-stage liver disease or HCC can be considered, especially from the original stem cell donor[61].

Patients with chronic hepatitis B may have atypical serologic course due to immunosuppression. They may benefit from clearance of antigenemia particularly if the donor has natural HBV immunity. Patients should be monitored for HBV DNA levels and alanine transaminase levels and considered for antiviral treatment at times of tapering of immunosuppressive therapy as well as initiation of new chemotherapy, as they are at risk of flares of hepatitis[62].

Iron overload

The etiology of iron overload in transplant survivors is usually multifactorial, including transfusion dependency and abnormal iron transport by the intestine due to bone marrow dysfunction. It can be an important contributor to chronic liver disease and should be considered in the differential diagnosis[63]. It affects cardiac, endocrine and pancreatic function as well as increasing the risk of opportunistic infections. HFE gene testing should be considered when patients have unexpectedly high levels of iron stores. Clinically significant iron overload usually occurs when serum ferritin exceeds 1000 μg/dL[64]. In the presence of other inflammatory conditions such as chronic GVHD, serum ferritin levels may be falsely elevated. Liver biopsy or noninvasive methods such as liver MRI or FerriScan can be utilized to document the severity of iron overload. Patients with severe iron overload may benefit from mobilization with improved hepatic and cardiac function[65]. If liver iron content is greater than 15 000 μg/g dry weight, both phlebotomy and chelation should be offered. The liver iron content of 7000-15 000 μg/g dry weight should be treated with phlebotomy only and if it is less than 7000 μg/g dry weight, treatment is needed only if there is liver disease[66].

Acute hepatocellular injury

Long term transplant survivors may present with acute elevations of transaminases. The differential diagnosis should include flares of chronic viral hepatitis, hepatitis presentation of chronic GVHD, VZV, HSV infection or drug-induced (antihypertensives, statins, hypoglycemic agents, antibiotics) liver injury.

Malignancies

The risk of new malignancies among transplant survivors increases significantly after 10 years. Patients with chronic hepatitis C have accelerated incidence of HCC[67] and lymphomas[68].

CONCLUSION

Gastrointestinal and hepatic complications count for a significant part of the morbidity during and after hematopoietic stem cell transplant. Recent advances in transplant approaches have changed the outcome and the post-transplant course of many patients[69]. As most transplant survivors are affected by multiple complications, it is imperative that they should receive long-term and systematic follow-up without compromising from individualized care.

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EDITORIAL

Surgical treatment of ulcerative colitis in the biologic therapy era

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Abstract

Recently introduced in the treatment algorithms and guidelines for the treatment of ulcerative colitis, biological therapy is an effective treatment option for patients with an acute severe flare not responsive to conventional treatments and for patients with steroid dependent disease. The reduction in hospitalization and surgical intervention for patients affected by ulcerative colitis after the introduction of biologic treatment remains to be proven. Furthermore, these agents seem to be associated with increase in cost of treatment and risk for serious postoperative complications. Restorative proctocolectomy with ileal pouch-anal anastomosis is the surgical treatment of choice in ulcerative colitis patients. Surgery is traditionally recommended as salvage therapy when medical management fails, and, despite advances in medical therapy, colectomy rates remain unchanged between 20% and 30%. To overcome the reported increase in postoperative complications in patients on biologic therapies, several surgical strategies have been developed to maintain long-term pouch failure rate around 10%, as previously reported. Surgical staging along with the development of minimally invasive surgery are among the most promising advances in this field.

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Key words: Ulcerative colitis; Inflammatory bowel disease; Infliximab; Surgery; Laparoscopy; Single incision laparoscopy; Total abdominal colectomy; Ileal pouch anal anastomosis; Restorative proctocolectomy

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INTRODUCTION

Ulcerative colitis (UC) is a mucosal inflammatory process affecting the rectum and the colon. It is characterized by contiguous inflammation starting in the rectum and progressing for variable distance proximally[1]. Intermittent exacerbations are typical, with symptoms characterized by bloody diarrhea associated with urgency and tenesmus[2]. The activity of disease can range from complete remission to fulminant symptoms along with systemic toxic effects[3].

Although the exact pathogenesis of UC remains poorly

understood, the most credited model states that the intestinal flora triggers and drives an aberrant intestinal immune response and subsequent inflammation in a genetically susceptible host[4]. Medical therapy aims at the control of symptoms and the resolution of the underlying inflammatory process, classically by a variety of agents in combination, such as 5-aminosalicylates, corticosteroids, and immunosuppressants, including purine antimetabolites and cyclosporine[5]. Treatment schemes are based on disease severity, (defined as mild, moderate or severe based on clinical ad laboratory parameters) and on the extent of the disease (pancolitis, left-sided colitis, rectosigmoiditis or proctitis)[6]. However, about a quarter of patients with UC end up needing a colectomy because of failure of medical therapy, onset of unacceptable side effects of chronic therapy, occurrence of acute complication of UC (fulminant colitis, severe bleeding, toxic megacolon, perforation), or development of malignancy[7].

For all of these patients, the removal of the colon and rectum represents a definitive cure for their disease, with cessation of symptoms, withdrawal of morbid medical therapy, and avoidance of the risk of developing a malignancy associated with the persistence of inflammation[8].

However, surgery is not without risks and can significantly affect patients' lifestyle, therefore, is traditionally deemed as a salvage treatment when medical therapy is ineffective[1].

During the last three decades astounding progress has been accomplished both in medical and surgical treatments, which might lead to substantial changes in the traditional principles for the management of UC patients. Medical therapy of UC has recently entered the era of biologic treatments with the approval by Food and Drug Administration (FDA) in 2005 of Infliximab, a monoclonal antibody directed against tumor necrosis factor-alpha. The initial enthusiasm raised by the promise to reduce the colectomy rate in acute presentations, was subsequently partially dampened by conflicting reports regarding Infliximab's safety and impact on the need for surgery in urgent/emergent setting[9-13].

As the number of available medications increases, more and more often patients are referred for surgery severely malnourished, immunocompromised, and experiencing the side effects of corticosteroids, immunomodulators, and biological agents. Whether they are referred for colectomy in an acute or chronic setting, these patients represent a unique challenge for colorectal surgeons, given the compromised general conditions and poor nutritional status in the former, and the side effects of long term corticosteroid use in the latter[14-16].

Together with the advances in medical therapy, the surgical treatment and techniques in UC has evolved as well. Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) is today considered the gold standard and, in experienced hands, can now be performed safely for UC with a low postoperative complication rate and a long-term pouch failure rate reported less than 10%[17-19]. Moreover, the introduction of minimally invasive techniques might further decrease postoperative morbidity and improve patients' satisfaction, with reduced impact on body image and better cosmesis[20-22].

The purpose of this report is to discuss the recent advances in medical and surgical treatment of UC patients addressing surgical concerns in the era of biologic therapy.

BIOLOGIC THERAPY IN UC: THE GASTROENTEROLOGIST'S VIEW

The primary goals of medical therapy in the treatment of UC are to induce remission of symptoms and maintain it on a long-term basis: by reducing the number of relapses, which occurs in 67% of patients and, at least, once over a 10-year period[23], medical therapy lowers the risk of long-term complications and improves patients' quality of life.

The majority of UC patients present with moderate- to severe disease (80%) rather than mild disease (20%)[24] and, during their illness, nearly 20% of patients afflicted with UC will experience a severe acute episode that requires hospitalization[25].

Despite the progress accomplished in medical therapy, which broadened the horizon of possible treatments after failure of corticosteroids[26,27], the need for surgery in this patient population seems to be unchanged or slightly decreased over time. Reported colectomy rates are steadily ranging between 20% and 30% in most of the epidemiological studies with additional risk for needing a resection as the extent and severity of the disease increase[8,28-31]. Beside a 10% who have surgery for cancer or pre-neoplastic degeneration, the vast majority of patients need an operation for acute colitis with severe complications not responsive to medical therapy[32,33]. The advantage of prolonged medical therapy *vs* surgery in patients with acute severe colitis failing initial high dose corticosteroids is still debated. About one third of these patients undergo a colectomy within one year, most likely in an emergency setting, and even if second-line medical therapy may reduce the need for immediate colectomy, most of them will require colectomy by 10 years[32,34]. In this setting, early subtotal colectomy and ileostomy combined with a late reconstructive surgery remains a safe alternative[19] since second-line medical therapy carries with it a not negligible mortality risk[35].

Additionally, about 20% of patients with UC have a persistent active disease often requiring several courses of systemic steroids, but followed by relapse of symptoms during steroid tapering or soon after their discontinuation, a condition known as steroid-dependency. Steroid dependency is associated with serious complications, which, for a significant proportion of patients, become an indication for surgery[36].

Although surgery is curative of the underlying inflammation and restorative proctocolectomy with IPAA preserves the normal anatomic route for defecation, the procedure may lead to new symptoms, such as diarrhea,

incontinence, nocturnal leakage, and in some patients does not obviate the need for medication. In several surgical series that follow patients a minimum of 5 years, up to 60% of patients are still having more than 8 bowel movements daily, with 55% of patients experiencing incontinence, and 50% nocturnal leakage[37-39]. Even if surgical techniques have dramatically evolved, surgery is still associated with significant early and late postoperative complications, e.g. anastomotic leak, pelvic sepsis, small bowel obstruction, pouchitis, sexual dysfunction, reduced fecundity in women and pouch failure[40,41]. Repeated surgery is sometimes necessary. A population-based study reported that approximately 20% of patients who had undergone IPAA required at least one additional surgery, and 15% of patients required at least two additional surgeries[42]. Pouch leak and the associated pelvic sepsis rate in large series have been reported to range from 5% to 15%[43]; incidence of late small-bowel resection after IPAA ranges from 12% to 35%. Pouchitis is the most frequent long-term complication of the IPAA[1]. It has been reported in 12% to 50% of patients postoperatively, and some patients (5%-19%) require chronic therapy. Finally, the risk of long-term pouch loss has been reported to range from 1% to 20% in different studies with an overall rate of pouch loss less than 10%, needing diverting ileostomy, pouch excision and end ileostomy, or pouch revision[17-19].

Acute severe ulcerative colitis

According to current treatment algorithms, in case of acute colitis, unless toxic megacolon, perforation or severe bleeding-which are absolute indication for surgery-occur, patients are started on high-dose iv steroids[44]. Response to treatment is assessed by objective measures (e.g., Oxford index or Sweden index) on day 3-4. Two different strategies have been developed in the attempt of avoiding surgery when a first course of steroids fails to control an acute flare. The standard approach in the '80s was to prolong the administration of steroids for other 7-10 d, which did not show any reduction in colectomy rates[45-47]. Ten years later, cyclosporine was found to be effective in patients with acute severe UC non responsive to steroids, and has been used as rescue therapy[44,48-51]. In a randomized controlled trial (RCT) 82% of patients on cyclosporine improved, while no patient improved in the placebo group[52]. However, as many as 50% of patients that responded to cyclosporine, required colectomy in subsequent studies with longer follow-up[35,53]. Moreover, the management of patients under cyclosporine can represent a real challenge, given the risk of severe and potentially fatal toxicities, which greatly limit the use of this medication.

Infliximab, an anti-tumor necrosis factor (TNF) antibody, has been approved recently by the United States FDA for the treatment of UC to reduce signs and symptoms, to induce clinical remission and healing of the intestinal mucosa, and to eliminate the use of corticosteroids in patients presenting with moderately-to-severely active UC without adequate response or who are intolerant or have medical contraindications to therapy with corticosteroids or immune modulators[54].

Response to infliximab has been assessed in RCTs with various endpoints such as clinical response, remission and colectomy rates. In patients with severe, steroid-refractory UC, the initial small trials demonstrated modest efficacy after single infusions when early clinical response was determined. The first published trial by Sands *et al*[55] in 2001 randomized 11 patients with steroid refractory UC to a single infliximab infusion or placebo, and noted a 50% (4/8) clinical response rate with infliximab at a week 2 evaluation. Subsequent studies by Probert *et al*[56] and Järnerot *et al*[51] also enrolled patients with steroid-refractory disease. The first study failed to show any significant difference between placebo and 2 infusions of infliximab 5 mg/kg in inducing remission as measured by endoscopy or clinical score. However, Järnerot *et al*[51] demonstrated in patients with moderate and severe steroid-refractory UC that only 7/24 (29%) patients who received a single infliximab infusion underwent colectomy within 90 d, compared with 14/21 (67%) who received placebo. The superiority of infliximab was only statistically significant in patients with moderate to severe disease, but not in those with more severe disease on the fulminant colitis score, although the study was not powered to detect differences between these two last groups. Even though a later report showed that at 2 years follow-up, the colectomy rate in patients who received infliximab had increased to 46%[57], these studies positioned infliximab as a therapeutic option for patients with steroid-refractory disease. The first controlled trial[58] involving patients who had moderate to severe disease that were neither steroid-dependent nor steroid-refractory, reported superior clinical response rates compared to those seen in steroid-refractory populations. These trials reported high response rates (100% and 83%, respectively), but follow-up was short (9.7 and 3 mo, respectively). The active ulcerative colitis trial (ACT) 1 and ACT 2 trials[59] each randomized 364 patients with moderate to severe UC who were failing conventional therapy (but did not require hospitalization) to either placebo or induction/maintenance infliximab 5 mg/kg or 10 mg/kg. Both in ACT 1 and ACT 2, eligible patients had moderate to severe UC despite concurrent treatment with corticosteroids, alone or in combination with azathioprine or mercaptopurine, but ACT 2 also required that the patient had failed 5-aminosalicylic acid (5-ASA) therapy. In ACT 1, both doses of infliximab (5 mg/kg and 10 mg/kg) resulted in a statistically significant clinical response at week 8 (68.4% and 61.5% respectively, $P < 0.01$, compared to a placebo response of 37.2%). This was similar in ACT 2, with clinical response at week 8 in 64.5% of patients in the infliximab 5 mg/kg group and 69.2% in the infliximab 10 mg/kg group, compared to a 29.3% response rate in the placebo group ($P < 0.001$). Clinical remission rates in the infliximab arms at week 8 ranged from 27.5% to 38.8% across both studies compared to

placebo-induced remission rates of 14.9% (ACT 1) and 5.7% (ACT 2). Mucosal healing and steroid-free remission rates were also superior in the infliximab arms of these studies. Sandborn *et al*[60] reported colectomy rates in ACT 1 and ACT 2 patients in a follow-up study. The cumulative colectomy rate at 54 wk was 10% in patients treated with infliximab, compared with 17% in those treated with placebo. These colectomy rates were not unexpected since the enrolled patients had moderate to severe disease, however in 13% of the enrolled patients the colectomy follow-up data was unavailable. The ACT 1 and ACT 2 studies were well-designed, large studies, with comprehensive assessment of clinical and secondary endpoints. They provided important data to support the use of infliximab in patients with moderate to severe UC who have failed other therapies such as steroids, immunomodulators and mesalamine. However, infliximab is not a panacea for all; the proportion of patients who started the study on steroids and were able to come off and remain in remission, was low (20%)[59].

In a recent study by Colombel *et al*[61], the association between early mucosal healing (defined as Mayo endoscopy subscore at week 8 endoscopy) and clinical outcomes in ACT-1 and ACT-2 patients was investigated. The authors observed that a low week 8 endoscopy subscore was significantly associated with a lower rate of colectomy at 54 wk follow-up ($P = 0.0004$; placebo $P = 0.47$) and better outcomes in terms of symptoms and need for steroids at weeks 30 and 54 ($P < 0.0001$, infliximab; $P < 0.01$, placebo), especially for those patients who did not achieve clinical remission at week 8[61].

A Cochrane meta-analysis of RCTs concluded that, when compared to placebo, treatment with infliximab is three-fold as effective in inducing clinical remission [relative risk (RR) 3.22; 95% CI: 2.18-4.76] and nearly twice as effective in inducing clinical response (RR: 1.99; 95% CI: 1.65-2.41) or endoscopic remission (RR: 1.88; 95% CI: 1.54-2.28) at week 8 in patients presenting with moderate-to-severe UC refractory to conventional treatment with corticosteroids and/or immune modulators[10].

Steroid dependent ulcerative colitis

Another specific pattern of UC disease is represented by steroid-dependent patients, in whom a response can be obtained with systemic steroids, but the relapse will occur as the dose is tapered or a few weeks or months after discontinuation, making it necessary to increase the dosage again or resume treatment to achieve control of symptoms[36]. As UC patients become dependent-upon or refractory to corticosteroids, the range of action from a medical standpoint become limited and a colectomy becomes a treatment option as the disease is deemed as refractory to medical treatment, or because of the occurrence of complications either related to the disease or associated with side effects of medications[1].

Often, immunomodulator therapies, such as azathioprine or mercaptopurine (6-mercaptopurine) are considered in these patients before surgery as a steroid-sparing treatment. However, the efficacy of azathioprine or mercaptopurine in UC is still debated[62]. Thiopurines are an effective maintenance therapy for patients who require repeated courses of steroids, however the quality of available data is quite poor, as stated in a recent Cochrane review[63]. Currently, the recommendation for using thiopurines in UC is based on the evidence shown by only one RCT of Ardizzone *et al*[64] which found steroid-free, clinical and endoscopic remission in 53% patients on azathioprine compared with 21% given only 5-ASA [odd ratio (OR) on intention to treat analysis 4.78, 95% CI: 1.57-14.5]. Azathioprine maintenance treatment of UC is beneficial for at least 2 years if patients have achieved remission while taking the drug, but not in those with chronic activity despite the drug[65].

When a steroid-dependent patient fails to benefit from thiopurines or shows intolerance to them, there are very few alternatives to conventional drugs, which lack of current definitive evidence of efficacy. Methotrexate has been tested and, although some uncontrolled studies suggested some benefit with its use[66-68], the only double-blind, placebo-controlled trial, showed no therapeutic benefit[69]. Therefore, current guidelines do not consider methotrexate as an evidence-based therapy in steroid-dependent UC.

After the demonstration of clinical efficacy of infliximab in the treatment of moderate-severe resistant UC, few small series have included steroid dependent patients. Only one study from Italy[70] specifically evaluated steroid dependent UC in an open-label study on 20 patients randomized to infliximab or methylprednisolone. This was the first RCT to implement a regimen of a triple infliximab infusion for induction followed by infusions to maintain remission. Even if this study was statistically underpowered, it demonstrated the benefit of infliximab therapy for responders, who were able to taper and then discontinue steroids during the maintenance phase (9 of 10 patients), as compared with the methylprednisolone group (8 of 10 patients), where responders required continued steroid therapy.

BIOLOGIC THERAPY IN UC: THE SURGEON'S VIEW

Biologic therapy has shown the ability to induce and maintain remission, but, as we stated above, its introduction in the therapeutic algorithm did not substantially affect the overall rate of colectomies, suggesting that it is effective only in delaying but not in avoiding surgery for a subgroup of patients who at some point will require an operation[54,59,60,71]. The clinical efficacy of infliximab in UC still remains unpredictable. Induction therapy is not always effective, and, to date, clinical and/or molecular predictors of response have not been identified. No RCT has been conducted comparing infliximab and cyclosporine in severe UC. Most of the current knowledge comes from the ACT 1 and ACT 2 trials. Those results are in part influenced by the heterogeneity of the sample (in-

Table 1 Literature-based comparison of postoperative complication risk associated with preoperative use of infliximab

Ref.	Year	Non-IFX/IFX patients	Infectious complication			Non-infectious complication		
			IFX group	Non-IFX group	OR (95% CI)	IFX group	Non-IFX group	OR (95% CI)
Selvasekar *et al*[13]	2007	254/47	13 (28%)	25 (10%)	3.50 (1.64-7.5)	16 (34%)	99 (39%)	0.81 (0.4-1.55)
Schluender *et al*[12]	2007	134/17	3 (18%)	11 (8%)	2.40 (0.6-9.63)	3 (18%)	26 (19%)	0.89 (0.24-3.33)
Kunitake *et al*[9]	2008	312/101	6 (6%)	32 (10%)	0.55 (0.22-1.36)	11 (11%)	17 (5%)	2.12 (0.96-4.69)
Mor *et al*[85]	2008	46/46	10 (22%)	1 (2%)	13.8 (1.82-105)	6 (13%)	6 (13%)	1.00 (0.3-3.37)
Ferrante *et al*[83]	2009	119/22	2 (9%)	29 (24%)	0.31 (0.07-1.141)	NR	NR	NR
Coquet-Reinier *et al*[84]	2010	13/13	NR	NR	NR	3 (23%)	4 (38%)	NR
Gainsbury *et al*[86]	2011	52/29	5 (17%)	14 (27%)	1.87 (0.46-7.57)	12 (41%)	16 (31%)	0.59 (0.19-1.87)

IFX: Infliximab; OR: Odd ratio; NR: Not reported; CI: Confidence interval.

cluding both steroid-dependent and/or immunomodulator-dependent and steroid responsive and/or immunomodulator-responsive patients). More studies are needed to assess the role of concomitant administration of immunosuppressants and infliximab[59]. Furthermore, data on maintenance therapy with infliximab in UC are scant and the benefits of continued maintenance therapy, as well as its long-term safety, are poorly known. The results of the ACT-1 and ACT-2 extension studies conducted on the 229 patients who achieved improvements with infliximab during the trials, showed that the benefits observed in the main studies are basically maintained up to 3 additional years, however an high drop-off rate was observed, due to adverse events (10.5%), lack of efficacy (4.8%), need for surgery (0.4%), or other reasons (14.8%)[72]. Furthermore, it is not clear to what extent postponing surgery by the means of a quite morbid medical therapy represents a safe and effective strategy.

Because of the early onset and chronic nature of inflammatory bowel diseases, patients can be expected to utilize considerable health care resources. Costs analysis are complicated, because they must calculate the impact that such therapies have on the direct costs of health care and the indirect costs for both the patient and their families and the health care system[73]. Surgeries and hospitalizations account for the majority of health care direct costs in inflammatory bowel disease (IBD), and medication costs, on the other hand, accounted for a quarter of total direct medical costs. Moreover, the cost data are right-skewed, with 25% of patients accounting for 80% of total costs[74]. This division of health care costs implies that the most effective cost-containment measure would be the one that reduces the number of hospitalizations and surgeries. With the improved response and remission rates seen with the use of infliximab for induction and maintenance treatment in IBD patients, the clinical benefits may likely translate into economic benefits as well[75]. Surprisingly, many of the cost-effectiveness and utility analyses suggested that infliximab use was associated with rather high incremental cost per quality adjusted year life[73] and the expanding use of infliximab has not significantly impacted the use of surgical procedures for patients with either UC or Crohn's disease, and rates of nonsurgical hospitalizations have actually increased[76,77]. This belief is supported by the observation that in the United States the hospitalization rates for IBD increased between 1998 and 2004, leading to a concurrent rise in the economic burden, with medical hospitalizations accounting for the largest proportion (58%) of inpatient services costs and biologic agents representing the most costly medications[78,79]. Further pharmaco-economic analyses are needed to accurately assess the impact of infliximab treatment on the costs associated with the treatment of UC.

Surgery in the biologic era: Treatment in evolution

The concept of pushing conservative treatment until surgery is strictly required may be risky, as it has been shown that mortality three years after elective colectomy for UC (3.7%) is significantly lower than that after admission without surgery (13.6%) or when an emergency operation is performed (13.2%)[80]. Moreover, a British study recently reported a significantly higher risk to develop major complications at a 5 year follow up for patients who received a longer course of medical therapy for acute severe UC before surgery, suggesting that the threshold for elective surgery may be too high in current practice[81].

While it's well known that high-dose systemic corticosteroid therapy (> 40 mg/d prednisolone-equivalent) is a widely recognized risk factor for pouch-related septic complications after restorative surgery[82], whether or not the preoperative administration of infliximab may increase the rates of septic complications is still controversial (Table 1)[9,12,13,83-86]. Nevertheless, the group from the Cleveland Clinic has found a covariate-adjusted risk of early complication for patients treated with infliximab 3.54 times higher, with the rate of sepsis increased by 13.8 folds, despite a significantly higher rate of three-stage procedures in the infliximab group[85]. Similar results have been shown in a paper by Mayo Clinic, where patients treated with infliximab prior to pouch surgery had a significantly higher incidence of anastomotic leaks, pouch specific and infectious complications, with the administration of anti-TNF-alpha as the only factor independently associated with septic complications (OR 3.5)[13]. In another study, a synergic interaction in increasing surgical morbidity was found between infliximab and cyclosporine A when administered together in the preoperative time[12]. These concerns are supported by a recent meta-analysis conducted including 5 studies and 706 patients, which revealed an

increased risk of short-term post-operative complications (OR 1.80, 95% CI: 1.12-2.87) associated with preoperative infliximab use, along with a trend towards increased post-operative infection[87].

Given the concern of increased rate of complications in patients on aggressive medical management, several different surgical approaches have been proposed. First described by Parks and Nichols in 1978, restorative proctocolectomy with IPAA has progressively gained acceptance to become the gold standard in the surgical treatment of UC for the last 25 years[88,89]. The introduction of this technique-most often fashioned as a J pouch created with the terminal ileum and anastomosed to the anal canal-was a real breakthrough, offering a curative treatment to these patients without the need for a permanent stoma, thus preserving their body image, achieving a quality of life comparable to that of the general population[38,90]. However, the procedure is technically demanding and is associated with a significant morbidity rate (around 30%), and an incidence of postoperative pelvic sepsis ranging between 5%-24%[91]. Since it has been shown that the occurrence of a pelvic infection can dramatically affect the functional outcome of the pouch, and considering that long-term steroid use and malnutrition are recognized risk factors for pelvic sepsis, surgical strategies have been developed in order to minimize the occurrence of infectious complications, especially in this subset of patients[92,93]. A total abdominal colectomy with end ileostomy is the operation of choice as first step of a restorative procedure, as it can be performed safely and quickly in the hands of an experienced colorectal surgeon, allowing the patient to overcome the colitis, wean off the medications, and return to an optimal health and nutritional status[94,95]. Moreover, as it is well known that a postoperative diagnosis of indeterminate colitis or Crohn's disease is not rare after colectomy in these patients[96], a multistep surgical procedure allows for selecting the most appropriate reconstructive surgery on the basis of the pathological findings of the colectomy specimen[19,94,97].

The removal of the rectum and the restoration of the bowel continuity with IPAA are performed as a second step when the patient has fully recovered, and the creation of a temporary ileostomy, although adding the need for one more operation, can further reduce the risk of local sepsis secondary to anastomotic leaks[98,99]. Albeit restorative surgery is not free from long term complications, such as incontinence and soiling (10%-60% of patients, depending on series and entity), pouchitis (about 50% of patients), and sexual dysfunction (20%-25% of cases), with a rate of pouch failure requiring excision ranging between 5%-15%, the majority of these conditions are manageable with medical and physical therapy, which explains the overall satisfaction in patients after IPAA, which exceeds the 90% in most series[40,98,100-105].

Indeed, most recent researches have shown that social and sexual function as well as overall quality of life is significantly improved after restorative surgery, when compared to the period with active UC or diverting ileostomy[106-109].

The application of minimally invasive techniques to the surgical treatment of UC at the beginning of the 1990s contributed in significantly improving the acceptance and tolerability of the procedure[110]. Numerous case series and, finally, two meta-analyses have been published since then, demonstrating the feasibility and safety of the laparoscopic approach, at the cost of longer operative times[110-117]. A subsequent RCT showed that operative time could be significantly reduced with the adoption of a hand-assisted technique, which at the same time allows for preserving the advantages of a minimal invasive approach[118]. Scant data is available so far regarding long-term outcomes, however the few series with adequate follow-up report laparoscopy pouch function results as good as the ones achieved with open surgery[21,119]. Laparoscopy has also been adopted with good results in the emergency setting[120,121], and similarly as for open surgery, a staged approach to a minimally invasive restorative procedure should be preferred which is as effective in significantly reducing the rate of post-operative pelvic sepsis[121-123]. Furthermore, when a staged procedure is planned, laparoscopy has been shown to decrease postoperative adhesion formation with less intraoperative adhesiolysis required during subsequent completion proctectomy and IPAA[124]. Similarly, a study by Indar and colleagues on 34 patients who underwent laparoscopic IPAA, where a laparoscopic exploration of the abdominal cavity was performed during the ileostomy closure, found that no patient had dense adhesion and only a minority of patients had filmy avascular adhesion to the abdominal wall (32%) and to the adnexa (29%), which represents a significant improvement compare to the rates reported for open surgery (as high as 90%)[125].

Despite the lack of strong evidence about the benefits attainable with laparoscopy in terms of short-term outcomes[21,126], it has been proven that patients treated laparoscopically are more satisfied with the cosmetic results and perceive a better body image-aspect anything but negligible in this usually young and socially active patient population-especially in the women's subset, as confirmed by the results of a RCT with a median follow-up of 2.7 years[21,119]. More recently, the quest for further minimizing surgical trauma and extent of incisions, has led to the development of single incision laparoscopy (SIL), which has already been applied in the field of colorectal diseases with proven benefits in terms of short-term outcomes over standard laparoscopy[127-131]. To date only few cases of SIL for UC has been reported, but preliminary results show that particularly for the total abdominal colectomy, this "no scar" approach have the potential for improving not only the cosmesis, but also the postoperative course, with less pain and reduced need for narcotics, which may translate in shorter hospital stay and faster return to normal activities[132-135]. Considering the excellent outcome of restorative surgery, heightened by the potentials of minimal invasive techniques, surgery should not be considered the last resort when everything has failed, but rather a valid alternative to an expensive and risky medical therapy[136].

CONCLUSION

Medical therapy in UC is rapidly evolving and the introduction of modern biological drugs has led to substantial changes in the traditional principles of management. Infliximab, the first biological agent used as rescue therapy after failure of steroids in UC, appears to be effective in reducing the need for urgent colectomy, although its efficacy in the long-term is not proven. In addition, concerns have been raised regarding the economic burden related to this drugs and the risk for serious postoperative complications.

Surgery continues to play an important role in UC treatment and its evolution keeps pace with the advance in medical therapy and the risk associated with it. Restorative proctocolectomy with IPAA, staged procedures, and minimally invasive surgery are important treatment tools to limit postoperative morbidity and achieve excellent long-term outcomes in these patients.

In an attempt at avoiding surgery, aggressive medical therapy is not without complications. A complex decision making process in a multidisciplinary fashion should take into consideration the excellent results of modern surgical therapies to avoid unnecessary morbidity.

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OBSERVATION

Colitis associated with biological agents

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Abstract

In the past, there has been considerable focus on a host of drugs and chemicals that may produce colonic toxicity. Now, a variety of new biological monoclonal antibody agents, usually administered by infusion, have appeared in the clinical realm over the last decade or so to treat different chronic inflammatory or malignant disorders.For some of these agents, adverse effects have been documented, including apparently new forms of immune-mediated inflammatory bowel disease. In some, only limited symptoms have been recorded, but in others, severe colitis with serious complications, such as bowel perforation has been recorded. In others, adverse effects may have a direct vascular or ischemic basis, while other intestinal effects may be related to a superimposed infection. Some new onset cases of ulcerative colitis or Crohn's disease may also be attributed to the same agents used to treat these diseases, or be responsible for disease exacerbation. Dramatic and well documented side effects have been observed with ipilimumab, a humanized monoclonal antibody developed to reduce and overcome cytotoxic T-lymphocyte antigen 4, a key negative feedback regulator of the T-cell anti-tumor response. This agent has frequently been used in the treatment of different malignancies, notably, malignant melanoma. Side effects with this agent occur in up to 40% and these are believed to be largely immune-mediated. One of these is a form of enterocolitis that may be severe, and occasionally, fatal. Other agents include rituximab (an anti-CD20 monoclonal antibody), bevacizumab (a monoclonal antibody against the vascular endothelial growth factor) and anti-tumor necrosis factor agents, including infliximab, adalimumab and etanercept.

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Key words: Biological agents; Colitis; Crohn's disease; Inflammatory bowel disease; Infliximab; Ipilimumab; Rituximab; Ulcerative colitis

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INTRODUCTION

Different patterns of inflammatory disease involving the small and large intestine have been historically recognized over the past century or so, and their features are well detailed in clinical textbooks especially focused on inflammatory bowel diseases. Crohn's disease, for example, is recognized as a pattern of inflammatory disease that may involve any site along the length of the gastrointestinal tract, usually in a segmental or focal distribution, typically with transmural involvement, and frequently, granulomas may be demonstrated. Ulcerative colitis has been traditionally defined by its colonic distribution, a more continuous pattern of mucosal involvement extending proximally within the colon from the rectum for variable distances. In reality, however, clinical and pathological overlap may occur frequently, and in many patients, precise differentiation of these different forms of chronic

inflammatory disease based on these descriptive parameters may not be so precise.

In the past, a variety of drugs have been used to control the inflammatory process in these disorders and improve quality of life. In addition, a host of biological agents have also emerged in recent years to treat a number of chronic inflammatory disorders, including Crohn's disease and ulcerative colitis, as well as a lengthening list of malignant disorders. Some of these biological agents have also been associated with the appearance of novel forms of colonic inflammatory disease, often severe and potentially fatal, as well as apparent paradoxical intestinal complications, including the *de novo* appearance or worsening of an underlying or unrecognized intestinal inflammatory disorder that may, in themselves, lead to serious complications.

Although a number of administered drugs and chemicals causing colonic toxicity have been enumerated elsewhere and reviewed in detail during the past 3 decades[1-3], this review focuses on newer agents, largely administered by the parenteral route, that interfere with key regulatory biological molecules. These include ipilimumab, rituximab, bevacizumab and a number of anti-tumor necrosis factor agents.

IPILIMUMAB-INDUCED COLITIS

A relatively novel strategy has emerged in cancer treatment in recent years to induce tumor regression and prolong patient survival involving control and reduction of the effect of specific immune regulatory molecules, such as the cytotoxic T-lymphocyte antigen 4 (CTLA-4). Ipilimumab is a fully human monoclonal antibody that has been developed to reduce and overcome cytotoxic CTLA-4, a key negative regulator of the T-cell anti-tumor immune response. In recent years, evidence has appeared showing tumor regression with prolonged time to progression in melanoma patients treated with CTLA-4 antibodies[4,5]. Ipilimumab plus dacarbazine showed improved survival in malignant melanoma compared to dacarbazine alone, a drug most frequently compared with new agents in randomized treatment trials on melanoma[5]. In addition to melanoma, prolonged effects with ipilimumab have been noted in other malignancies including ovarian cancer[6], prostate cancer[7] and renal cell cancer[8]. Inhibition of CTLA-4 with this antibody is also associated with characteristic side effects in an estimated 40%[4]. These are believed to be largely immune-mediated and include an ever-lengthening list of adverse effects such as dermatitis, endocrinopathies, particularly hypophysitis, uveitis, nephritis, inflammatory myopathies, hepatitis, and diarrhea or colitis[9,10]. Similar immune-related adverse events may result from another monoclonal CTLA-4 antibody, tremelimumab,used for the treatment of metastatic melanoma[11].

Colonic toxicity has been recorded in about 20% and appears to occur relatively rapidly after administration of ipilimumab, sometimes within days marked by the onset of abdominal cramping pain and profuse diarrhea, often bloody[9,12].In others with few or mild symptoms, colitis could still be present since only those with more severe symptoms were recorded[12]. Up to 5% of patients may suffer a fatal outcome attributed to a significant complication, a protracted clinical course or failure of prompt treatment, sometimes related to limited compliance[12]. Colonoscopy and ileoscopy as well as upper endoscopy with duodenal biopsies have documented both small bowel and colonic inflammatory changes. In some, a diffuse, but non-specific colitis may occur, in the absence of any detectable infectious agent, while in others, the inflammatory process may be patchy or segmental in distribution. The appearances may not be distinguishable by endoscopy from other forms of inflammatory bowel disease. Endoscopic biopsies may show a non-specific acute and chronic inflammatory infiltrate, including cryptitis as well as crypt abscess formation. Colon biopsy samples show a colitis that has an abundant T-cell infiltrate[13]. Granulomatous inflammation has not been recorded.

Treatment for this enterocolitis largely based upon-supportive measures, specifically, fluid and electrolyte replenishment and, sometimes, parenteral nutrition. In addition, the colitis has often been treated with intravenous high dose steroids (or oral budesonide) and, if the response to steroids fails or has been limited, infusions of infliximab have been used[14,15]. If no response for the colitis is evident, diverting ileostomy or partial/complete colectomy has been recommended. The incidence of life-threatening colon perforation has been recorded at 4 in 700 cases with doses of ipilimumab of 3 mg/kg or more (i.e., less than 1%). Even during treatment with steroids or infliximab for the colitis, the anti-tumor response for metastatic melanoma still appears to be sustained. In a recent study of ipilimumab with dacarbazine for previously untreated metastatic melanoma, rates of intestinal adverse events were reported to be lower, while the rates of altered liver chemistry test changes were higher[5].

RITUXIMAB-ASSOCIATED COLITIS

Rituximab is an anti-CD20 monoclonal antibody that has been used in the management of nephrotic syndrome in children and adults[16-18] as well as a form of B-cell targeted therapy in rheumatoid arthritis[19,20]. It appears to result in depletion of systemic as well as intestinal B cell populations. Although the agent appears to be efficacious, adverse effects have been noted in about 27% of children treated with rituximab for refractory nephrotic syndrome[21]. Some of the reported adverse effects have included fever and chills, mucocutaneous reactions, fatal infusion reactions, progressive multifocal leukoencephalopathy, and bowel perforation[18,22]. New onset ulcerative colitis[23] and an exacerbation of previously documented colitis have been recorded[24]. In a later report, it was hypothesized that a severe colitis that developed after rituximab therapy may have been related to an infectious torovirus agent[22].

BEVACIZUMAB-ASSOCIATED COLONIC ULCERATION

Bevacizumab is a humanized monoclonal antibody against

the vascular endothelial growth factor receptor. This antibody has shown promise in the treatment of recurrent and metastatic colorectal cancer as well as metastatic non-small cell lung cancer. The agent has also been used to treat other malignancies, including ovarian cancer. Several mechanisms of action have been proposed, including an ability to restrict or deprive tumors of their neovascularity required to permit tumor progression and growth.

A number of cases have now been reported describing bowel perforation following bevacizumab treatment[25-29]. There also appears to be an especially increased risk of leakage at anastomotic suture sites following surgery for either ulcerative colitis or colorectal cancer[26]. In some, delayed anastomotic complications have been observed, some more than 1 year after surgery. Some hypothesized risk factors included anastomotic leakage during the original operative procedure or prior pre-operative pelvic irradiation[27]. Other mechanisms that have been recorded include an ischemic pathogenesis with anastomotic perforation after a partial colectomy[28] or more diffuse perforation associated with histological evidence of ischemia in a patient with non-small cell lung cancer[29].

ANTI-TUMOR NECROSIS FACTOR ADVERSE EVENTS

Most intriguing is the recent increased recognition of paradoxical adverse events following therapy with anti-tumor necrosis factor. In selected patients with inflammatory bowel disease, improved symptoms and mucosal changes may result. Similar treatment effects have been recorded for different agents including the chimeric monoclonal agent, infliximab, along with more humanized forms, such as adalimumab. Interestingly, in some treated with these agents for other disorders, in particular spondyloarthropathies or rheumatoid arthritis, so-called "paradoxical" adverse effects have been recorded, including flares or new onset inflammatory bowel disease[30]. Initially, in this early evaluation, intestinal effects appeared to occur more often with etanercept than either of the monoclonal antibody agents[30]. Later, however, other effects appeared to develop during arthritis treatment, particularly the skin disorder, *pyoderma gangrenosum*[31,32]. Later, new onset ulcerative colitis was initially recorded during infliximab treatment[33] as well as adalimumab[34]. Similar cases of new onset inflammatory bowel disease, specifically, Crohn's disease have also been recorded usually after etanercept therapy administered for spondyloarthropathy (as opposed to Crohn's disease where etanercept has not been effective)[35-39]. Some have suggested that the *de novo* appearance of inflammatory bowel disease following anti-tumor necrosis factor therapy may simply be related to "unmasking" of an underlying inflammatory disease process[40]. Others have documented a superimposed infectious agent[41,42]. A large retrospective study concluded that paradoxical adverse events of anti-tumor necrosis factor therapy may occur, but none were agent specific[43].

CONCLUSION

Several intestinal, particularly colonic complications have been recorded with the emerging armamentarium of monoclonal antibody agents used in the management of different inflammatory or malignant disorders. For some, immune-mediated adverse events may occur regularly, while for others, a complication may be rare and the mechanism not so evident. The precise frequency of colonic complications after treatment with these agents has been difficult to determine. In large part, this has been related to the clinical focus being largely directed to the most severely symptomatic cases. Although selected patients treated for either ulcerative colitis or Crohn's disease with these biological agents has increased over the past decade, in retrospect, it may be that some labeled as "refractory" or not responsive to these agents may simply have been made worse. Published clinical trials may not always detail a failed therapeutic event as an adverse event. Future awareness of the possible adverse intestinal effects of monoclonal agents may be important.

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ORIGINAL ARTICLE

Probiotic metabolites from *Bacillus coagulans* GanedenBC30™ support maturation of antigen-presenting cells *in vitro*

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Abstract

AIM: To study the effects of probiotic metabolites on maturation stage of antigen-presenting immune cells.

METHODS: Ganeden *Bacillus coagulans* 30 (GBC30) bacterial cultures in log phase were used to isolate the secreted metabolite (MET) fraction. A second fraction was made to generate a crude cell-wall-enriched fraction, by centrifugation and lysis, followed by washing. A preparation of MET was subjected to size exclusion centrifugation, generating three fractions: < 3 kDa, 3-30 kDa, and 30-200 kDa and activities were tested in comparison to crude MET and cell wall in primary cultures of human peripheral blood mononuclear cell (PBMC) as a source of antigen-presenting mononuclear phagocytes. The maturation status of mononuclear phagocytes was evaluated by staining with monoclonal antibodies towards CD14, CD16, CD80 and CD86 and analyzed by flow cytometry.

RESULTS: Treatment of PBMC with MET supported maturation of mononuclear phagocytes toward both macrophage and dendritic cell phenotypes. The biological activity unique to the metabolites included a reduction of CD14+ CD16+ pro-inflammatory cells, and this property was associated with the high molecular weight metabolite fraction. Changes were also seen for the dendritic cell maturation markers CD80 and CD86. On $CD14^{dim}$ cells, an increase in both CD80 and CD86 expression was seen, in contrast to a selective increase in CD86 expression on $CD14^{bright}$ cells. The co-expression of CD80 and CD86 indicates effective antigen presentation to T cells and support of T helper cell differentiation. The selective expression of CD86 in the absence of CD80 points to a role in generating T regulatory cells.

CONCLUSION: The data show that a primary mechanism of action of GBC30 metabolites involves support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making.

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Key words: Mononuclear phagocytes; Dendritic cell maturation; Co-stimulatory molecules; Antigen-presentation; Probiotics; Metabolites

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INTRODUCTION

Bacteria are ubiquitous in the environment, having colonized every extreme of nature. This includes the human body where they outnumber human cells by an order of magnitude. The biggest reservoir of these symbiotic bacteria on the human body is the lower gastrointestinal tract[1] where large numbers of coexisting (commensal) bacteria participate in nutrient assimilation including the breakdown of indigestible carbohydrates. They also produce amino acids and vitamins for their host and play a key role in healthy immune system development.

The immune system recognizes both pathogenic and commensal bacteria through a family of pattern recognition receptors known as the toll-like receptor (TLR) family[2]. These receptors interact with molecules present on the exterior surface of bacteria and include lipopolysaccharide (LPS), flagellin, lipoteichoic acid and lipoproteins as well as bacterial DNA. Toll-like receptors are present on cells participating in both innate and adaptive immunity such as monocytes/macrophages and dendritic cells (DC) as well as epithelial cells of the intestinal mucosa.

The emerging picture is that commensal bacteria have an enormous impact on health. While a healthy microbiota can aid the host by increasing nutrient absorption and training the immune system to not respond to self, conversely an unhealthy (i.e., unbalanced) microbiota can lead to malabsorption, inflammation and disease[3-5]. A growing body of evidence suggests that these effects, both positive and negative, of the microbiota on the host are mediated by the immune system. Probiotics are defined as microorganisms that when ingested in a sufficient amount confer a health benefit upon the host and are known to interact with the immune system. Probiotic microorganisms have a long history of human consumption in the form of fermented foods and have shown health benefits in treating dysbiosis, irritable bowel syndrome, and eczema[6]. GanedenBC30™(*Bacillus coagulans* GBI-30, 6086) (GBC30) is a proprietary strain of the gram positive, lactic acid producing spore-forming bacteria known as *Bacillus coagulans*. This strain of *B. coagulans* can survive extremes of heat and pressure in manufacturing as well as the harsh, acidic environment of the human gastrointestinal tract, leading to a very high survival rate and germination in the lower intestinal tract. The safety of consumption of this strain was documented in acute and sub-chronic studies in rats[7].

One way in which commensal bacteria modulate the immune response is by the secretion of certain bioactive compounds. This suggests that metabolites of commensal bacteria have effects of their own and that there may be unique health benefits to be derived from the consumption of live probiotic cultures or probiotic metabolite preparations. Recent studies on the bacterial compound polysaccharide A from *Bacteroides fragilis* have shown the ability of this molecule to prevent intestinal inflammation caused by *Helicobacter pylori* infection and to correct the symptoms of encephalomyelitis in mice, an animal model for human multiple sclerosis[8-10]. The recent sequencing data from 178 commensal microbial genomes has identified over 30 thousand potential protein-coding sequences of which 97% are unique[11]. This suggests a vast untapped reservoir of novel genes including those coding for potential secreted compounds.

The work presented here build on a previous study that showed both enhancement of innate immune responses as well as anti-inflammatory effects of GBC30 *in vitro*[12]. In particular, the data presented here has aimed at investigating the differences between a crude preparation versus the metabolite fraction in more detail with a particular focus on modulation of key regulatory immune cells by specific size-selected fractions of GBC30 metabolite (MET) compounds.

MATERIALS AND METHODS

Reagents

The following buffers and reagents were obtained from Sigma-Aldrich (St. Louis, MO): Histopaque 1077 and 1119, phosphate-buffered saline (PBS), RPMI-1640 culture medium, fetal calf serum, L-glutamine 200 mmol/L, penicillin-streptomycin 100X solution, and bovine serum albumin. CD80-FITC, CD86-PE, CD16-PE and CD14-PerCP were obtained from BD Biosciences (San Jose, CA). Sodium Azide (NaN_3) was obtained from LabChem Inc. (Pittsburgh, PA). Low-binding 100 μm zirconium beads were obtained from OPS Diagnostics (Lebanon, NJ) and 0.2 μm cellulose acetate filters from Whatman (Florham Park, NJ). The *Bacillus coagulans* strain (GanedenBC30™) was obtained from Ganeden Biotech Inc. (Mayfield Height, OH).

Preparation of Bacillus coagulans metabolite fractions

Using sterile technique, two separate samples of 2.0 g of GanedenBC30™ spores were each placed into 25 mL PBS and heated at 70 ℃ for 30 min. Spores were then centrifuged at 2400 rpm for 5 min, PBS was removed and each tube of spores re-suspended and placed in culture flasks containing 25 mL of RPMI-1640 culture medium. The cultures were incubated at 37 ℃ for 24 h at which time an additional 20 mL of RPMI-1640 was added and the cultures incubated for an additional 24 h. Following 48 h of incubation, the bacterial cultures contained 5×10^7 bacteria/mL.

Preparation of GanedenBC30™ culture supernatant as

a source of metabolites (MET): Cultures were transferred to 50 mL centrifuge tubes and initially spun at 1000 rpm for 2 min to remove any remaining spores. The liquid containing the bacteria and metabolites was decanted into new tubes and centrifuged at 3500 rpm for 20 min. The supernatant was decanted from the large bacterial pellets and combined in a single tube followed by filtration twice through a 0.2 μm cellulose acetate syringe filter. This filtrate was either frozen directly as 250 μL aliquots (crude metabolite fraction) or spun through Amicon Ultra protein size separation columns from Millipore (Bedford, MA) followed by aliquot preparation and storage at -20 ℃. Separation of the metabolites into different molecular weight fractions was performed in the following manner: for the fraction that is < 3 kDa, the crude metabolite preparation was placed onto a 3 kDa molecular weight cutoff centrifuge column and spun at 2500 rpm for 10 min. The filtrate that passed through the filter contained material that was less than 3 kDa. Aliquots were made from this material and frozen (metabolites < 3 kDa fraction). The material that did not pass through the column was then placed into a tube containing a 30 kDa molecular weight cutoff filter and spun at 2500 rpm for 10 min. The filtrate that passed through this filter contained material that was 3-30 kDa. Aliquots were made from this material and frozen (metabolites 3-30 kDa fraction). The remaining metabolite material that did not pass through the 30 kDa molecular weight filter was also aliquoted and frozen and this fraction was called metabolites 30-200 kDa fraction. It is important to note that these size separations are not exact and that while large molecules are excluded from the fractions containing the smaller molecules, some small molecules may still remain in the fractions containing the larger molecules.

Preparation of GanedenBC30™ crude cell wall (CW): The two bacterial pellets were each processed separately and then combined after the final bead milling step. Following centrifugation of the bacterial culture at 3500 rpm for 20 min and decanting of the supernatant, the bacterial pellets were washed twice in 45 mL of PBS with subsequent pelleting by centrifugation at 2500 rpm for 10 min. The washed pellets went through one freeze/thaw cycle followed by multiple bead milling cycles. In brief, the pellets were resuspended in 4 mL of PBS and then 4 mL of 100 μm low-binding zirconium beads were added. One cycle of bead milling consisted of 60 one-second pulses of the bacteria/bead mixture on a vortex mixer. Ten of these cycles were performed. The bacteria/bead mixture was placed on ice in between bead milling cycles. At the end of the 10 bead-milling cycles, the beads were allowed to settle in the tubes and the liquid removed from the two tubes and combined. The liquid containing the fragmented bacteria was spun at 3500 rpm for 20 min followed by transfer of the liquid to Eppendorf tubes and centrifugation at 14 000 rpm for 5 min. The high speed centrifugation was necessary to remove any large fragments of bacteria that were not disrupted by the bead milling. The final solution was brought up to 45 mL with PBS and filtered through a 0.2 μm cellulose acetate filter and stored directly at -20 ℃ as 250 μL aliquots (crude cell wall). It is important to note that the cell wall preparation will also contain some GBC30 cellular contents in addition to cell wall components.

Purification of peripheral blood mononuclear cells

Healthy human volunteers between the age of 20 years and 60 years served as blood donors after obtaining written informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board. Isolation of peripheral blood mononuclear cell (PBMC) was performed as previously described[13].

Cell surface staining of CD14 positive mononuclear phagocytes

Complete cell culture media used for the culture of PBMC consisted of RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Peripheral blood mononuclear cells were cultured for 3 d in the presence of serial dilutions of BC30 metabolite fractions or crude cell wall followed by cell surface immunostaining with CD14, CD80, CD86 and CD16 monoclonal antibodies. Processing of cells for immunostaining was performed as previously described[13] with the following modifications: optimal amounts of monoclonal antibodies per sample were 3 μL for CD14-PerCP and 4 μL for CD80-FITC, CD86-PE and CD16-PE. Experiments were performed three times using PBMC isolated from three different blood donors. Each test condition was performed in duplicate and untreated and LPS-treated controls were tested in quadruplicate and triplicate, respectively.

Statistical analysis

Statistical significance was tested using Student's *t*-test performed with Microsoft Excel. All *P* values were two-sided and were considered significant when $P < 0.05$ and highly significant when $P < 0.01$. Only statistically significant *P* values are reported.

RESULTS

GBC30 effects on mononuclear phagocyte differentiation

Mononuclear phagocyte differentiation in 3-d primary PBMC cultures was examined by cell surface staining for proteins expressed by monocytes/macrophages and dendritic cells. These included CD14 (Figure 1) and CD80 and CD86 (Figures 2 and 3). CD14 expression on CD-14^{bright} cells was increased following exposure of cells to GBC30 crude MET and CW fractions (Figure 1A). Treatment of cells with crude MET showed a strong dose-dependent response with statistically significant increases occurring with the 4 highest doses. As expected, LPS treatment of cells greatly increased CD14 expression[14]. Crude CW also led to statistically significant increases in CD14 expression. When the effects of crude and size-selected MET fractions of GBC30 at a 1:200 dilution

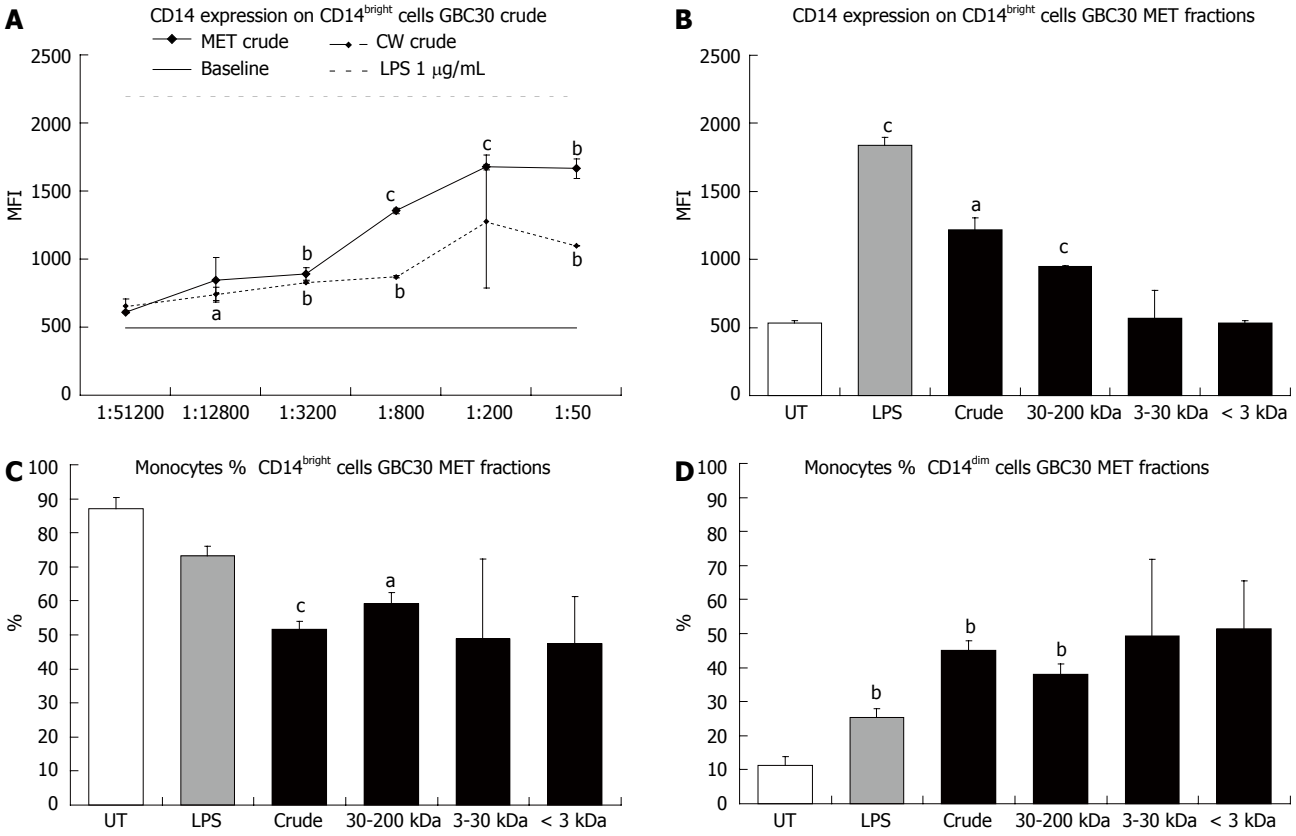

Figure 1 CD14 expression on mononuclear phagocytes. Mononuclear phagocytes present in 3-d peripheral blood mononuclear cell cultures exposed to either the Ganeden *Bacillus coagulans* 30 (GBC30) metabolites (MET), cell wall-enriched (CW), or MET fractions, were identified using electronic gating of the flow cytometry data by gating on forward scatter/side scatter followed by gating for CD14 positivity. A comparison was made between cells that were untreated (UT), exposed to lipopolysaccharide (LPS) or to the different GBC30 fractions. A: Comparison of CD14 mean fluorescence intensity showed a dose-dependent increase in CD14 expression in cells treated with crude MET. A milder increase was seen for cells treated with crude CW. The baseline indicates CD14 expression on untreated cells; B: The increase in CD14 expression was primarily caused by high molecular weight compounds present in MET; C: The percent of $CD14^{bright}$ cells in the mononuclear phagocyte population was decreased by all fractions of MET; D: The percent of $CD14^{dim}$ cells in the mononuclear phagocyte population was increased by treatment of cells with all MET fractions. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity.

were compared (Figure 1B), high molecular weight fractions (crude and 30-200 kDa) increased CD14 expression while PBMC treated with either the 3-30 kDa or < 3 kDa fractions showed CD14 expression levels similar to untreated cultures.

Reduction of $CD14^{bright}$ cells

Because CD14 expression on mononuclear phagocytic cells varies and expression levels have been correlated with different cell populations, the percent of $CD14^{bright}$ versus $CD14^{dim}$ cells was determined for PBMC cultures exposed to different GBC30 MET fractions. All fractions of MET at a 1:200 dilution led to decreased numbers of $CD14^{bright}$ cells (Figure 1C) while an inverse pattern of response was seen regarding changes in $CD14^{dim}$ cell numbers (Figure 1D). In this case all fractions of MET led to increases in the percent of $CD14^{dim}$ cells.

$CD14^{bright}$ cells: Effects of GBC30 metabolite and cell wall on CD80 and CD86 expression

Next, CD80 and CD86 expression was determined for the $CD14^{bright}$ cell population. Both MET and CW crude fractions led to statistically significant decreases in CD80 expression on $CD14^{bright}$ cells (Figure 2A) while only MET crude increased CD86 expression (Figure 2C). When crude and size selected fractions of MET were compared at the 1:200 dilution all fractions of MET led to similar statistically significant decreases in CD80 expression (Figure 2B). A comparison of the effect of MET fractions on CD86 expression showed that the 3-30 kDa and < 3 kDa fractions led to a decrease but these changes were not statistically significant (Figure 2D).

$CD14^{dim}$ cells: Effects of GBC30 metabolite and cell wall on CD80 and CD86 expression

When expression of the co-stimulatory molecules CD80 and CD86 was determined for the $CD14^{dim}$ cell population, both MET and CW crude increased CD80 (Figure 3A) and CD86 (Figure 3C) expression with MET crude having the biggest effect, particularly on CD86 expression. When crude and size selected fractions of MET were compared at the 1:200 dilution, only the crude and 30-200 kDa fractions led to statistically significant increases in CD80 expression (Figure 3B). A comparison

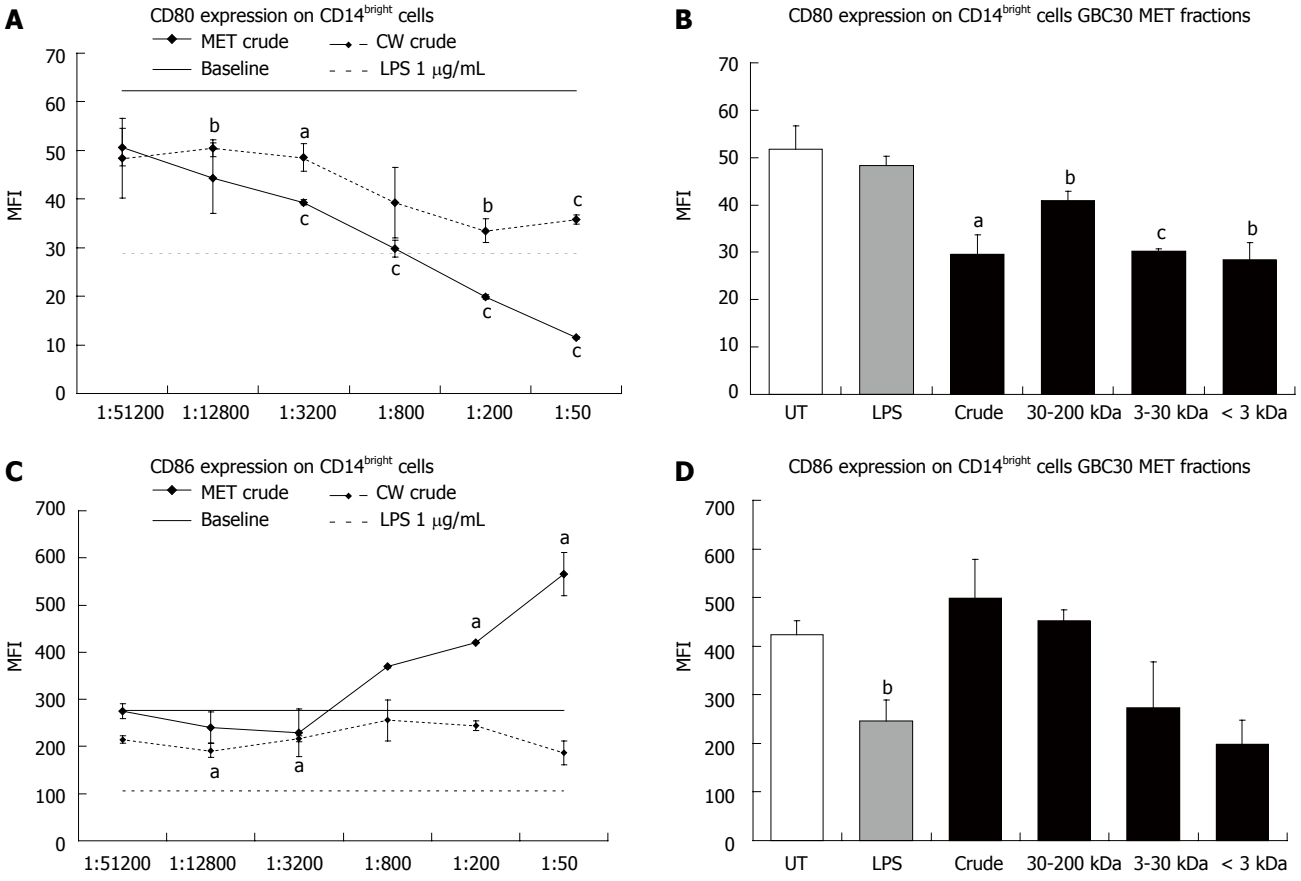

Figure 2 Expression of the co-stimulatory molecules CD80 and CD86 on CD14^{bright} mononuclear phagocytes from 3-d peripheral blood mononuclear cell cultures. A: Comparison between the effects of serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) or cell wall enriched (CW) fractions on CD80 expression on CD14^{bright} cells showed dose-dependent decreases in CD80 expression. Both MET and CW reduced CD80 expression to levels similar to those seen with Lipopolysaccharide (LPS) treatment; B: A comparison of the effects of size-fractionated MET on CD80 expression on CD14^{bright} cells shows that all MET fractions reduce expression; C: Comparison between the effects of serial dilutions of crude MET or CW on CD86 expression on CD14^{bright} cells showed dose-dependent increases in CD86 expression when cells were exposed to the three most concentrated dilutions of MET. Treatment of cells with CW resulted in a uniform modest decrease in CD86 expression; D: The effect on increased CD86 expression is present only in the crude preparation of MET. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity; UT: Untreated.

of the effect of MET fractions on CD86 expression showed that crude MET increased CD86 expression while the 3-30 kDa and < 3 kDa fractions decreased expression (Figure 3D).

Reduction in CD14+ CD16+ cells

Mononuclear phagocytes have also been classified according to expression of the cell surface protein CD16 with CD14+ CD16+ cell subsets considered to be pro-inflammatory[15]. The effect of crude MET and CW fractions on the percent of CD14+ CD16+ and CD14+ CD16- cells in 3-d PBMC cultures was investigated. Crude MET treatment of cells resulted in a dose dependent decrease in CD14+ CD16+ cells (Figure 4A) and an increase in CD14+ CD16- cells (Figure 4C). Crude CW had a much milder effect that mirrored that of crude MET. When cells were exposed to the crude or size fractionated preparations of MET at a 1:200 dilution, it was the fractions with the largest compounds (crude and 30-200 kDa) that showed the greatest effect on CD14+ CD16+ (Figure 4B) and CD14+ CD16- (Figure 4D) cell numbers although the 3-30 kDa and < 3 kDa fractions also produced statistically significant reductions in the number of CD14+ CD16+ cells.

DISCUSSION

The work presented here investigated the effects of MET and CW fractions of the GBC30 probiotic strain on mononuclear phagocyte phenotypes in primary PBMC cultures. The cellular model for examining the immune effects was carefully chosen, and primary PBMC cultures were used because this allows the simultaneous interaction of multiple cell types and has been shown to support the survival of blood dendritic cells without the addition of exogenous cytokines[16]. One of the main findings was the biological activities of the metabolites and the data showed that a primary mechanism of action of BC30 metabolites involved support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making.

Compounds present in the MET crude fraction con-

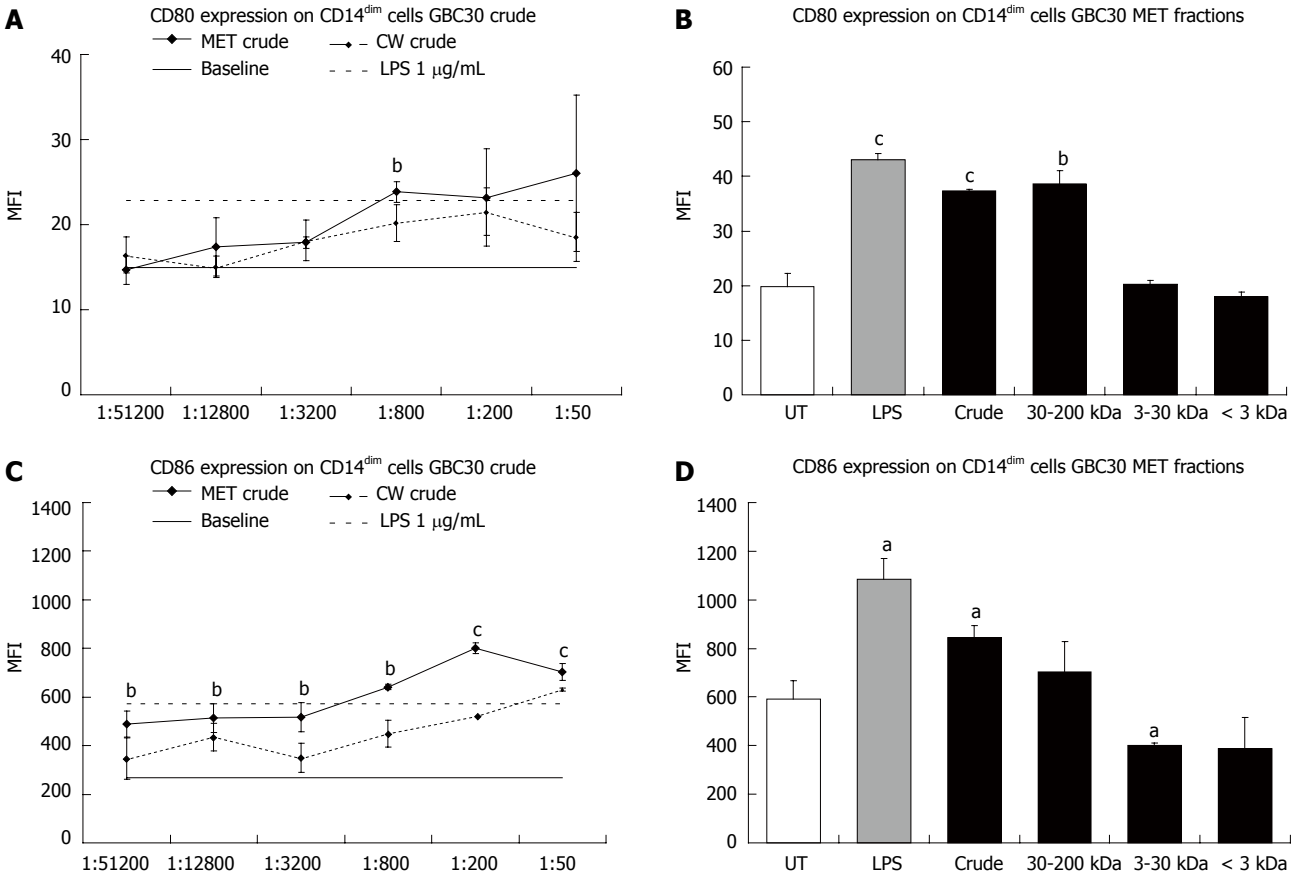

Figure 3 Expression of the co-stimulatory molecules CD80 and CD86 on CD14^{dim} mononuclear phagocytes from 3-d peripheral blood mononuclear cell cultures. A: Comparison between the effects of serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) or cell wall enriched (CW) fractions on CD80 expression on CD14^{dim} cells showed that both MET and CW led to increased expression; B: The increase in CD80 expression following treatment of cells with MET was due to high molecular weight compounds; C: Comparison of CD86 expression on CD14^{dim} cells exposed to crude fractions of MET or CW resulted in increased CD86 expression; D: Size-selected fractions of MET did not have uniform effects on CD86 expression on CD14^{dim} cells. Crude MET increased expression while 3-30 kDa and < 3 kDa fractions decreased expression. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. MFI: Mean fluorescence intensity; UT: Untreated; LPS: Lipopolysaccharide.

sisted entirely of compounds that were secreted by GBC30 into the culture media. The CW crude fraction was isolated from whole bacteria and may contain some compounds present in the MET preparation in addition to compounds unique to the cell wall. Size fractionation of crude MET was used to evaluate immune modulating compounds based on MW and their association with one or more fractions.

Probiotic organisms support mucosal immunity and similar to commensal bacteria in the human gut, they interact with mononuclear phagocytic cells such as dendritic cells and macrophages[17-19]. The expression levels of CD80 and CD86 co-stimulatory molecules can be used to indicate the differentiation of mononuclear phagocytes to that of antigen presenting cells such as dendritic cells. While CD14 is still present on some subsets of dendritic cells, typically when mononuclear phagocytes adopt a dendritic cell identity, CD14 expression is down regulated with the concurrent up regulation of CD80 and CD86[20]. The differential roles of the co-stimulatory molecules CD80 and CD86 suggests that co-expression of both molecules on dendritic cells leads to T helper cell differentiation, whereas the predominant expression of CD86 support T regulatory cells, and supports an anti-inflammatory cytokine profile by decreasing Interferon-gamma production and increasing interleukin (IL)-4 production[21]. Since the current literature suggests that mononuclear phagocytes present in the circulation are already committed in their developmental path[22], the changes seen in CD14 expression suggest that MET and CW simultaneously enhance the maturation of two separate subpopulations of mononuclear phagocytic cells (CD14^{bright} and CD14^{dim}) towards their corresponding macrophage and dendritic cell phenotypes. The effect of GBC30 on putative DC maturation in PBMC cultures, suggests that DC may be responsible for the IL-6 production that was previously shown *in vitro*[12], and this increased IL-6 production may reflect normal physiological interactions between DC and commensal bacteria in the human gut[17,23].

The data suggest that live GBC30 in the gut lumen would provide metabolites from GBC30, different from the immune modulating compounds associated with the cell wall enriched fraction, and support the interpretation that the live metabolically active GBC30 has stronger immune modulating activity than accounted for by its cell wall alone. Immune modulating activity has been identified from the supernatant of the probiotic strains *Lactoba-*

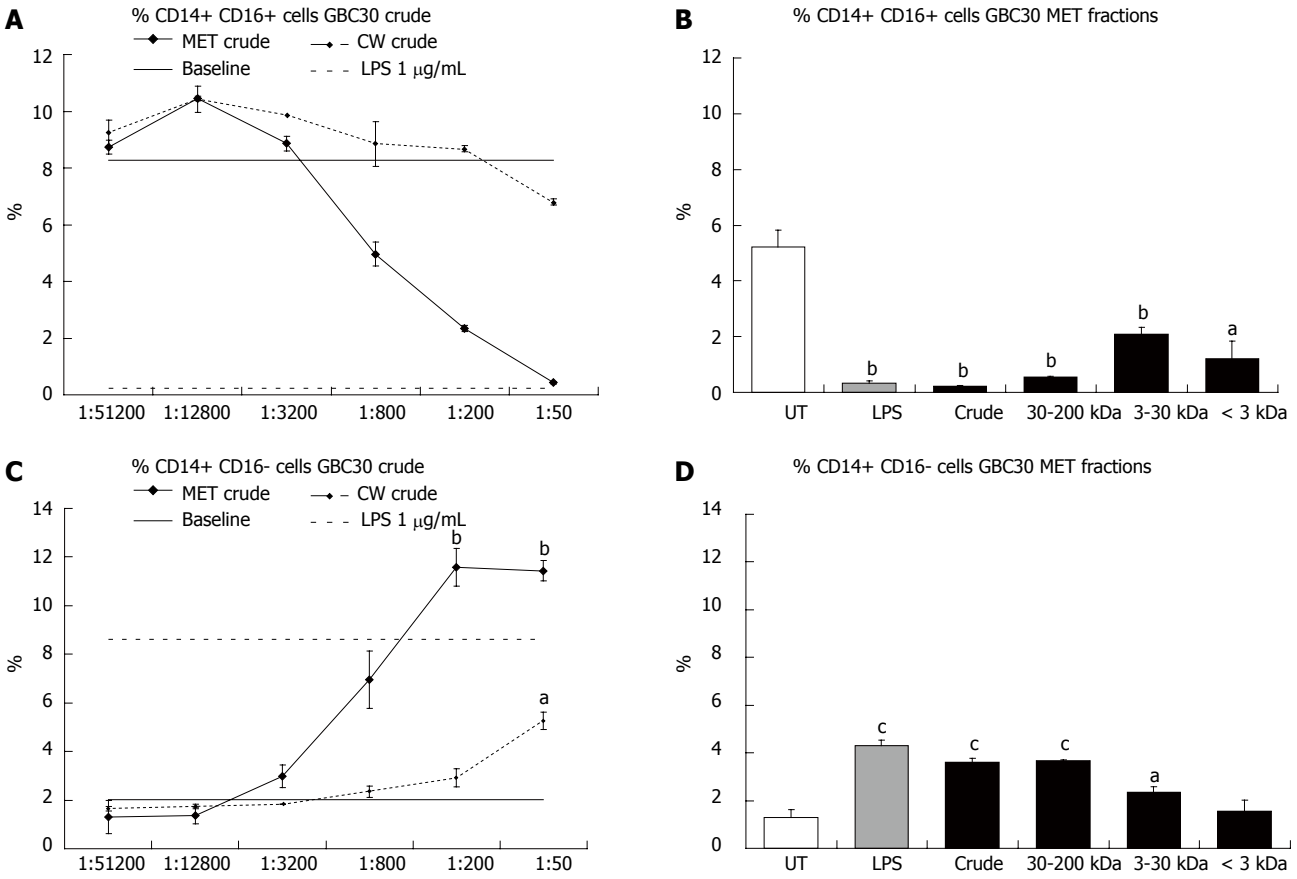

Figure 4 Changes in the percent of CD14+ CD16+ and CD14+ CD16- cell populations following exposure of 3-d peripheral blood mononuclear cell cultures to Ganeden *Bacillus coagulans* 30. A: Exposure of cells to serial dilutions of Ganeden *Bacillus coagulans* 30 (GBC30) crude metabolites (MET) led to a strong dose-dependent decrease in CD14 CD16 double positive cells while exposure to cell wall enriched (CW) did not reduce this cell population; B: Treatment of cells with size-selected MET fractions show that all fractions of MET reduce the number of CD14+ CD16+ cells; C: The percent of CD14+ CD14- cells in peripheral blood mononuclear cell cultures increased in cultures treated with crude MET and CW. Treatment with MET resulted in a very strong dose-dependent increase while CW treatment produced a milder increase at the two highest concentrations; D: Treatment of cells with size-selected MET fractions show that only fractions containing high molecular weight compounds increase the number of CD14+ CD16- cells. Bar graphs show data from 1:200 dilutions of each MET fraction and lipopolysaccharide (1 μg/mL). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$. For each data point, the mean ± SD are shown for each duplicate data set. Graphs show data representative of 1 out of 3 experiments. UT: Untreated; LPS: Lipopolysaccharide.

cillus casei Shirota[24] and *Bifidobacterium breve*[25], the probiotic yeast *Saccharomyces boulardii*[26], the commensal bacterium *Faecalibacterium prausnitzii*[27] and gut-derived lactobacilli and bifidobacteria[28]. In the case of *Faecalibacterium prausnitzii*, injection of the supernatant completely protected mice from trinitrobenzenesulphonic acid induced colitis while live bacteria provided only partial protection[27]. Most of these studies focused on cytokine production in monocyte-derived dendritic cell cultures[26,27] and have determined this to occur through a TLR2 dependent mechanism. In one study, it was determined that the active component in the supernatant from *Lactobacillus casei* was a polysaccharide peptidoglycan complex[24] while another study has suggested that the immune boosting effect of common botanical extracts is through effects of bacterial lipoproteins and lipopolysaccharides (derived from endophytes, the resident bacteria present in all plants) on macrophage activation[29].

Thus, due to direct effects on mononuclear phagocyte differentiation, GBC30 metabolites lend support to two important cell types responsible for antigen recognition, presentation to cells within the adaptive immune system, and execution of regulatory functions, including immunological memory. The effect of dried/reconstituted material was tested in three different bioassays previously reported to show bioactivity[12], including anti-inflammatory effects (data not shown), and no significant difference was seen between this and frozen/thawed material. The stability of the bioactive compounds in the metabolite fraction holds promise for development of a consumable product.

Results from the GBC30 MET fractions suggest that the metabolic activity of this probiotic organism is an intregral part of its immune modulating functions, and that multiple different compounds act in synergy to support key aspects of mucosal immune protection. These results suggest specific mechanisms of action and may give insight into some aspects of previous clinical studies showing reduced symptoms from irritable bowel syndrome[30]. We suggest that further studies include *ex vivo* evaluation of mononuclear cells isolated from lamina propria and Peyer's patches, in terms of antigen presentation, dendritic cell and B lymphocyte maturation, and IgA production. Further clinical work is warranted, not only

in populations with inflammatory syndromes, but also in populations with reduced mucosal immune protection, and should include assessment of inflammatory markers in serum, as well as secretion of IgA.

In conclusion, the biological activities reported here for the metabolites point to a unified mechanism of action directed at the differentiation and maturation state of antigen-presenting cells such as the macrophage/dendritic cells. In terms of immune regulation, this plays a pivotal role in decision-making, for example in whether T lymphocytes are induced into immunological anergy (unresponsiveness, tolerance) or whether they are triggered into proliferation, cytokine production, and other mechanism of inter-cellular communication. It is conceivable that metabolites are absorbed into the mucosal immune tissue along the intestinal track and help direct more efficient antigen-recognition, while reducing immune reactivity towards harmless food-borne antigens. This may provide a mechanism to explain the improved immune protection, while also seeing a reduction in food allergies and associated inflammatory reactions with consumption of certain probiotic strains.

COMMENTS

Background

The mucosal surface of the human gastrointestinal tract is an interface between the external and internal environments, separated by a single epithelial cell layer. On the one side are food antigens, commensal bacteria and potential pathogens while cells of the immune system reside on the other. Oral tolerance refers to the ability of the immune system to not react towards food and commensal bacterial antigens while still evoking a robust immune response towards pathogens. Probiotic bacteria interact with the host immune system and elicit beneficial immune modulating effects that include a reduction in inflammation in inflammatory bowel disease, amelioration of antibiotic-induced diarrhea, and protection from pathogen infection.

Research frontiers

Recent evidence suggest that the interaction of commensal bacteria and probiotics with the immune system is more than a mechanical engagement of bacterial cell wall components with immune cell receptors and includes an active cross-talk between live bacteria and the host through secreted substances (metabolites). This is an active area of research and data from microbiome genomic sequencing suggests that the majority of predicted genes encode proteins with unknown functions.

Innovations and breakthroughs

Most of the published work on probiotics interacting with the immune system has focused on the bacterial cell wall activating the immune system through engagement of the Toll-like receptor (TLR) family, in particular TLR2 and TLR4. Much less research has focused on secreted metabolites and very little is known about what these secreted compounds are. The data presented here showed that a primary mechanism of action of Ganeden *Bacillus coagulans* 30 (GBC30) metabolites involved support of more mature phenotypes of antigen-presenting cells, important for immunological decision-making. An immature antigen presenting cell may fail in triggering an appropriate immune defense reaction, while either inducing immunological unresponsiveness (anergy) towards the antigen, or induce an allergic reaction to the antigen.

Applications

The support of antigen-presenting cells *in vitro* by GBC30 metabolites suggests that consumption of GBC30 may lead to *in vivo* effects of improved decision-making in the gut-associated lymphoid tissue (GALT), translating into clinical observations of improved immunity against infections, and reduced immunological anergy and allergy.

Terminology

Cluster of differentiation 14 is a monocyte marker and functions as a co-receptor for bacterial lipopolysaccharide recognition. It is highly expressed on the cell surface of monocytes and macrophages; GALT is a mucosa-associated lymphoid tissue lining the gastrointestinal tract from the esophagus to the colon. It contains immune cells and plays an important part in preventing the immune system from reacting to the resident microflora as well as defence from pathogens; Anergy refers to the absence of a normal immune response to a specific antigen or allergen.

Peer review

The authors present a paper that aimed to investigate any differences between the effects of a crude preparation of GBC30 bacterial culture metabolites compared to the fractionated preparations on the maturation of peripheral blood mononuclear cell. It demonstrates that probiotic bacteria produce metabolites that activate cells of the immune system, beyond what is expected from simple bacterial cell wall components.

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ORIGINAL ARTICLE

Antithrombin Ⅲ injection *via* the portal vein suppresses liver damage

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Abstract

AIM: To investigate the effects of antithrombin Ⅲ (AT Ⅲ) injection *via* the portal vein in acute liver failure.

METHODS: Thirty rats were intraperitoneally challenged with lipopolysaccharide (LPS) and D-galactosamine (GalN) and divided into three groups: a control group; a group injected with AT Ⅲ *via* the tail vein; and a group injected with AT Ⅲ *via* the portal vein. AT Ⅲ (50 U/kg body weight) was administrated 1 h after challenge with LPS and GalN. Serum levels of inflammatory cytokines and fibrin degradation products, hepatic fibrin deposition, and hepatic mRNA expression of hypoxia-related genes were analyzed.

RESULTS: Serum levels of alanine aminotransferase, tumor necrosis factor-α and interleukin-6 decreased significantly following portal vein AT Ⅲ injection compared with tail vein injection, and control rats. Portal vein AT Ⅲ injection reduced liver cell destruction and decreased hepatic fibrin deposition. This treatment also significantly reduced hepatic mRNA expression of lactate dehydrogenase and heme oxygenase-1.

CONCLUSION: A clinically acceptable dose of AT Ⅲ injection into the portal vein suppressed liver damage, probably through its enhanced anticoagulant and anti-inflammatory activities.

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Key words: Antithrombin Ⅲ; Acute liver failure; Intravascular coagulation; Portal vein

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INTRODUCTION

In some patients with acute liver injury (ALI), the liver disease proceeds to acute liver failure (ALF); a severe condition associated with a high mortality rate. Liver transplantation is an effective treatment for patients with

severe ALF[1], whereas artificial liver support systems such as plasma exchange and hemodiafiltration are less effective[2,3]. The difficulties associated with the development of effective treatments for ALF may be attributed to the incomplete understanding of the mechanisms involved in disease progression.

ALF is pathologically characterized by massive hepatocellular necrosis, therefore, intrahepatic microcirculatory disturbances are involved in the pathogenesis and progression of liver disease. Observation of sinusoidal fibrin deposition, increased fibrinogen catabolism and decreased platelet counts suggest that the intrahepatic coagulation system may be activated in ALF, and the following microcirculatory disturbances may play a role in the formation of massive hepatocellular necrosis[4]. The hypothesis that activation of the intrahepatic coagulation system is a fundamental pathogenic factor underlying the development of ALF has prompted researchers to develop new treatments using anticoagulants in experimental animal models and in clinical trials. Intravenous injection of antithrombin Ⅲ (AT Ⅲ), which inhibits serine proteases involved in the coagulation cascade, has been reported to attenuate the progression of liver disease in animal models of ALF induced by concanavalin A, dimethylnitrosamine (DMN) and endotoxin[5-8]. However, the need for high doses of AT Ⅲ (200-400 U/kg body weight) to suppress liver damage makes it difficult to apply this treatment in clinical practice. Therefore, the development of regimens using clinically acceptable doses of AT Ⅲ is necessary.

It has been shown that direct drug delivery into the target organs is more efficient than systemic administration. Direct delivery of 5-fluorouracil and cisplatin into the hepatic artery has been reported to control tumor progression and to extend the median survival time of patients with unresectable hepatocellular carcinoma, which is resistant to systemic chemotherapy[9]. Similarly, we have reported that the progression of severe ALI toward fulminant liver failure is inhibited by transcatheter arterial steroid injection, in which methylprednisolone is directly delivered into the diseased liver *via* the hepatic artery[10]. The effectiveness of direct steroid delivery into the liver has been confirmed in an experimental animal model. Injection of steroids *via* the portal vein in rats with lipopolysaccharide (LPS)- and D-galactosamine (GalN)-induced ALF more effectively suppresses hepatic inflammation and improves survival than injection *via* the tail vein[11]. These observations suggest that the direct delivery of AT Ⅲ into the liver, *via* the hepatic artery or portal vein, may improve liver damage more effectively than peripheral injection of AT Ⅲ.

In this study, we administered a clinically acceptable dose of AT Ⅲ *via* the portal vein or a peripheral vein (tail vein) in rats with LPS/GalN-induced ALF. The suppressive effects of AT Ⅲ on hepatic inflammation were estimated based on the serum levels of transaminase and inflammatory cytokines, and hepatic histology. The extent of damage to the intrahepatic coagulation system was estimated by determining sinusoidal fibrin deposition. Hypoxia in the diseased liver, which is caused by hepatic microcirculatory disturbances, was estimated by analyzing the hepatic mRNA expression of hypoxia-related genes. These parameters were compared among three groups: a control group; rats injected with AT Ⅲ *via* the tail vein; and rats injected *via* the portal vein. Our observations suggest that the injection of AT Ⅲ *via* the portal vein suppresses liver damage more effectively than *via* the tail vein, because of its enhanced anticoagulant and anti-inflammatory activities.

MATERIALS AND METHODS

Chemicals

Human concentrated AT Ⅲ (Anthrobin P500) was purchased from CSL Bering (King of Prussia, PA, United States). LPS (*Escherichia coli*, 055:B5), GalN and other chemicals were purchased from Sigma (St. Louis, MO, United States). All experiments were performed using the same lot of LPS.

Animals

Eight-week-old male Wistar rats weighing 200 g were purchased from Japan SLC (Hamamatsu, Japan). Rats were maintained under controlled conditions with free access to standard chow and water. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Animal Care Committee of Kyushu University.

Animal treatment

LPS (5 μg/kg body weight) and GalN (500 mg/kg body weight) dissolved in 500 μL phosphate buffered solution (PBS) were injected intraperitoneally into rats. One hour after the injections, the animals were anesthetized with pentobarbital sodium, and AT Ⅲ (50 U/kg body weight) dissolved in 200 μL of PBS was injected into the portal or tail vein. Control animals underwent sham injections. Each group consisted of 10 rats.

Transaminase and cytokine assays

Blood samples were taken from the tail vein at 6 h, 12 h and 24 h after injection of LPS and GalN. The serum levels of alanine aminotransferase (ALT) were estimated by Transaminase *C*-test (Wako Pure Chemical Industry, Osaka, Japan). Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-6 (IL-6) were measured using enzyme linked immunosorbent assay (ELISA) kits (Endogen, Rockford, IL, United States).

Assay of serum fibrin degradation products

Blood samples were taken from the tail vein 24 h after injection of LPS and GalN. Serum fibrin degradation products (FDPs) levels were measured using an ELISA kit (Cusabio Biotech, Barksdale, DE, United States).

Histology

Liver tissue samples were collected 24 h after injecting LPS and GalN, fixed in 10% formalin, and embedded in

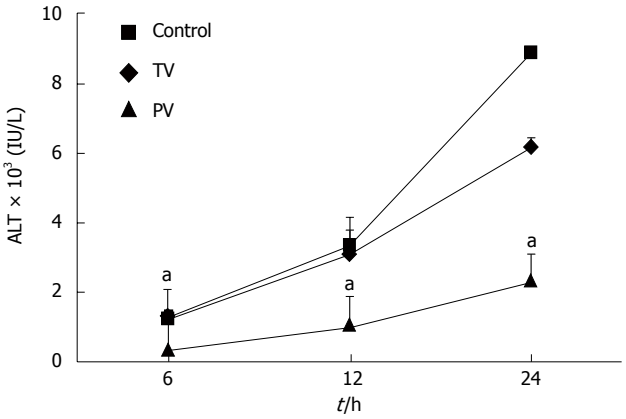

Figure 1 Effects of antithrombin Ⅲ on serum alanine aminotransferase levels in rats with acute liver failure. Lipopolysaccharide (LPS) and D-galactosamine (GalN) were injected intraperitoneally into 8-wk-old Wistar rats. One hour after the challenge, antithrombin (AT) Ⅲ (50 U/kg body weight) was injected into the portal or tail vein. Serum alanine aminotransferase (ALT) levels were measured at 6 h, 12 h and 24 h after injection of LPS and GalN. Control: Untreated; TV: AT Ⅲ injection *via* the tail vein; PV: AT Ⅲ injection *via* the portal vein. Values are mean ± SD (n = 10 rats/group). $^{a}P < 0.01$ *vs* the control group.

paraffin. The sections were stained with hematoxylin and eosin to assess hepatic damage. To determine intrasinusoidal fibrin deposition, the sections were stained with phosphotungstic acid-hematoxylin[12].

Reverse transcription polymerase chain reaction

Total RNA from liver tissue was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and cDNA was synthesized from 1.0 μg RNA by GeneAmp RNA polymerase chain reaction (PCR) (Applied Biosystems, Branchburg, NJ, United States) using random hexamers. Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche, Basel, Switzerland). The reaction mixture (20 μL) contained Master SYBR Green I, 4 mmol $MgCl_2$, 0.5 μmol upstream and downstream PCR primers, and 2 μL first-strand cDNA as a template. To control variations in reactions, all PCR data were normalized against glyceraldehyde 3-phosphate dehydrogenase expression. The forward and reverse PCR primers were 5'-ACTTTCAGAAGGGTCAGGTGTCC-3' and 5'-TTGAGCAGGAAGGCGGTCTTAG-3', respectively, for heme oxygenase-1 (HO-1) and 5'-AGACTGCCGTCCCGAACAAC-3' and 5'-ACATCCACCAGGGCAAGCTC-3', respectively, for lactate dehydrogenase (LDH), respectively.

Statistical analysis

All results are expressed as means ± SD. Significant differences between two groups were assessed using Wilcoxon's rank-sum test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Portal vein AT Ⅲ injection reduced liver cell destruction more effectively than tail vein injection

In the control group, the serum levels of ALT increased over time, reaching 1262 ± 240, 3381 ± 808 and 8906 ± 766 U/L (Figure 1) at 6, 12 and 24 h, respectively. Injection of AT Ⅲ into the tail vein did not affect ALT levels at 6 h or 12 h after the injection of LPS and GalN. However, at 24 h, the ALT levels in the tail vein injection group were significantly lower than those in the control group (8906 ± 766 U/L *vs* 6181 ± 823 U/L, $P < 0.01$). This suggests that the suppressive effects of AT Ⅲ injected *via* the tail vein may be limited to the late stage of liver disease. In contrast, in rats injected with AT Ⅲ *via* the portal vein, ALT levels were reduced during the early stage (i.e., 6 h, 369 ± 141 U/L), which was maintained at all time-points. At 24 h, the ALT levels in this group were significantly lower than those in the control group (2352 ± 760 U/L *vs* 8906 ± 766 U/L, $P < 0.01$).

To support the effects of these treatments on the suppression of liver damage, the serum levels of inflammatory cytokines were measured. The cytokine levels demonstrate the greater anti-inflammatory effects of AT Ⅲ injected *via* the portal vein. TNF-α levels in the tail vein injection group were similar to those in the control group. In contrast, TNF-α levels in the portal vein injection group were significantly lower than those in the control group (235 ± 79 pg/mL *vs* 500 ± 127 pg/mL, $P < 0.01$, Figure 2A). AT Ⅲ injection *via* the tail vein reduced the IFN-γ levels compared with the controls, but the difference was not significant. As with other cytokines, IFN-γ was significantly reduced in the portal vein group compared with the control group (21 ± 12 pg/mL *vs* 89 ± 45 pg/mL, $P < 0.05$, Figure 2B). The IL-6 levels showed similar trends to those observed for TNF-α. AT Ⅲ injection *via* the tail vein did not suppress IL-6 levels, whereas its injection *via* the portal vein significantly reduced IL-6 levels compared with those in the control group (368 ± 120 pg/mL *vs* 572 ± 47 pg/mL, $P < 0.01$, Figure 2C).

Effects of AT Ⅲ on liver damage analyzed by liver histology

Histological examination showed extensive hepatocellular necrosis and hemorrhaging in the control liver (Figure 3A). In tail-vein-injected rats, extensive hepatocellular necrosis was not found but areas with confluent necrosis were scattered throughout the liver (Figure 3B). Consistent with the suppression of ALT and inflammatory cytokine levels, prominent histological improvement was noted in the liver of rats injected with AT Ⅲ *via* the portal vein, because relatively few, scattered areas of necrosis with disordered hepatic cords were observed (Figure 3C).

Effects of AT Ⅲ on hepatic mRNA expression of hypoxia-related genes in ALF

To evaluate the extent of hepatic hypoxia, as induced by microcirculatory disturbances, we determined the hepatic expression of hypoxia-related genes such as LDH and HO-1. LDH is an essential enzyme for anaerobic respiration, and its expression increases in cells exposed to hypoxia[13-15]. The transcriptional expression of HO-1 is also increased in hypoxia, resulting in increased produc-

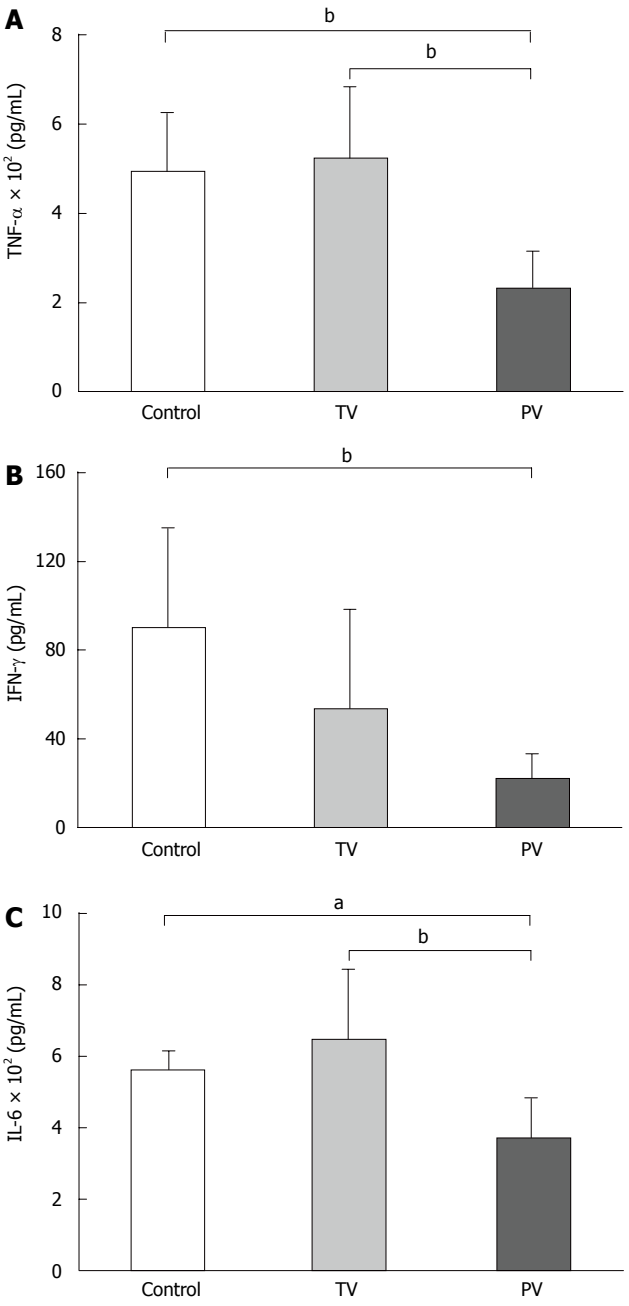

Figure 2 Effects of antithrombin Ⅲ on serum inflammatory cytokine levels. The serum levels of tumor necrosis factor-α (TNF-α) (A), interferon-γ (IFN-γ) (B) and interleukin (IL)-6 (C) were determined 24 h after injection of lipopolysaccharide and D-galactosamine. Control: Untreated; TV: Antithrombin (AT) Ⅲ injection *via* the tail vein; PV: AT Ⅲ injection *via* the portal vein. Values are means ± SD (n = 10 rats/group). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

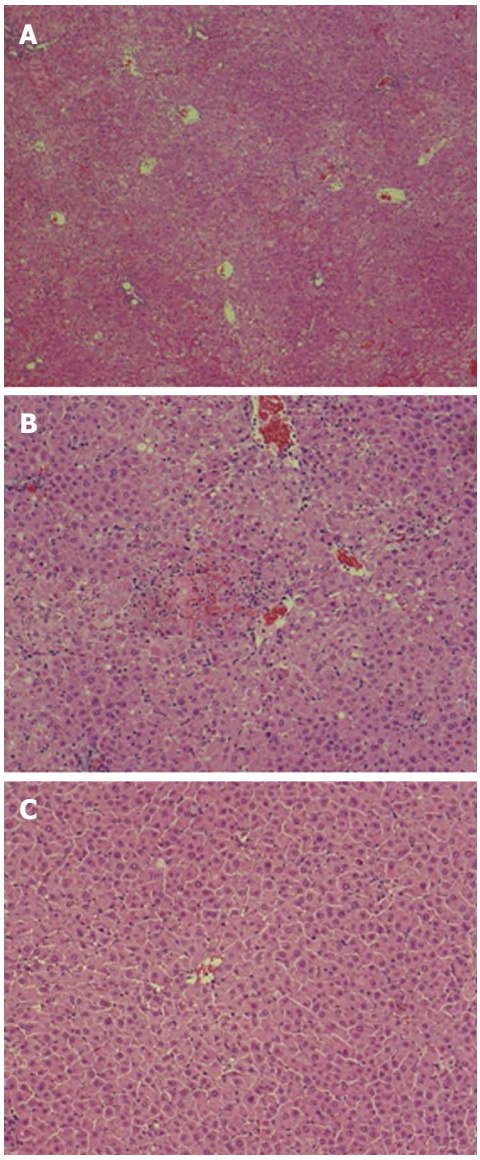

Figure 3 Effects of antithrombin Ⅲ on liver histology. Liver samples were obtained 24 h after lipopolysaccharide and D-galactosamine injection and stained with hematoxylin and eosin (magnification, × 200). A: Control; B: Antithrombin (AT) Ⅲ injected *via* the tail vein; C: AT Ⅲ injected *via* the portal vein.

tion of carbon monoxide, a vasodilator, and bilirubin, an antioxidant[16,17]. Increased serum LDH levels and hepatic expression of HO-1 have been reported in patients with ALF and might be useful to predict prognosis[18,19]. Therefore, we speculated that the expression of these genes could reflect the extent of hypoxia in the diseased liver. Tail vein AT Ⅲ injection did not affect the expression of LDH, suggesting that peripheral administration of AT Ⅲ does not improve hepatic hypoxia. In contrast, LDH expression was significantly reduced in rats injected with AT Ⅲ *via* the portal vein compared with the tail vein, and the control group (Figure 4A). The expression patterns of HO-1 differed from those of LDH. AT Ⅲ injection *via* the tail vein significantly reduced HO-1 expression levels compared with those in the control group, and its expression was further reduced by AT Ⅲ injection *via* the portal vein (Figure 4B). These observations suggest that AT Ⅲ injection *via* the portal vein reduces intrahepatic hypoxia, probably by controlling the microcirculatory disturbances.

Portal vein injection of AT Ⅲ injection improved activity of the deteriorated coagulation system

Serum levels of FDPs are a marker for the extent of deterioration in the coagulation system in ALF[20,21]. Injection of AT Ⅲ *via* the tail vein did not change FDP levels, whereas injection of AT Ⅲ *via* the portal vein significant-

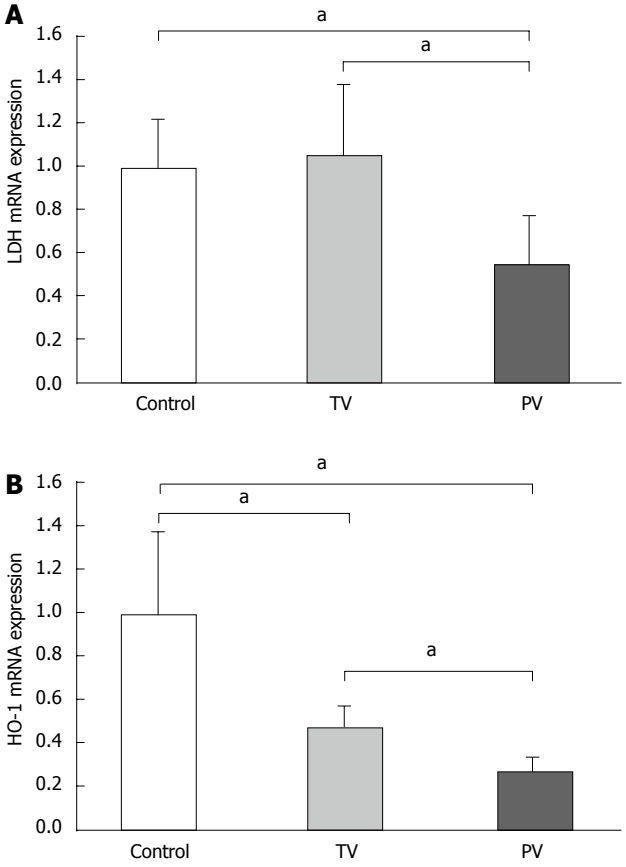

Figure 4 Effects of antithrombin Ⅲ on hepatic mRNA expression of lactate dehydrogenase and heme oxygenase-1. Hepatic mRNA expression of lactate dehydrogenase (LDH) (A) and heme oxygenase (HO)-1 (B) was determined by real-time polymerase chain reaction. Reactions were normalized for glyceraldehyde-3-phosphate dehydrogenase expression and the relative expression in the untreated liver was used as a control. Control: Untreated; TV: Antithrombin (AT) Ⅲ injection *via* the tail vein; PV: AT Ⅲ injection *via* the portal vein. Values are means ± SD (n = 10 rats/group). $^{a}P < 0.05$ *vs* the tail vein group and the control group.

ly reduced FDP levels compared with those in the control and tail vein groups (Figure 5). Taken together, improvements in the coagulation system were only achieved by injecting AT Ⅲ directly into the diseased liver.

Effects of AT Ⅲ on intrahepatic fibrin deposition

Fibrin deposition in hepatic sinusoids has been observed in ALF. It is recognized as a manifestation of disturbances in the intrahepatic coagulation system and is mainly caused by sinusoidal endothelial cell injury[22]. In our study, phosphotungstic acid-hematoxylin staining revealed that fibrin was diffusely deposited in the sinusoids in the control liver, suggesting intrahepatic coagulation (Figure 6A). In rats treated with AT Ⅲ *via* the tail vein, hepatic fibrin deposition was reduced but it was still sparsely distributed (Figure 6B). Meanwhile, injection of AT Ⅲ *via* the portal vein diminished fibrin deposition in the liver parenchyma, which suggests that it may affect the maintenance of the intrahepatic coagulation system (Figure 6C).

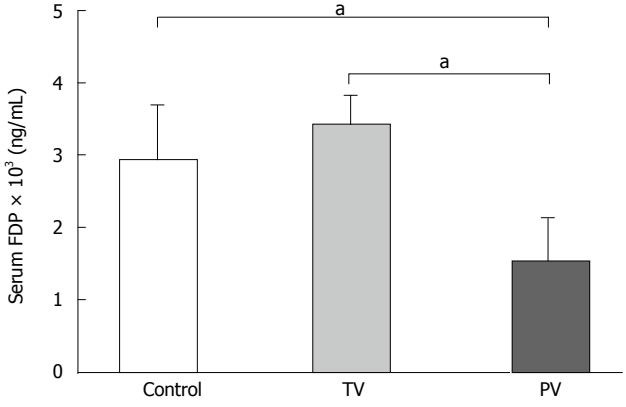

Figure 5 Effects of antithrombin Ⅲ on serum fibrin degradation product levels. The serum fibrin degradation product (FDP) levels were determined 24 h after injection of lipopolysaccharide and D-galactosamine. Control: Untreated; TV: Antithrombin (AT) Ⅲ injection *via* the tail vein; PV: AT Ⅲ injection *via* the portal vein. Values are means ± SD (n = 10 rats/group). $^{a}P < 0.05$ *vs* control and tail vein groups.

DISCUSSION

We demonstrated that directly injecting AT Ⅲ into the portal vein improved liver damage in a rat model of ALF induced by LPS and GalN. AT Ⅲ injection *via* the portal vein suppressed the increases in serum ALT and inflammatory cytokine levels, and intrahepatic fibrin deposition, and reduced the mRNA expression of hypoxia-related genes associated with ALF. These effects were accomplished by administering a relatively low dose of AT Ⅲ that should be acceptable in clinical practice.

Microcirculatory disturbances are involved in the pathogenesis of ALF. Activation of the inflammatory cascade may affect the coagulation system and the resulting intrahepatic coagulation may worsen sinusoidal blood flow, leading to massive liver necrosis; a histological feature of ALF[23]. Activation of macrophages, as represented by the elevated serum levels of CD163 and osteopontin in ALF patients, seems to be a trigger for this inflammatory process[24]. Following the onset of inflammation, disturbances in the intrahepatic coagulation system may enhance the destruction of liver cells. In patients with ALF, electron microscopy has revealed disorders in sinusoidal endothelial cells (SECs)[25]. Moreover, reduced mRNA expression of anticoagulants such as tissue factor pathway inhibitor and thrombomodulin are observed in SECs. Following the activation of tissue factor, intrahepatic coagulation causes sinusoidal fibrin deposition and thus restricts blood circulation in the diseased liver[26-28].

Based on these observations, AT Ⅲ treatment has been tested in animal models of ALF because of its anticoagulant activity. Systemic injection of AT Ⅲ (500 U/kg body weight) prevented Con-A-induced liver injury by inhibiting macrophage inflammatory protein-2 release and endothelial cell production of prostacyclin[7]. Meanwhile, a similar dose of AT Ⅲ (400 U/kg body weight) reduced

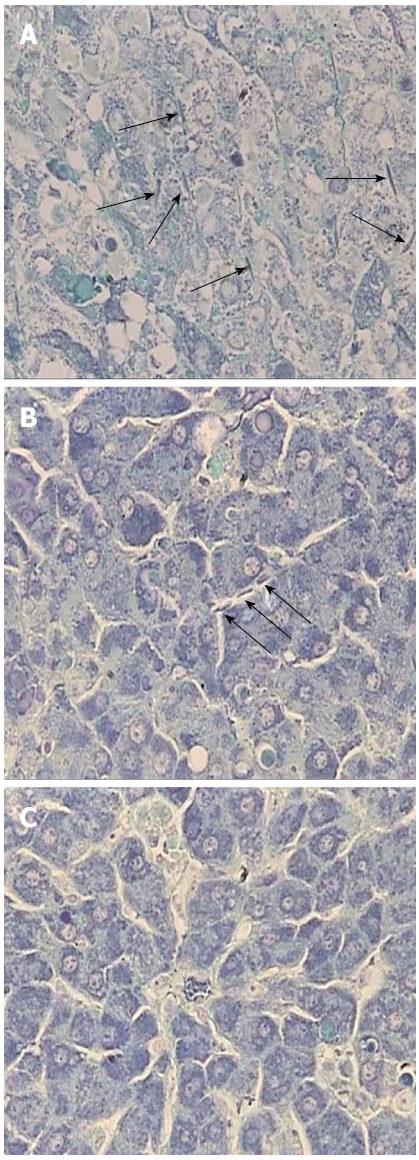

Figure 6 Effects of antithrombin Ⅲ on hepatic phosphotungstic acid-hematoxylin staining. To estimate the extent of intrasinusoidal coagulation, fibrin deposition was analyzed by phosphotungstic acid-hematoxylin staining (magnification, × 400). Fibrin deposition was observed as a dense rod-like structure (arrow). A: Control; B: Antithrombin (AT) Ⅲ injected *via* the tail vein; C: AT Ⅲ injected *via* the portal vein.

liver damage in an ALF model with coagulopathy induced by DMN, which was characterized by marked intrasinusoidal fibrin deposition and elevated serum fibrin monomer complexes[5]. However, large doses of AT Ⅲ, which are unacceptable in clinical practice, were used to suppress liver injury in these experimental models.

The effects of AT Ⅲ for the treatment of ALF patients seem to be limited. Fujiwara *et al*[29] treated 26 patients with fulminant hepatic failure with daily infusion of 3000 U AT Ⅲ. Notably, survival time was longer in patients with plasma AT Ⅲ levels within the normal range compared with levels beyond the normal range. However, the survival rates were not significantly different between patients treated with AT Ⅲ and control patients. Another research group treated 13 ALF patients with 3000 U AT Ⅲ, followed by a further 1000 U every 6 h. However, survival time was not improved by AT Ⅲ and the extent of intravascular coagulation was similar between AT-Ⅲ-treated and control patients[30]. These different outcomes of clinical trials and animal experiments might be due to the insufficient concentration of AT Ⅲ in the patients' liver. Indeed, the relative doses of AT Ⅲ per body weight used in patients were < 10% of those used in animals. Moreover, even though the plasma concentrations of AT Ⅲ were maintained within the normal ranges, the survival rate was not improved, which suggests that the intrahepatic concentration of AT Ⅲ did not reach the levels needed to maintain the integrity of the coagulation system in the diseased liver because of impaired sinusoidal circulation.

From our previous experience of treating ALF rats with methylprednisolone, we have demonstrated that direct delivery of steroid into the liver suppresses liver damage more effectively than does systemic injection. We found that injecting methylprednisolone *via* the portal vein significantly increased the survival rate, reduced serum cytokine levels, and decreased the number of apoptotic liver cells compared with tail vein injection[11]. Therefore, we speculated that the anticoagulant activity of AT Ⅲ would be more effective when injected *via* the portal vein than *via* a peripheral vein. In our study, significant reductions in the serum FDP levels and fibrin deposition were only observed in rats injected with AT Ⅲ *via* the portal vein, suggesting that direct drug delivery is necessary to achieve therapeutic concentrations of AT Ⅲ in the diseased liver. Of particular interest is that, using this method, we reduced the dose of AT Ⅲ to levels acceptable for clinical practice.

Anti-inflammatory activities of AT Ⅲ have been reported in addition to its anticoagulant activity. In septic patients, AT Ⅲ improves lung injury by suppressing the production of inflammatory cytokines, and prevents liver and kidney failure[31,32]. The mechanisms involved in the anti-inflammatory activities of AT Ⅲ have been analyzed in rats treated with endotoxin[33]. AT Ⅲ prevents pulmonary vascular injury by inhibiting leukocyte activation mediated by the enhanced release of prostacyclin from endothelial cells. Additionally, AT Ⅲ has been reported to inhibit the activation of inflammatory signaling cascades in several cell types, including the activation of nuclear factor (NF)-κB in human monocytes and vascular endothelial cells[34]; the production of TNF-α and IL-6 in LPS-stimulated murine macrophages[35]; and human neutrophil migration[36]. In our study, portal vein injection of AT Ⅲ significantly reduced serum TNF-α, IFN-γ and IL-6 levels compared with tail vein injection, and the control group. These results support two possible actions of AT Ⅲ injected *via* the portal vein to suppress inflammation: (1) the anti-inflammatory activity of AT Ⅲ was enabled because the tissue concentration reached effective levels following direct drug delivery; and (2) the reduced liver cell destruction mediated by the anticoagulant activity of AT Ⅲ suppressed the activation of surrounding inflammatory cells. We are currently unable to postulate

which action of AT Ⅲ might be dominant; however, it seems reasonable to suggest that the anticoagulant and anti-inflammatory activities of AT Ⅲ may act together to suppress tissue inflammation.

In patients with ALF, it has been reported that microcirculatory disturbances induce hypoxia in the liver[18,19]. Increased serum LDH levels and hepatic HO-1 expression are markers for the extent of hypoxia, reflecting the damage to the hepatic microcirculation. In this study, serum LDH levels were significantly reduced in rats injected with AT Ⅲ *via* the portal vein, whereas hepatic HO-1 expression was decreased in both groups of rats injected with AT Ⅲ compared with the control group. Expression of LDH and HO-1 are induced by hypoxia-inducible factor-1, therefore, the different expression patterns of these genes are unlikely to be due to hypoxia-mediated transcriptional regulation[37]. In contrast, the expression of HO-1 is transactivated by activator protein-1 and NF-κB, which are transcriptional factors that can activate various inflammatory signals[38]. In this context, we speculate that the reduced HO-1 expression in rats injected with AT Ⅲ *via* the tail vein may partly reflect decreased hepatic inflammation; however, the hypoxia may only be improved by injecting AT Ⅲ *via* the portal vein.

In conclusion, we demonstrated that injecting AT Ⅲ *via* the portal vein suppressed liver damage in a rat model of ALF. The increased concentration of AT Ⅲ in the diseased liver following direct drug delivery might enhance its anticoagulant and anti-inflammatory activities. Furthermore, the dose of AT Ⅲ used in this method was < 10% of that used in previous studies where AT Ⅲ was injected *via* peripheral veins. We believe that further studies are needed to establish this method as an effective treatment for ALF.

COMMENTS

Background

Acute liver damage occasionally progresses to acute liver failure (ALF) with extremely high mortality. Liver transplantation is the only effective treatment for patients with ALF. Plasma exchange and hemodiafiltration have been used as artificial liver support systems for affected patients but are only partially effective.

Research frontiers

Intravascular coagulation is thought to be involved in the pathogenesis of ALF. Anticoagulation therapy using antithrombin (AT) Ⅲ effectively suppresses liver damage in experimental models of ALF; however, extremely high doses of AT Ⅲ (200-500 U/kg body weight) are necessary. In this study, the authors demonstrated that injection of AT Ⅲ into the portal vein may help to improve the efficiency of AT Ⅲ compared with injected into peripheral vein.

Innovations and breakthroughs

The authors found that injection of AT Ⅲ *via* the portal vein showed superior effects to those achieved by tail vein injection in terms of lowering the serum levels of transaminase and inflammatory cytokines, reducing damage to the intrahepatic coagulation system, and improving hypoxia in the diseased liver. A clinically acceptable dose of AT Ⅲ injected *via* the portal vein suppressed liver damage, therefore, direct delivery of AT Ⅲ into the diseased liver could enhance the anticoagulant and anti-inflammatory activities of AT Ⅲ.

Applications

The development of injection of AT Ⅲ *via* the portal vein might be useful to enhance the effects of AT Ⅲ and ultimately improve the outcomes of patients with ALF, such as increasing the survival rate and reducing the number of patients who need liver transplantation.

Terminology

AT Ⅲ inhibits serine proteases involved in the coagulation cascade. Additionally, AT Ⅲ is reported to inhibit activation of inflammatory signaling cascades, including the activation of nuclear factor-κB, production of tumor necrosis factor-α and interleukin-6 in various cell types.

Peer review

The manuscript is clearly written and the authors discuss their findings in an adequate way. Moreover, the reduction of the dose of AT Ⅲ *via* injection in the portal vein has clinical implications.

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ORIGINAL ARTICLE

Over-expression of uPA increases risk of liver injury in pAAV-HBV transfected mice

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Abstract

AIM: To investigate the relationship between over-expression of urokinase plasminogen activator (uPA) and hepatitis B virus (HBV) related liver diseases in a transgenic mouse model.

METHODS: Albumin-tetracycline reverse transcriptional activator and tetO-uPA transgenic mice were generated respectively through pronuclear injection and crossed to produce the double transgenic in-alb-uPA mice, for which doxycycline (Dox)-inducible and liver-specific over-expression of uPA can be achieved. Hydrodynamic transfection of plasmid adeno-associated virus (AAV)-1.3HBV was performed through the tail veins of the Dox-induced in-alb-uPA mice. Expression of uPA and HBV antigens were analyzed through double-staining immunohistochemical assay. Cytokine production was detected by enzyme linked immunosorbent assay and α-fetoprotein (AFP) mRNA level was evaluated through real-time quantitative polymerase chain reaction.

RESULTS: Plasmid AAV-1.3HBV hydrodynamic transfection in Dox-induced transgenic mice not only resulted in severe liver injury with hepatocarcinoma-like histological changes and hepatic AFP production, but also showed an increased serum level of HBV antigens and cytokines like interleukin-6 and tumor necrosis factor-α, compared with the control group.

CONCLUSION: Over-expression of uPA plays a synergistic role in the development of liver injury, inflammation and regeneration during acute HBV infection.

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Key words: Tet-on system; Albumin promoter; Urokinase-type plasminogen activator; Hydrodynamic transfection; Liver injury

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INTRODUCTION

Hepatitis B virus (HBV) infection causes a necroinflammatory liver disease of variable duration and severity, with a high risk of developing cirrhosis and hepatocellular carcinoma. The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during HBV infection[1]. However,

the roles of urokinase plasminogen activator (uPA)/uPA's receptor (uPAR) systems as important inflammatory mediators have not yet been well investigated in acute and chronic hepatitis B, a common inflammatory disease in China[2]. Clinical studies almost focused on the correlation of uPA levels with the liver disease severity in hepatitis B patients. And the role of uPA in the HBV-induced liver injury, especially in the early stage, is less investigated.

uPA is one kind of plasminogen activator that catalyzes the conversion of plasminogen to plasmin. Together with uPAR, uPA participate in fibrinolysis, innate and adaptive immunity, and pathology[3]. In cancer cells, the effects of uPA and uPAR were thought to be related to cell migration[4], metastasis[5], and a more recent role of uPA in cancer growth has emerged[6]. The levels of uPA and uPAR have been found to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis, such as in patients with colon adenocarcinoma[7], lymphomas and leukemia[8].

The tetracycline (Tet)-inducible expression system is one of the most prominent and widely-used systems, which allows relatively stringent, reversible, and quantitative regulation of transgene expression in a wide range of cells in culture as well as in transgenic animals[9,10]. It consists of two parts: the ligand-dependent transactivator tetracycline reverse transcriptional activator (rtTA) as the effector and a tetO-cytomegalovirus (CMV) minimal promoter cassette regulating the expression of the transgene as the responder[11]. When doxycycline (Dox) is present, rtTA binds to the tetO-sequence and induces expression of the target gene[12].Together with a tissue-specific promoter, it can result in transgene expression in a temporally and spatially defined fashion.

In this study, an effective inducible and liver specific uPA expression mouse model was constructed in which the murine uPA expression was controlled by rtTA which is regulated by murine albumin enhancer/promoter. Through administration of Dox, the inducible expression of uPA specifically in mouse liver can be achieved with lower mortality. Then hydrodynamic injection of pAAV-1.3HBV, which contained inverted terminal repeat elements of adeno-associated virus (AAV) and 1.3 copies of HBV genome(ayw subtype), was performed to mimic the acute HBV infection. The mouse liver showed specific and inducible expression of uPA. Plasmid AAV-1.3HBV transfection in Dox-induced transgenic mice resulted in severer liver injury, higher HBV antigen and cytokine expression compared to the control group. These data further indicated for the first time in mice that the overexpression of uPA may have accelerative role in the development of liver injury, inflammation and liver regeneration during acute HBV infection.

MATERIALS AND METHODS

Plasmid construction

For liver-specific expression of rtTA, the transgenic construct albumin-rtTA was generated, which has rtTA gene under the control of the liver-specific albumin promoter and was based the plasmid pTet-on (Clontech Lab, Inc). To introduce appropriate restriction sites in pTet-on, linker sequences were designed as follows, Tet-on-linker-F: 5'-CTAGGATATCACTAGTGGTACCGGGCCCGCGGCCGCG-3'and Tet-on-linker-R: 5'-AATTCGCGGCCGCGGGCCCGGTACCACTAGTGATATC-3'. The linkers were annealed at 95 ℃ for 10 min and then were digested with *Eco*R I and *Spe* I and ligated to pTet-on digested with the same restriction enzymes, and the construct was named pTet-on-link. The albumin promoter fragment and enhancer fragment (Genbank accession no. AC140220.4) were separately amplified by polymerase chain reaction (PCR) using genomic DNA extracted from C57BL/6 mouse liver as the template. The primers for albumin enhancer were Alb-En-FP: 5'-GCCGAGCTCCTGCCGGCTAGCTTCCTTAGCATG-3' and Alb-En-RP: 5'-GGGTTAAGGATCCCAAG CTGGAG-3'. The primers for albumin promoter were Alb-Pro-FP: 5'-CGGGATCCACAGCTCCAGATGGCAAACATAC-3'and Alb-Pro-RP: 5'-TTTGCCAGAGGCTAGTGGG GTTG-3'.

The albumin enhancer PCR product was digested with *Bam*H I and cloned into pGEM-7ZF, then the albumin promoter sequence was inserted behind the enhancer at the site of *Bam*H I , and the plasmid was named p7ZF-Albumin, which was confirmed by restriction enzyme digestion analysis and DNA sequence analysis. Then p7ZF-Albumin was digested by *Sac* I and *Kpn* I , and the released 2233bp fragment was ligased to pTet-on-link digested by *Eco*R V and *Kpn* I , to yield the recombinant construct named pTet-on-Albumin.

For rtTA responsive expression of uPA, the transgenic construct pTRE$_2$-uPA was generated which is based on the plasmid pTRE$_2$ containing tetO. The uPA cDNA and uPA exon 11 was amplified by reverse transcription polymerase chain reaction (RT-PCR) and PCR from the total RNA and genomic DNA extracted from the kidney of C57BL/6 mouse, respectively. For uPA DNA, the primers were uPA-cDNA-F: 5'-CGGGATCC ATGAAAGTCTGGCTGGCGAGCCTG-3' and uPA-cDNA-R: 5'-CGGTCGACCATCAGAAGGCCAGACCTTTCTCTTC-3'. The RT-PCR product was ligated to pMD18T and the construct pMD18T-uPA cDNA was confirmed by sequence analysis. The uPA exon 11 was amplified by PCR from the genomic DNA template, the primers were uPA-3'-F: 5'-CGGTCGACGCCCTCAGGTAGCTGAGGGAAG-3' and uPA-3'-R: 5'-CGGTCGACGTGAAACCGACATTTAGTGCTAGTC-3'. The PCR product was ligated to the *Sal* I site of pMD18T-uPA cDNA to yield the construct pMD18T-uPA and sequence was confirmed. Then the uPA (cDNA + exon 11) fragment was subcloned into the *Pvu* Ⅱ and *Xba* I sites of pTRE$_2$ to yield pTRE$_2$-uPA.

Generation and PCR analysis of the albumin-rtTA and tetO-uPA transgenic mice

The albumin-rtTA and tetO-uPA transgenic mice were generated in C57BL/6 × CBA F1 zygotes using standard

pronuclear injection, which was performed by Shanghai Research Center for Biomodel Organisms. For microinjection, the 6034 bp fragment of transgene albumin-rtTA and the 5739 bp fragment of transgene tetO-uPA were excised from the vector backbone of pTet-on-albumin by *Xho* I digestion and pTRE2-uPA by *Pvu* I digestion, respectively, isolated and purified using QIA quick gel extraction kit (Qiagen), and then microinjected into the pronuclei of one cell-stage fertilized embryos. The DNA injected fertilized eggs were implanted into the oviducts of 12 pseudopregant recipient mice. All together 9 positive albumin-rtTA transgenic mice and 5 tetO-uPA positive ones were confirmed by PCR. One upstream pair and one downstream pair of primers, which were designed to amplify the sequences between vector and inserted fragment were designed for albumin-rtTA as follows, 1-up-F: 5'-GTGCAGCTTGGCTTGAACTCGTTC-3'; 1-up-R: 5'-GAGTATGGTGCCTATCTAACATCTC-3'; 1-down-F: 5'-GACGCGCTAGACGATTTCGATCTG-3'; 1-down-R: 5'-ACCTTGCACAGATAGCGTGGTC-3'. By the same way, one upstream pair and one downstream pair of primers were designed for tetO-uPA as follows 2-up-F: 5'-GTTTAGTGAACCGTCAGATCGCCTG-3'; 2-up-R: 5'-CTAGGCTAATAGCATCAGGTCTG-3'; 2-down-F: 5'-GGTAGCTTG AGGAGTAGAGACACT-3'; 2-down-R: 5'-GACAATGGTTGTCAACAGAGTAG-3'. The PCR conditions were the same for each of the primer pairs: 34 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, 72 ℃ for 30 s. Genomic DNA from wild-type mice was amplified as negative control. The PCR positive mice were the transgenic founders.

Mouse propagation and PCR analysis

At 6-8 wk of age, founder mice were backcrossed with wild-type C57BL/6J mice to generate F1. Genomic DNA were isolated from tail biopsy samples of F1 mice at 4 wk and analyzed by PCR, for which the protocols were mentioned above.

rtTA expression in different tissues of albumin-rtTA F1 transgenic mice

The isolation of total RNA from different tissues of 6-8 wk old F1 PCR-positive and negative offsprings of the founders was performed using the RNeasy Mini Kit (Qiagen) following the instructions. Purified RNA was eluted in 40 μL DNA-free water. 400 ng of total RNA reverse transcribed with the Takara RNA LA PCR Kit (AMV)Ver1.1 (TaKaRa), the reaction condition was 30 ℃ for 10 min, 42 ℃ for 30 min, 99 ℃ for 5 min, 5 ℃ for 5 min. The oligonucleotide primers used for RT-PCR were rtTA-F: 5'-GACGCGCTAGACGATTTCGATCTG-3'; rtTA-R: 5'-ACCTTGCACAGATAGCGTGGTC-3', the PCR reaction condition was 34 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, 72 ℃ for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control, the primers were GAPDH-F: 5'-TTCACCACCATGGAGAAGGC-3' and GAPDH-R: 5'-CCT CAGTGTAGCCCAAGATGC-3', PCR reaction condition was 34 cycles of 94 ℃ for 30 s, 48 ℃ for 30 s, 72 ℃ for 30 s.

The total protein was isolated from different tissues of 6-8 wk old F1 PCR-positive and negative offsprings of the founders by using the Tissue Lysis Buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate) supplemented with aprotinin (2 μg/mL), pepstatin A (0.7 μg/mL), leupeptin (0.5 μg/mL), phenylmethanesulfonyl fluoride (PMSF) (1 mmol/L). Aprotinin, Pepstatin A, Leupeptin, PMSF were purchased from Amresco. For Western blotting analysis, 25 μg of the total protein was used for each loading; the primary antibody for rtTA was TetR monoclonal antibody (Clontech) (used in 1:1000 dilution), and the primary antibody for GAPDH was an anti-GAPDH polyclonal antibody (Sigma) (1:10000 dilution); and the second antibodies were HRP-labeled goat anti-mouse IgG and goat anti-rabbit IgG (both in 1:5000 dilution), respectively. For imaging results, the SuperSignal WestDura Trial Kit (Pierce) was used following the instructions.

Generation of double transgenic mice in-alb-uPA and Doxycycline administration

Double transgenic in-alb-uPA and wild type female offspring were generated from a cross between the albumin-rtTA F1 transgenic positive mice and the tetO-uPA F1 positive mice. 20 d after born, these mice were given two intramuscular injection of 2 mg Dox in 0.2 mL 0.9% NaCl-solution each week for a period of 3 wk. Another group of each type of mice was maintained off doxycycline administration.

Hydrodynamic transfection of AAV-HBV and histological analysis

After 3 weeks' induction, a 20 μg pAAV-HBV1.3 DNA was injected hydrodynamically into the tail veins of the in-alb-uPA mice within 5 seconds. A control group of in-alb-uPA mice was injected with pAAV-internal ribosome entry site (IRES). At 20 d post transfection, mice were sacrificed and the livers were fixed with 4% (v/v) phosphate-buffered formalin, and paraffin-embedded liver sections were prepared and stained with hematoxylin and eosin. Semi-quantitative assessment of liver injury in each group was evaluated by the area of liver necrosis on the whole slide in each group. NP for no necrosis; P1 for < 10% area of necrosis; P2 for 10%-30% area of necrosis; P3 for > 30% area of necrosis. All the evaluation of liver damage was conducted by two independent observers. The average score of three mice in each group was taken as score for that group.

For uPA and HBV antigens detection, the expression of uPA protein and hepatitis B core antigen (HBcAg) were identified by double-staining with a polyclonal rabbit anti-rodent urokinase (uPA) antibody (American diagnostica Inc) and monoclonal anti-HBcAg antibody (Thermo Scientific). Diamino-benzidine and alkaline phosphatase substance (ZhongShan Goldenbridge biotech, Beijing, China) were used to visualize the uPA and HBV antigens.

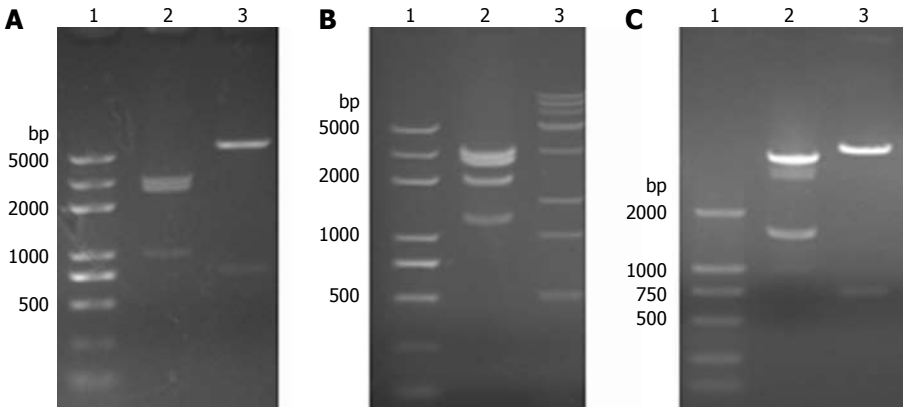

Figure 1 Identification of pTet-on-link, pTet-on-Albumin and pTRE$_2$-urokinase plasminogen activator by restriction endonucleases. A1 and B1: 2K plus DNA Marker; A2: pTet-on-link/*Eco*R Ⅴ + *Sal* Ⅰ; A3: pTet-on-link/*Eco*R Ⅴ + *Bam*H Ⅰ; B2: pTet-on-Albumin/*Bam*H Ⅰ; B3:15K DNA marker; C1: 2K DNA marker; C2: pTRE$_2$-urokinase plasminogen activator (uPA)/*pvu* Ⅱ; C3: pTRE$_2$-uPA/*Sal* Ⅰ.

Enzyme linked immunosorbent assay for HBV antigens and cytokine production

At 10 d and 20 d post transfection, mouse serum samples from different groups were harvested. The HBeAg and HBsAg enzyme linked immunosorbent assay (ELISA) kit (Wantai Biotech, Beijing) were used for the detection of the serum HBV antigens respectively. And interleukin (IL)-6 and tumor necrosis factor (TNF)-α ELISA kit (Dakewei Biotech, Beijing) were used for the quantitation of the serum cytokines. Serum ALT were measured with an Olympus Model 640 automated analyzer.

qRT-PCR analysis of α-fetoprotein mRNA expression in the livers of AAV-HBV transfected in-alb-uPA mice

The isolation of total RNA from livers of the AAV-HBV transfected in-alb-uPA mice was performed using the RNeasy Mini Kit (Qiagen) following the instructions. Purified RNA was eluted in 40 μL DNA-free water and 400 ng of total RNA in a 10 μL reaction mixture was reverse transcribed with the Takara RNA LA PCR Kit (AMV) Ver1.1 (TaKaRa). Relative quantization of the α-fetoprotein (AFP) mRNA level was performed using RNA Master SYBR Green1 (Roche Diagnostics) by Eppendorf Replex. The primers used for amplification were AFP-real-F: 5'-TCTGCTGGCACGCAAGAAG-3' and AFP-real-R: 5'-TCGGCAGGTTCTGGAAACTG-3'. GAPDH serves as a control and the primers were GAPDH-real-F: 5'-TCACCACCATGGAGAAGGC-3' and GAPDH-real-R: 5'-GCTAAGCAGTTGGTGGTGCA-3'. The amplification conditions included initial denaturation at 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 15 s, annealing at 55 ℃ for 15 s, extension at 68 ℃ for 30 s.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed using Student's *t* test.

RESULTS

Construction and identification of pTet-on-albumin and pTRE$_2$-uPA

For inserting the albumin enhancer and promoter sequence into pTet-on in place of the CMV promoter, a linker as designed and the following restriction sites were introduced: *Spe* Ⅰ, *Eco*R Ⅴ, *Spe* Ⅰ, *Kpn* Ⅰ, *Apa* Ⅰ, *Not* Ⅰ, *Eco*R Ⅰ. pTet-on-link was identified by *Eco*R Ⅴ/*Sal* Ⅰ and *Eco*R Ⅴ/*Bam*H Ⅰ double digestion respectively (Figure 1A), and results showed that the linker was introduced into pTet-on. pTet-on-Albumin was identified by *BamH* Ⅰ digestion (Figure 1B), the expected 5022 bp, 2689 bp and 1284 bp fragment can be observed. pTRE$_2$-uPA was identified by *pvu* Ⅱ and *Sal* Ⅰ respectively (Figure 1C). In addition, DNA sequence analysis of the albumin enhance/promoter and uPA sequence shows complete accordance with those in the National Center for Biotechnology Information database (data not shown).

Generation of the albumin-rtTA and tetO-uPA transgenic mice and PCR analysis

The albumin-rtTA expression unit contains the mouse albumin enhancer/promoter, rtTA coding sequence and SV40 polyA, and the tetO-uPA expression unit contains the TRE$_2$-PminCMV, uPA cDNA and uPA exon11 (Figure 2A). In the end, 9 albumin-rtTA transgenic founder mice and 5 tetO-uPA transgenic founder mice were confirmed positive by PCR for both the upstream and downstream primers (Figure 2B).

Specific expression of rtTA in the livers of albumin-rtTA and in-alb-uPA transgenic mice

To identify the liver-specific expression of uPA in the livers of transgenic mice, RT-PCR and Western blotting analysis was performed. The data showed that rtTA mRNA expressed specifically in the livers of F1 albumin-rtTA transgenic positive mice (Figure 3A, right image), while there was no rtTA mRNA expression in all the tissues of the albumin-rtTA transgenic negative mouse (Figure 3A, left image). GAPDH mRNA was expressed equally in different tissues of these mice (Figure 3A). Results from Western blotting analysis (Figure 3B) were in accordance with those from RT-PCR analysis. By Western blotting analysis, rtTA expression was also confirmed specifically in the livers of in-alb-uPA transgenic mice and albumin-rtTA transgenic mice (Figure 3C). The cell

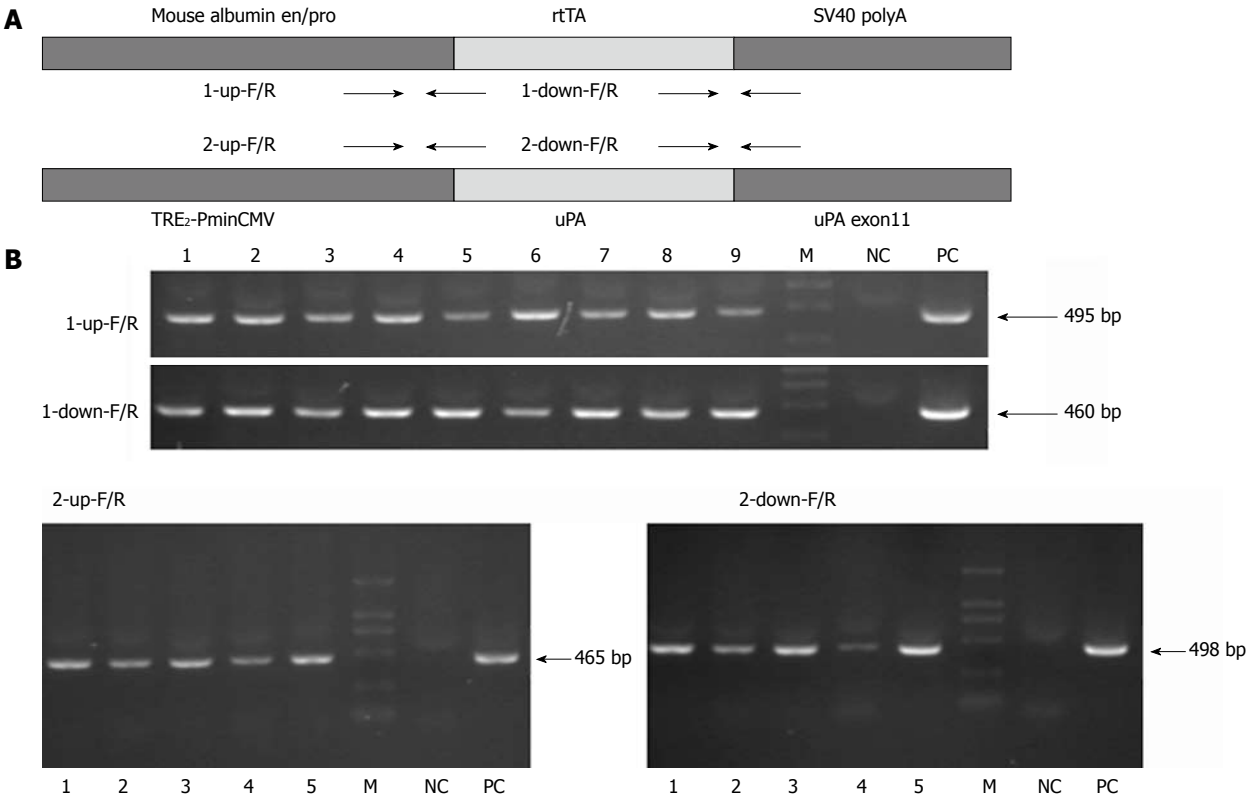

Figure 2 Establishment of albumin-tetracycline reverse transcriptional activator and tetO-urokinase plasminogen activator transgenic mice. A: The albumin-tetracycline reverse transcriptional activator (rtTA) unit contains the mouse albumin enhancer/promoter, rtTA coding sequence, and SV40 polyA. The tetO-urokinase plasminogen activator (uPA) unit contains the TRE2-PminCMV, uPA cDNA, uPA exon11. Arrowheads depict the positions and directions of the polymerase chain reaction (PCR) primers; B: PCR identification of the transgenic founders. 1-9, PCR identification for the nine albumin-rtTA transgenic founder mice; 1-5, PCR identification for the five tetO-uPA transgenic founder mice. CMV: Cytomegalovirus; M: Marker; NC: Negative control; PC: Positive control.

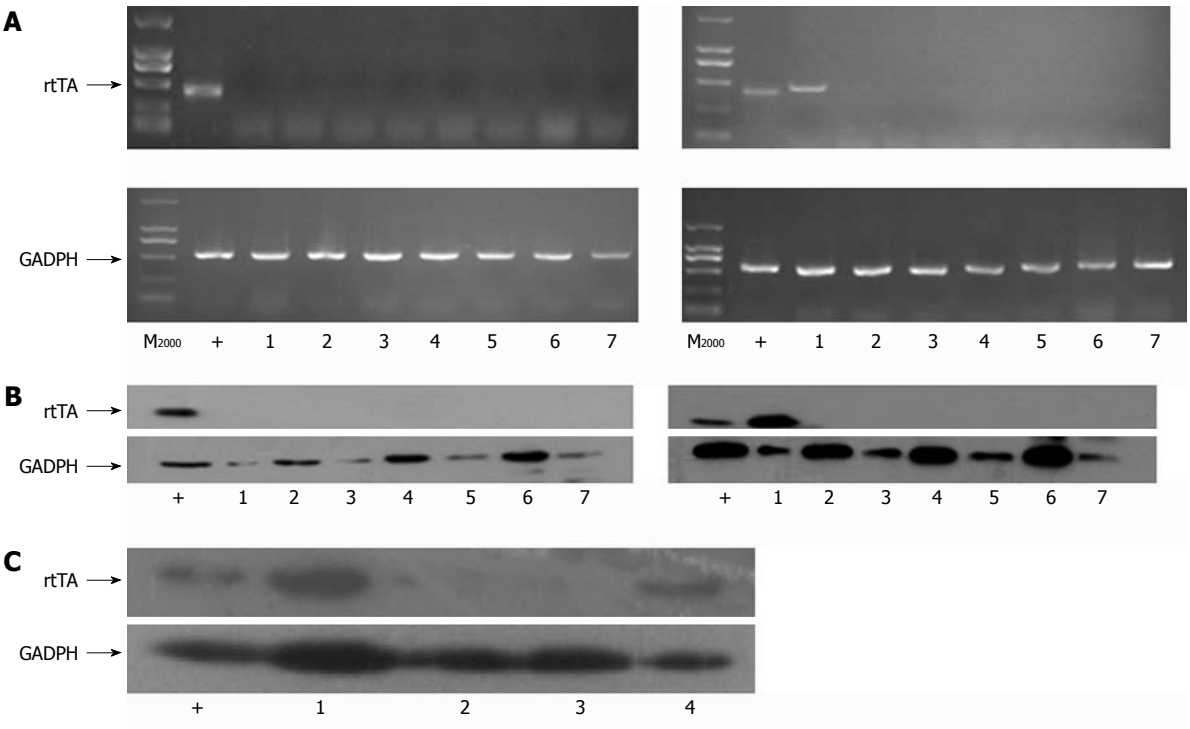

Figure 3 The specific expression of tetracycline reverse transcriptional activator in the livers of albumin-tetracycline reverse transcriptional activatortransgenic mice and in-alb-urokinase plasminogen activator transgenic mice. A, B: Reverse transcription polymerase chain reaction (A) and Western blotting (B) analysis of tetracycline reverse transcriptional activator (rtTA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in different tissues of the 6-8 wk old F1 albumin-rtTA PCR-negative (left for A, B) or positive (right for A, B) transgenic mice. 1: Liver; 2: Brain; 3: Thymus; 4: Heart; 5: Lung; 6: Kidney; 7: Spleen; C: Western blotting analysis of rtTA and GADPH expression in the liver extracts of mice with different genotypes.1: In-alb-urokinase plasminogen activator (uPA) mice group; 2: Liver extracts of wild type mice group; 3: tetO-uPA mice group; 4: Albumin-rtTA mice group. pTet-on transfected Huh7 cell extracts were used as positive control (+).

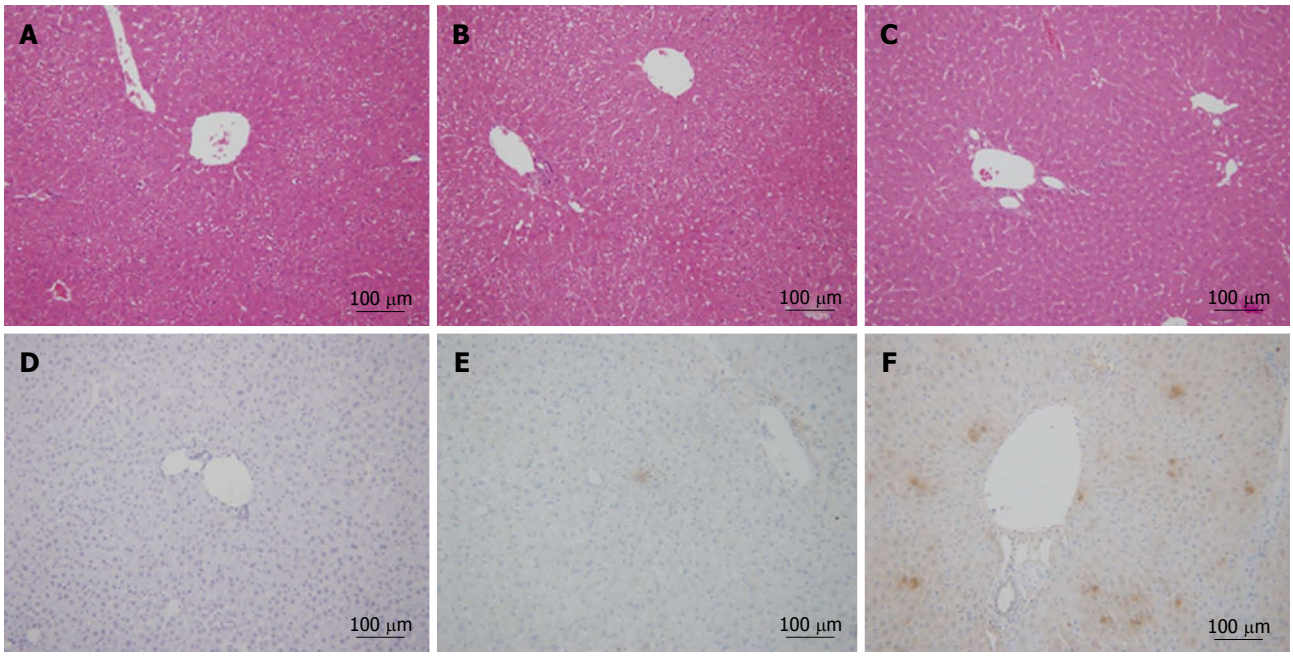

Figure 4 The expression of urokinase plasminogen activator in the livers of in-alb-urokinase plasminogen activator transgenic mice. A: Histology of livers from wild type (WT) mice; B: Histology of livers from in-alb-urokinase plasminogen activator (uPA) mice without doxycycline (Dox) induction; C: Histology of livers from in-alb-uPA mice with Dox induction; D: uPA expression in hepatocytes from from WT mice; E: uPA expression in hepatocytes from in-alb-uPA mice without Dox induction; F: uPA expression in hepatocytes from in-alb-uPA mice with Dox induction. Magnification, × 20.

extract from Huh7 transfected with pTet-on was used as positive control while tetO-uPA and WT mice served as negative control.

Histological change of liver in in-alb-uPA transgenic mice after Dox induced uPA expression

To confirm the expression of uPA in liver and its role on the hepatocytes, uPA expression and the histological changes of liver was analyzed with immunohistochemistry and HE staining respectively. The results showed light degeneration of hepatocytes and mild inflammation in the livers with in-alb-uPA double transgenic mice after 3 wk of Dox induction when compared to that of double transgenic mice without Dox or the control group mice (Figure 4A-C), which was coincident with that of uPA expression with immunohistochemistry in the livers of in-alb-uPA double transgenic mice after Dox induction while almost no expression of uPA detected in double transgenic mice without Dox. The data showed that the specific expression of uPA after Dox induction induced slight histological changes in the liver of this in-alb-uPA double transgenic mice.

Synergistic liver injury in in-alb-uPA transgenic mice after AAV-1.3HBV transfection

Although uPA plays critical role in hepatic repair via proteolysis of matrix elements and clearance of cellular debris from the field of injury, clinical data showed that the levels of uPA and uPAR in patients with acute and chronic hepatitis B significantly higher than that in healthy controls, which indicated that uPA level was closely related to the degree and period of inflammation and liver injury[13]. To confirm if the coexisting uPA expression and HBV replication induced serious acute liver injury, the in-alb-uPA transgenic mice were transfected with pAAV-1.3HBV, a plasmid which could mediated the production of replicative HBV virus[14] *in vivo*. Large area necrosis was observed 20 d later in the liver of Dox-induced in-alb-uPA double transgenic mice that were transfected with pAAV-1.3HBV (Figure 5C), compared with that of non-induced in-alb-uPA mice transfected with pAAV-1.3HBV (Figure 5B) or with the control plasmid pAAV-IRES (Figure 5A). Double-staining immunohistochemical analysis confirmed both uPA expression (in brown) and HBcAg expression (in red) in the AAV-HBV transfected Dox-induced in-alb-uPA mice (Figure 5F). Interestingly, the coexpression of uPA and HBcAg exist in the most of the necrosis areas and the hepatocytes with HBcAg expression alone were morphological intact. The severe liver damage in the mice after HBV transfection indicated that the expression of uPA accelerated the liver injury. The result was confirmed by a statistics analysis that about 86.7% of the AAV-HBV transfected Dox-induced in-alb-uPA mice experienced severe liver pathogenic changes compared with the 20% AAV-HBV transfected non-induced in-alb-uPA mice, which could be explained by the leaky expression of uPA due to the tet-on inducible system. And about 40% of the AAV-HBV transfected Dox-induced in-alb-uPA mice experienced severe liver injury (Table 1).

Comparison of the serum HBV antigens and cytokines produced in mice from different groups

Previous reports have shown that HBV infection is associated with the production of a broad range of pro-inflammatory cytokines and chemokines such as IL-1β,

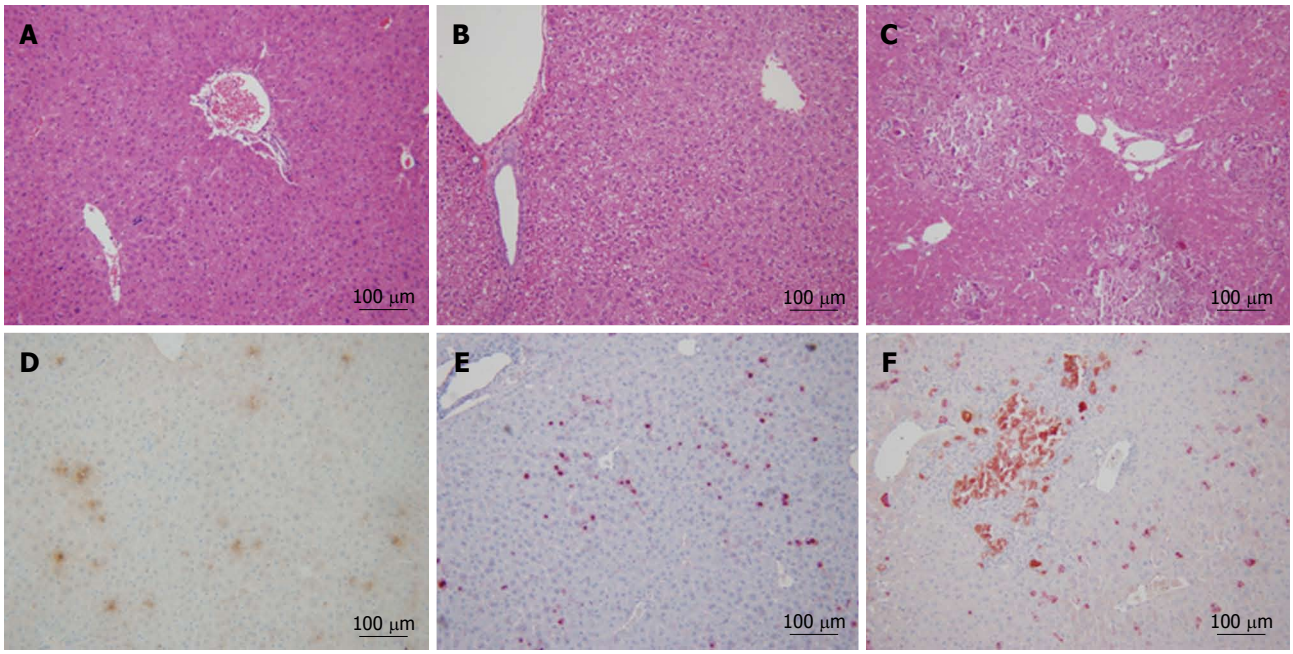

Figure 5 Synergistic injury of liver in in-alb-urokinase plasminogen activator transgenic mice after adeno-associated virus-1.3hepatitis B virus transfection. Histological and immunohistochemical staining for hepatitis B core antigen in the livers of different groups of mice 20 d later after adeno-associated virus (AAV)-1.3hepatitis B virus (HBV) transfection. A: Histology of the AAV-internal ribosome entry site (IRES) transfected doxycycline (Dox)-induced in-alb-urokinase plasminogen activator (uPA) mice; B: Histology of the AAV-HBV transfected non-induced in-alb-uPA mice; C: Histology of the AAV-HBV transfected Dox-induced in-alb-uPA mice; D: Double-staining immunohistochemical analysis of the AAV-IRES transfected Dox-induced in-alb-uPA mice; E: Double-staining immunohistochemical analysis of the AAV-HBV transfected non-induced in-alb-uPA mice; F: Double-staining immunohistochemical analysis of the AAV-HBV transfected Dox-induced in-alb-uPA mice. Magnification, × 20.

Table 1 Statistical analysis of the liver pathogenic rates for mice in different groups (%)

Group	1	2	3
NP	8 (100)	8 (80)	2 (13.3)
P1	0	2 (20)	2 (13.3)
P2	0	0	5 (33.3)
P3	0	0	6 (40)
Total	8	10	15

Group 1: Adeno-associated virus (AAV)-internal ribosome entry site transfected doxycycline (Dox)-induced in-alb-urokinase plasminogen activator (uPA) mice; Group 2: AAV-hepatitis B virus (HBV) transfected non-induced in-alb-uPA mice; Group 3: AAV-HBV transfected Dox-induced in-alb-uPA mice. NP stands for number of the non-pathogenic mice; P1 stands for number of mice in which the liver pathogenic area is below 10%; P2 stands for number of mice in which the liver pathogenic area ranges between 10% and 30%; P3 stands for number of mice in which the liver pathogenic area is above 30%.

IL-6, IL-8, IL-12, TNF-α and IFN-γ[15-17], among which IL-6 and TNF-α are important components of the early signaling pathway that lead to liver regeneration[18]. In this study, results also confirmed the elevation of serum IL-6 and TNF-α levels in the AAV-HBV transfected in-alb-uPA mice (Figure 6). 10 d and 20 d after AAV-HBV transfection, the serum IL-6 level for the Dox-induced in-alb-uPA mice was 47.28 ± 0.57 and 96.97 ± 2.91 (pg/mL), while the level for the non-induced in-alb-uPA mice was 18.32 ± 2.38 (pg/mL) and 45.83 ± 1.50 (pg/mL) ($P < 0.01$) (Figure 6D). The serum TNF-α level for the Dox-induced in-alb-uPA mice was 50.55 ± 2.01 (pg/mL) and 46.72 ± 2.01 (pg/mL), while the level for the non-induced in-alb-uPA mice was 14.58 ± 3.05 (pg/mL) and 18.17 ± 3.63 (pg/mL) ($P < 0.01$) (Figure 6E). Compared with the non-induced in-alb-uPA mice, the average serum HBeAg level of the Dox-induced in-alb-uPA mice was significantly higher both at 10 d and 20 d after AAV-HBV transfection ($P < 0.01$)) (Figure 6A), while there was no significant difference between the average serum HBsAg level of the Dox-induced and non-induced mice (Figure 6B). Compared with the non-induced in-alb-uPA mice, the average serum ALT level of the Dox-induced in-alb-uPA mice was slightly higher at 20 d after AAV-HBV transfection ($P < 0.01$)) (Figure 6C).

Relative quantitative analysis of AFP mRNA expression in the livers of AAV-HBV transfected in-alb-uPA mice

It has been reported that AFP level *in vivo* decreases abruptly soon after bith and remains at a low level throughout life. And reactivation of AFP production occurs during liver regeneration[19]. In this study, we found that, compared with the non-induced in-alb-uPA mice that were transfected by AAV-HBV, the AFP mRNA level for the Dox-induced in-alb-uPA mice that were transfected by AAV-HBV was greatly higher. 10 d after AAV-HBV transfection, the average level of the AFP mRNA for those induced in-alb-uPA mice increased about 21.8 times,while the fold change further increased to 142.1 times at 20 d after AAV-HBV transfection (Figure 6F). The data further confirmed our hypothesis that uPA expression and HBV

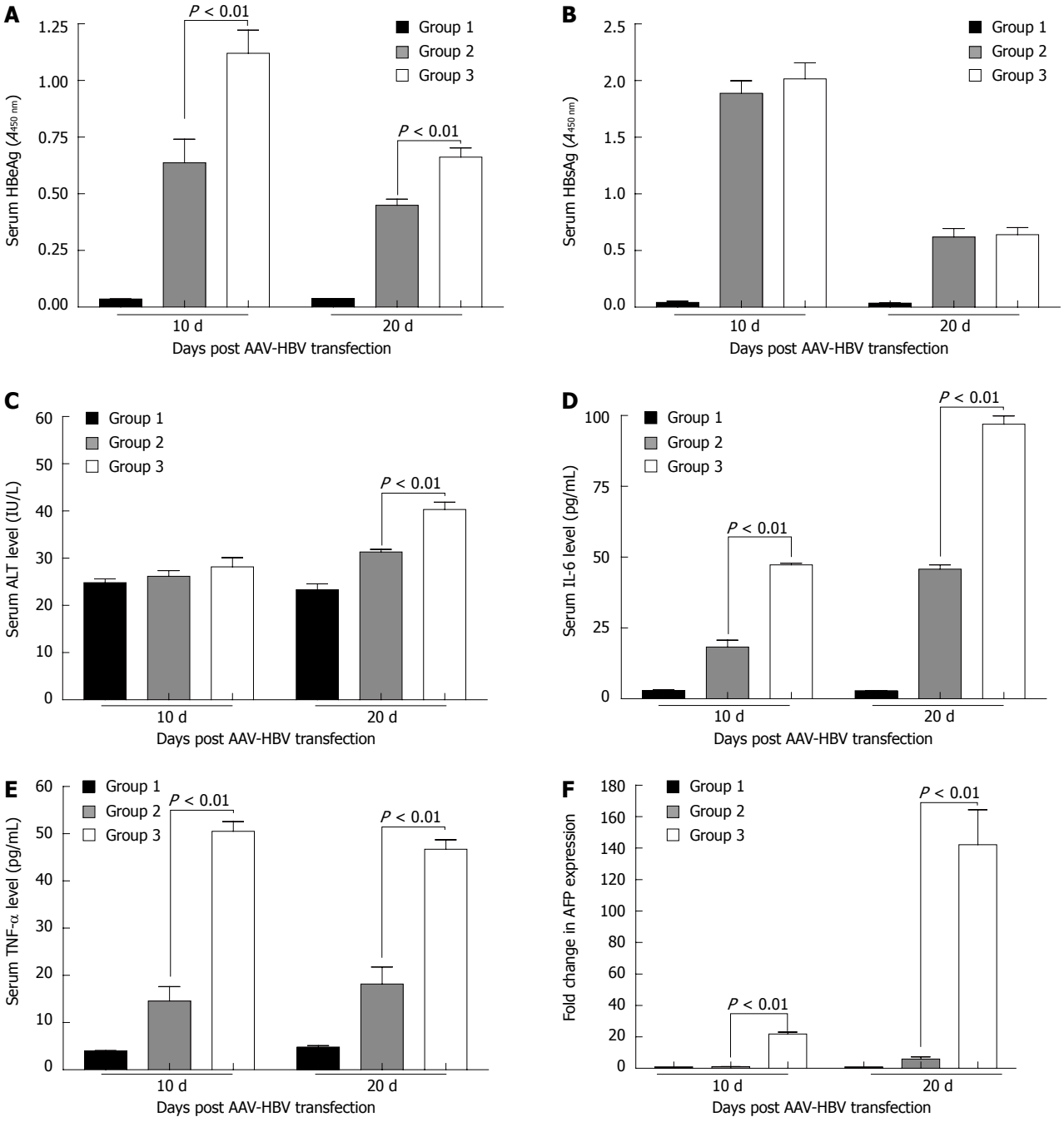

Figure 6 Comparison of the serum hepatitis B e antigen, hepatitis B surface antigen, interleukin-6, tumor necrosis factor-α, alanine aminotransferase levels and hepatic α-fetoprotein mRNA levels between mice from different groups. Group 1: Adeno-associated virus (AAV)-internal ribosome entry site transfected doxycycline (Dox)-induced in-alb-urokinase plasminogen activator (uPA) mice (n = 6); Group 2: AAV-hepatitis B virus (HBV) transfected non-induced in-alb-uPA mice (n = 6); Group 3: AAV-HBV transfected Dox-induced in-alb-uPA mice (n = 6). HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; IL: Interleukin; TNF: Tumor necrosis factor; AFP: α-fetoprotein.

infection have close relations and HBV infection further accelerated liver injury and regeneration when uPA was overexpressed.

DISCUSSION

Tet-inducible expression system is one of the most suitable inducible systems which could be used to investigate the function of a given gene *in vivo*, which including the tTA (Tet-off) system[20] and rtTA (Tet-on) system[21], and facilitates not only the understanding of gene function in development and pathogenesis, but also in transgenic mouse modeling[22-24]. On the other hand, tissue-specific expression of a target gene relies on tissue-specific promoters. Tissue-specific expression is vital for gene function research in organism development and can reduce immunological response and side effects in gene therapy applications. Many liver-specific promoters have been

identified so far, such as the AFP promoter[25], the albumin promoter, mouse major urinary protein promoter[26], hSAP promoter (human serum amyloid P component promoter)[27], and apoE promoter (human apolipoprotein E promoter)[28], which have been applied in liver-specific expression of target genes. In addition, elements like enhancers influence the transcriptional activity of the these tissue-specific promoters[29].

The urokinase plasminogen activator (uPA) is a serine protease that can activate the plasminogen into plasmin, and perform multiple functions in fibrinolysis, immunity and pathology[3]. Previous studies showed uPA diverse functions in tissue remodeling, angiogenesis,wound healing and protective effects in liver diseases[30]. The levels of uPA have been found to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis. And in human immunodeficiency virus (HIV)-infected patients, the serum levels of uPA have been found to be increased[2]. Also, the abnormal levels of plasma uPA in the patients with acute or chronic hepatitis B were observed, and it seems that the plasma levels of uPA are closely related to the degree and period of inflammation for these patients[13]. Although the clinical significance of uPA in viral chronic hepatitis B, hepatitis induced liver cirrhosis and HCC has been evaluated, the role of uPA in the process is less well understood, especially in the early stage.

In 1990, an Alb-uPA transgenic mouse which carried the mouse uPA gene under the control of the mouse albumin enhancer/promoter, was developed by Dr. Brinster's team to study the pathophysiology of plasminogen hyperactivation[31]. The over-expression of the uPA gene in the liver resulted in high plasma uPA levels and hypofibrinogenemia, which led to severe and sometimes abdominal bleeding soon after birth. And the high mortality also increases the difficulty for the generation of human liver chimeric mice[32,33] and the study of hepatitis C virus infections *in vivo*[34-36]. In this study, we established an uPA inducible double transgenic mouse in-alb-uPA, in which uPA can be expressed specifically in the liver only after Dox induction. Hypofibrinogenemia and neonatal hemorrhaging were not observed in the Dox-induced in-alb-uPA mice, which greatly brought down the mortality rate. Also the inducible expression of uPA makes it possible for us to study and illuminate the relations of uPA over-expression and HBV infection clinically.

Hydrodynamic transfection method was suitable for the AAV-mediated delivery of HBV genome *in vivo*[37]. To investigate the risk of HBV induced liver injury in the case of uPA over-expression, the hydrodynamic transfection of pAAV-HBV1.3, which could mediated the production of replicative HBV virus *in vivo*, was performed. In the Dox-induced in-alb-uPA mice that were hydrodynamically transfected by AAV-1.3HBV, severe liver histological changes were observed in the liver (Figure 5). Also uPA over-expression in the liver resulted in higher HBV antigen expression, higher IL-6 and TNF-α production and slight elevation of serum ALT level (Figure 6). Our results also found a significant increase of the AFP mRNA level in the AAV-HBV transfected Dox-induced in-alb-uPA mice (Figure 6). Produced by the embryonic yolk sac and tetal liver, the AFP level decreases abruptly soon after bith and remains at a low level throughout life. And reactivation of AFP production occurs during liver regeneration[19]. As IL-6 and TNF-α are proinflammatory cytokines that lead to liver regeneration, we came to the conclusion that the uPA over-expression in AAV-HBV transfected mice increased the liver necrosis injury, inflammation and liver regeneration, which reflects a process that may eventually lead to hepatocellular carcinoma.

It is generally considered that cell-mediated immunity and inflammation are the main mediators of the hepatic pathology induced by HBV infection. In this study, we found that HBV infection further accelerated liver injury and regeneration when uPA was overexpressed, indicating a close relation between uPA expression and HBV infection. Also as clinical data showed that the increased level of uPA in HIV infected patients, this study may in part explain the increased risk of liver disease during HIV and HBV coinfection.

COMMENTS

Background

Hepatitis B virus (HBV) infection causes a high risk of developing liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC). The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during HBV infection. The urokinase plasminogen activator (uPA) is a serine protease that can activate the plasminogen into plasmin, and perform multiple functions in fibrinolysis, immunity and pathology. However, the roles of uPA/uPA's receptor (uPAR) systems as important inflammatory mediators have not yet been well investigated in acute and chronic hepatitis B, a common inflammatory disease in China. Clinical studies almost focused on the correlation of uPA levels with the liver disease severity in hepatitis B patients. And the role of uPA in the HBV-induced liver injury, especially in the early stage, is less investigated.

Research frontiers

Various researchers have found the levels of uPA to be increased in tissues, plasma and other body fluids of cancer patients and to be markers of cancer development and metastasis. And in human immunodeficiency virus (HIV)-infected patients, the serum levels of uPA have been found to be increased. Also, the abnormal levels of plasma uPA in the patients with acute or chronic hepatitis B were observed, and it seems that the plasma levels of uPA are closely related to the degree and period of inflammation for these patients. Although the clinical significance of uPA in viral chronic hepatitis B, hepatitis induced liver cirrhosis and HCC has been evaluated, the role of uPA in the process is less well understood, especially in the early stage.

Innovations and breakthroughs

In this study, an inducible liver-specific uPA transgenic mice model was developed. Plasmid adeno-associated virus-1.3HBV transfection in doxycycline (Dox)-induced transgenic mice resulted in severer liver injury, higher HBV antigen and cytokine expression compared to the control group. These data further indicated for the first time in mice that the over-expression of uPA may have accelerative role in the development of liver injury, inflammation and liver regeneration during acute HBV infection Also as clinical data showed that the increased level of uPA in HIV infected patients, this study may in part explain the increased risk of liver disease during HIV and HBV coinfection.

Applications

This study deepens our knowledge of uPA function in HBV-induced liver diseases, which may not only facilitate the elucidation of the molecular mechanism

of HBV pathogenesis, but also provide a basis for the uPA-targeted anti-HBV therapies.

Terminology

uPA is one kind of plasminogen activator that catalyzes the conversion of plasminogen to plasmin. Together with uPAR, uPA participate in fibrinolysis, innate and adaptive immunity, and pathology. Tetracycline (Tet)-inducible expression system consists of two parts: the ligand-dependent transactivator rtTA as the effector and a tetO-CMV minimal promoter cassette regulating the expression of the transgene as the responder. When Dox is present, rtTA binds to the tetO-sequence and induces expression of the target gene. Together with a tissue-specific promoter, it can result in transgene expression in a temporally and spatially defined fashion.

Peer review

The authors studied the role of uPA and found that the over-expression of uPA may have a synergistic role in the development of liver injury, inflammation and liver regeneration during acute HBV infection. They added as such new information to the field on the knowledge about uPA function in HBV-induced liver diseases.

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ORIGINAL ARTICLE

siRNA targeting of Cdx2 inhibits growth of human gastric cancer MGC-803 cells

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Abstract

AIM: To investigate the effects of small interference RNA (siRNA) targeting of Cdx2 on human gastric cancer MGC-803 cells *in vitro* and *in vivo*.

METHODS: The recombinant pSilencer 4.1-Cdx2 siRNA plasmids were constructed and transfected into gastric cancer MGC-803 cells *in vitro*. The stable transfectants were selected. The effects of Cdx2 siRNA on growth, proliferation, cell cycle, apoptosis, migration and invasiveness of human gastric cancer MGC-803 cells were evaluated and the expression of phosphatase and tensin homolog (PTEN), caspase-9 and caspase-3 was observed *in vitro* by reverse transcription polymerase chain reaction (RT-PCR) and Western blotting analysis. We also investigated the effect of Cdx2 siRNA on growth of MGC-803 cells in nude mice *in vivo*.

RESULTS: Cdx2 siRNA led to inhibition of endogenous Cdx2 mRNA and protein expression as determined by RT-PCR and Western blotting analysis. Cdx2 siRNA significantly inhibited cell growth and proliferation, blocked entry into the S-phase of the cell cycle, induced cell apoptosis, and reduced the motility and invasion of MGC-803 cells. Cdx2 siRNA also increased PTEN expression, and activated caspase-9 and caspase-3 in MGC-803 cells *in vitro* . In addition, siRNA targeting of Cdx2 inhibited the growth of MGC-803 cells and promoted tumor cell apoptosis *in vivo* in nude mice tumor models.

CONCLUSION: Cdx2 was involved in regulating progression of human gastric cancer cells MGC-803. Manipulation of Cdx2 expression may be a potential therapeutic strategy for gastric cancer.

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Key words: Cdx2; Gastric cancer; Growth; Small interference RNA

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INTRODUCTION

The transcription factor, Cdx2, is a member of the caudal-related homeobox gene family, and is mainly expressed in

the intestine. Cdx2 plays important roles in early differentiation, proliferation, and maintenance of intestinal epithelial cells, and in the transcription of genes such as multidrug resistance 1[1,2]. Overexpression of Cdx2 in the small intestine is associated with reduced postnatal growth, early epithelial maturation, alterations in the development of a differentiated phenotype in crypt base organization, and changes in paneth and goblet cell lineages[3].

Initially, Cdx2 was reported to be a tumor suppressor. Several investigators reported that low levels of Cdx2 is a characteristic feature of human colon and squamous esophageal cancer[4,5], and overexpression of Cdx2 could decrease mobility and antagonize metastasis of colon cancer cells[6]. However, other studies showed that strong and robust expression of Cdx2 was found in > 80% of colorectal cancers and non-small cell lung cancer[7,8]. In addition, Cdx2 was found to enhance proliferation and have tumorigenic potential in the human colon cancer cell lines, LoVo and SW48[9]. These studies suggested that Cdx2 also had oncogenic property. Together, these conflicting findings point to a complex role for Cdx2 in the regulation of cell proliferation.

Gastric cancer is the third most common cancer in China, and is one of the most frequent causes of cancer-related mortality in China, with an incidence of 0.4 million new cases and 0.3 million deaths annually[10]. Intestinal metaplasia has been shown to be a precursor of intestinal-type gastric adenocarcinoma. Since intestinal metaplasia cannot be eradicated, it is important to determine how to reduce the morbidity from intestinal metaplasia to gastric cancer. However, the histogenesis of intestinal metaplasia and factors in the metaplastic epithelium that lead to its development into carcinoma is still in dispute[11,12]. In adult humans, Cdx2 has been reported to be associated with intestinal metaplasia in the stomach in which ectopic expression of Cdx2 is speculated to cause the gastric epithelial cells to trans-differentiate and take the intestinal phenotype[13]. In addition, Cdx2 transgenic mice have been shown to induce intestinal metaplasia and have a high incidence of gastric carcinoma[14,15]. This indicates a direct relationship between Cdx2-induced intestinal metaplasia and gastric carcinogenesis.

In the present study, we constructed small interference RNA (siRNA) sequences targeting of Cdx2, transfected them into the human gastric cancer cell line MGC-803, selected the stable transfectants, and explored changes in growth, proliferation, cell cycle, apoptosis, metastasis and invasiveness. We also observed the effect of Cdx2 siRNA on the expression of phosphatase and tensin homolog (PTEN), caspase-9, and caspase-3. Moreover, we investigated the effects of Cdx2 downregulation on the growth and apoptosis of MGC-803 cells in nude mice.

MATERIALS AND METHODS

Cell culture

The human gastric carcinoma cell line, MGC-803, was supplied by the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD, United States). All media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were cultured in an incubator with 5% CO_2 at 37 ℃ with medium changes every 3 d.

Antibodies

Anti-Cdx2, anti-β-actin and secondary antibody were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States. Antibodies specific for PTEN, pro-caspase-9, cleaved caspase-9, pro-caspase-3, cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology, Beverly, MA, United States.

Plasmid construction and transfection

Double strand siRNA oligonucleotides were obtained from Gima Biotechnology Company (China). There were two reversed repeated sequences with 21 inserted sequences (GACAAATATCGAGTGGTGTAC, TACCCGCGATCTGTTCTGCA) in the complementary sequence, with *Bam*H Ⅰ and *Hin*dⅢ sites for ligation into the pSilencer 4.1 vector, which contained a neomycin resistance marker for the selection of stable transfectants in the presence of G418. The siRNA targeting site of the transcripted product was nucleotides 115-818 of Cdx2 mRNA (GeneBank No. NM-001265). The negative control was the siRNA sequence with no homology to any human gene sequence.

After ligation, the plasmid was transformed into Escherichia coli TOP10 cells, and then cultured on solid LB medium (LB solid medium containing 50 ng/L ampicillin and 2% agarose gel) at 37 ℃ for 16 h. Positive clones were identified by DNA sequence analysis (Majorbio Biotech Co., Ltd), and the resulting plasmid was named pSilencer 4.1-Cdx2(+) or pSilencer 4.1-Cdx2(-). Six-well plates were inoculated with MGC-803 cells (1×10^5), and cells were transfected with pSilencer 4.1-Cdx2(+) recombination plasmids. For selection of stable transfectants, G418 (Life Technologies) was added to the cells 48 h after transfection. The concentration of G418 for selection was gradually decreased as follows: 1 mg/mL for 4 d; 750 μg/mL for 4 d; 500 μg/mL for 4 d; and 250 μg/mL as a sustaining dose. At day 20 after transfection, G418-resistant clones were isolated. The selected cell colonies were transferred from 10-mm dishes to 96-well plates, and then from 96-well plates to 24-well plates. The transformants selected by G418 were analyzed by measuring the expression of Cdx2 mRNA and protein. The negative control cells were transfected with vector pSilencer 4.1-Cdx2(-) alone, and maintained under identical conditions. In the case of cells that were selected in medium containing G418, antibiotics were routinely included in their growth medium until 1 to 2 d before experiments were carried out. The cells were divided into 3 groups: MGC-803/Cdx2 siRNA, MGC-803/Cdx2 negative control and MGC-803 group.

Semi-quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the positive cell clone using TRIzol Reagent (Invitrogen). Neo gene segments were amplified and verified by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Complementary deoxyribonucleic acids (cDNAs) were reverse-transcribed from 2 μg of total RNA. Primers used in this study were as follows: Cdx2 forward primer (5'-CGGCAGCCAAGTGAAAAC-3') and reverse primer (5'-GATGGTGATGTAGCGACTGTAGTG-3'), PCR product: 100 base pairs (bp); β-actin forward primer (5'-AACTCCATCATGAAGTGTGA-3') and reverse primer (5'-ACTCCTGCTTGCTGATCCAC -3'), PCR product: 247 bp. The PCR products were checked by agarose gel electrophoresis, and the abundance of each mRNA was detected and normalized to that of β-actin mRNA.

Western blotting analysis

Cell lysates were prepared in a buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-Cl (pH 7.6), 1 mmol/L ethylenediaminetetraacetic acid (pH 8.0), 1 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. After protein quantitation using the Lowry protein assay, equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three buffer system. The membrane was blocked with 5% bovine serum albumin in phosphate buffer solution Tween-20 (PBST) (PBS, pH 7.5, containing 0.1% Tween-20), and incubated with a 1:500 dilution of primary antibody (anti-Cdx2) overnight at 4 ℃. The membrane was then washed with PBST and incubated with a peroxidase-conjugated secondary antibody (1:1000) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, Rockford, IL, United States), according to the manufacturer's recommendations. Western blotting film was scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, United States). After development, the membrane was stripped and reprobed with antibody against β-actin (1:1000) to confirm equal sample loading.

Cell growth and proliferation assay

The growth of MGC-803 cells was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using a CellTiter 96 AQueous assay system (Promega, Madison, WI, United States), according to the manufacturer's instructions. This assay measures dehydrogenase enzyme activity in metabolically active tumor cells, as reflected by the conversion of MTT to formazan, which is soluble in tissue culture medium and is detected by absorbance (A) at 490 nm. The production of formazan is proportional to the number of living cells, with the intensity of the produced color serving as an indicator of cell viability. Briefly, MGC-803 cells were plated at a density of 5×10^3 cells/well in 96-well plates, and cultured for 72 h. The percentage of cell survival was calculated using the background-corrected absorbance: % proliferation rate = 100 × A of experimental well/A of untreated control well. All experiments were performed at least three times.

Colony formation assay

Cell suspensions from each group were diluted in DMEM with 10% FBS, and immediately re-plated in 6-well plates at a density of 20 cells/cm^2. The plates were incubated until cells in control wells formed sufficiently large colonies. After that, the colonies were fixed in 6% glutaraldehyde and stained with 0.5% crystal violet. The plates were photographed and their digital images were analyzed manually to determine colony number.

Cell cycle analysis by flow cytometry

For cell cycle analysis, MGC-803 cells (1×10^6) were washed twice with ice-cold PBS, treated with trypsin, and then fixed in 70% cold ethanol at 4 ℃ for 30 min. The cell pellet was incubated in a solution containing 50 ng/mL propidium iodide, 0.2 mg/mL RNase, and 0.1% Triton X-100 at room temperature for 30 min, and then analyzed by flow cytometry using a FACscan (Becton Dickinson, Mountain View, CA, United States). Data were analyzed with the MultiCycle for Windows (Phoenix Flow Systems, San Diego, United States).

Apoptosis assay by flow cytometry

Apoptotic cells were determined using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Jingmei Biotech Co., Shenzhen, China) and an EPICS XL-MCL flow cytometer (Becton Dickinson) according to the manufacturer's instructions. Briefly, 1×10^6 cells were stained with Annexin V/FITC for 30 min at 4 ℃ in the dark and then stained with propidium iodide for 10 min before flow cytometric analysis.

Wound healing assay

The cells were cultured to confluence in 6-well plates, and were then treated with mitomycin C to inhibit cell proliferation. A central linear wound was made with a 200 μL sterile pipet tip. Media were changed gently to remove any floating cells. Phase micrographs of the wound cultures were taken at 0 and 36 h. The photographs were analyzed by measuring the distance from the wound edge of the cell sheet to the original wound site. Migratory activity was calculated as the mean distance between edges of three points in 12 fields per well. Relative motility = (mean original distance - mean distance at a time point)/mean original distance × 100%. Each test group was assayed in triplicate.

Cell invasion assay

Cell invasion was assessed using Transwell chambers (6.5 mm; Corning, New York, United States) with 50 μL serum-free DMEM containing 1 μg/mL Matrigel (De-

partment of Biology, Beijing University, China) in the upper chamber. The lower chamber was filled with 50 μL DMEM containing 0.1 μg/mL fibronectin (Beijing University). Cells (1×10^5) were suspended in 100 μL DMEM with 1% fetal calf serum and plated into the upper chamber. PBS (5%) 500 μL was added in the lower chamber. After a 24 h incubation with 5% CO_2 at 37 ℃, the number of cells with Giemsa staining on the undersurface of the polycarbonate membranes was scored visually in five random fields at a 400 × magnification by light microscopy.

Analyses of PTEN, caspase-9 and caspase-3 expression

Semi-quantitative RT-PCR was performed as previously described. Primers used in this study were as follows: (1) PTEN forward primer (5'-CTGGAAAGGGACGAACTG-3') and reverse primer (5'-AGGTAACGGCTGAGGGA-3'), PCR product: 368 bp; (2) Caspase-9 forward primer (5'-GGCTGTCTACGGCACAGATGGA-3') and reverse primer (5'-CTGGCTCGGGGTTACTGCCAG-3'), PCR product: 200 bp; (3) Caspase-3 forward primer (5'-AAGCGAATCAATGGACTC-3') and reverse primer (5'-TTCCTGACTTCATATTTCAA-3'), PCR product: 192 bp; (4) GAPDH (a) forward primer (5'-ACAGCAACAGGGTGGTGGAC-3') and reverse primer (5'-TTTGAGGGTGCAGCGAACTT-3'), PCR product: 252 bp; and (5) GAPDH (b) forward primer (5'-ACCACAGTCCATGCCATCAC-3') and reverse primer (5'-TCACCACCCTGTTGCTGTA-3'), PCR product: 450 bp. Western blotting analysis was carried out as previously described.

Animal studies

BALB/c male nude mice at 5 wk old were obtained from Guangxi Animal Center, China. All animals were kept under specific pathogen-free conditions and tended to in accordance with institutional guidelines. All experimental studies were approved by the Guangxi Medical University Animal Care and Use Committee. MGC-803/Cdx2 siRNA cells, MGC-803/Cdx2 negative control cells and MGC-803 cells were used for tumor implantation. There were six mice in each group. Approximately 2×10^6 tumor cells were implanted subcutaneously into the flanks of the nude mice. Tumor sizes were measured every 4 d with a caliper, and the diameters were recorded. The tumor volume (TV) was calculated by the formula: $TV = W^2 \times L/2$, where L was the length and W was the width of the tumor. The relative tumor volume (RTV) was calculated by the formula: $RTV = Vt/V_0$ (V_0 is the TV at the day when the chemicals were given, and Vt is the TV of subsequent measurement). After mice were killed, total RNA and protein were extracted from tumor tissues. The expression of Cdx2 mRNA and protein were detected by semi-quantitative RT-PCR and Western blotting analysis, respectively. Tumor cells were assessed for apoptosis using *in situ* terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick end labeling assays (TUNEL). Apoptosis was evaluated by counting the positive cells (brown-stained cells) as well as the total number of cells in 10 arbitrarily selected fields at 400 × magnifications in a double-blinded manner. The apoptotic index (per 400 × microscopic field) was calculated as the number of apoptotic cells × 100/total number of cells. Brown-stained nuclei immediately at the edge of a tissue section were excluded from cell counts to minimize false positives.

Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined using χ^2 test, student's *t* test, or one-way analysis of variance. Statistical analysis were carried out using SPSS, version 13.0 (SPSS Inc., Chicago, IL, United States) or Origin 7.5 software programs (OriginLab Co., Northampton, MA, United States). A value of $P < 0.05$ was considered as statistically significant.

RESULTS

pSilencer 4.1-Cdx2(+) inhibits Cdx2 mRNA and protein expression

Recombinant pSilencer 4.1-Cdx2(+) and pSilencer 4.1-Cdx2(-) sequences were verified by DNA sequenced analysis (data not shown) which demonstrated that the inserted siRNA coding frames and frame sequences were correct. This confirmed that the construction of Cdx2 siRNA expression plasmid was successful.

The transfection of pSilencer 4.1-Cdx2(+) plasmid into MGC-803 cells led to remarkable inhibition of Cdx2 mRNA and protein expression. Densitometric analysis showed that Cdx2 mRNA and protein in MGC-803/Cdx2 siRNA cells were about 11- and 7-fold lower, respectively, than those in the two control groups ($P < 0.05$), while no differences were found between MGC-803/Cdx2 negative control cells and MGC-803 cells (Figure 1).

Cdx2 siRNA inhibits cell growth and proliferation in gastric cancer MGC-803 cells

Next, we determined the *in vitro* survival rates of gastric tumor cells stably transfected with pSilencer 4.1-Cdx2(+) plasmids, using the gastric carcinoma cell line, MGC-803, as a model for gastric cancer. As shown in Figure 2, Cdx2 siRNA significantly reduced cell survival, as assessed by the MTT assay. We observed that MGC-803/Cdx2 siRNA cells obviously grew slower than MGC-803/Cdx2 negative control cells and MGC-803 cells ($P < 0.05$), which was consistent with the decreased levels of Cdx2 in MGC-803/Cdx2 siRNA cells. Additionally, MGC-803/Cdx2 negative control cells and MGC-803 cells exhibited about 3-fold higher mean proliferation rates than MGC-803/Cdx2 siRNA cells ($P < 0.05$). These results indicate a suppressive effect of Cdx2 siRNA on MGC-803 cell growth and survival.

To confirm the inhibitory effect of Cdx2 siRNA on the growth of MGC-803 cells, we performed colony formation assays to measure the capability of the cells to grow in an anchorage-independent environment by

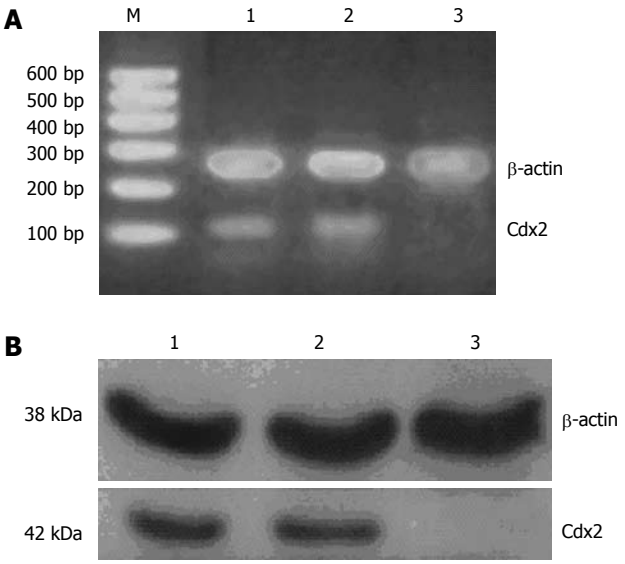

Figure 1 Cdx2 small interference RNA significantly reduced Cdx2 mRNA and protein expression in MGC-803 cells. A: Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. The RNA samples (2 μg in each) extracted from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were subjected to RT-PCR for Cdx2 and β-actin mRNAs. RT-PCR for β-actin was performed in parallel to show an equal amount of total RNA in the sample; B: Western blotting analysis. Whole protein extracts (100 μg in each) were prepared from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 siRNA cells. The expression of Cdx2 protein was determined by Western blotting with an anti-Cdx2 antibody. The β-actin expression levels were determined as a control for equivalent protein loading. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group; M: 600 bp marker.

culturing the cells in soft agarose. As shown in Figure 3, three cell lines were able to form colonies in soft agarose, but the number of colony formation in MGC-803/Cdx2 siRNA cells after 3 wk was 51.4 ± 3.2, with a 60.1% and 57.6% decrease, compared to the two control groups, respectively ($P < 0.05$). Together, these data suggest that Cdx2 siRNA inhibits cell growth and proliferation in gastric cancer cells.

Effect of Cdx2 siRNA on cell cycle control in gastric cancer MGC-803 cells

We used flow cytometry to determine whether the inhibitory effect of Cdx2 siRNA on MGC-803 cell proliferation was mediated, at least in part, through affecting cell cycle progression. We found that MGC-803/Cdx2 siRNA cells were 73.1% in G0/G1 phase and 18.2% in S phase, with a 13.8% and 16.2% increase in the G0/G1 phase cell population, and a 17% and 18% decrease in the S phase cell population, compared to MGC-803 cells and MGC-803/Cdx2 negative control cells ($P < 0.05$) (Figure 4). These data indicate that cell growth inhibition by Cdx2 siRNA is associated with significant cell cycle arrest in G0/G1 phase, and suggest that siRNA directed against the Cdx2 gene suppresses cell proliferation by controlling the G1 and S checkpoints and inducing a specific block in cell cycle progression.

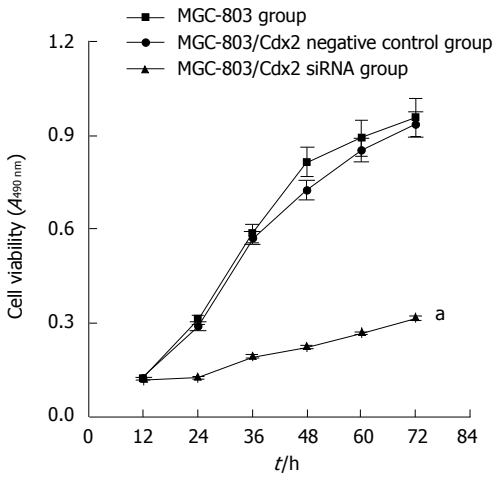

Figure 2 Cdx2 small interference RNA inhibits cell proliferation in MGC-803 cells. MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at days 1-3. The cell viability in each group was presented. Each time point represented the mean of cell viability for each group. $^aP < 0.05$ for MGC-803/Cdx2 siRNA group *vs* MGC-803 and MGC-803/Cdx2 negative control group.

Cdx2 siRNA induces cellular apoptosis

To further study the effect of Cdx2 siRNA on MGC-803 cell apoptosis, cells were stained with Annexin V-FITC and propidium iodide, and then subsequently analyzed by flow cytometry. The dual parameter fluorescent dot plots showed that the viable cells were in the lower left quadrant, and the apoptotic cells were in the right quadrant. As shown in Figure 5, the apoptotic percentage of MGC-803/Cdx2 siRNA cells was 11.7% ± 2.2%, which was significantly higher than that of MGC-803/Cdx2 negative control (5.3% ± 1.3%) and MGC-803 cells (5.6% ± 1.1%) ($P < 0.05$). This implies that inhibition of Cdx2 is able to induce apoptosis in gastric cancer MGC-803 cells.

Cdx2 siRNA decreases migration and invasion of gastric cancer cells

We measured the migratory ability of three cell groups using the wound healing assay by scratching the single-layer cells. As shown in Figure 6, the distance between the wound edges was determined at 0 and 36 h and the healing rate was calculated in the three groups. MGC-803/Cdx2 siRNA cells showed a lower migratory ability at 36 h than MGC-803/Cdx2 negative control and MGC-803 cells. The healing rate of MGC-803/Cdx2 siRNA cells after 36 h was 53.7% ± 7.2%, with a 39.9% and 40.8% decrease, as compared to MGC-803/Cdx2 negative control cells and MGC-803 cells ($P < 0.05$).

Since siRNA targeting of Cdx2 inhibited the expression of Cdx2 gene in gastric cancer cells, we assessed its ability to inhibit cell invasion. After incubation for 24 h in the invasion assay, the numbers of MGC-803/Cdx2 negative control and MGC-803 cells invaded through the membrane of Matrigel chamber were 2.9- and 3.0-fold greater than that of MGC-803/Cdx2 siRNA cells, re-

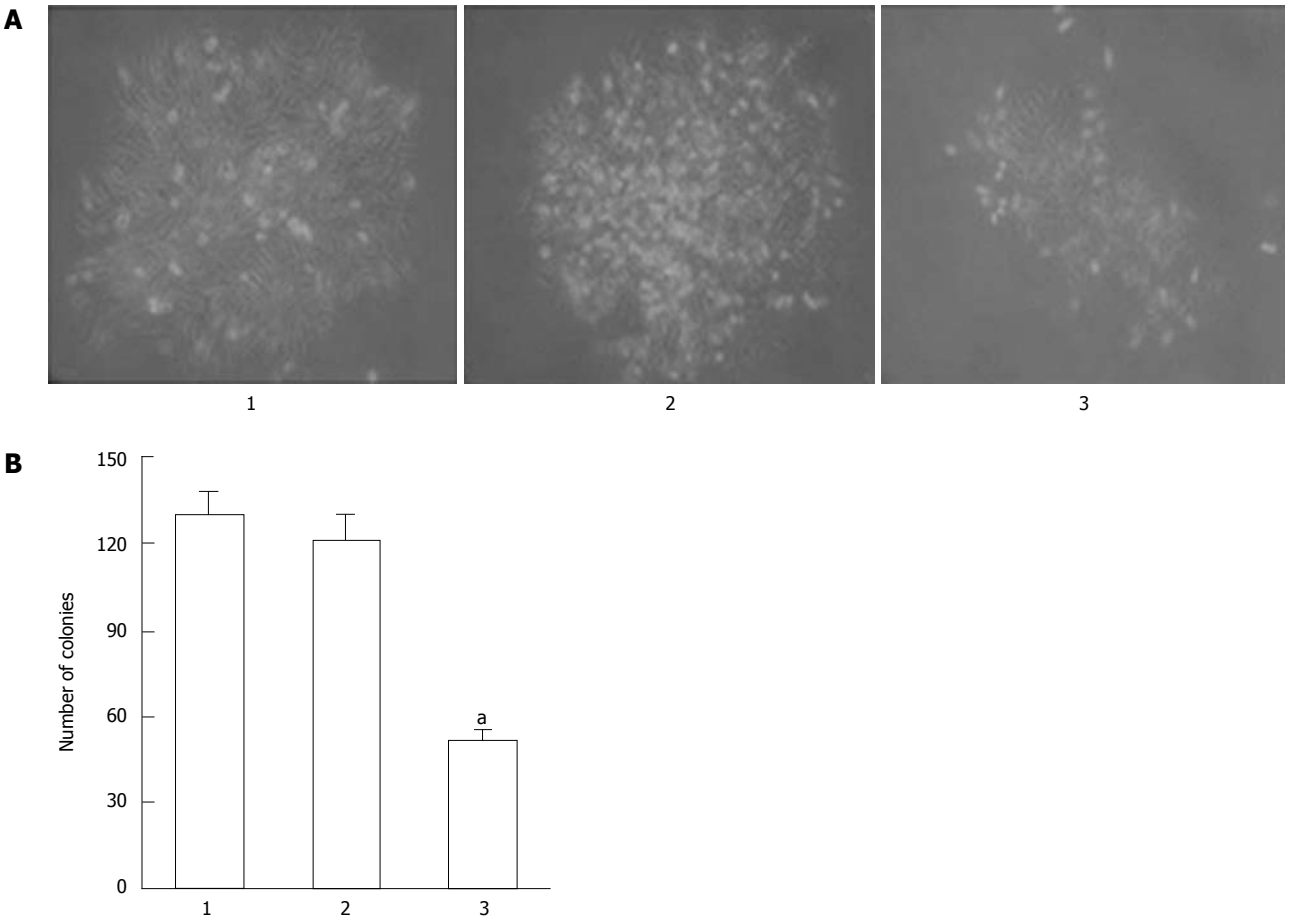

Figure 3 MGC-803/Cdx2 small interference RNA cells exhibited fewer colonies than MGC-803/Cdx2 negative control cells or MGC-803 cells. A: MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were plated in 6-well plates at a density of 20 cells/cm^2, and the colonies were observed under optical microscope at 13 d (×100); B: The surviving fraction of cells (visible colonies) was stained with gentian violet, and counted manually. MGC-803/Cdx2 siRNA cells exhibited fewer colonies than MGC-803/Cdx2 negative control cells or MGC-803 cells. Each column presents as mean ± SE from 3 independent experiments. $^aP < 0.05$ for MGC-803/Cdx2 siRNA group *vs* MGC-803 and MGC-803/Cdx2 negative control group. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group.

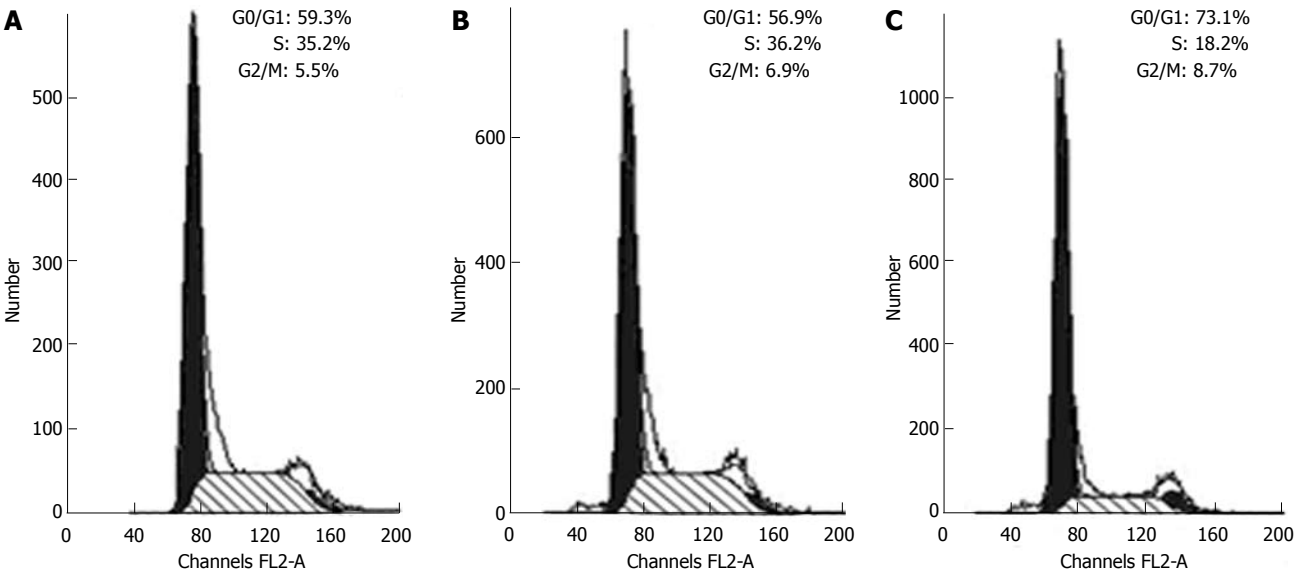

Figure 4 Cdx2 small interference RNA caused cell cycle arrest in the G0/G1 phase. Cell cycle was analyzed by flow cytometry in MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells. The data were representative of 3 independent experiments. A: MGC-803 group; B: MGC-803/Cdx2 negative control group; C: MGC-803/Cdx2 siRNA group.

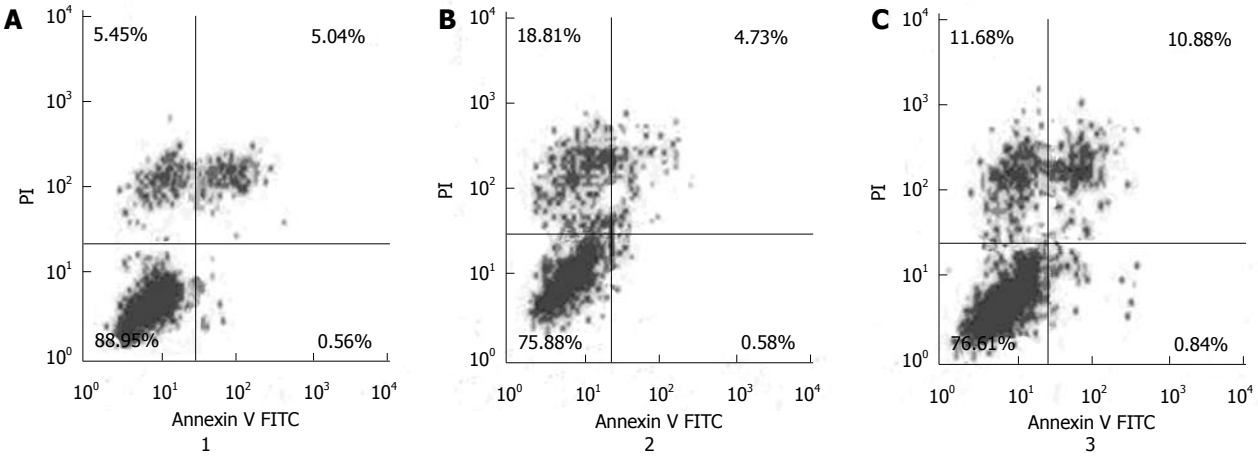

Figure 5 The mean apoptotic rate in MGC-803/Cdx2 small interference RNA cells was significantly higher than that in MGC-803/Cdx2 negative control or MGC-803 cells. Percentages of apoptotic cells analyzed by flow cytometry. Numbers in the quadrants reflected the percentage of cells. A: MGC-803 group; B: MGC-803/Cdx2 negative control group; C: MGC-803/Cdx2 small interference RNA group. PI: Propidium iodide; FITC: Fluorescein isothiocyanate.

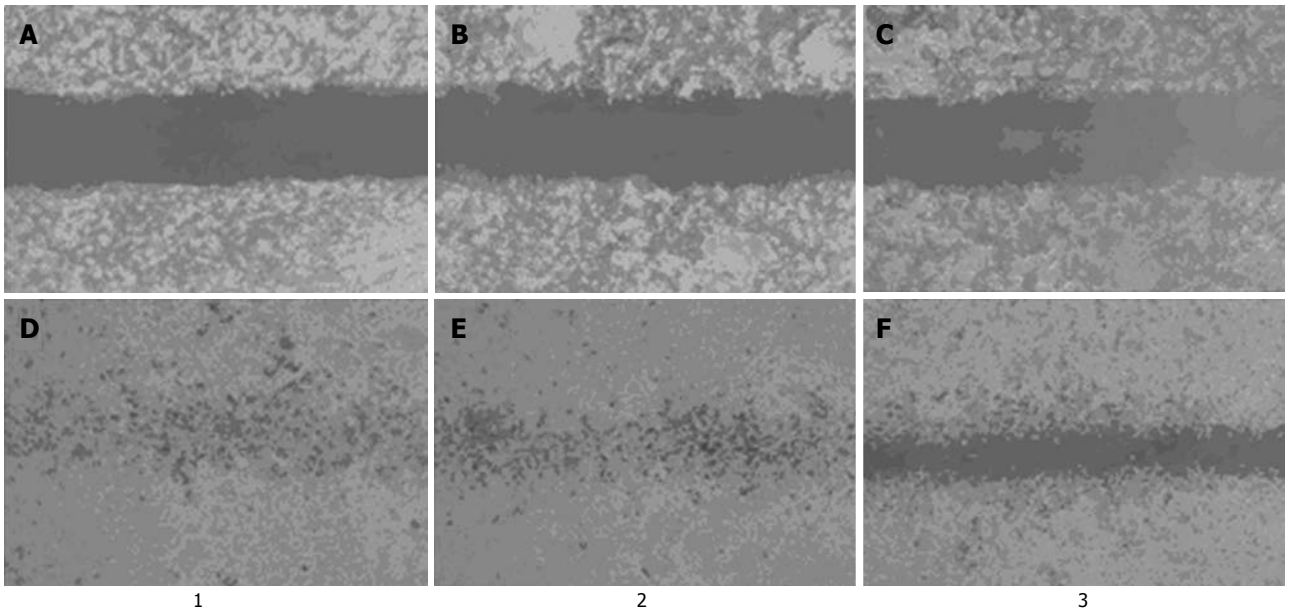

Figure 6 Cdx2 small interference RNA decreased migration of MGC-803 cells in wound healing assay. MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were cultured to confluence on 6-well plates, a central linear wound was made with a 200 μL sterile pipet tip. The central linear was photographed at different intervals (× 100). A: MGC-803 cells at 0 h; B: MGC-803/Cdx2 negative control cells at 0 h; C: MGC-803/Cdx2 siRNA cells at 0 h; D: MGC-803 cells at 36 h; E: MGC-803/Cdx2 negative control cells at 36 h; F: MGC-803/Cdx2 siRNA cells at 36 h. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group.

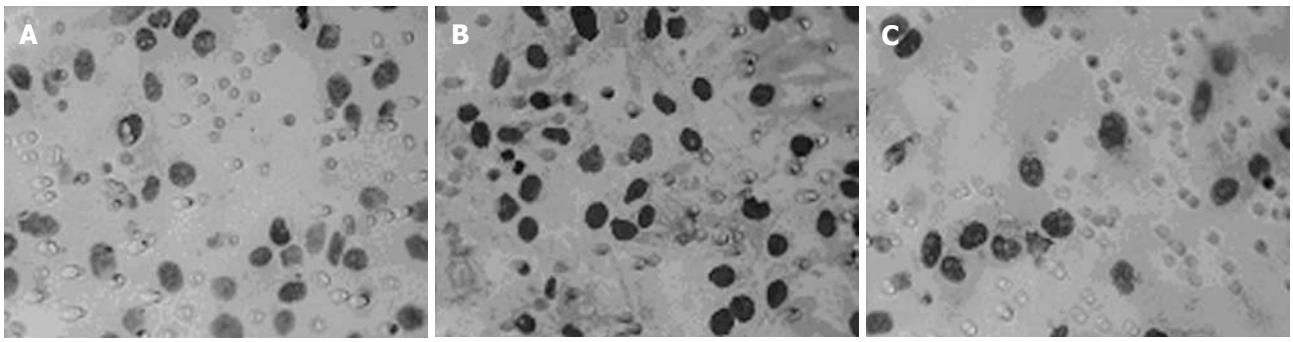

Figure 7 Cdx2 small interference RNA decreased invasion of MGC-803 cells. MGC-803 cells (A), MGC-803/Cdx2 negative control cells (B) and MGC-803/Cdx2 small interference RNA cells (C) were loaded onto Matrigel-coated upper chambers of Transwell plates. Filtrated cells on the undersurface of the polycarbonate membranes were stained and counted under a optical microscope at 24 h (× 200).

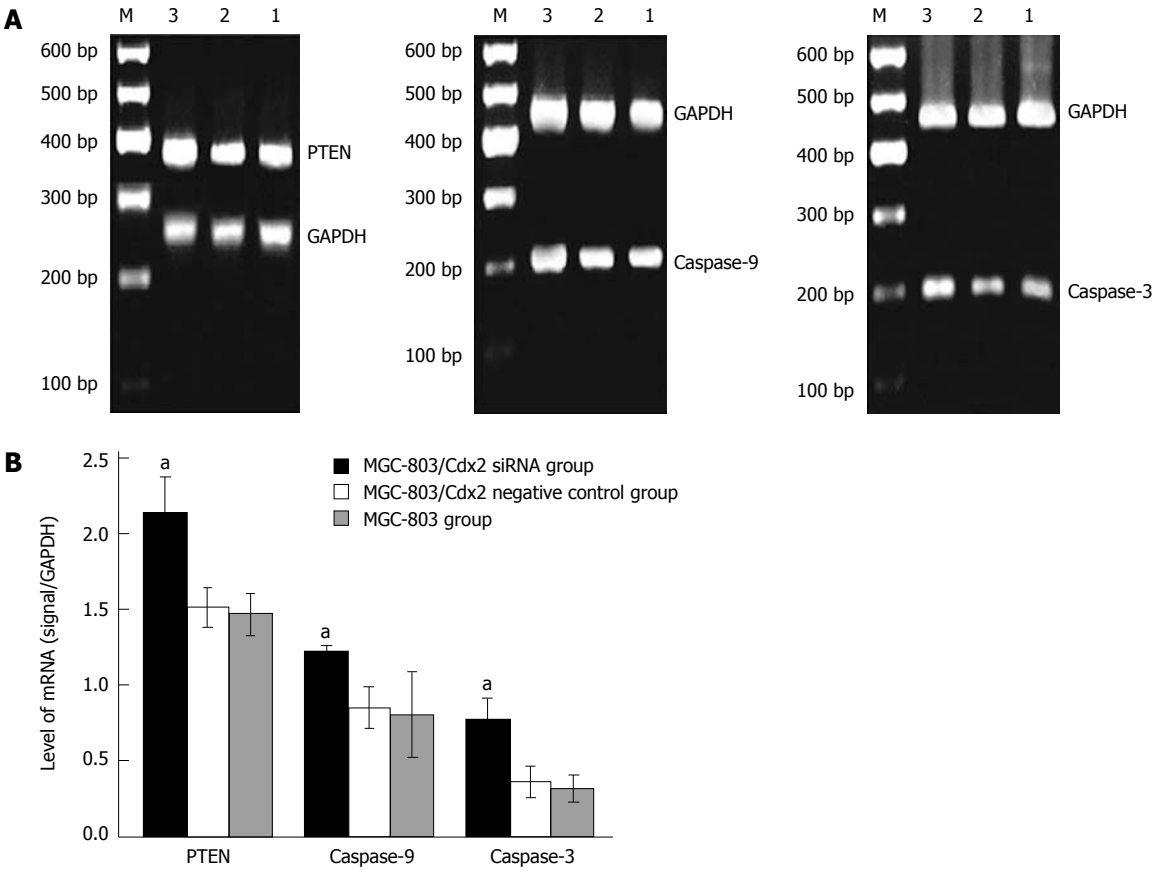

Figure 8 Cdx2 small interference RNA upregulated phosphatase and tensin homolog, caspase-9 and caspase-3 mRNA expression. A: Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. The RNA samples (2 μg in each) extracted from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were subjected to RT-PCR for phosphatase and tensin homolog (PTEN), caspase-9, caspase-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. RT-PCR for GAPDH was performed in parallel to show an equal amount of total RNA in the sample. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group; M: 600 bp marker; B: PTEN, caspase-9 and caspase-3 mRNA levels were measured at three groups, normalized to those of GAPDH and presented as mean ± SE. $^{a}P < 0.05$ for MGC-803/Cdx2 siRNA group *vs* MGC-803 and MGC-803/Cdx2 negative control group.

spectively ($P < 0.05$) (Figure 7). The results indicate that Cdx2 siRNA reduces the migratory and invasion ability of gastric cancer MGC-803 cells.

Cdx2 siRNA increases PTEN expression, and activates caspase-9 and caspase-3

To investigate the mechanism by which Cdx2 siRNA induces apoptosis in MGC-803 cells, we detected expression levels of several apoptotic family members including PTEN, caspase-9, and caspase-3 by semi-quantitative RT-PCR and Western blotting analysis. As shown in Figure 8, densitometric analysis showed that PTEN, caspase-9, and caspase-3 mRNA of MGC-803/Cdx2 siRNA cells were higher than that in MGC-803 cells and MGC-803/Cdx2 negative control cells ($P < 0.05$), while no differences were found between MGC-803/Cdx2 negative control cells and MGC-803 cells. As shown in Figure 9, Cdx2 siRNA led to the cleavage of pro-caspase-9 (47 kDa) and pro-caspase-3 (35 kDa) into other multiple, cleaved, maturation products (data not shown), but only the 37-kDa form of cleaved caspase-9 and the 17-kDa form of cleaved caspase-3 were observed. Densitometric analysis showed that PTEN, p37 cleaved caspase-9, and p17 cleaved caspase-3 protein of MGC-803/Cdx2 siRNA cells were higher, while pro-caspase-9 and pro-caspase-3 were lower than that in MGC-803 cells and MGC-803/Cdx2 negative control cells ($P < 0.05$). No differences were found between MGC-803/Cdx2 negative control cells and MGC-803 cells.

Inhibitory effect of Cdx2 siRNA in vivo

We also examined the effect of Cdx2 siRNA on growth of MGC-803 cells *in vivo* by implanting MGC-803/Cdx2 siRNA cells subcutaneously into the flanks of BALB/c nude mice. Four weeks after implantation, tumor weight from MGC-803/Cdx2 siRNA cells was 0.773 ± 0.054 g, which was significantly less than 2.334 ± 0.087 g from MGC-803 cells, and 2.356 ± 0.092 g from MGC-803/Cdx2 negative control cells ($P < 0.05$). As shown in Figure 10, the tumor growth curves indicate the significant growth inhibition in MGC-803/Cdx2 siRNA cells ($P < 0.05$). Densitometric analysis showed that Cdx2 mRNA expression in MGC-803/Cdx2 siRNA cells (0.305 ± 0.053) was lower than that in MGC-803 cells (1.524 ±

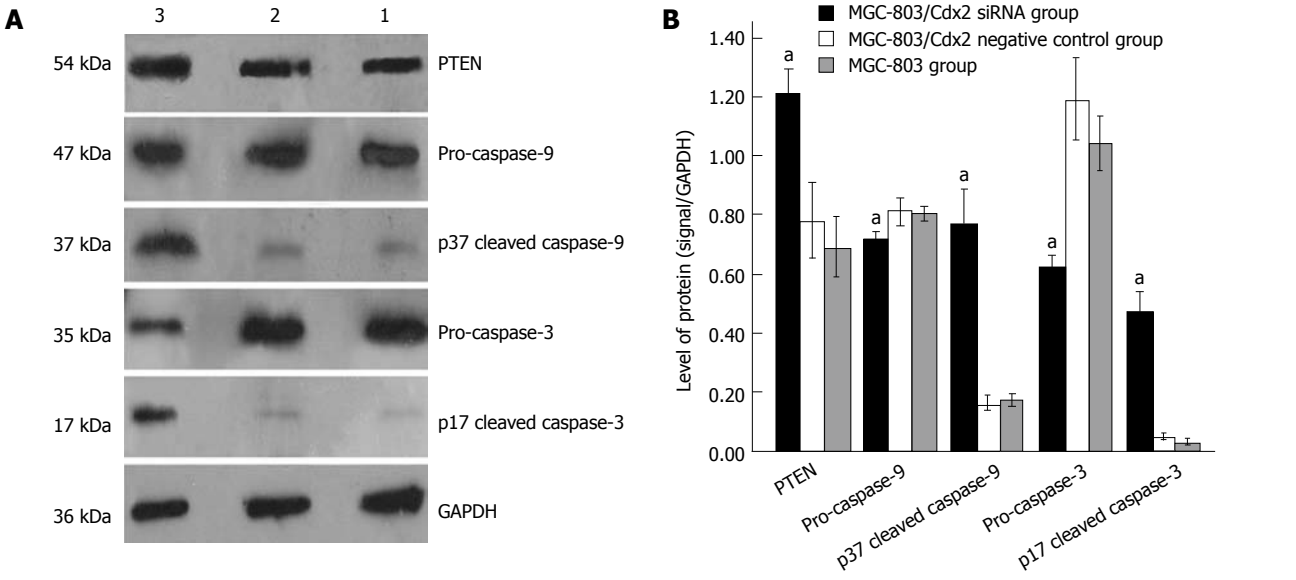

Figure 9 Cdx2 small interference RNA significantly increased phosphatase and tensin homolog, cleaved caspase-9 and cleaved caspase-3 protein concentrations while pro-caspase-9 and pro-caspase-3 are decreased. A: Western blotting analysis. Whole protein extracts (100 μg in each) were prepared from MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells. The expression of phosphatase and tensin homolog (PTEN), pro-caspase-9, p37 cleaved caspase-9, pro-caspase-3, and p17 cleaved caspase-3 was determined by Western blotting with an anti-PTEN, pro-caspase-9, cleaved caspase-9, pro-caspase-3 and cleaved caspase-3 antibody. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression levels were determined as a control for equivalent protein loading. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group; B: PTEN, pro-caspase-9, p37 cleaved caspase-9, pro-caspase-3 and p17 cleaved caspase-3 protein levels were measured at three groups, normalized to those of GAPDH and presented as mean ± SE. $^{a}P < 0.05$ for MGC-803/Cdx2 siRNA group *vs* MGC-803 and MGC-803/Cdx2 negative control group.

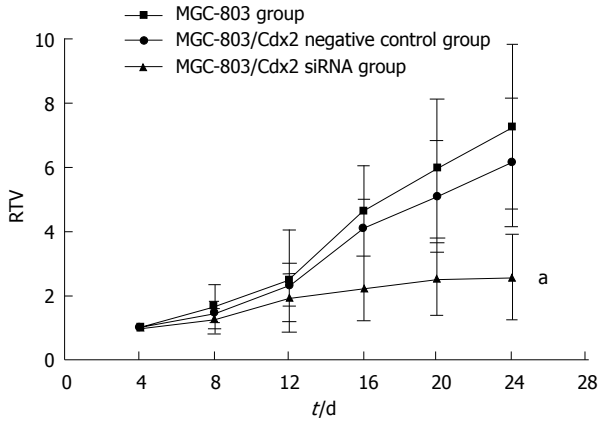

Figure 10 Tumor growth curve showed a significant growth tendency in MGC-803 cells and in MGC-803/Cdx2 negative control cells, while the tumor growth in MGC-803/Cdx2 small interference RNA cells was obviously inhibited. MGC-803 cells, MGC-803/Cdx2 negative control cells and MGC-803/Cdx2 small interference RNA (siRNA) cells were implanted subcutaneously into the flanks of the nude mice. The relative tumor volume (RTV) of nude mice in each group were presented. Each time point represented the mean of RTV for each group; $^{a}P < 0.05$ for MGC-803/Cdx2 siRNA group *vs* MGC-803 and MGC-803/Cdx2 negative control group.

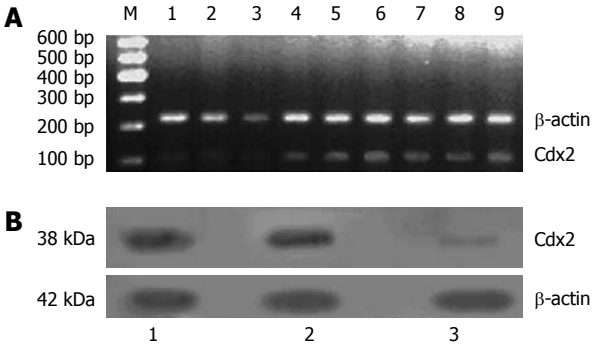

Figure 11 Cdx2 mRNA and protein expression was suppressed in MGC-803/Cdx2 small interference RNA tumor tissue. A: Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Total RNAs (2 μg in each) extracted from tumor tissue were subjected to RT-PCR for Cdx2 and β-actin mRNAs. RT-PCR for β-actin was performed in parallel to show an equal amount of total RNA in the sample; Lanes 1-3: MGC-803/Cdx2 small interference RNA (siRNA) group; Lanes 4-6: MGC-803/Cdx2 negative control group; Lanes 7-9: MGC-803 group; M: 600 bp marker; B: Western blotting analysis. Equal amounts of protein extracts (100 μg in each) were prepared from tumor tissue. The expression of Cdx2 protein was determined by Western blotting with an anti-Cdx2 antibody. The β-actin expression levels were determined as a control for equivalent protein loading. Lane 1: MGC-803 group; Lane 2: MGC-803/Cdx2 negative control group; Lane 3: MGC-803/Cdx2 siRNA group.

0.323) and MGC-803/Cdx2 negative control cells (1.441 ± 0.269), as determined by semi-quantitative RT-PCR ($P < 0.05$) (Figure 11A). In addition, the relative protein expression of Cdx2 in MGC-803/Cdx2 siRNA cells (0.134 ± 0.087) also significantly decreased ($P < 0.05$), when compared to MGC-803 cells (0.634 ± 0.156) and MGC-803/Cdx2 negative control cells (0.569 ± 0.167), as determined by Western blotting analysis (Figure 11B). As shown in Figure 12, the percent of apoptotic tumor cells in MGC-803/Cdx2 siRNA cells was 16.7% ± 5.6%, which was more than 10.5% ± 4.1% in MGC-803/Cdx2 negative control cells and 11.2% ± 4.3% in MGC-803 cells, as determined by the TUNEL method.

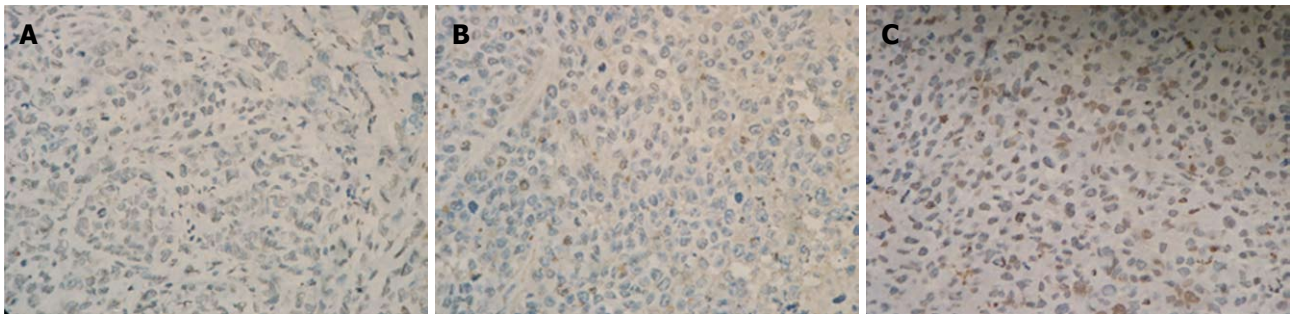

Figure 12 Cdx2 small interference RNA promoted tumor cells apoptosis. Tumor cells were assessed for apoptosis using terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine, 5'-triphosphate nick end labeling assay. The apoptotic cells were brown-stained and counted under a optical microscope (× 400). A: MGC-803 group; B: MGC-803/Cdx2 negative control group; C: MGC-803/Cdx2 small interference RNA group.

DISCUSSION

The Cdx2 homeobox gene, which is homologous to the *Drosophila* gene caudal, has an essential role during early development[4]. In adults, Cdx2 expression is restricted to intestinal epithelial cells. Overexpression of Cdx2 in human colon cancer cells induces a less malignant phenotype, inhibiting proliferation, invasion, and migration[16], and Cdx2 expression is progressively reduced in gastric cancer[17]. Moreover, heterozygous-null Cdx2 mice are more sensitive to azoxymethane-induced colonic adenocarcinomas[18], and mice that are compound heterozygotes for Cdx2 and the tumor suppressor Adenomatous Polyposis Coli (Apc) developed more adenomatous polyps in the colon than their heterozygous Apc littermates[19]. These studies suggested that Cdx2 is a putative tumor suppressor.

However, other reports have shown that Cdx2 plays a pivotal role in the development of intestinal metaplasia[20,21]. The implication of Cdx2 in intestinal metaplasia has been demonstrated in intestinal metaplasia of the stomach where Cdx2 was ectopically overexpressed, suggesting that it could play a major role during intestinal metaplasia formation in the stomach[21]. Intestinal metaplasia is a precursor of intestinal-type gastric adenocarcinoma. Long-term intestinal metaplasia induced gastric adenocarcinoma in the Cdx2-transgenic mouse stomach, and no significant changes were noted in wild-type littermates[14]. The tumor incidence was 100% at 100 wk after birth[15]. It can thus be concluded that Cdx2-induced intestinal metaplasia itself is a precancerous lesion leading to gastric carcinoma. Furthermore, Cdx2 is overexpressed in most colorectal tumors compared to matched normal mucosa in adults[7]. Dang *et al*[22] showed that Cdx2 does not suppress tumorigenicity in the human gastric cancer cell line, MKN45. It can be concluded that, in contrast to the prevailing paradigm, Cdx2 does not serve as a tumor suppressor in the development of most sporadic colorectal tumors. Rather, in the context of earlier observations of its role in promoting the neoplastic phenotype in some cells and tissues, many observations suggest the intriguing possibility that Cdx2 could serve as an oncogene in the gastrointestinal tract[9,23]. This suggests that the level of Cdx2 expression may contribute to its function[9], thereby raising the possibility that intervening with Cdx2 expression in gastric cancer cells with RNA interference may control their growth rate.

Our study indicated that Cdx2 siRNA led to remarkable inhibition of Cdx2 mRNA and protein expression in MGC-803 cells, inhibited cell growth, caused cell cycle arrest in the G0/G1 phase, and induced cell apoptosis. Furthermore, RNAi-directed targeting of Cdx2 in MGC-803 cells reduced the capability of cell motion, invasion, and colony formation. Moreover, a strong anti-tumor effect of Cdx2-siRNA *in vivo* was observed, as tumor growth was suppressed and tumor apoptosis was increased in nude mice when Cdx2 mRNA and protein was silenced by Cdx2 siRNA. These findings suggest that Cdx2 has tumorigenic potential in the human gastric cancer cell lines MGC-803.

However, our previous study showed that Cdx2 overexpression in human gastric cancer MGC-803 cells produce similar results as Cdx2 siRNA[24]. Moreover, Cdx2 overexpression was associated with cell cycle arrest in the G0/G1 phase which was the same as Cdx2 siRNA. This suggests that Cdx2 plays a double role in the regulation of MGC-803 cell growth and death. Thus, we can only speculate on potential explanations for these observed contrasts. First, appropriate activity and expression levels of Cdx2 are necessary for the normal cell cycle, even in promoting tumor proliferation and regression. Just like E2F-1, both the upregulation and downregulation of E2F-1 can suppress human gastric cancer MGC-803 cell growth *in vitro*[25,26]. Second, these two conflicting results may involve different mechanisms. Our previous data showed that overexpression of Cdx2 inhibits MGC-803 cell progression *via* the Wnt signaling pathway (unpublished data). In this result, PTEN, caspase-9 and caspase-3 expression were all increment when Cdx2 was downregulated. The PTEN protein product is a lipid phosphatase that antagonizes PI3K function and consequently inhibits downstream signaling transduction through Akt[27]. Caspase-9, a member of the protease family, is intimately associated with the initiation of apoptosis, and is thought to be activated while

Akt is inhibited[28]. Activated caspase-9 is able to cleave caspase-3 *in vitro*, leading to apoptosis[29]. Therefore, in the present study, inhibition of Cdx2 expression may increase PTEN expression directly or indirectly, leading to activation of caspase-9 and caspase-3 *via* the PI3K/Akt signaling pathway, which is responsible for inhibition of MGC-803 cell growth *in vitro* and *in vivo*. Further studies are needed to confirm our results.

Gastric cancer is a worldwide problem. Besides the undetermined etiological factors, there are also limitations in surgery, chemotherapy and radiotherapy, which to date, are the major therapies for gastric cancer[30]. Many patients lose the chance of surgery because of their systemic condition, while many cannot tolerate the side effects of chemotherapy or radiotherapy. It is important to find a new way to effectively inhibit cancer cell growth and avoid the side effects of drugs or radioactive rays. Gene target therapies have proved to be a promising way to achieve this goal[26]. In this study, we showed that Cdx2 plays a critical role in gastric cancer cell proliferation, invasion, and apoptosis. The down-regulation of Cdx2 using RNAi successfully reduced the progression of gastric cancer MGC-803 cells *in vitro* and *in vivo*. In conclusion, this study lays the foundation for treatment of gastric cancer through manipulation of Cdx2 expression.

COMMENTS

Background

Gastric cancer is a worldwide problem. Besides the undetermined etiological factors, there are also limitations in surgery, chemotherapy and radiotherapy, which to date, are the major therapies for gastric cancer. It is important to find a new way to effectively inhibit cancer cell growth and avoid the side effects of drugs or radioactive rays. Gene target therapies have proved to be a promising way to achieve this goal. The caudal-type homeobox gene, Cdx2, plays an important role in intestinal metaplasia, and is a precursor of intestinal-type gastric carcinoma. However, the effect of Cdx2 in gastric cancer is still not very clear.

Research frontiers

Cdx2 plays important roles in early differentiation, proliferation and maintenance of intestinal epithelial cells. The role of Cdx2 as an oncogene or a tumor suppressor gene is still in dispute at the present time. The Cdx2 research hotspot is how it affect the progression of human cancer.

Innovations and breakthroughs

This study for the first time demonstrated that Cdx2 small interference RNA (siRNA) significantly inhibited cell growth and proliferation, blocked entry into the S-phase of the cell cycle, induced cell apoptosis, and reduced the motility and invasion of MGC-803 cells. Cdx2 siRNA also increased phosphatase and tensin homolog expression, and activated caspase-9 and caspase-3 in MGC-803 cells *in vitro* as determined by reverse transcription polymerase chain reaction and Western blotting analysis. In addition, siRNA targeting of Cdx2 inhibited the growth of MGC-803 cells and promoted tumor cell apoptosis *in vivo* in nude mice tumor models.

Applications

This study lays the foundation for treatment of gastric cancer through manipulation of Cdx2 expression.

Terminology

The transcription factor, Cdx2, is a member of the caudal-related homeobox gene family, and is mainly expressed in the intestine. It is also known to be a key factor in the development of intestinal metaplasia.

Peer review

In this study, the authors constructed recombinant pSilencer 4.1-Cdx2 siRNA plasmids and transfected them into human gastric cancer MGC-803 cells *in vitro*. The authors demonstrated that Cdx2 siRNA led to inhibition of endogenous Cdx2 mRNA and protein expression and Cdx2 siRNA significantly inhibited cell growth and proliferation, blocked entry into the S-phase of the cell cycle, induced cell apoptosis, and reduced the motility and invasion of MGC-803 cells. The authors conclude that Cdx2 is involved in the regulation of tumor growth, proliferation, apoptosis and invasion of gastric cancer cells. Overall this is a well-conducted pilot study.

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BRIEF ARTICLE

Caspase-cleaved cytokeratin-18 and tumour regression in gastro-oesophageal adenocarcinomas treated with neoadjuvant chemotherapy

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Abstract

AIM: To examine cytokeratin-18 (CK-18) and caspase-cleaved CK-18 expression in tumours and correlate with clinicopathological outcomes including tumour regression grade (TRG) response.

METHODS: Formalin-fixed human gastro-oesophageal cancers were constructed into tissue microarrays. The first set consisted of 122 gastric/gastro-oesophageal cancer cases not exposed to neoadjuvant chemotherapy and the second set consisted of 97 gastric/gastro-oesophageal cancer cases exposed to pre-operative platinum-based chemotherapy. Expression of CK-18 and caspase-cleaved CK-18 was investigated using immunohistochemistry.

RESULTS: CK18 was commonly expressed in gastro-oesophageal tumours (92.6%). Fifty-six point seven percent of tumours previously exposed to neoadjuvant chemotherapy were positive for caspase-cleaved CK-18 expression compared to only 24.6% of tumours not previously exposed to neoadjuvant chemotherapy (P = 0.009). In patients who received neoadjuvant chemotherapy, caspase-cleaved cytokeratin-18 expression correlated with favourable TRG response (TRG 1, 2 or 3, P = 0.043).

CONCLUSION: This is the largest study to date of CK-18 and caspase-cleaved CK-18 expression in gastro-oesophageal tumours. We provide the first evidence that caspase-cleaved CK-18 predicts tumour regression with neoadjuvant chemotherapy.

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Key words: Tumour regression grade; Gastro-oesophageal cancers; Chemotherapy; Full length cytokeratin-18; Caspase-cleaved cytokeratin-18

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INTRODUCTION

Neoadjuvant platinum-based chemotherapy followed by surgery is the standard of care for patients with gastro-oesophageal adenocarcinoma[1,2]. However, there is an urgent need to develop predictive markers to individualize patient therapy[3]. We have recently shown that tumour regression grade (TRG) is a marker of histopathological response and tumour down-staging in tumours receiving neoadjuvant chemotherapy[4]. TRG was defined as per Mandard's criteria[5]. TRG1 (complete regression) showed absence of residual cancer and fibrosis extending through the different layers of the oesophageal wall; TRG2 was characterised by the presence of rare residual cancer cells scattered through the fibrosis; TRG3 was characterised by an increase in the number of residual cancer cells but fibrosis predominated; TRG4 showed residual cancer outgrowing fibrosis; and TRG5 was characterised by the absence of regressive changes[5]. In patients who received neoadjuvant chemotherapy (CS group), 46.7% of gastric/gastro-oesophageal junction adenocarcinomas and 45.5% of lower third oesophageal adenocarcinomas had TRG 1, 2 or 3 compared to 13.7% in patients who did not receive neoadjuvant chemotherapy but proceeded to primary surgery. In the CS group, responders (TRG 1, 2 or 3) showed significant tumour down-staging [early ypT-stage disease (P = 0.002)]. In gastric cancers specifically, additional associations were seen with negative nodal disease (P = 0.044) and absence of vascular invasion (P = 0.027)[4]. More recently, we have also demonstrated that favourable tumour regression predicts better clinical outcomes in patients receiving neoadjuvant chemotherapy in gastro-oesophageal adenocarcinomas[6].

The anticancer activity of chemotherapeutic agents is directly related to the induction of apoptosis in tumours. Whilst the apoptotic pathway is complex, the intrinsic mitochondrial pathway is the predominant apoptotic pathway in cancer cells. In the intrinsic pathway, the mitochondrial release of cytochrome c activates caspase-9, which in turn activates caspase-3 and caspase-7[7,8]. Among the several cellular substrates of the capsases, members of the cytokeratin family, including cytokeratin-18 (CK-18), contribute to cellular collapse and apoptosis. Caspase-cleaved CK-18 is a specific marker of epithelial cell death and correlates with apoptosis in gastrointestinal epithelial cancers[9-11].

In the current study we have evaluated full length CK-18 and caspase-cleaved CK-18 protein expression using immunohistochemistry. We show for the first time that caspase-cleaved CK-18 expression in tumours correlates with histopathological tumour regression in early stage gastro-oesophageal adenocarcinomas.

MATERIALS AND METHODS

Patients

We identified patients referred to our centre with resectable gastric, gastro-oesophageal junction (GOJ) and lower third oesophageal adenocarcinomas between January 2001 and May 2008. GOJ tumours were defined as per Siewert's classification[12]. The Union for International Cancer Control TNM staging system for oesophageal and gastric cancer was used in this study. The study was approved by the Ethics Committee of Nottingham University Hospitals.

Construction of tissue micro-array

Tissue micro-arrays (TMAs) were constructed. In brief, HE-stained slides (5 μm) were used to identify and mark out representative areas of viable tumour tissue. Then 0.6 mm-diameter needle core biopsies from the relevant areas of corresponding paraffin-embedded blocks were placed at defined coordinates in the recipient paraffin array blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). Array blocks were constructed at a density of 80-150 cores per array. Two broad sets of TMA blocks were constructed. An array set of 97 patient cores to include gastric and gastro-oesophageal tumours that had received neoadjuvant chemotherapy and an array set of 122 cores of patients who had received no neoadjuvant chemotherapy were constructed. These TMA blocks were constructed in triplicate, each containing one sample from a different region of the tumour.

Immunohistochemistry

A standard streptavidin-biotin complex technique was used. In brief, 5 μm TMA sections were deparaffinised with xylene and rehydrated through graded alcohol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min. Antigen retrieval was carried out by microwave treatment of the slides in sodium citrate buffer (pH 6) for 10 min at 750 W followed by 10 min at 300 W. The slides were rinsed in phosphate buffer saline (PBS) and incubated with Vectastain blocking serum diluted in PBS to block non-specific absorption. The slides were incubated for 30 min with the primary antibody M30 to detect caspase-cleaved CK-18 (Peviva, Bromma, Sweden) at a dilution of 1:75 and primary antibody M6 to detect full length CK-18 (Peviva, Bromma, Sweden) at a dilution of 1:150 at room temperature. After washing with PBS, sections were incubated with secondary antibody (Vectastain) for 30 min followed by avidin-biotin complex for a further 30 min. 3-3' Diaminobenzidine tetrahydochloride was used as a chromogen. All sections were counterstained with Gill' s haematoxylin, dehydrated and mounted using DPX (a mixture of disterene, plasticizer, and xylene; Sigma).

Evaluation of staining

Evaluation of staining was performed with the observer blinded to the corresponding clinicopathological data. For

Table 1 Patient demographics *n* (%)

	Neoadjuvant chemotherapy group	Primary surgery group
Total number of patients	97	122
Median age (yr)	64	74.5
Sex		
Male	74 (76)	92 (75.4)
Female	23 (24)	30 (24.6)
T stage		
T1	6 (6.1)	12 (9.8)
T2	29 (29.8)	41 (33.6)
T3	50 (51.5)	65 (53.2)
T4	8 (8.2)	4 (3.2)
TX	1 (1)	
N stage		
N0	31 (31.9)	32 (26.2)
⩾ N1	66 (68.1)	90 (73.8)
M stage		
M0	97 (100)	122 (100)
M1	-	-
Tumour type		
Adenocarcinoma	83 (85.5)	122 (100)
Squamous cell carcinoma	12 (12.3)	-
Adenosquamous	2 (2)	-
Site of tumour		
Gastric	20 (20.6)	122 (100)
GOJ	47 (48.4)	-
Lower third of oesophagus	36 (37.1)	-
Surgery		
Total gastrectomy	22	70
Partial gastrectomy	5	39
Oesophagectomy/ Oesophago-gastrectomy	70	13
Survival status		
Alive	47 (48.4)	47 (38)
Dead	50 (51.6)	75 (62)

GOJ: Gastro-oesophageal junction.

full length CK-18 expression, cytoplasmic expression in cancer cells was considered positive. For caspase-cleaved CK-18 expression, TMA cores from tumour showing any positively stained apoptotic cells were considered positive. Caspase-cleaved CK-18 positive apoptotic cells were counted in TMA cores and the total number of positive cells from each tumour was taken as the number of positive cells.

Statistical analysis

All statistical analyses were carried out using SPSS package (version 15 for Windows, SPSS, Inc.). Associations between categorical variables were examined using cross-tabulation and the Pearson χ^2 test. Kaplan Meier curves were derived to assess disease-specific survival, and the significance of differences in disease-specific survival between groups was calculated using the log-rank test. Patients whose death related to their oesophago-gastric cancer were considered in the disease-specific survival calculations. This was determined by death certification entries. Deaths resulting from non-oesophago-gastric cancer-related causes were censored. Survival rates were calculated from the date of diagnosis until the 13th January 2009, when any remaining survivors were censored and Kaplan Meier curves were plotted. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Patient demographics

There were 2 groups of patients: those who received neoadjuvant chemotherapy (neoadjuvant group) and those who underwent primary surgery only (primary group). There were 97 patients in the neoadjuvant group with a median age of 64 years; 76% (n = 74) were male and 51.5% (n = 50) of cases were T3 tumours. There were 122 cases in the primary group with a median age of 74.5 years; 75.4% (n = 92) were male and 53.2% (n = 65) had T3 tumours. Patients in the primary surgery group did not receive any adjuvant chemotherapy after surgery. In the neoadjuvant group, 78% of patients had received all the planned three cycles of neoadjuvant ECF/ECX chemotherapy (adenocarcinomas) and 96.4% had received all the planned two cycles of neoadjuvant CF chemotherapy (squamous cell carcinomas). Of the patients who received all three cycles of ECF/ECX chemotherapy, 42% went on to receive a further three cycles of ECF/ECX chemotherapy. There was no significant difference between the primary surgery group and the perioperative chemotherapy group (gastric/GOJ) with regards to T stage [T2 (33.6% *vs* 29.8%), T3 (53.2% *vs* 51.5%)] and N stage [N0 (26.2% *vs* 31.9%), > N0 (73.8% *vs* 68.1%)]. Only adenocarcinomas were included in the immunohistochemical and survival analyses in this study (Table 1).

Full length cytokeratin-18 expression

All 122 tumours in the primary surgery group were available for CK-18 analyses. One hundred and thirteen tumours stained positive for CK-18 (92.6%) (Figure 1A) and 9 cores were negative for CK-18 expression. There was no statistically significant correlation between tumour differentiation, T stage, N stage, vascular/perineural invasion, resection margin involvement and full length CK-18 expression in tumours.

Caspase-cleaved cytokeratin-18 expression

All tumours were suitable for caspase-cleaved CK-18 expression analyses (Figure 1B). In tumours previously exposed to neoadjuvant chemotherapy (neoadjuvant TMA, n = 97), 56.7% of tumours (55/97) were positive compared to 24.6% (30/122) of tumours not previously exposed to neoadjuvant chemotherapy (primary TMA). This was statistically significant (P = 0.009). The mean total number of caspase-cleaved CK-18 positive cancer cells per tumour was 4.16 in the neoadjuvant group (range: 1-92) compared to 2.7 in the primary surgery group (range: 1-51).

We have previously demonstrated that TRG as assessed using Mandard's criteria is a useful tool to assess response to neoadjuvant chemotherapy in gastro-oesophageal adenocarcinomas; favourable TRG correlated with tumour down-staging in that study[4]. In the current study we

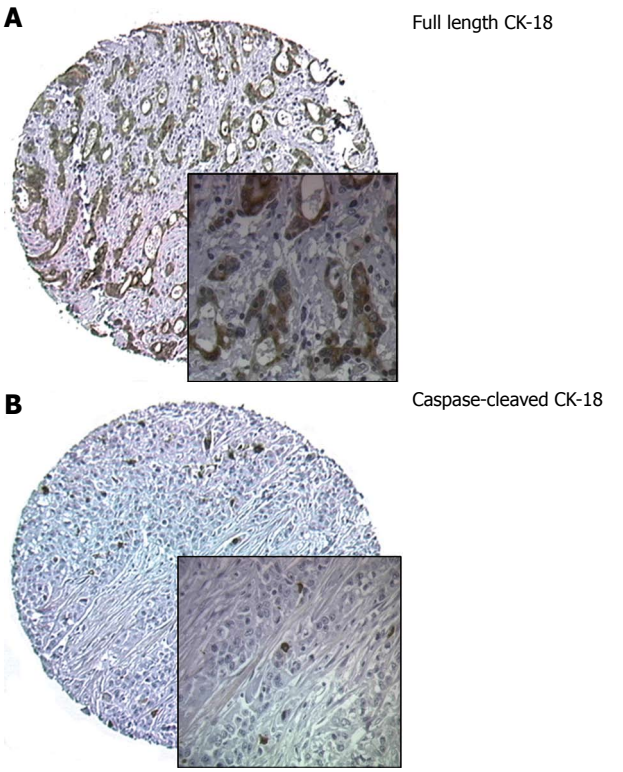

Figure 1 Immunohistochemical staining of full length cytokeratin-18 and caspase-cleaved cytokeratin-18. A: Immunohistochemical staining of full length cytokeratin-18 (CK-18) showing strong cytoplasmic staining; B: Immunohistochemical staining for caspase-cleaved CK-18. Cores from tumour showing positively-stained apoptotic cells. Original magnification × 100; insets × 400.

Table 2 Caspase-cleaved cytokeratin-18 and tumour regression in tumours exposed to neoadjuvant chemotherapy *n* (%)

Caspase-cleaved CK-18	TRG1, TRG2, TRG3	TRG4, TRG5	Total
Negative	10 (23.8)	32 (76.2)	42 (100)
Positive	24 (43.6)	31 (56.4)	55 (100)
Total	34 (35.1)	63 (64.9)	97 (100)

CK-18: Cytokeratin-18; TRG: Tumour regression grade.

evaluated whether caspase-cleaved CK-18 expression correlated with TRG. We found that 43.6% of tumours that were positive for caspase-cleaved CK18 also had a favourable tumour response (TRG 1-3) compared to 23.8% that were negative for caspase-cleaved CK-18 expression (Table 2). This was statistically significant ($P = 0.043$). There was no statistically significant correlation between tumour differentiation, T stage, N stage, vascular/perineural invasion, resection margin involvement and caspase-cleaved CK-18 positivity.

Clinicopathological correlations were also observed in tumours not exposed to neoadjuvant chemotherapy. Well-differentiated tumours were more likely to be caspase-cleaved CK-18 positive (62.5%) compared to poor- and moderately-differentiated tumours which were only positive in 23.6% and 19% of tumours respectively ($P = 0.031$). However, no differences between T, N stage, vascular/perineural invasion, resection margin involvement

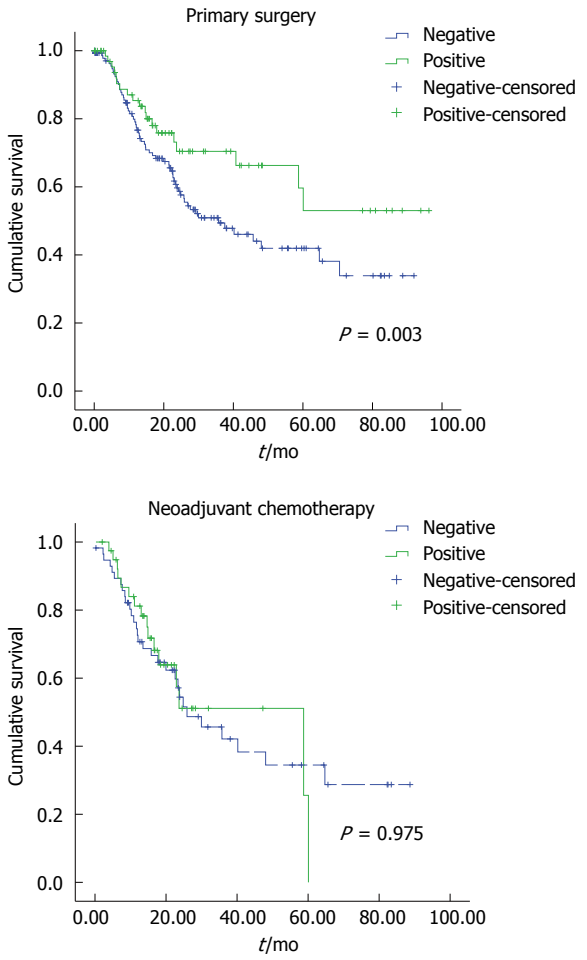

Figure 2 Kaplan Meier curves representing the relationship between caspase-cleaved cytokeratin-18 and disease-specific survival. A: In months from time of diagnosis in patients who received primary surgery only; B: In patients who received neoadjuvant chemotherapy.

and caspase-cleaved CK-18 positivity were observed. Regarding patients who had not received neoadjuvant chemotherapy, Kaplan Meier plot showed that in patients whose tumours stained positive for caspase-cleaved CK-18, a longer disease-specific survival was observed compared to patients whose tumours were negative (mean 84 mo *vs* 51 mo, $P = 0.003$) (Figure 2). However, in patients who had received neoadjuvant chemotherapy, no statistically significant differences were observed (mean 37.3 mo *vs* 41.6 mo, $P = 0.975$) (Figure 2).

Those factors found to be significant in univariate analyses were also included in a multivariate logistic regression analysis to estimate the independent effect of each factor after adjusting for the contributions of other factors. There was no one factor in the cohort of patients studied that showed significance on multivariate analysis (Table 3).

DISCUSSION

The ability to predict response to chemotherapy and individualize patient treatment is a high priority in gastro-oesophageal adenocarcinomas. Whilst the role of multi-

Table 3 Univariate and multivariate analyses showing predictive factors for disease-specific and overall survival in neoadjuvant and surgery only groups

	Univariate analysis		Multivariate analysis
	DSS	OS	OS
Neoadjuvant group			
Caspase-cleaved CK-18 positivity	0.975	0.865	1.32
TRG 1-3 *vs* 4-5	0.038	0.136	0.109
Tumour diff. (well/mod *vs* poor)	0.087	0.101	0.32
T stage (T1, 2 and 3, 4)	0.07	0.09	0.101
N stage (N0 and > N1)	0.106	0.124	0.23
M stage (M0 and > M0)	0.23	0.14	1.72
Vascular invasion	0.87	0.76	0.86
Perineural invasion	1.32	1.455	1.76
Resection margin involvement	2.34	1.98	0.24
Surgery only group			
Caspase-cleaved CK-18 positivity	0.003	0.668	0.10
TRG 1-3 *vs* 4-5	0.87	0.003	0.23
Tumour diff. (well/mod *vs* poor)	0.39	0.34	0.21
T stage (T1, 2 and 3, 4)	0.04	0.08	0.19
N stage (N0 and > N1)	0.16	0.10	0.43
M stage (M0 and > M0)	1.27	1.64	1.99
Vascular invasion	1.98	1.67	1.34
Perineural invasion	1.56	1.99	2.43
Resection margin involvement	0.34	0.25	0.34

DSS: Disease-specific survival; OS: Overall survival; CK-18: Cytokeratin-18; TRG: Tumour regression grade; diff.: Differentiation.

modality therapy in improving patient outcomes is well established, these treatments are toxic and have a considerable impact on patient morbidity. In the United Kingdom, neoadjuvant chemotherapy followed by surgery is routinely offered to patients who are fit with no significant co-morbidities. Accordingly, these patients generally tend to be young and can tolerate toxic chemotherapy. Patients considered not suitable for chemotherapy are usually elderly with significant co-morbidities. Moreover, until 2006 in the United Kingdom, the standard treatment for patients with early stage gastro-oesophageal adenocarcinoma was surgery only and patients were not routinely offered adjuvant chemotherapy. With the publication of results from a large United Kingdom trial of perioperative chemotherapy[1], neoadjuvant chemotherapy was established as a standard treatment option for patients. This is reflected in the differences in mean age between the two groups in our study.

In the current study we have evaluated the potential role of full length CK-18 and caspase-cleaved CK-18 as biomarkers in gastro-oesophageal adenocarcinomas. To evaluate the prognostic significance of full length CK-18 in gastro-oesophageal adenocarcinomas, we first evaluated CK-18 expression in tumours not exposed to neoadjuvant chemotherapy (n = 122). We found that CK-18 was commonly expressed in tumours. This is consistent with a previously reported study[13]. Although Xu *et al*[13] demonstrated that CK18 mRNA expression correlated with lymph node metastasis and tumour differentiation in gastric cancer, we were unable to demonstrate any positive clinicopathological correlations in our study.

Caspase-cleaved CK-18 has recently emerged as a promising marker of apoptosis in gastrointestinal cancers[11]. We therefore evaluated caspase-cleaved CK-18 expression in gastro-oesophageal adenocarcinomas. Fifty-six point seven percent of tumours previously exposed to neoadjuvant chemotherapy were positive for caspase-cleaved CK-18 expression, compared to only 24.6% of tumours not previously exposed to neoadjuvant chemotherapy (P = 0.009). This provides direct evidence that chemotherapy exposure leads to apoptosis-induced increased caspase-cleaved CK-18 expression in gastro-oesophageal tumours. We then demonstrated, for the first time, that the caspase-cleaved CK-18 expression correlated well with favourable tumour regression in patients receiving neoadjuvant chemotherapy (P = 0.043). Factors found to be significant in univariate analyses were not significant in a multivariate logistic regression analysis. This may be because the current study is a small retrospective study and a larger prospective study is required to confirm our observations. However, our study provides evidence that caspase-cleaved CK-18 may be a promising predictive biomarker in gastro-oesophageal adenocarcinomas. Moreover, our study also supports a rational hypothesis for evaluating serial blood caspase-cleaved CK18 secretion, using a recently developed assay[11,14,15], as a promising non-invasive biomarker in operable gastro-oesophageal adenocarcinomas where patients routinely receive three cycles of platinum-based neoadjuvant chemotherapy[1,2]. In a recent study of advanced colorectal cancer, serum levels of caspase-cleaved CK18 were significantly higher in patients who responded to chemotherapy compared to those who did not[11]. Similar results were also reported in advanced colorectal, oesophageal and gastric adenocarcinoma patients receiving palliative chemotherapy[16]. Although these results are promising, whether tumour tissue and serum caspase CK-18 levels are related to each other is not clear. Moreover, whether similar results could be achieved in early stage gastro-oesophageal adenocarcioma patients receiving neoadjuvant chemotherapy is an area of ongoing investigation in our laboratory.

We also made interesting clinicopathological observations in tumours not exposed to chemotherapy. Caspase-cleaved CK18 expression correlated with better differentiation and improved survival in this group. Although the reason for this unexpected finding is not clear, whether host immune factors such as lymphocytic infiltration could contribute to cancer cell death is currently unknown and is an area of ongoing investigation.

In summary, we have conducted a study of full length CK-18 and caspase-cleaved CK-18 expression in gastro-oesophageal cancer. We provide evidence that caspase-cleaved CK-18 is a promising predictive biomarker in patients who receive platinum-based neoadjuvant chemotherapy.

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COMMENTS

Background

Neoadjuvant chemotherapy followed by surgery is a standard treatment option in patients with early stage gastro-oesophageal adenocarcinomas. However, only 40% of patients respond to chemotherapy. There is an urgent need to develop predictive biomarkers.

Research frontiers

Development of a serum biomarker test that can predict response to chemotherapy is highly desirable in gastro-oesophageal adenocarcinomas.

Innovations and breakthroughs

Cytokeratin-18 (CK-18) is commonly expressed in epithelial tumours. Caspase-cleaved CK-18 is expressed in cancer cells undergoing apoptosis following chemotherapy. Caspase-cleaved CK-18 is also secreted by tumour cells and can be evaluated using a blood test. In early stage gastrooesophageal cancers, the authors show that caspase-cleaved CK-18 is prevalent in tumour tissue following chemotherapy and that this correlates with tumour regression. The study suggests that serum testing of caspase-cleaved CK-18 may be feasible to predict response in early stage gastro-oesophageal adenocarcinomas.

Applications

A prospective study of serial serum testing of caspase-cleaved CK-18 elevation and correlation with tumour response to chemotherapy is required to evaluate CK-18 as a promising predictive biomarker in early stage gastro-oesophageal adenocarcinomas.

Terminology

CK-18 is widely expressed in epithelial cancers. Caspase-cleaved CK-18: in epithelial cells undergoing apoptosis, caspase-cleaved CK-18 is expressed and can be detected by immunohistochemistry.

Peer review

This is a study to evaluate whether caspase-cleaved CK-18 can predict response to chemotherapy in gastro-oesophageal adenocarcinomas receiving neoadjuvant chemotherapy. Data presented here support a rational hypothesis to test if secretion of caspase-cleaved CK-18 in blood can be used as a marker of response to chemotherapy in patients.

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BRIEF ARTICLE

Helpfulness of the combination of acetic acid and FICE in the detection of Barrett's epithelium and Barrett's associated neoplasias

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Abstract

AIM: To investigate the mucosal morphology in Barrett's oesophagus by chromo and magnifying endoscopy.

METHODS: A prospective pilot study at a tertiary medical centre was conducted to evaluate the use of acetic acid pulverisation combined with virtual chromoendoscopy using Fujinon intelligent chromoendoscopy (FICE) for semiological characterization of the mucosal morphology in Barrett's oesophagus and its neoplastic complications. Upper endoscopy using high definition white light, 2% acid acetic pulverisation and FICE with high definition videoendoscopy were performed in 20 patients including 18 patients who presented with aspects of Barrett's oesophagus at endoscopy examination. Two patients used as controls had normal endoscopy and histological results. Prospectively, videos were watched blind from histological results by three trained FICE technique endoscopists.

RESULTS: The videos of patients with high-grade dysplasia showed an irregular mucosal pattern in 14% using high definition white light endoscopy and in 100% using acid acetic-FICE combined. Videos did not identify irregular vascular patterns using high definition white light endoscopy, while acid acetic-FICE combined visualised one in 86% of cases.

CONCLUSION: Combined acetic acid and FICE is a promising method for screening high-grade dysplasia and early cancer in Barrett's oesophagus.

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Key words: Acetic acid; Barrett's metaplasia; Chromoendoscopy; Fujinon intelligent chromoendoscopy

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INTRODUCTION

Barrett's oesophagus is a premalignant lesion of oesophageal adenocarcinoma and endoscopic surveillance has been proposed for the diagnosis of this condition[1,2]. A stepwise four quadrant biopsy protocol is considered the gold standard procedure[3]. Current guidelines advise that biopsies should be obtained from any visible abnormality and that four random quadrant biopsies every 2 cm should be taken to detect inconspicuous dysplasia during endoscopic surveillance[4,5]. In theory, a high sensitivity endoscopic technique for the detection of high-grade dysplasia or early carcinoma is warranted and targeting biopsies and a four quadrant biopsy protocol will be unnecessary to improve Barrett's oesophagus cancer detection.

Chromoendoscopy with methylene blue, acetic acid, or virtual chromoendoscopy have been proposed to improve the detection of preneoplastic lesions of Barrett's oesophagus. Although acid acetic pulverisation improves visibility of the mucosal pattern by removing the superficial mucus and enhancing the pit pattern, virtual chromoendoscopy was developed to identify abnormalities from superficial mucosal or vascular patterns, and, therefore, facilitate diagnosis of Barrett's associated neoplasias[6,7]. Virtual chromoendoscopy using narrow band imaging (NBI) was first studied in this indication and abnormalities of pit and vascular patterns have been described[6]. Improvements in endoscopic material allows functional imaging to be incorporated, which, in turn, permits visualisation of more detail in mucosal and vascular patterns and may complement high-resolution endoscopy to increase the sensitivity of the endoscopic detection of early neoplasia in Barrett's oesophagus. Kim *et al*[8] showed that NBI was not reproducible and had a sensitivity of 89% in detecting preneoplastic lesions of Barrett's oesophagus. Virtual chromoendoscopy using Fujinon intelligent chromoendoscopy (FICE) has been shown to be a useful tool in identifying gastric lesions[9]. Pohl *et al*[10] failed to show, in a single prospective study, significant differences between FICE and acetic acid combined with conventional chromoendoscopy for the detection of high-grade dysplasia or early cancer in patients with Barrett's oesophagus. No study has shown any benefit with the combination of acetic acid and FICE. These two techniques could be complementary, since acetic acid enhances visualisation of the pit pattern and FICE allows detection of vascular abnormalities in Barrett's oesophagus. With the Pohl *et al*[10] study in mind, we conducted a pilot study to evaluate the combination of 2% acetic acid pulverisation and FICE for semiological characterization of the mucosal morphology in Barrett's oesophagus.

These two techniques could be complementary, since acetic acid enhances visualisation of the pit pattern[11] and FICE allows detection of vascular abnormalities in Barrett's oesophagus.

MATERIALS AND METHODS

Ethics

All patients enrolled in this study gave written informed consent. The study was in accordance with the Declaration of Helsinki and the Institutional review board (Centre de protection des personnes d'Ile de France Ⅲ) approved the study (ref: CPP: AT102).

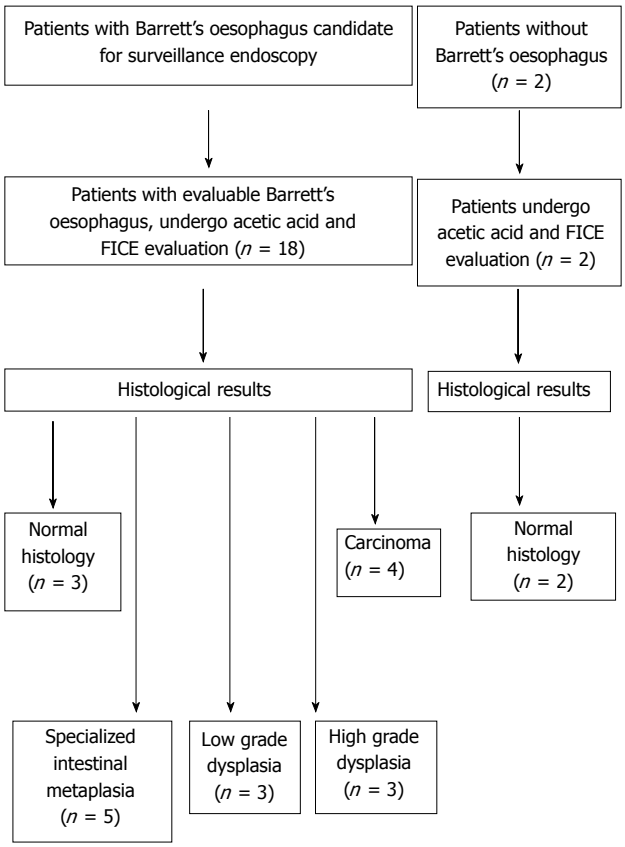

Figure 1 Patient selection for the present study. FICE: Fujinon intelligent chromoendoscopy.

Patients

The study population consisted of patients with Barrett's oesophagus, as confirmed by pathological analysis. None of the patients had received previous therapy for Barrett's oesophagus. The eligibility criteria also included a four-quadrant biopsy protocol, a standardized procedure as described above and a video record of the overall procedure. Patients were required to have at least one dysplastic lesion that could be evaluated. Data were collected retrospectively from a review of the electronic medical records and endoscopy database of our institution from November 2006 to September 2009. Eighteen patients had endoscopic Barrett's oesophagus (Figure 1). Of these patients, fifteen had specialized intestinal metaplasia, dysplasia or carcinoma and three patients had normal histology results. In addition, two patients with no history of Barrett's oesophagus or endoscopic lesions, and normal biopsies were included as controls.

Endoscopy procedure

All explorations were performed using a high definition Fujinon 1.3-million-pixel EG 490 ZW5 gastroscope zoom with optical magnification up to 100 times equipped with

Table 1 Clinical characteristics of patients undergoing combined acetic acid and Fujinon intelligent chromoendoscopy endoscopy

	Normal mucosa	SIM or LGD	HGD or carcinoma	All patients
Number of patients (%)	5 (25)	8 (40)	7 (35)	20
Age (yr) (mean ± SD)	56.4 ± 21.7	65 ± 10.5	71 ± 13.8	65 ± 15
Sex (men/women)	5/0	7/1	7/0	19/1
Concomitant therapy PPI (%)	60	100	100	90
Median Barrett's oesophagus Length (cm) ± IQR	1 (0-1)	2.3 (2-3.3)	3.5 (2.5-3.6)	2 ± 1.3

SIM: Specialized intestinal metaplasia; LGD: Low-grade dysplasia; HGD: High-grade dysplasia; PPI: Proton-pump inhibitor; IQR: Interquartile range.

a short soft transparent hood by one expert endoscopist and were video recorded. In each case, videos of the oesophagus were taken before biopsies. Endoscopies were carried out following total intravenous anaesthesia of the patient using Propofol. The endoscopic technique was standardized as follows: first the oesophagus was examined with high definition white light endoscopy, followed by 6-10 mL of 2% acetic acid pulverisation, and, finally, FICE was activated. Acetic acid pulverisation was performed with a spray catheter PW-1V-1 (Olympus Optical Co., Ltd., Japan) and the endoscopist gently sucked up excess acetic acid from the oesophageal lumen before inspection. In the case of macroscopic abnormalities in colour or pit-pattern, the zoom was used with a magnification of 10-15. Videos were made with high definition white light and with the combination of acetic acid and FICE. FICE channels 4, 7 and 0 were used following previous studies using FICE in upper endoscopy[9,10]. In the case of macroscopic abnormalities, separate biopsy samples were performed and then systematic biopsies were used in "normal macroscopic areas" of Barrett's oesophagus using Radial Jaw® Single-Use Biopsy Forceps (Boston Scientific, Fremont, CA, United States) with jumbo capacity.

Histological analysis

All biopsies were evaluated by two pathologists with extensive experience in Barrett's neoplasia, and reviewed by a third pathologist in cases of dysplasia. Histological results were classified according to the revised Vienna classification[12]. The highest grade of dysplasia obtained from any biopsy sample was used to determine the diagnosis in each patient.

Evaluation of Barrett's oesophagus

Retrospectively, videos were watched blind from histological results by three FICE trained endoscopists. For each patient, the most severe lesion was selected for evaluation. Experts noted the characteristics of Barrett's oesophagus and any other abnormalities with and without combined acetic acid-FICE: macroscopic appearance of the lesion using the Paris classification[12], type of mucosal pattern (regular: ridged/villous, circular, irregular), vascular pattern characteristics (regular, irregular) using the Sharma classification[13], raised lesion, ulcerous lesion, pigmented lesion; and spontaneous bleeding lesion. The last four items were considered on clinical findings.

Statistical analysis

Means and standard deviations were used to summarize continuous variables with an apparently Gaussian distribution, whereas the median and the interquartile range (IQR) were used to summarize variables with a skewed distribution. Kappa statistics with their 95% confidence intervals (CI) were used to test for inter-observer agreement of the 3-step classification system using arbitrary interpretation by Landis and Koch (0, poor agreement; 0.00-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; 0.80-1.00, almost perfect agreement)[14]. Because Kappa statistics can only be calculated with pair-wise observations, Kappa values were calculated for all pair-wise combinations obtained by the observers.

RESULTS

Patient characteristics

Twenty patients were included. The patients' characteristics are shown in Table 1. Histological results were normal histology, specialized intestinal metaplasia, low-grade dysplasia, high-grade dysplasia, and carcinoma in 25%, 25%, 15%, 15%, and 20%, respectively. With high definition white light endoscopy, abnormalities in mucosal or vascular pattern were detected in one patient (6%) out of the 18 patients with suspected Barrett's oesophagus. With combined acetic acid-FICE, seven patients (39%) out of the 18 with suspected Barrett's oesophagus had a visible irregular mucosal pattern (Table 2). All of these patients had high-grade dysplasia or carcinoma (sensitivity: 100%). With combined acetic acid-FICE, six patients (33%) out of the 18 with suspected Barrett's oesophagus had a visible irregular vascular pattern. All had high-grade dysplasia or carcinoma (sensitivity: 100%). No patient with metaplasia or low-grade dysplasia had visible irregular mucosal vascular patterns, raised lesions, or spontaneous bleeding (Figure 1).

An irregular mucosal pattern was identified in patients with high-grade dysplasia or carcinoma using high definition white light endoscopy or the combination of acetic acid-FICE in 14% and 100%, respectively. An irregular vascular pattern was identified in patients with high-grade dysplasia or carcinoma using high definition white light endoscopy or the combination of acetic acid-FICE in 0% and 86%, respectively (Figures 2 and 3). An irregular mucosal or vascular pattern was not identified in patients without high-grade dysplasia or carcinoma using the combination of acetic acid and FICE (specificity 100%).

Table 2 Correlation of the predominant mucosal and vascular patterns with histological results during high resolution white light endoscopy or after 2% acetic acid pulverisation and Fujinon intelligent chromoendoscopy 0, 4 and 7

	High resolution white light			Acetic acid pulverisation and FICE		
	Normal histology	SIM or LGD	HGD or carcinoma	Normal histology	SIM or LGD	HGD or carcinoma
Number of patients (%)	5	8	7	5	8	7
Regular or not visualized mucosal pattern[1] (%)	100	100	86	100	100	0
Irregular mucosal pattern (%)	0	0	14	0	0	100
Regular or not visualized vascular pattern[1] (%)	100	100	100	100	100	14
Abnormal blood vessels (%)	0	0	14	0	0	86
Raised lesion (%)	0	0	14	0	0	71
Pigmented lesion (%)	0	0	0	0	37.5	0
Bleeding lesion (%)	0	0	14	0	0	57
Ulcerous lesion (%)	0	14	14	0	14	14

FICE: Fujinon intelligent chromoendoscopy; SIM: Specialized intestinal metaplasia; HGD: High-grade dysplasia; LGD: Indicate Low grade dysplasia. [1]Endoscopists were not able to exactly classify the mucosal pattern according to Sharma's classification of mucosal pattern due to lack of visibility, but they judged that the mucosa was regular.

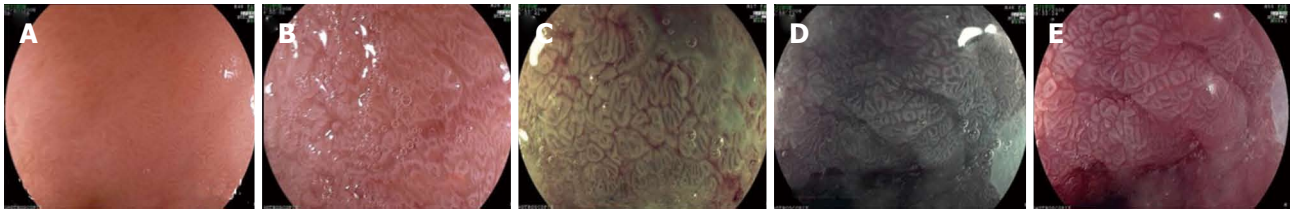

Figure 2 Acetic acid and Fujinon intelligent chromoendoscopy image of the oesophagus. Specialized intestinal metaplasia using high definition white light (A), 2% acetic acid (B), and the combination of acetic acid with Fujinon intelligent chromoendoscopy (FICE) 4 (C), FICE 0 (D) and FICE 7 (E).

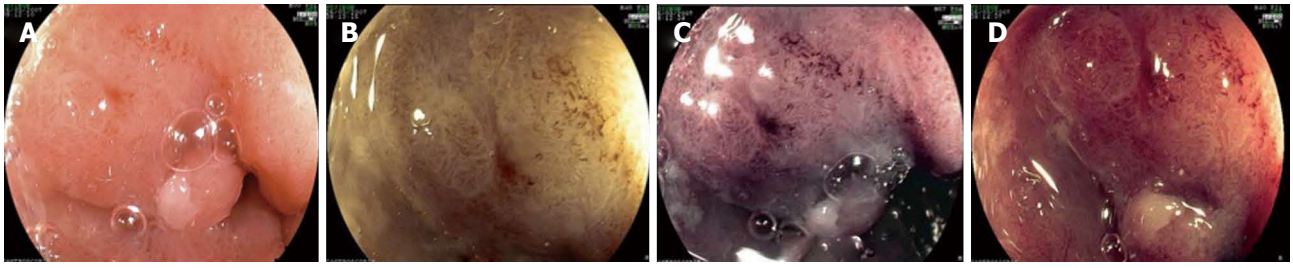

Figure 3 Acetic acid and Fujinon intelligent chromoendoscopy image of an oesophageal carcinoma. Irregular pit pattern and abnormal vascularisation is shown with 2% acetic acid (A), or following the combination of acetic acid and Fujinon intelligent chromoendoscopy (FICE) 4 (B), FICE 0 (C) and FICE 7 (D).

Among the 7 patients with high-grade dysplasia, 4 patients (57%) presented with early carcinoma on biopsy. The sizes of the lesions were 1 cm, 0.5 cm and 0.4 cm. The lesion size was not recorded in one patient. All patients underwent a mucosectomy within 2 mo of the primary endoscopy.

Definitive histological staging was intramucosal carcinoma (pT1m) for 3 of 4 patients following mucosectomy histological analysis, and resection margins were healthy. Only high-grade dysplasia was found in the fourth patient.

Interobserver agreement for mucosal morphology

When the evaluations by the experts were grouped together, the interobserver agreement for mucosal morphology assessed on high definition white light images was substantial on 5 items (mucosal pattern: $\kappa = 0.97$, raised lesion: $\kappa = 1.00$, pigmented lesion: $\kappa = 1.00$, bleeding lesion: $\kappa = 1.00$, and ulcerous lesion: $\kappa = 1.00$) and moderate on 2 items (vascular pattern: $\kappa = 0.73$, abnormal blood vessels: $\kappa = 0.76$). There was no difference in interobserver agreement between high definition white light images and combined acetic acid-FICE images, except for 3 evaluations (abnormal blood vessels, vascular pattern and pigmented lesion). In the evaluation of abnormal blood vessels using high definition white light images, the interobserver agreement ($\kappa = 0.76$; 95% CI: 0.64-1.00) was lower than that of combined acetic acid-FICE images ($\kappa = 0.91$; 95% CI: 0.86-1.00). In the evaluation of vascular pattern using high definition white light images, the interobserver agreement ($\kappa = 0.73$; 95% CI: 0.64-0.91) was lower than that of combined acetic acid-FICE images ($\kappa = 0.83$; 95% CI: 0.76-0.88). In the evaluation of pigmented lesion using high definition white light images, the interobserver agreement ($\kappa = 1.00$; 95% CI: 0.64-1.00) was better than that of combined acetic acid-FICE images ($\kappa = 0.37$; 95% CI: 0.28-0.54).

COMMENTS

Background

Barrett's oesophagus is a pre-neoplastic lesion with an estimated rate of transformation to carcinoma of approximately 0.3%-0.6% each year. Guidelines from the American College of Gastroenterology proposed a surveillance programme to detect high-grade dysplasia or carcinoma in Barrett's oesophagus. This screening programme recommends performing 4 quadrant biopsies every 2 cm at 1 year in low-grade dysplasia and every 3 mo in high-grade dysplasia.

Research frontiers

Acetic acid pulverisation is a simple and inexpensive method of improving visibility of the pit pattern, but does not allow appreciation of the vascular pattern. Acetic acid instillation increased the detection of cancer compared to white light endoscopy with or without high resolution endoscopy. Whereas acetic acid instillation, indigo carmine chromoendoscopy, narrow-band imaging and chromoendoscopy by Fujinon intelligent chromoendoscopy (FICE) seem to be interesting new techniques, each technique alone seems to be insufficient to warrant abandonment of the Seattle protocol of multiple blind sample biopsies. The authors showed significant differences between FICE and chromoendoscopy with acetic acid for the detection of high-grade dysplasia or early cancer in patients with Barrett's oesophagus. Both the acetic acid and FICE techniques showed separate per-lesion sensitivity of up to 87% for the detection of high-grade neoplasia and early cancer in patients with Barrett's oesophagus. The authors conducted a video study to evaluate the combined acetic acid and FICE technique.

Innovations and breakthroughs

Virtual chromoendoscopy has begun to receive greater attention as a potential technique in the diagnosis of Barrett's oesophagus. In the series, the study highlight the positive effect of the combination of acetic acid and virtual chromoendoscopy. The study confirms the usefulness of FICE technique combined with acetic acid using video reviews. However, the generalization of the results is made difficult by the author's small population size. Therefore, a larger study is warranted to confirm the author's results. The data suggest a high sensitivity and specificity with this combination, however, data are lacking on a real-time basis rather than relying on subsequent video reviews. The report, for the first time, on the benefit of the combination of acetic acid and FICE in identifying high-grade dysplasia or early oesophageal neoplasia.

Applications

By using the presented procedure of acetic acid and FICE, this may represent a future strategy for the diagnosis of Barrett's oesophagus lesions. In the study, taking into account two criteria (irregular mucosal and/or vascular patterns), 100% of patients with high-grade dysplasia or carcinoma were identified by endoscopy, with no errors in the diagnosis of high-grade dysplasia or carcinoma. The study showed that combined acetic acid and FICE had a positive benefit in the identification of high-grade dysplasia or carcinoma. By combining the two techniques of acetic acid and FICE, the results showed improvement in the quality of endoscopic images obtained and visualisation of both mucosal and vascular patterns or irregularities. The endoscopic detection of high-grade dysplasia or carcinoma was enhanced by this combination method as compared to using high definition white light imaging alone. In order to further verify the high sensitivity and specificity findings in this study, future prospective multi-centre studies of patients presenting for endoscopic evaluation of Barrett's oesophagus with all levels of early neoplasia are required to definitively compare the combination imaging algorithm of acetic acid and FICE to Seattle Protocol random biopsies.

Peer review

This is an interesting study, which adds information to how to tackle detection and surveillance of Barrett's esophagus.

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BRIEF ARTICLE

Factors associated with the overall survival of elderly patients with hepatocellular carcinoma

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Abstract

AIM: To identify the factors associated with overall survival of elderly patients with hepatocellular carcinoma (HCC).

METHODS: A total of 286 patients with HCC (male/female: 178/108, age: 46-100 years), who were diagnosed and treated by appropriate therapeutic procedures between January 2000 and December 2010, were enrolled in this study. Patients were stratified into two groups on the basis of age: Elderly (≥ 75 years old) and non-elderly (< 75 years old). Baseline clinical characteristics as well as cumulative survival rates were then compared between the two groups. Univariate and multivariate analyses were used to identify the factors associated with prolonged overall survival of patients in each group. Cumulative survival rates in the two groups were calculated separately for each modified Japan Integrated Stage score (mJIS score) category by the Kaplan-Meier method. In addition, we compared the cumulative survival rates of elderly and non-elderly patients with good hepatic reserve capacity (≤ 2 points as per mJIS).

RESULTS: In the elderly group, the proportion of female patients, patients with absence of hepatitis B or hepatitis C viral infection, and patients with coexisting extrahepatic comorbid illness was higher (56.8% *vs* 31.1%, $P < 0.001$; 27.0% *vs* 16.0%, $P = 0.038$; 33.8% *vs* 22.2%, $P = 0.047$; respectively) than that in the non-elderly group. In the non-elderly group, the proportion of hepatitis B virus (HBV)-infected patients was higher than that in the elderly group (9.4% *vs* 0%, $P = 0.006$). The cumulative survival rates in the elderly group were 53.7% at 3 years and 32.9% at 5 years, which were equivalent to those in the non-elderly group (55.9% and 39.4%, respectively), as shown by a log-rank test ($P = 0.601$). In multivariate analysis, prolonged survival was significantly associated with the extent of liver damage and stage ($P < 0.001$ and $P < 0.001$, respectively), but was not associated with patient age. However, on individual evaluation of factors in both groups, stage was significantly ($P < 0.001$) associated with prolonged survival. Regarding mJIS scores of ≤ 2, the rate of female patients with this score was higher in the elderly group when compared to that in the non-elderly group ($P = 0.012$) and patients ≥ 80 years of age tended to demonstrate shortened survival.

CONCLUSION: Survival of elderly HCC patients was associated with liver damage and stage, but not age, except for patients ≥ 80 years with mJIS score ≤ 2.

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Key words: Age; Hepatocellular carcinoma; Liver damage; Stage; Survival

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INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common causes of mortality worldwide, usually occurs in a cirrhotic liver[1-3]. Moreover, the number of elderly HCC patients is increasing, and the average age of patients with hepatitis C is increasing in Japan[4-8]. This trend indicates the need to investigate and identify the optimal treatment of HCC in elderly patients. Elderly patients have a high incidence of comorbid illnesses and the risks of major surgery are usually higher in these patients when compared to those in younger patients. Therefore, radical surgical resection of HCC is less feasible in elderly patients than in younger patients.

Some studies have indicated that treatment outcomes in elderly patients are essentially similar to those in non-elderly patients; moreover, there are several studies comparing different treatment procedures[9-16]. However, the prognostic factors for survival in elderly patients remain obscure. This article aims to review a retrospective cohort of elderly (≥ 75 years) and non-elderly (< 75 years) patients in order to clarify the characteristics of elderly HCC patients and reveal the factors associated with prolonged survival of these patients.

MATERIALS AND METHODS

Study population

A total of 286 patients with HCC treated at Aiseikai-YamashinaHospital between January 2000 and December 2010 were enrolled in this study. A follow up study of patient survival was performed until the end of December 2010. This study was conducted in accordance with the Declaration of Helsinki. We explained our therapeutic strategy and other regimens to each patient prior to every treatment procedure and obtained written informed consent from all patients.

The 286 patients who fulfilled the inclusion criteria were categorized into two groups as elderly (≥ 75 years) and non-elderly (< 75 years old). The breakpoint of 75 years old was chosen because it enabled comparison with other relevant reports. Moreover, in Japan, patients ≥ 75 years of age are covered by a health insurance system which is different from that of patients < 75 years.

Etiology of liver disease

The etiology of liver disease was classified as follows: (1) hepatitis B virus (HBV), if patients were hepatitis B surface antigen (HBsAg) positive; (2) HCV, if patients were anti-HCV positive; (3) HCV and HBV, if patients were both HBsAg and anti-HCV positive; and (4) non-B non-C, if patients were negative for both HBsAg and anti-HCV.

Diagnosis of hepatic reserve capacity

The severity of liver damage was scored according to the Liver Damage Classification scheme proposed by the Liver Cancer Study Group of Japan (LCSGJ)[17].

Diagnosis and staging of hepatocellular carcinoma

The diagnosis of HCC was based on histopathology and/or imaging studies such as ultrasonography (US), computed tomography (CT) scans, angiography, CT angiography, and magnetic resonance imaging. The final diagnosis was confirmed when at least two diagnostic modalities identified the presence of HCC. The tumor stage was defined on the basis of the LCSGJ. To compare treatment outcomes with those of other institutions, the modified Japan Integrated Stage score (mJIS score) was selected as the integrated staging system for HCC[17].

Treatment

After diagnosis of HCC, the most appropriate therapeutic procedure was selected according to the tumor status and underlying hepatic reserve capacity of each patient. As a general rule, we treated all patients except for those with uncontrollable ascites or hepatic encephalopathy and those who rejected any treatment. Hepatic resection (HR) was particularly considered in patients with localized HCC and preserved hepatic reserve capacity. Nonsurgical treatments, such as transcatheterarterial chemoembolization (TACE), transcatheterarterial infusionchemotherapy (TAI),percutaneous ethanol injection therapy (PEIT), radiofrequency ablation therapy (RFA), and hepatic arterial infusion chemotherapy (HAIC) were considered when the patients refused surgical treatment or HR was not feasible. TACE was performed using doxorubicin hydrochloride or cisplatin with iodized oil (Lipiodol Ultra Fluide; LaboratoireGuerbet, Roissy, France) and gelatin sponge particles. TAI was performed using doxorubicin hydrochloride or cisplatin. Locoregional ablative therapies such as PEIT and RFA were considered in patients with one to three tumor nodules that were devoid of vascular invasion and not associated with extrahepatic metastases. All locoregional ablative therapies were CT- or US-assisted. HAIC was performed using intra-arterial hepatic injections with low-dose 5-fluorouracil/cisplatin[18]. In many cases, patients were treated by a combination of several procedures. These therapies were repeated when HCC relapsed until patients reached maximum tolerability. The best supportive care was considered when the pa-

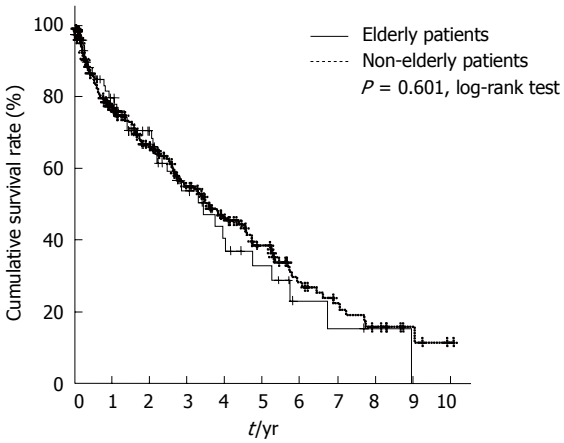

Figure 1 Cumulative survival rates by Kaplan-Meier method in elderly (*n* = 74, thin line) and non-elderly groups (*n* = 212, thick line).

tient had compromised hepatic reserve capacity or when he/she refused any treatment for HCC.

Comorbid illnesses

The presence of malignant tumors other than HCC, cardiovascular diseases, renal diseases, pulmonary diseases, and neurological diseases, all of which could have potential impact on the prognosis, were recorded.

Statistical analysis

To evaluate the differences in clinical features of the patients and tumor characteristics, the Mann-Whitney *U* and Pearson χ^2 tests were used for continuous and discrete data, respectively. The analytical data were first collected on the day of initial HCC diagnosis. The patients were followed-up as long as they lived or, in some cases, until their last visit to the hospital. The primary outcome was overall survival. Cumulative survival rates were calculated using the Kaplan-Meier method and compared using the logrank test. Patient survival was followed up to 31 December, 2010. For the analysis of predictors of survival, a Cox proportional hazards model was used, in which the following parameters were evaluated: age (≥ 75 years or < 75 years), gender, presence of comorbid illnesses, HCV positivity, serum alfa-fetoprotein ≥ 200 ng/mL, serum des-gamma-carboxyprothrombin ≥ 200 mAU/mL, and liver damage classification and stage. A *P* value of ≤ 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS 19.0 statistical package (SPSS Incorporated, Chicago, Illinois, United States).

RESULTS

Clinical features of patients

The clinical profiles of 286 patients, divided into elderly (≥ 75 years) and non-elderly (< 75 years) groups, are shown in Table 1. In the elderly group, the number of female patients, patients with comorbid illness, and patients with absence of HBV and HCV infection (non-B non-C) were significantly higher ($P < 0.001$, $P = 0.047$, $P = 0.038$, respectively) than those in the non-elderly group. In the non-elderly group, the proportion of patients with HBV infection was significantly ($P = 0.006$) higher than that in the elderly group.

Table 1 Clinical characteristics of the patients

	Elderly patients (*n* = 74)	Non-elderly patients (*n* = 212)	*P* value
Age (yr) (range)	80.5 (75.4-100.0)	65.8 (46.0-74.8)	-
Male/female (%)	32/42 (43.2/56.8)	146/66 (68.9/31.1)	$P < 0.001$
Extrahepaticcomorbidity (%)	25 (33.8)	47 (22.2)	$P < 0.05$
Malignant tumors except HCC	6	12	
Cardiovascular	10	15	
Renal	3	7	
Pulmonary	3	4	
Neurological	3	9	
Cause of liver dysfunction			
HBV (%)	0	20 (9.4)	$P < 0.01$
HCV (%)	54 (73.0)	155 (73.2)	NS
HBV HCV (%)	0	3 (1.4)	NS
Non-B Non-C (%)	20 (27.0)	34 (16.0)	$P < 0.05$
Liver damage A/B/C	39/31/4	97/86/29	NS
Stage Ⅰ/Ⅱ/Ⅲ/Ⅳ	13/24/23/14	45/71/51/45	NS
mJIS score 0/1/2/3/4/5	9/16/22/18/8/1	24/53/59/36/27/13	NS
Death except hepatic disease/total death	10/35	18/113	NS

NS: Not significant; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; mJIS: Modified Japan Integrated Stage.

Overall survival rates

Over a median follow-up of 1.8 years, 148 patients died, of whom 35 were elderly and 113 were non-elderly patients. The median survival period was comparable in the two groups [elderly: 3.46 years, 95% confidence interval (CI), 2.26-4.66; non-elderly: 3.56 years, 95% CI, 2.58-4.55; $P = 0.167$] (Figure 1). The survival rates at one, three, five, and 10 years were 79.7%, 53.7%, 32.9%, and 0.0% in the elderly group, and 77.9%, 55.9%, 39.4%, and 12.4% in the non-elderly group, respectively. There were no significant differences in survival rates between the two groups ($P = 0.601$).

With regard to the cause of mortality, 10 patients (28.6%) in the elderly group and 18 patients (16.8%) in the non-elderly group died from causes other than hepatic diseases (tumor progression, hepatic failure, variceal bleeding, or other complications of cirrhosis), and there were no significant differences between the two groups ($P = 0.095$, Table 1). In addition, we performed an analysis of survival rates after excluding patients who died from causes other than hepatic diseases. As a result, the survival rates at one, three, five, and 10 years were 86.8%, 64.0%, 42.3%, and 0.0% in the elderly group, and 80.8%, 60.7%, 44.9%, and 18.6% in the non-elderly group, respectively. There were no significant differences in survival rates between the two groups ($P = 0.779$, data not shown).

Table 2 Univariate and multivariate analyses of the relative risks for overall survival

	Univariate analysis relative risk	*P* value	Multivariate analysis relative risk	*P* value
Age ⩾ 75 yr	1.107	NS	1.161	NS
Gender (male)	1.155	NS	1.135	NS
Comorbid illness	1.335	NS	1.144	NS
HCV+	0.636	< 0.05	1.036	NS
AFP (ng/mL) ⩾ 200	2.098	< 0.001	1.229	NS
DCP (mAU/mL) ⩾ 200	1.763	< 0.05	1.229	NS
Liver damage				
A/B	2.345	< 0.001	2.506	< 0.001
B/C	7.674	< 0.001	10.463	< 0.001
Stage				
Ⅰ/Ⅱ	3.168	< 0.001	3.126	< 0.001
Ⅱ/Ⅲ	3.818	< 0.001	6.323	< 0.001
Ⅲ/Ⅳ	20.064	< 0.001	35.498	< 0.001

NS: Not significant; HCV: Hepatitis C virus; AFP: α-fetoprotein; DCP: Des-gamma-carboxy prothrombin.

Table 3 Univariate and multivariate analyses for overall survival in elderly and non-elderly patients

	Elderly		Non-Elderly	
	Univariate analysis *P* value	Multivariate analysis *P* value	Univariate analysis *P* value	Multivariate analysis *P* value
Gender (male)	NS	NS	NS	NS
Comorbid illness	NS	NS	NS	NS
HCV+	NS	NS	< 0.05	NS
AFP (ng/mL) ⩾ 200	NS	NS	< 0.001	NS
DCP (mAU/mL) ⩾ 200	NS	NS	< 0.05	NS
Liver damage				
A/B	NS	NS	< 0.001	< 0.001
B/C	< 0.001	NS	< 0.001	< 0.001
Stage				
Ⅰ/Ⅱ	NS	< 0.001	< 0.05	< 0.05
Ⅱ/Ⅲ	NS	< 0.001	< 0.05	< 0.001
Ⅲ/Ⅳ	NS	< 0.001	< 0.001	< 0.001

NS: Not significant; HCV: Hepatitis C virus; AFP: α-fetoprotein; DCP: Des-gamma-carboxy prothrombin.

Factors affecting survival

The factors affecting survival in all patients were calculated by multivariate analysis, and liver damage and stage were selected as the significant factors (Table 2). In elderly patients, multivariate analysis demonstrated that stage was independently associated with survival. In non-elderly patients, multivariate analysis demonstrated that liver damage and stage were independently associated with survival (Table 3). Gender, comorbid illness, HCV positivity, and tumor markers were not associated with survival in both groups.

Survival curve according to the mJIS score

Because survival was influenced by both liver damage and stage, we applied mJIS scores to both patient groups, and reached the conclusion that there was no clear association between these scores and survival in elderly patients (Figure 2A), whereas there was a clear association between the two in non-elderly patients (Figure 2B).

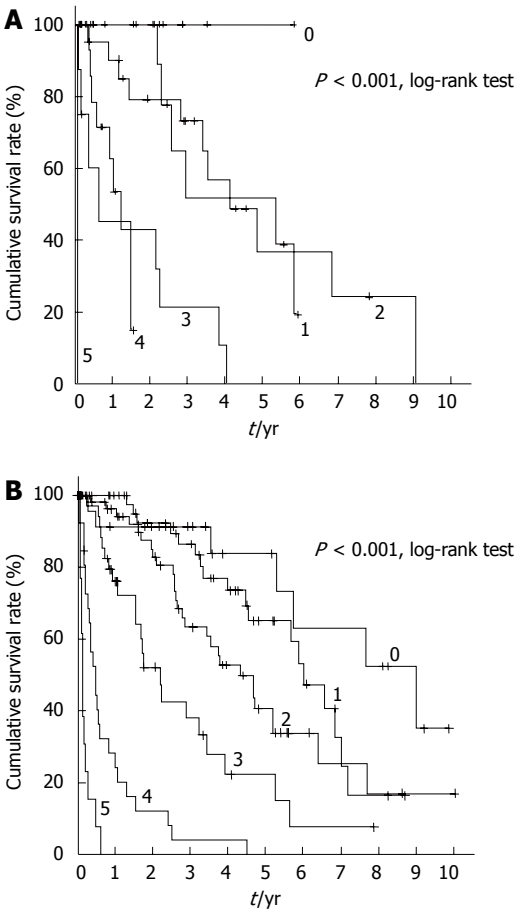

Figure 2 Cumulative survival rates by Kaplan-Meier method in each modified Japan Integrated Stage score group in elderly and non-elderly patients are shown. A: Elderly patients; B: Non-elderly patients.

Furthermore, we analyzed the clinical profile of patients with mJIS scores of ⩽ 2 in both groups (Table 4). The 5-year survival rate of these patients was expected to be ⩾ 50%. In the elderly group, the proportion of females with this score was higher than that in the non-elderly group. In the non-elderly group, the proportion of patients with HBV infection was higher than that in the elderly group. Other clinical factors were not statistically different. There was no significant difference in survival rates between patients with this score in both groups ($P = 0.386$, Figure 3A). Furthermore, we analyzed the survival rate of elderly patients with mJIS scores ⩽ 2 by dividing them into two subgroups, one comprising patients between 75 and 80 years of age and the other comprising patients ⩾ 80 years of age. The survival rates tended to deteriorate in the latter group of patients, although the difference was not significantly different ($P = 0.335$, Figure 3B).

DISCUSSION

In this study, we reviewed HCC cases by dividing them into two groups and demonstrated that the survival rates of elderly patients ⩾ 75 years of age were generally

Table 4 Clinical characteristics of the patients whose modified Japan Integrated Stage score were two or less

	Elderly patients (n = 47)	Non-elderly patients (n = 136)	P value
Age (yr) (range)	80.3 (75.4-87.7)	65.8 (46.0-74.8)	-
Male/female (%)	21/26 (43.2/56.8)	89/47 (68.9/31.1)	$P < 0.05$
Extrahepaticcomorbidity (%)	16 (34.0)	33 (24.3)	NS
Malignant tumors except HCC	5	9	
Cardiovascular	5	8	
Renal	2	4	
Pulmonary	2	4	
Neurological	2	8	
Cause of liver dysfunction			
HBV (%)	0	11 (8.1)	$P < 0.05$
HCV (%)	37 (78.7)	109 (80.1)	NS
HBV HCV (%)	0	2 (1.5)	NS
non-B non-C (%)	10 (21.3)	14 (10.3)	NS
Liver damage A/B/C	32/14/1	87/45/4	NS
Stage Ⅰ/Ⅱ/Ⅲ/Ⅳ	13/24/10	45/64/27	NS

NS: Not significant; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

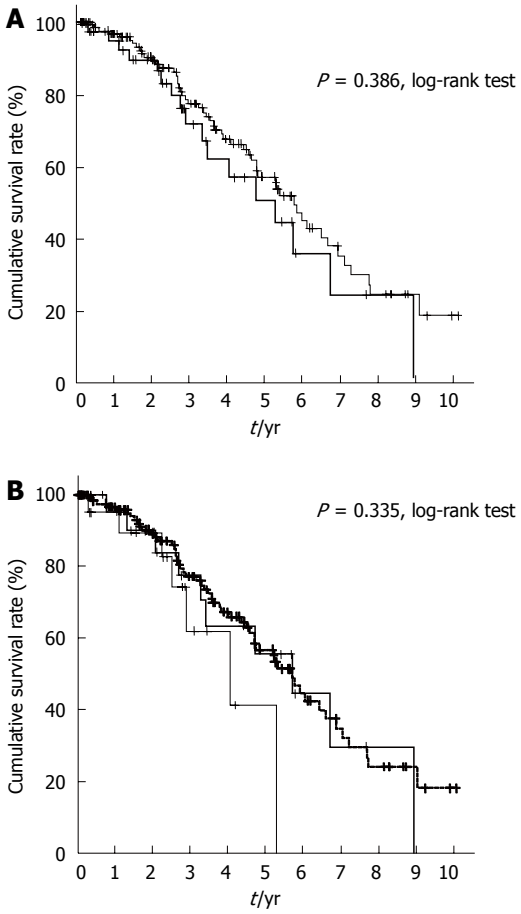

Figure 3 The analysis of the survival rate with modified Japan Integrated Stage score two points or less. A: Cumulative survival rates by Kaplan-Meier method in elderly (n = 47, thick line) and non-elderly groups(n = 136, thin line) whose modified Japan Integrated Stage (mJIS) score is two points or less; B: Cumulative survival rates by Kaplan-Meier method in 75-80 year-olds (n = 25, thick line), 80 years old or more (n = 22, thin line) and non-elderly groups (n = 136, dotted line) whose mJIS score is two points or less are shown.

equivalent to those of non-elderly patients < 75 years of age. There have been many previous studies reporting the efficiency and safety of each treatment modality for HCC in elderly patients, and most reports have shown similar survival rates and safety when compared with those of non-elderly patients[9-16]. However, in clinical practice, HCC is treated with several modalities in Japan. A previous paper, which evaluated the total survival rates of older HCC patients in comparison with those of younger HCC patients[19-21], reported cumulative survival rates similar to those reported in our present study.

Our elderly and non-elderly HCC patients differed in several clinical characteristics. Elderly patients were more likely to be female and negative for both HBV and HCV. This was expected since all these factors are known to influence the age of HCC development. The peak age of HCC occurrence in females is 5 years later than that in males[22]. HCV infection is generally acquired during adult life[23], while HBV infection frequently occurs in childhood[24]. A multifactorial etiology accelerates the progression of chronic liver disease, hence anticipating the appearance of HCC[22,25].

Despite a higher prevalence of comorbid illnesses and a difference of 14.7 years in the mean age, elderly patients demonstrated an overall 5-year survival similar to that of their younger counterparts. This unexpected result could be because of the low survival rate of both groups (overall survival of approximately < 40% at 5 years). The Liver Cancer Study Group of Japan has reported the 5-year overall survival rate after initial HCC diagnosis as 35.4%[5]. There may be many specific factors that influence the treatment strategy for elderly patients. Aggressive and risky treatments in these patients may be avoided due to comorbid illnesses. However, the impact of HCC occurrence on life expectancy outweighs that of both comorbid illnesses and age. We suggest that comorbid conditions and therapeutic procedures (including hepatectomy) had little effect on the survival of the patients especially the elderly ones.

Before analyzing the prognostic factors in each group, it was expected that the presence of comorbid illnesses may be a poor prognostic factor for elderly patients. However, this factor was not statistically associated with survival rates in either the elderly or non-elderly patient groups. The analysis of prognostic factors revealed that liver damage and stage were significantly associated with survival rates in both groups. Furthermore, stage was a very strong factor in elderly patients. Patients who have a sufficient hepatic reserve capacity may survive long enough for HCC to develop. The association of other factors with survival in elderly HCC patients seems insignificant in comparison with that of stage.

We also analyzed patients with a good hepatic reserve capacity (≤ 2 points as per mJIS), whose 5-year survival rate was expected to exceed 50% (Figure 3A). However, there was no statistical difference between survival rates of patients ≥ 75 and < 75 years of age. Interestingly,

when divided into subgroups, the survival of patients ⩾ 80 years of age was shorter than that of patients in the other subgroup (Figure 3B). In other words, extreme old age influenced the survival rate even in patients with good hepatic reserve capacity.

With regard to the cause of death, there was no difference between elderly and non-elderly patients (Table 1). Similar to non-elderly patients, elderly patients also died from liver-associated diseases. If the HCC patients ⩾ 80 years of age were divided into two subgroups (treated and untreated), there were obvious differences in the prognosis(data not shown). Further studies with a larger sample of patients will clarify this issue in future.

In conclusion, survival of elderly HCC patients (⩾ 75 years old) was associated with liver damage and stage. The effectiveness of treatment for HCC was equivalent in elderly and non-elderly patients. Survival was unaffected by age; however, when individually evaluated for patients with a mJIS score of ⩽ 2, those ⩾ 80 years of age tended to demonstrate shortened survival.

COMMENTS

Background

Hepatocellular carcinoma (HCC), one of the most common causes of mortality worldwide, usually occurs in a cirrhotic liver. At present, the number of elderly HCC patients is increasing, as is the average age of hepatitis C is increasing in Japan. However, the factors associated with overall survival of patients with HCC especially the elderly (⩾ 75 years old) are not adequately investigated.

Research frontiers

Some studies have indicated that treatment outcomes in elderly patients are essentially similar to those in non-elderly patients. In addition, there are several studies comparing different treatment procedures. The research hotspot is to clarify general prognostic factors for survival in elderly HCC patients in Japan.

Innovations and breakthroughs

In the elderly HCC patients of Japan, the proportion of female patients, patients with absence of hepatitis B or hepatitis C viral infection, and patients with co-existing extrahepatic comorbid illness was higher than that in the non-elderly group. The cumulative survival rates in the elderly group were 53.7% at 3 years and 32.9% at 5 years, which were equivalent to those in the non-elderly group, as shown by a log-rank test. In multivariate analysis, prolonged survival was significantly associated with the extent of liver damage and stage, but was not associated with patient age. Patients ⩾ 80 years of age tended to demonstrate shortened survival.

Applications

In the present study, the authors have reached the conclusion that survival of the HCC patients treated by appropriate procedures depends on liver damage and stage, but not on patient age. Only patients ⩾ 80 years of age tended to demonstrate shortened survival.

Terminology

The modified Japan Integrated Stage score (mJIS score) is the integrated staging system which combined the degree of liver damage and the degree of tumor stage.

Peer review

This is a good descriptive study in which authors analyze the factors associated with overall survival of patients with HCC especially the elderly (⩾ 75 years old). The results are interesting and give an useful information to the hepatologists in the world.

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BRIEF ARTICLE

Is hepatic arterial infusion chemotherapy effective treatment for advanced hepatocellular carcinoma resistant to transarterial chemoembolization?

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Abstract

AIM: To evaluate the effectiveness of hepatic arterial infusion chemotherapy (HAIC) for advanced hepatocellular carcinoma (HCC) resistant to transarterial chemoembolization (TACE).

METHODS: This study was conducted on 42 patients who received HAIC for advanced HCC between 2001 and 2010 at our hospital. 5-fluorouracil (5-FU) was administered continuously for 24 h from day 1 to day 5 every 2-4 wk *via* an injection reservoir. Intra-arterial cisplatin or subcutaneous interferon was administered in combination with the 5-FU. The patients enrolled in this retrospective study were divided into two groups according to whether or not they fulfilled the criteria for resistance to TACE proposed by the Japan Society of Hepatology in 2010 (written in Japanese); one group of patients who did not fulfill the criteria for TACE resistance (group A, n = 23), and another group who fulfilled the criteria for TACE resistance (group B, n = 19). We compared the outcomes in terms of the response and survival rates between the two groups.

RESULTS: Both the response rate and tumor suppression rate following HAIC were significantly superior in group A than in group B (response rate: 48% *vs* 16%, P = 0.028, tumor suppression rate: 87% *vs* 53%, P = 0.014). Furthermore, both the progression-free survival rate and survival time were significantly superior in group A than in group B (3-, 6-, 12-, and 24-mo = 83%, 70%, 29% and 20% *vs* 63%, 42%, 16% and 0%, respectively, P = 0.040, and 9.8 mo *vs* 6.2 mo, P = 0.040). A multivariate analysis (Cox proportional hazards regression model) showed that resistance to TACE was an independent predictor of poor survival (P = 0.007).

CONCLUSION: HAIC administrating 5-FU was not effective against advanced HCC resistant to TACE. Other tools for treatment, i.e., molecular-targeting agents may be considered for these cases.

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Key words: Hepatocellular carcinoma; Hepatic arterial infusion chemotherapy; 5-fluorouracil; Transarterial chemoembolization

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases around the world, and the number of HCC-related deaths has been increasing worldwide[1-5]. HCC has a poor prognosis due to its rapidly-infiltrating growth characteristic and occurrence in a background of liver cirrhosis (LC). Surgical treatment is only indicated in a small proportion of patients, due to the frequently large tumor size, presence of multiple tumors, and poor hepatic function[6,7]. Regional interventional therapies have led to major breakthroughs in the management of HCC; transarterial chemoembolization (TACE) has been reported as an effective treatment modality for patients with advanced HCC, especially those with multiple nodules[8-15], therefore, it is often repeated several times for the treatment of recurrent HCC. Furthermore, advances in implantable drug delivery systems have made it possible to administer repeated arterial infusions of anticancer agents, and recent studies, including our previous reports, have shown the effectiveness of combined therapy with intra-arterial 5-fluorouracil (5-FU) plus cisplatin or subcutaneous interferon (IFN) therapy in patients with advanced HCC[16-24]. We previously reported a case of unresectable advanced HCC with portal vein tumor thrombosis (PVTT) who was treated successfully by combined intra-arterial 5-FU plus subcutaneous pegylated interferon-α2b (PEG-IFN-α2b) therapy[23], and also a retrospective cohort study of this combined hepatic arterial infusion chemotherapy (HAIC)[24]. However, the precise efficacy of HAIC in patients with advanced HCC resistant to TACE still remains unclear.

In the present cohort study, we evaluated the effectiveness and outcomes, in terms of the overall survival rate, median survival time and response to therapy, of HAIC in patients with unresectable advanced HCC with and without a resistance to TACE.

MATERIALS AND METHODS

Patients and eligibility

The subjects of this study were 42 patients with HCC in whom the diagnosis was made on the basis of the pathological or radiological findings between January 2001 and December 2010 at Yokohama City University Hospital, Kanagawa, Japan. Of the 42 patients, 5 had not received any treatment before enrollment in this study, 27 had been treated by TACE, 8 had undergone hepatic resection, and 2 had been treated by local ablation therapy before enrollment in this study. All the patients satisfied the following criteria: Child-Pugh class A or B, white blood cell > 2000/μL, neutrophil count > 1000/μL, Plt > 50 000/μL, total bilirubin < 3.0 mg/dL, serum creatinine < 1.5 mg/dL, unresectable or unsuitable for local ablation therapy, 4 or more lesions throughout the liver or presence of vessel invasion, Eastern Cooperative Oncology Group Performance Status, 0-2[25], absence of extra-hepatic metastases, and absence of past history of treatment with 5-FU. The PVTT grade and tumor stage were determined according to the criteria of the Liver Cancer Study Group of Japan[26]. All patients gave written informed consent for participation in this study, and the study was conducted with the approval of the Ethics Committee of Yokohama City University Graduate School of Medicine. The patients enrolled in this retrospective study were divided into two groups according to whether or not they fulfilled the criteria for resistance to TACE proposed by the Japan Society of Hepatology in 2010 (written in Japanese) (Table 1); one group of patients who did not fulfill the criteria (group A, $n = 23$), and another group of patients who fulfilled the criteria for TACE resistance (group B, $n = 19$). We compared the outcomes in terms of the response and survival rates between the two groups. A comparison of the patient characteristics between the two groups before the start of HAIC is shown in Table 2. The duration of treatment from the first detection of HCC to the time of the HAIC (i.e., to enrollment in this study) was significantly longer in group B than in group A (36.2 mo *vs* 16.3 mo, $P = 0.004$). The liver function parameters did not differ significantly between the two groups.

Table 1 Criteria for transarterial chemoembolization resistance

The evaluation was performed on the day of TACE and 1 mo after the TACE; the following were observed at least two times
Staining with the injected agent (lipiodol-anticancer agent emulsion) was considered insufficient with evaluation CT [the occupation rate was less than 50% of lesion(s)]
Appearance of multiple new recurrent lesions on the evaluation CT
Appearance of vessel invasion after TACE
Appearance of distal metastasis after TACE
Persistent elevation of tumor marker(s) regardless of TACE

TACE: Transarterial chemoembolization; CT: Computed tomography.

Arterial catheterization

The arterial catheter was inserted into the right or left femoral artery by the Seldinger method. A heparin-coated catheter (Clinical Supply, Gifu, Japan) was inserted into the femoral artery and its tip was advanced to the

Table 2 Comparison of the patient characteristics in the two groups prior to hepatic arterial infusion chemotherapy *n* (%)

	Group A	Group B	*P* value
Patients	23	19	
Age (yr)	66.6 ± 6.9	65.5 ± 7.3	NS ($P = 0.635$)
Gender			
Male/female	20 (87)/3 (13)	15 (79)/4 (21)	NS ($P = 0.488$)
Etiology of LC			
HCV	13 (57)	11 (58)	NS ($P = 0.070$)
HBV	2 (9)	6 (32)	
HCV + HBV	0 (0)	1 (5)	
Alcohol	4 (17)	0 (0)	
NonB-nonC	4 (17)	1 (5)	
Albumin (g/dL)	3.6 ± 0.6	3.5 ± 0.6	NS ($P = 0.503$)
Total bilirubin (mg/dL)	1.1 ± 0.7	1.3 ± 0.5	NS ($P = 0.397$)
PT (INR)	1.19 ± 0.13	1.17 ± 0.10	NS ($P = 0.607$)
AST (U/L)	64 ± 33	79 ± 51	NS ($P = 0.256$)
ALT (U/L)	47 ± 30	53 ± 38	NS ($P = 0.569$)
GGT (U/L)	155 ± 169	76 ± 76	NS ($P = 0.067$)
WBC (/μL)	4600 ± 1400	4400 ± 900	NS ($P = 0.431$)
Hb (g/dL)	13.1 ± 2.0	12.8 ± 1.0	NS ($P = 0.521$)
Plt ($\times 10^4$/μL)	14.3 ± 6.5	12.1 ± 5.8	NS ($P = 0.262$)
AFP (median, ng/mL)	7550	3116	NS ($P = 0.434$)
DCP (median, mAU/mL)	12314	3363	NS ($P = 0.159$)
Child-Pugh A/B	12 (52)/11 (48)	6 (32)/13 (68)	NS ($P = 0.219$)
Child-Pugh score	6.8 ± 1.7	7.1 ± 1.4	NS ($P = 0.582$)
Number of tumor (s) ≤ 5/6-10/> 10	5 (22)/7 (30)/11 (48)	5 (26)/8 (42)/6 (32)	NS ($P = 0.515$)
Size of the largest tumor (cm)	7.3 ± 5.2	3.8 ± 1.3	$P = 0.008$
Vessel invasion presence/absence	12 (52)/11 (48)	7 (37)/12 (63)	NS ($P = 0.320$)
Clinical stage Ⅰ/Ⅱ/Ⅲ/ⅣA	0 (0)/0 (0)/11 (48)/12 (52)	0 (0)/0 (0)/13 (68)/6 (32)	NS ($P = 0.180$)
Duration of treatment received prior to HAIC (mo)	16.3 ± 20.7	36.2 ± 21.5	$P = 0.004$
Previous number of TACE session (s)	0.9 ± 0.6	4.5 ± 1.8	$P < 0.0001$
HAIC regimens			
5-FU, cisplatin	8 (35)	7 (37)	NS ($P = 0.923$)
5-FU, natural IFN-α	4 (17)	4 (21)	
5-FU, PEG-IFN-α2b	11 (48)	8 (42)	

HCV: Hepatitis C virus; HBV: Hepatitis B virus; LC: Liver cirrhosis; PT: Prothrombin time; INR: International ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ glutamyl transferase; AFP: α-fetoprotein; WBC: White blood cell; DCP: Des-γ-carboxyprothrombin; HAIC: Hepatic arterial infusion chemotherapy; TACE: Transarterial chemoembolization; 5-FU: 5-fluorouracil; IFN: Interferon; PEG-IFN-α2b: Pegylated interferon-α2b.

common hepatic artery or proper hepatic artery. The other end of the catheter was connected to the injection reservoir, already implanted into a subcutaneous pocket created in the right or left lower quadrant of the abdomen. The gastroduodenal and right gastric arteries were occluded with coils to prevent potential gastroduodenal injury by the anticancer agents.

Treatment protocol

Patients received arterial infusions of the anticancer agents *via* the injection reservoir. Each chemotherapy cycle lasted 2-4 wk. 5-FU (300 mg/m^2 per day, Kyowa Hakko, Tokyo, Japan) was administered continuously for 24 h *via* the infusion pump on days 1 to 5 of each of the two weeks. PEG-IFN-α2b (PEG-INTRON, MSD KK, Tokyo, Japan) on Day 1 of every week or natural IFN-α (OIF, Otsuka Pharmaceuticals, Tokyo, Japan) on Days 1, 3, 5 of every week was administered by the subcutaneous route. The administered dose of PEG-IFN-α2b was adjusted by the weight of each patient (50 μg-100 μg), and the dose of natural IFN-α was fixed at 5.0 × 10^6 unit. In another HAIC regimen, cisplatin (10 mg/body per day, Nihon-Kayaku Pharmaceuticals, Tokyo, Japan) was combined with 5-FU (250 mg/body per day) administered continuously for 24 h *via* the infusion pump on days 1 to 5 of each of the four weeks. Each of the HAIC therapy regimens was repeated for a total of at least 2 cycles until the response changed to progressive disease (PD) or a severe adverse reaction appeared.

Evaluation

The duration of the progression-free survival was measured from the date of start of HAIC to the date on which the response was judged to have changed to PD. The response to the HAIC was evaluated by contrast-enhanced computed tomography (CT) after every 2 cycles of treatment. The response criteria of the Response Evaluation Criteria in Solid Tumors were used[27]. The duration of the response was measured from the date of start of treatment to the date of documented progression. Adverse reactions were assessed every week during therapy based on the United States National Cancer Institute Common Toxicity Criteria (NCI-CTC; version 3.0)[28].

Statistical analysis

The statistical analysis was performed using the StatView software, version 5.0 (SAS, Cary, NC). Group comparisons were performed by the chi-square test for independence or by Fisher's exact test for comparison of more than two independent groups. The overall survival rate of each group was evaluated by the Kaplan-Meier method and the logrank test from the start of HAIC until the patient's death, and the progression-free survival rate was evaluated until the effect of the HAIC changed to PD. *P* values of < 0.05 were considered to denote significance in all the statistical tests. The closing date of the study was May 31, 2011.

RESULTS

Response to the HAIC

In group A, 2 patients (8.7%) showed complete response (CR), 9 patients (39.1%) showed partial response (PR), 9 patients (39.1%) showed stable disease (SD), and the remaining 3 patients (13.1%) showed PD. On the other hand, in group B, none of the patients (0%) showed CR, 3 patients (15.8%) showed PR, 7 patients (36.8%) showed SD, and the remaining 9 patients (47.4%) showed PD.

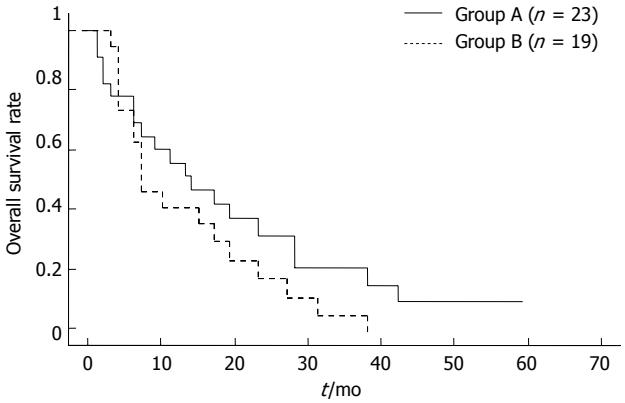

Figure 1 The overall survival rate tended to be superior in group A (a solid line) than in group B (a dotted line) (3-, 6-, 12-, 24-, and 36 mo = 82.6%, 78.3%, 56.5%, 32.8% and 21.9% *vs* 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively, *P* = 0.203).

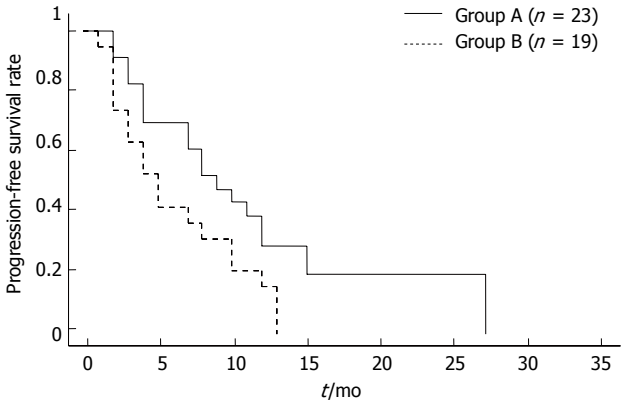

Figure 2 The progression-free survival rate was significantly superior in group A (a solid line) than in group B (a dotted line) (3-, 6-, 12-, and 24 mo = 82.6%, 69.6%, 29.3%, and 19.6% *vs* 63.2%, 42.1%, 15.8% and 0%, respectively, *P* = 0.040).

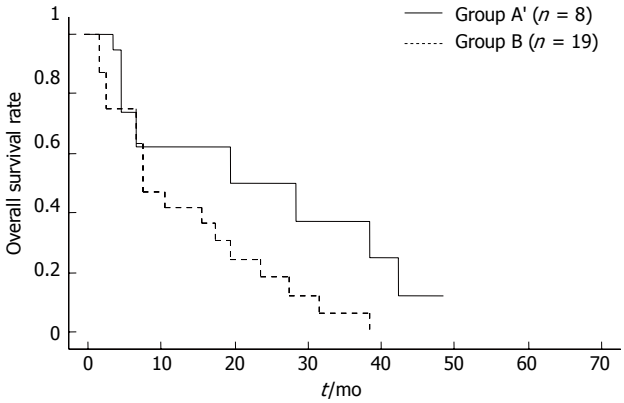

Figure 3 The overall survival rate and survival time tended to be superior in group A' (a solid line) than in group B (a dotted line) (3-, 6-, 12-, 24-, and 36 mo = 75.0%, 75.0%, 62.5%, 50.0% and 37.5% *vs* 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively, *P* = 0.095).

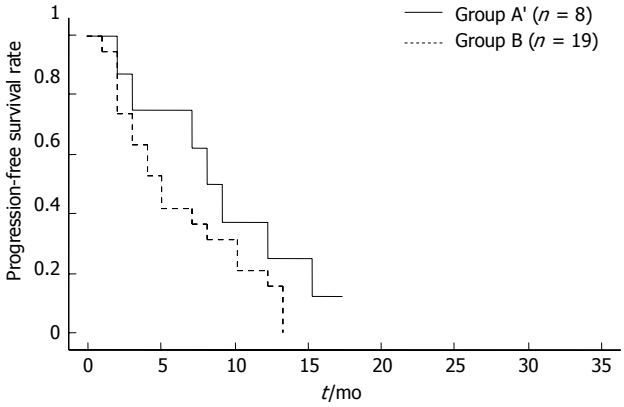

Figure 4 The progression-free survival rate and time tended to be superior in group A' (a solid line) than in group B (a dotted line) (3-, 6-, 12-, and 24 mo = 75.0%, 75.0%, 25.0%, and 0% *vs* 63.2%, 42.1%, 15.8% and 0%, respectively, *P* = 0.192).

Both the response rate [CR and PR patients/all patients × 100(%)] and the tumor suppression rate [CR, PR, and SD patients/all patients × 100(%)] following HAIC were significantly superior in group A than in group B (response rate: 47.8% *vs* 15.8%, $P = 0.028$, tumor suppression rate: 86.9% *vs* 52.6%, $P = 0.014$).

Survival

The overall survival rate and survival time tended to be superior in group A than in group B (3-, 6-, 12-, 24-, and 36 mo = 82.6%, 78.3%, 56.5%, 32.8% and 21.9% *vs* 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively, $P = 0.203$ (Figure 1), and 18.8 mo *vs* 14.0 mo, $P = 0.267$). Furthermore, the progression-free survival rate and time were significantly superior in group A than in group B (3-, 6-, 12-, and 24 mo = 82.6%, 69.6%, 29.3%, and 19.6% *vs* 63.2%, 42.1%, 15.8% and 0%, respectively, $P = 0.040$ (Figure 2), and 9.8 mo *vs* 6.2 mo, $P = 0.040$).

Subgroup analysis

In group A, both the patients who received TACE once or twice ($n = 8$) and who did not receive TACE ($n = 15$) were included. Therefore, to evaluate the effectiveness of HAIC after TACE, we performed subgroup analysis compared the patients who received TACE in group A (group A', $n = 8$) to group B. In group A', 1 patient (12.5%) showed CR, 3 patients (37.5%) showed PR, 3 patients (37.5%) showed SD, and the remaining 1 patient (12.5%) showed PD. Both the response rate and the tumor suppression rate following HAIC tended to be superior in group A' than in group B (response rate: 50.0% *vs* 15.8%, $P = 0.064$, tumor suppression rate: 87.5% *vs* 52.6%, $P = 0.087$).

The overall survival rate and survival time tended to be superior in group A' than in group B (3-, 6-, 12-, 24-, and 36 mo = 75.0%, 75.0%, 62.5%, 50.0% and 37.5% *vs* 94.7%, 73.7%, 42.1%, 18.4%, and 6.1%, respectively, $P = 0.095$ (Figure 3), and 24.0 mo *vs* 14.0 mo, $P = 0.086$). Furthermore, the progression-free survival rate and time also tended to be superior in group A' than in group B (3-, 6-, 12-, and 24 mo = 75.0%, 75.0%, 25.0%, and 0% *vs* 63.2%, 42.1%, 15.8% and 0%, respectively, $P = 0.192$ (Figure 4), and 9.1 mo *vs* 6.2 mo, $P = 0.143$). These results of comparison between group A' and group B was similar to that between group A and group B.

Table 3 Multivariate analysis (Cox proportional hazards regression model) to identify factors influencing the survival

	Odds ratio	95% CI	*P* value
Age > 66 (yr)	0.284	0.077-1.044	NS (*P* = 0.058)
Gender: female	3.995	0.704-22.662	NS (*P* = 0.118)
Resistance to TACE	8.264	1.770-38.461	*P* = 0.007
AFP > 200 (ng/mL)	0.385	0.121-1.230	NS (*P* = 0.107)
DCP > 200 (mAU/mL)	1.181	0.218-6.390	NS (*P* = 0.847)
Albumin > 3.5 (g/dL)	0.012	0.001-0.181	*P* = 0.001
Total bilirubin > 1.0 (mg/dL)	4.000	1.004-15.933	*P* = 0.049
PT (INR) > 1.20	0.490	0.155-1.551	NS (*P* = 0.225)
ALT > 50 (U/L)	1.229	0.378-3.999	NS (*P* = 0.732)
Plt > 15.0 (× 10^4/μL)	1.251	0.330-4.736	NS (*P* = 0.742)
Number of tumors > 6	0.403	0.090-1.794	NS (*P* = 0.233)
Size of the largest tumor > 5.0 cm	0.913	0.215-3.884	NS (*P* = 0.902)
Clinical stage: ⅣA	13.800	1.638-116.257	*P* = 0.016
Response to HAIC: CR, PR	0.024	0.004-0.160	*P* = 0.0001
Child-Pugh: B	0.251	0.019-3.307	NS (*P* = 0.293)
Hepatic encephalopathy: presence	0.643	0.123-3.347	NS (*P* = 0.599)
Ascites: presence	3.471	0.835-14.419	NS (*P* = 0.087)

TACE: Transarterial chemoembolization; AFP: α-fetoprotein; DCP: Des-γ-carboxyprothrombin; PT: Prothrombin time; INR: International ratio; ALT: Alanine aminotransferase; HAIC: Hepatic arterial infusion chemotherapy; CR: Complete response; PR: Partial response; CI: Confidence interval.

Multivariate analysis to identify factors influencing the survival

A multivariate analysis (Cox proportional hazards regression model) was performed to identify factors that might influence the survival following HAIC, which identified resistance to TACE [odds ratio (OR): 8.264, *P* = 0.007], serum albumin > 3.5 g/dL (OR: 0.012, *P* = 0.001), serum total bilirubin > 1.0 mg/dL (OR: 4.000, *P* = 0.049), clinical stage ⅣA (OR: 13.800, *P* = 0.016), and CR, PR to HAIC (OR: 0.024, *P* = 0.0001) as significant independent predictors influencing of the survival (Table 3).

Adverse reactions

The common systemic adverse reactions were fever, loss of appetite and general fatigue, however, none exceeded Grade 1 to 2 in severity. Furthermore, no case of serious leukopenia or thrombocytopenia was observed, with the severity of these adverse reactions not exceeding Grade 1 to 2 in any of the cases; none of the patients required administration of granulocyte-colony-stimulating factor or blood transfusion. On the other hand, among the 42 patients, there were 3 patients who developed Grade 2 generalized skin rash, 3 patients who developed obstruction of hepatic artery, and 2 patients who developed infection of reservoir. There were no cases of adverse event-related death.

DISCUSSION

According to the treatment algorithm for hepatocellular carcinoma in the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan[15], TACE and HAIC are recommended when the number of HCCs is four or more, with preserved liver function. In a large prospective cohort study of 8510 patients with a long follow-up period of 8 years, Takayasu *et al*[14] reported that TACE using an anticancer agent-lipiodol emulsion with or without gelatin sponge particles improved the survival of patients with advanced HCC, with overall 1-, 3-, 5-, and 7-year survival rates of 82%, 47%, 26%, and 16%, respectively, and a median survival duration of 34 mo. We also reported the superior effectiveness of TACE using a cisplatin- or epirubicin-lipiodol emulsion as compared with that of palliative treatment in a recent study of patients with advanced HCC[13]; both the overall survival rate and median survival time in patients who received TACE were significantly superior to those in patients who received only palliative treatment (1-, 2-, 5-, and 8-year survival rates of 98%, 90%, 56% and 16% *vs* 47%, 39%, 23% and 0%, respectively; median survival duration, 25 mo *vs* 10 mo). However, repeat sessions of TACE were often required which can potentially result in deterioration of the liver function[29]. Another group reported that selective TACE using conventional doses of anticancer drugs can cause persistent, serious worsening of the liver function[30].

Several recent studies have reported the effectiveness and survival benefit of combined therapy with intra-arterial 5-FU plus cisplatin or systemic various IFN in patients with unresectable advanced HCC[16-24]. Ando *et al*[21] investigated the outcomes of HAIC using a combination 5-FU plus cisplatin for HCC patients with complicating PVTT (*n* = 48), and reported a response rate of 48%, median survival time of 31.6 mo, and 1-, 2-, 3- and 5-year survival rates of 45%, 31%, 25% and 11%, respectively. Obi *et al*[18] reported an objective response rate of 52.6% (61/116 patients) in 116 patients with advanced HCC and Vp 3 or 4 treated with a combination of 5-FU plus natural IFN-α. A recent study conducted by us demonstrated the effectiveness of combined therapy with 5-FU plus subcutaneous PEG-IFN-α2b for unresectable advanced HCC (*n* = 18); the response rate was 33.3%, the median survival time was 17.7 mo, and the 6-, 12-, 24- and 36-mo survival rates were 89%, 71%, 39% and 29%, respectively[24]. However, few reports have investigated the effectiveness of HAIC in patients with advanced HCC resistant to TACE. This study revealed that HAIC yielded an unsatisfactory survival rate and survival time in patients with HCC resistant to TACE, and a multivariate analysis identified resistance to TACE as one of the independent predictors of poor survival in these patients.

Recently, a multikinase inhibitor, sorafenib, was approved as the first molecular targeted agent for advanced HCC, and two global phase Ⅲ trials[31,32] showed survival benefit with this drug administered orally for advanced HCC patients with preserved liver function. The SHARP Study was a randomized double-blind placebo-controlled multicenter study conducted in western countries, which showed that both the overall survival and the time to progression were significantly superior in the sorafenib group (*n* = 299) than in the placebo group (*n* = 303) (10.7 mo *vs* 7.9 mo, and 5.5 mo *vs* 2.8 mo, respectively). Interestingly, 86 patients (29% of sorafenib group) and 90 patients (30%

of placebo group) who had previously received TACE were included in the SHARP Study. Galle *et al*[33] reported that among 176 patients after TACE, the overall survival and the time to progression were superior in the sorafenib group (n = 86) than in the placebo group (n = 90) (11.9 mo *vs* 9.9 mo, and 5.8 mo *vs* 4.0 mo, respectively) in subanalysis of the SHARP Study. These results suggest that sorafenib may be an effective treatment agent for patients with advanced HCC resistant to TACE. Furthermore, the Asia-Pacific Study, performed in eastern Asian countries, also showed, similar to the SHARP study, significant survival prolongation in the sorafenib group as compared with that in the placebo group. Therefore, in Japan, sorafenib has recently been recommended for the treatment of patients with advanced HCC and extra-hepatic metastasis or major vessel invasion with preserved liver function, e.g., Child-Pugh class A[34,35].

In conclusion, although the evaluation needs to be conducted in a larger number of patients and the study was a retrospective cohort study, the results of this study revealed that HAIC administered with 5-FU exerted insufficient effect against advanced HCC resistant to TACE. Molecular-targeting agents may need to be considered in the future for patients with HCC resistant to TACE.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases around the world, and interventional therapies such as transarterial chemoembolization (TACE) or hepatic arterial infusion chemotherapy (HAIC) has been performed for patients with advanced HCC, especially those with multiple nodules, therefore, it is often repeated several times for the treatment of recurrent HCC. However, the precise efficacy of HAIC in patients with advanced HCC resistant to TACE still remains unclear.

Research frontiers

Advances in implantable drug delivery systems have made it possible to administer repeated arterial infusions of anticancer agents, and recent studies, including our previous reports, have shown the effectiveness of combined therapy with intra-arterial 5-fluorouracil (5-FU) plus cisplatin or subcutaneous interferon (IFN) therapy in patients with advanced HCC which have multiple intra-hepatic lesions or portal vein tumor thrombosis.

Innovations and breakthroughs

The study was considered the first report which investigated the effectiveness of HAIC administrating 5-FU for advanced HCC resistant to TACE. The patients enrolled in their study were divided into two groups according to whether or not they fulfilled the criteria for resistance to TACE proposed by the Japan Society of Hepatology in 2010 (written in Japanese) (Table 1); one group of patients who did not fulfill the criteria for TACE resistance (group A, n = 23), and another group who fulfilled the criteria for TACE resistance (group B, n = 19). They compared the outcomes in terms of the response and survival rates between the two groups. Both the response rate and tumor suppression rate following HAIC were significantly superior in group A than in group B. Furthermore, both the progression-free survival rate and survival time were significantly superior in group A than in group B. A multivariate analysis (Cox proportional hazards regression model) showed that resistance to TACE was an independent predictor of poor survival.

Applications

The results of this study revealed that HAIC administered with 5-FU exerted insufficient effect against advanced HCC resistant to TACE.

Terminology

HAIC administrating 5-FU was not effective against advanced HCC resistant to TACE, and our study showed the limitation of interventional therapies to prolong the survival for advanced HCC and consideration of new strategy including other tools for treatment, i.e., molecular-targeting agents.

Peer review

In this study, the authors report that patients with HCC resistant to TACE exhibit a poorer response to HAIC. This paper is clearly written and the topic material is important.

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BRIEF ARTICLE

Preoperative microcoil embolization of the common hepatic artery for pancreatic body cancer

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Abstract

AIM: To evaluate safety and feasibility of microcoil embolization of the common hepatic artery under proper or distal balloon inflation in preoperative preparation for en bloc celiac axis resection for pancreatic body cancer.

METHODS: Fifteen patients (11 males, 4 females; median age, 67 years) with pancreatic body cancer involving the nerve plexus surrounding the celiac artery underwent microcoil embolization. To alter the total hepatic blood flow from superior mesenteric artery (SMA), microcoil embolization of the common hepatic artery (CHA) was conducted in 2 cases under balloon inflation at the proximal end of the CHA and in 13 cases under distal microballoon inflation at the distal end of the CHA.

RESULTS: Of the first two cases of microcoil embolization with proximal balloon inflation, the first was successful, but there was microcoil migration to the proper hepatic artery in the second. The migrated microcoil was withdrawn to the CHA by an inflated microballoon catheter. Microcoil embolization was successful in the other 13 cases with distal microballoon inflation, with no microcoil migration. Compact microcoil embolization under distal microballoon inflation created sufficient resistance against the vascular wall to prevent migration. Distal balloon inflation achieved the requisite 1 cm patency at the CHA end for vascular clamping. All patients underwent en bloc celiac axis resection without arterial reconstruction or liver ischemia.

CONCLUSION: To impede microcoil migration to the proper hepatic artery during CHA microcoil embolization, distal microballoon inflation is preferable to proximal balloon inflation.

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Key words: Embolization; Microcoil; Balloon inflation; En bloc celiac axis resection; Pancreas body cancer

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INTRODUCTION

The overall 5-year survival rate after surgical resection for pancreatic cancer is extremely poor (< 10%)[1-3]. There is an ongoing effort toward treating this difficult disease and improving survival. Surgery plays a main role for a complete

cure of pancreatic body cancer. En bloc celiac axis resection (modified Appleby operation) has been introduced to expand the surgical treatment for pancreatic body cancer with celiac axis involvement[4,5], and Hirano *et al*[6] report a promising estimated 5-year survival rate of 42% for locally advanced pancreatic body cancer. This surgical procedure has the aim of en bloc lymphadenectomy together with resection of the spleen, pancreatic body, and tail by ligation of the celiac trunk artery and common hepatic artery (CHA). The safety of this operation is based on the rationale that hepatic arterial blood is supplied from the superior mesenteric artery *via* the pancreatico-duodenal arcades following ligation of the CHA. However, weak pulsation of the proper hepatic artery was observed in some patients during surgery, immediately after surgical ligation of the CHA[7-9]. When poor pulsation of the proper hepatic artery is observed after clamping of the CHA, arterial reconstruction is necessary because liver necrosis is fatal once it occurs[10-13]. To avoid this complicated procedure, Kondo *et al*[14] reported the preparatory technique of enlarging the collateral pathways from the SMA before surgery, by preoperative embolization of the CHA using interlocking detachable coils. Following this report, surgeons first asked interventional radiologists to embolize the CHA in preoperative management to enlarge the collateral pathways from the superior mesenteric artery (SMA). However, exact microcoil embolization of the short segment of the CHA is not easy, even for experienced interventional radiologists, because of its rapid arterial flow and, of particular concern, the possibility of coil migration to the proper hepatic artery. In our case, the surgeon also asked that we retain vascular lumen patency 1 cm from the distal end of the common hepatic artery and the proximal celiac artery trunk, to enable clamping of these vessels. In response to these requirements, we conducted microcoil embolization of the CHA under either proximal or distal microballoon inflation. The purpose of this clinical study is to describe these techniques and to evaluate their safety and feasibility.

MATERIALS AND METHODS

Patients

Approval of the Institutional Ethics Committee of our institution was obtained for this clinical trial prior to initiation of the study. All patients were fully informed of the extent of their diseases and of the risks and benefits associated with preoperative CHA embolization and en bloc resection.

In cases of right or common hepatic artery branching from the SMA, it was not necessary to conduct microcoil embolization of the CHA. Between May 2007 and January 2010, 15 patients with pancreatic body cancer involving the nerve plexus surrounding the celiac artery were scheduled for surgical radical pancreatectomy and underwent microcoil embolization preoperatively. Tumor stage was T4 in 14 patients and T3 in 1 patient according to the tumor, node and metastasis classification of the Union for International Cancer Control (UICC)[15]. Eleven patients were male and 4 were female; age ranged from 46 to 79 years (median, 67 years) (Table 1). All patients suffered from severe back pain and/or abdominal pain. Enhanced computed tomography using contrast medium revealed tumors sized 10-76 mm located in the body to the tail of the pancreas and involving the celiac, splenic, and/or common hepatic arteries, but with no evidence of liver metastases or invasion to the superior mesenteric artery. Microcoil embolization of the common hepatic artery was performed 7 d to 14 d before surgery. Two interventional radiologists, each with more than 7 years experience in transcatheter arterial embolization, conducted the following procedure.

Interventional procedure

Microcoil embolization under proximal balloon inflation: A 5F balloon catheter (balloon diameter 10 mm; Selecon MP, Catheter Rosch Ⅱ; Terumo, Tokyo, Japan) was inserted into the celiac artery through a 6F long sheath (Terumo) *via* the right femoral artery. After celiac arteriography, a 5F balloon catheter was advanced to the CHA using a guide wire (0.035, angle type; Radiofocus, Terumo). Under balloon inflation at the proximal end of the CHA, a microcatheter with two markers for detachable coil embolization (Rapidtransit, Johnson and Johnson, New Brunswick, NJ) was inserted coaxially and advanced to the distal CHA, 1 cm before the branching of the gastroduodenal artery. Detachable microcoils (interlocking detachable coil, Boston Scientific, Boston, MA) of diameter at least 1 mm greater than that of the CHA were deployed by making a lengthwise and/or sidewise frame (Figure 1A).

Microcoil embolization under distal balloon inflation: 6F and 4F long sheaths (Terumo) were inserted *via* the right and left femoral arteries, respectively. A 6F guiding catheter (Elway, Terumo Clinical Supply, Tochigi, Japan) was advanced through a 6F sheath to the celiac artery. After celiac arteriography, a 3.3F microballoon catheter (8 mm in maximum inflated diameter, 1 cm in length; Iiguman, Fuji System, Tokyo, Japan) was inserted into the common hepatic artery through a 6F guiding catheter and placed at the distal end of the CHA (Figure 1B). The microballoon was inflated, taking care not to interrupt blood flow in the gastroduodenal artery (Figure 2A). A 4F catheter (Rosch celiac type, Medikit, Tokyo, Japan) was advanced through a 4F sheath to catheterize the celiac artery, and a microcatheter with two markers was then advanced to the proximal end of the balloon inflation (Figure 2A). Detachable coils of diameter greater than that of the CHA were used initially, and fiber coils (Tornado, Boston Scientific) were added to fill the space if necessary. Microcoils were placed in the CHA from the proximal end of the microballoon inflation to the inlet of the left gastric artery (LGA) branching from the celiac trunk artery (Figure 2B). In fluoroscopic guidance, the tube angle that enabled the best visualization of the CHA or LGA was used. After microcoil embolization, the microballoon catheter was deflated and withdrawn.

Table 1 Backgrounds of patients with pancreatic body cancer for common hepatic artery microcoil embolization

N	Age (yr)	Sex	Tumor		Balloon catheter inflation proximal/distal	Coil migration	CHA diameter (mm)	Coil used	
			Major axis (mm)	Stage (UICC TNM ver.6)				Detachable coil diameter (mm)/length (cm) × N	Fiber coil proximal/distal diameters (mm) × N
1	51	F	10	T4	+/-	-	7.0	8/20	
2	70	M	35	T4	+/-	+	6.7	10/10	
3	67	M	40	T4	+/+	-	6.1	8/20	4/8 × 3
4	56	M	46	T4	-/+	-	5.6	7/10	4/8 × 2
5	64	M	30	T4	-/+	-	7.4	10/20, 7/20, 6/10	
6	71	M	61	T4	-/+	-	6.4	9/20, 7/20, 6/10	
7	71	M	35	T4	-/+	-	7.7	10/10	7/3 × 2
8	54	M	31	T4	-/+	-	4.5	6/10 × 2, 5/10	6/2 × 2
9	69	F	76	T3	-/+	-	5.1	7/10	7/3 × 2, 8/4 × 3
10	46	M	35	T4	-/+	-	4.4	6/10	6/2 × 4
11	78	F	42	T4	-/+	-	6.4	8/10, 6/10	6/2
12	64	M	45	T4	-/+	-	2.1	3/10	3/2
13	74	F	37	T4	-/+	-	3.6	5/10	6/2, 5/2
14	62	M	34	T4	-/+	-	4.4	6/10	7/3 × 3
15	79	M	45	T4	-/+	-	5.5	7/10	8/4 × 3

N: Number; F: female; M: Male; UICC: Union for International Cancer Control; CHA: Common hepatic artery; TNM: Tumor, node and metastasis.

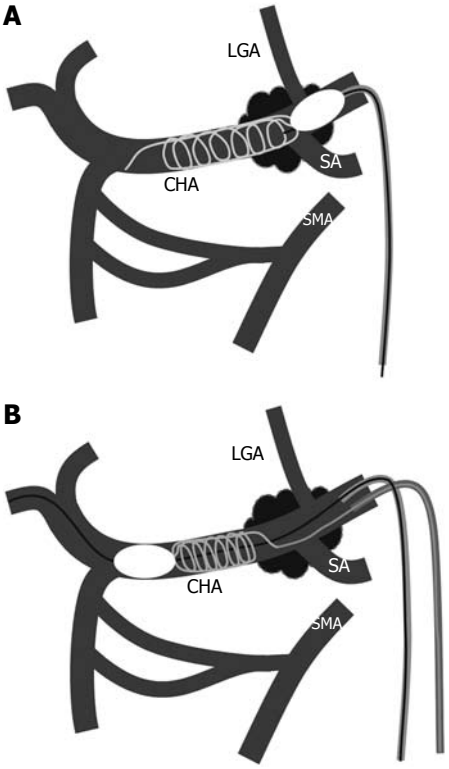

Figure 1 Schematic drawing of microcoil embolization under proximal balloon inflation (A) and distal balloon inflation (B) in the common hepatic artery. LGA: Left gastric artery; CHA: Common hepatic artery; SA: Splenic artery; SMA: Superior mesenteric artery.

Microcoil embolization under distal balloon inflation and proximal balloon inflation at the time of withdrawal: After microcoil embolization of the CHA under distal balloon inflation, as described above, a 5F balloon catheter (Selecon MP, Catheter Rosch Ⅱ, Terumo) was inserted *via* the femoral artery to the celiac artery through a 6F long sheath. This 5F balloon catheter was used to prevent microcoil migration from the CHA to the celiac trunk artery. Specifically, when the distal balloon catheter was deflated and withdrawn, the 5F balloon catheter was inflated at the CHA proximal end or celiac trunk artery. After confirming no proximal coil migration, the proximal balloon catheter was deflated, and both balloon catheters were removed.

After coil embolization in each procedure, superior mesenteric arteriography was conducted to confirm the alteration of blood flow from the SMA to the hepatic artery (Figure 2C). We aimed for total hepatic arterial blood flow to be supplied from the superior mesenteric artery. When the left hepatic artery branched from the LGA, microcoil embolization of left gastric artery was also performed.

RESULTS

In the present series, the first two patients underwent microcoil embolization of the CHA under proximal balloon inflation. Successful microcoil embolization of the CHA was completed in the first case. However, in the second case, distal migration of the microcoils occurred from the CHA to the proper hepatic artery after deflation of the proximal balloon catheter following microcoil placement. When this occurred, we immediately inserted the deflated microballoon catheter into the proper hepatic artery, advanced the catheter beyond the migrated microcoil, inflated the balloon catheter, and successfully withdrew the migrated coil to the common hepatic artery (Figure 3).

The third case underwent microcoil embolization under distal microballoon inflation. At the time of withdrawing the distal deflated balloon catheter located at the distal end of the CHA, we inflated the proximal balloon at the proximal end of the CHA to prevent microcoil migration from the CHA to the celiac trunk artery. However, we realized that the second (proximal) balloon catheter was not necessary because of the rigid fixation of the microcoils against the vascular wall. Thereafter, the distal

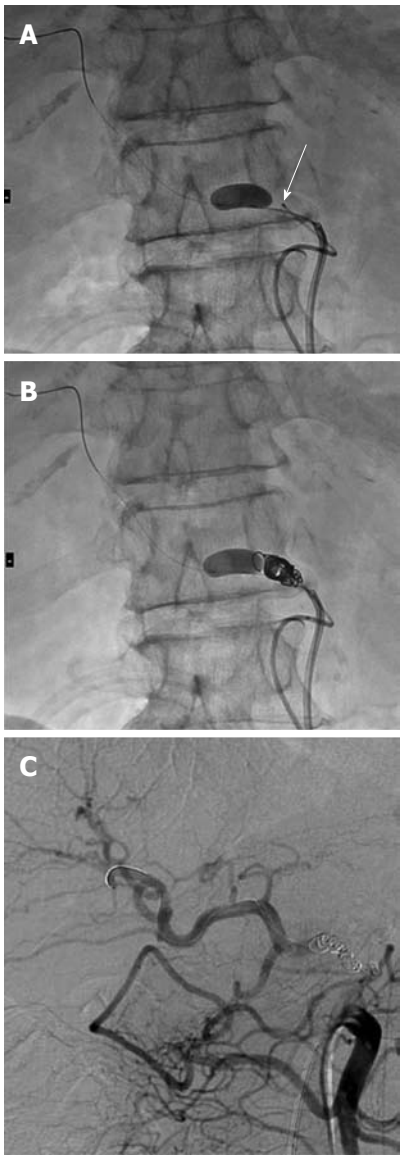

Figure 2 Microcoil embolization under distal balloon inflation. A: Radiograph during catheterization shows microcatheter (arrow) insertion to the common hepatic artery (CHA) *via* the celiac artery under distal microballoon inflation in the CHA; B: Radiograph during microcoil embolization shows a tight widthwise frame; C: Superior mesenteric arteriography after microcoil embolization shows blood flow from the superior mesenteric artery to the proper hepatic artery *via* the pancreatico-duodenal arcades.

deflated microballoon catheter was withdrawn without the assistance of proximal balloon inflation. There was no microcoil migration in the following 12 cases under distal microballoon inflation (Table 1). Accordingly, distal balloon inflation achieved the 1 cm patency at the end of the CHA required for vascular clamping.

In three cases having the variation of left hepatic artery branching from the LGA, we performed additional embolization of the LGA trunk using microcoils. In two of these three cases, total hepatic arterial blood flow was confirmed in superior mesenteric arteriography to come from the SMA *via* intrahepatic communication between the right and left hepatic arteries. In the remaining case, there was communication between the LGA and the

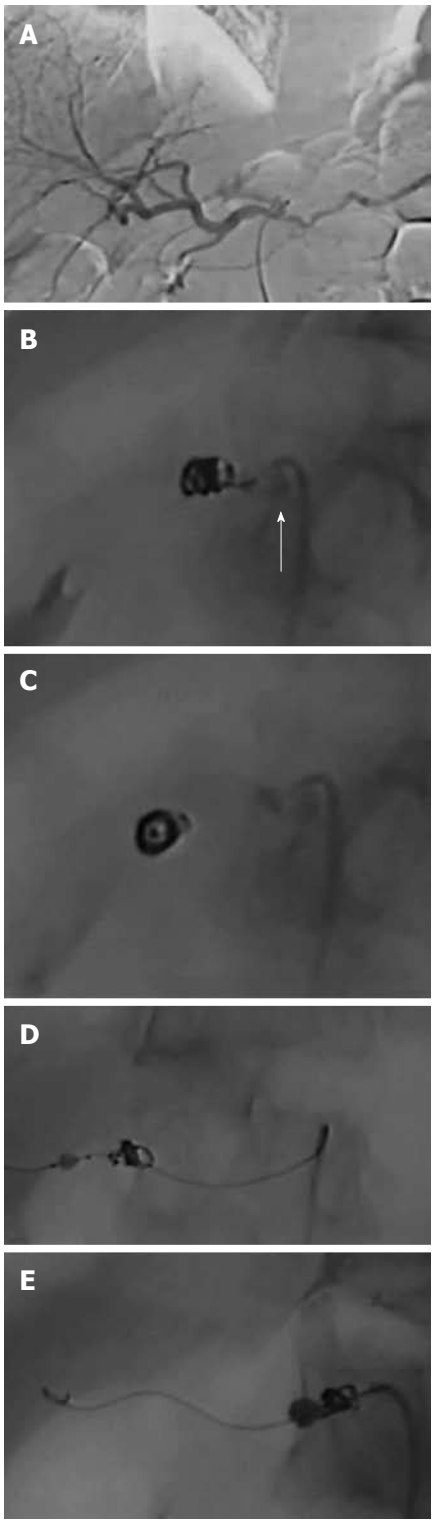

Figure 3 Distal migration of the microcoils and the successful withdraw. A: Following celiac arteriography; B: Microcoil embolization under proximal balloon inflation (arrow) was performed; C: Microcoil migration from the common hepatic artery (CHA) to the proper hepatic artery occurred after deflation of the proximal balloon catheter; D: Under fluoroscopic guidance using the tube angle that enabled the best visualization of the CHA and with the assistance of a microwire, a microballoon catheter was then inserted through the migrated coil and inflated; E: The migrated coils were withdrawn to their original position in the CHA by the inflated balloon catheter.

short gastric artery and left gastroepiploic artery, which

branched from the splenic artery. In this case, LGA and the splenic artery were embolized, aiming to achieve total hepatic blood flow from the SMA. However, following these embolizations, hepato-petal blood flow did not come from the SMA but from the inferior phrenic artery. No evidence of liver ischemia was observed during surgery, enabling radical pancreatectomy to be performed.

Superior mesenteric arteriography after embolization showed good hepato-petal blood flow from the SMA to the proper hepatic artery in all cases except that described above. All patients successfully underwent radical pancreatectomy without liver or gastric ischemia, and experienced no problems or complications related to the microcoil embolization.

DISCUSSION

In the present series, microcoil embolization under distal balloon inflation was superior to that under proximal balloon inflation in terms of impeding distal embolization. White[16] described two techniques of coil placement in the pulmonary artery: an anchor technique in which the microcoil tip was hooked into the small branch, and a scaffold technique in which a long frame was created lengthwise to increase friction against the vessel wall and fill the feeding artery. There are no small branch arteries from the CHA; therefore, using the scaffold technique we made a lengthwise and sidewise frame, by pushing and pulling the coils. If the microcoils continued to push out under proximal balloon inflation, then they tended to move to a more peripheral site than intended; in this case, the microcoils needed to be pulled back. In the second case of the present series, under proximal balloon inflation the microcoils migrated to the proper hepatic artery despite the microcoils having a diameter 3 mm greater than that of the CHA; this probably occurred because the loose frame of placed microcoils did not create sufficient friction against the vascular wall. The migrated microcoils were pulled back by the distal inflated balloon catheter to the original CHA site. We found that microcoils placed under distal balloon inflation became more compact than those under proximal balloon inflation, creating enough friction against the vascular wall to prevent migration.

We anticipated that the microcoils could migrate from the CHA to the celiac trunk artery or abdominal aorta after retrieval of the deflated distal microballoon catheter following microcoil embolization. For this reason, in the third case of this series, an additional balloon catheter was inserted and inflated in the celiac trunk artery, with the aim of blocking proximal coil migration from the CHA to the celiac trunk. However, this precaution proved unnecessary. The rigid friction of the microcoils against the vessel wall resulted in no proximal migration when the distal deflated microballoon catheter was retrieved. The use of detachable coils of diameter 1-2 mm greater than that of the CHA was sufficient to create increased friction in the subsequent 12 cases under distal microballoon inflation.

As an additional positive outcome of the described technique, the long dimension of the inflated microballoon (10 mm) enables patency to be maintained at the distal CHA end. Accordingly, the distal microballoon inflation method fulfilled the surgeon's requirement to retain 1 cm patency at the distal CHA end for vascular clamping.

It is a weakness of the distal microballoon catheter method that performing the procedure is somewhat complicated. However, the dual femoral artery approach is minimally invasive, and maneuvering the microballoon catheter does not have a steep learning curve.

In conclusion, the distal microballoon inflation method in CHA microcoil embolization was preferable to the proximal balloon inflation method, in terms of creating a compact microcoil frame that caused no coil migration to the proper hepatic artery, and of supplying a sufficient length of CHA patency to enable vascular clamping.

COMMENTS

Background

In preoperative management to avoid liver ischemia during surgery for pancreatic body cancer, the surgeons requested the interventional radiologists to embolize the common hepatic artery, to enlarge the collateral pathways from the superior mesenteric artery.

Research frontiers

This surgical procedure has the aim of en bloc lymphadenectomy together with resection of the spleen, pancreatic body, and tail by ligation of the celiac trunk artery and common hepatic artery (CHA). The safety of this operation is based on the rationale that hepatic arterial blood is supplied from the superior mesenteric artery *via* the pancreatico-duodenal arcades following ligation of the CHA.

Innovations and breakthroughs

The previous method using microcoils was conducted to occlude CHA without a balloon catheter. The present study occluded the CHA using microcoil embolization with the assistance of a microballoon catheter.

Applications

As the actual application for CHA occlusion, the distal microballoon inflation method in CHA microcoil embolization was preferable to the proximal balloon inflation method, in terms of creating a compact microcoil frame that caused no coil migration to the proper hepatic artery, and of supplying a sufficient length of CHA patency to enable vascular clamping.

Terminology

For surgical treatment for pancreas body cancer, en bloc lymphadenectomy is performed together with resection of the spleen, pancreatic body, and tail by ligation of the celiac trunk artery and CHA. Because of the risk of liver ischemia following ligation of the CHA during surgery, preoperative occlusion of the CHA using microcoil embolization is necessary.

Peer review

This study describes an interesting variant of interventional radiology that allows to minimize ischemic hepatic damage in Appleby operation. All technical steps are well and clearly described. Results are encouraging and convincing. Figure are clear and pertaining. The study is important for surgeons and interventional radiologists, even if only 15 patients have been enrolled.

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BRIEF ARTICLE

Hepatocellular carcinoma in Budd-Chiari syndrome: A single center experience with long-term follow-up in South Korea

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Abstract

AIM: To evaluate long-term clinical course of Budd-Chiari syndrome (BCS) and predictive factors associated with the development of hepatocellular carcinoma (HCC) and survival.

METHODS: We analyzed 67 patients with BCS between June 1988 and May 2008. The diagnosis of BCS was confirmed by hepatic venous outflow obstruction shown on abdominal ultrasound sonography, computed tomography, magnetic resonance imaging, or venography. The median follow-up period was 103 ± 156 [interquartile range (IQR)] mo.

RESULTS: The median age of the patients was 47 ± 16 (IQR) years. At diagnosis, 54 patients had cirrhosis, 25 (37.3%) Child-Pugh class A, 23 (34.3%) Child-Pugh class B, and six (9.0%) patients Child-Pugh class C. During the follow-up period, HCC was developed in 17 patients, and the annual incidence of HCC in patients with BCS was 2.8%. Patients in HCC group (n = 17) had higher hepatic venous pressure gradient (HVPG) than those in non-HCC group (n = 50) (21 ± 12 mmHg *vs* 14 ± 7 mmHg, P = 0.019). The survival rate of BCS patients was 86.2% for 5 years, 73.8% for 10 years, and 61.2% for 15 years. In patients with BCS and HCC, survival was 79% for 5 years, 43.1% for 10 years, and 21.5% for 15 years.

CONCLUSION: The incidence of HCC in patients with BCS was similar to that in patients with other etiologic cirrhosis in South Korea. The HVPG is expected to provide additional information for predicting HCC development in BCS patients.

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Key words: Budd-Chiari syndrome; Hepatocellular carcinoma; Prognosis

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Park H, Yoon JY, Park KH, Kim DY, Ahn SH, Han KH, Chon CY, Park JY. Hepatocellular carcinoma in Budd-Chiari syndrome: A single center experience with long-term follow-up in South Korea. *World J Gastroenterol* 2012; 18(16): 1946-1952 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i16/1946.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i16.1946

INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare hepatic disease caused by occlusion of the hepatic venous outflow. Thrombogenic conditions among the known causes of BCS are well documented, such as coagulopathy, chronic intake of contraceptives, myeloproliferative diseases, autoimmune diseases, and others[1]. Hepatic venous outflow block can also be caused by malignant tumors[2]. BCS was initially defined as a symptomatic occlusion of the hepatic veins, but with increasing reports on various obstructive cases in the hepatic portion of the inferior vena cava (IVC), it became to include obstructive IVC lesions as well as the major hepatic veins. BCS induces chronic liver congestion so that it causes hepatomegaly, ascites, leg edema, collateral venous dilatation in the body trunk and portal hypertension[3]. Several studies have suggested that hepatic congestion caused by obstruction of hepatic venous outflow can lead to cirrhosis and hepatocellular carcinoma (HCC)[4,5]. The incidence of HCC in patients with BCS has varied according to regions and investigators[3,6-9]. Japan and South Africa showed relatively higher incidences compared to those of United States and France: 6.4%-47.5% *vs* 4%-20%[5,7,9,10]. Prognosis of HCC in BCS has varied as well[5,6,11].

We followed BCS patients treated at our hospital for more than 20 years, and our long term follow-up data can be helpful to understand the prognosis of BCS patients and natural course of the disease. Thus, we (1) evaluated the incidence and cumulative annual risk of HCC in BCS patients; (2) analyzed the characteristics associated with the development of HCC in BCS patients; and (3) investigated the prognosis of BCS and HCC in BCS patients.

MATERIALS AND METHODS

Patients

From June 1988 to May 2008, 95 consecutive patients who were diagnosed with BCS at Severance Hospital were studied retrospectively. Among them, 28 patients were excluded based on the criteria as follows: patients with secondary BCS [occlusion of the hepatic venous outflow by an outside structure (HCC, a klatskin tumor, and renal cell carcinoma)], hematologic diseases which could result venous obstructive disease, hepatic septic emboli of colon cancer, or post-operative complication of HCC. Finally 67 patients who were diagnosed with primary BCS were investigated. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by the institutional review board of our institute.

Methods

We retrospectively reviewed patients' age, gender, and presenting symptoms of decompensate liver cirrhosis such as ascites, encephalopathy and variceal bleeding. We also investigated their history of medication which can induce thrombosis such as oral contraceptive, herbal medication, and steroid. Patients were assessed for other risk factors of cirrhosis and HCC such as alcohol, hepatitis B virus and hepatitis C virus (HCV) infections.

Laboratory tests included complete blood count, prothrombin time, alanine aminotransferase (ALT), bilirubin, albumin, creatinine, viral markers such as hepatitis B virus surface antigen (HBsAg) and anti-HCV antibody. Based on laboratory and physical examination results, Model for End-Stage Liver Disease (MELD) score and Child Pugh score were calculated to evaluate liver function. Every 3-6 mo, imaging studies such as computed tomography (CT), ultrasonography or magnetic resonance imaging (MRI) were performed, and alpha-fetoprotein (AFP) level was checked for HCC surveillance.

Diagnosis of BCS and HCC

The diagnosis of BCS was confirmed by hepatic venous outflow obstruction shown on ultrasound sonography (US), contrast-enhanced CT, MRI, or venography. Patients were confirmed to have HCC according to the American Association for the Study of Liver Disease practice guidelines for the HCC diagnosis[12]. Briefly, patients were diagnosed with HCC if they had a tumor with a maximum diameter of > 2 cm and the typical features of HCC on dynamic CT (defined as enhancement in the arterial phase and early washout in the portal phase), and an AFP > 200 ng/mL[12]. If the maximum diameter of the tumor was 1 cm to 2 cm, dynamic CT and MRI were performed. HCC was diagnosed if coincidental typical features of HCC were noted. If the tumor did not satisfy above criteria, a biopsy was performed. During hospital stay, abdominal ultrasonography was carried out to evaluate the presence of cirrhosis and hepatocellular carcinoma.

Measurement of hepatic venous pressure gradient

The hepatic venous pressure gradient (HVPG) was measured through catheterization during venography or angioplasty. Forty-five patients underwent venography or angioplasty when diagnosed with BCS or during follow up period. Patients were diagnosed with clinical liver cirrhosis if they satisfied one or more of the following 3 conditions: (1) a platelet count < 100 000/μL and ultrasonographic findings suggestive of cirrhosis including a blunted, nodular liver edge accompanied by splenomegaly (> 12 cm)[13,14]; (2) the presence of esophageal or gastric varices; and (3) overt complications of liver cirrhosis, including ascites, variceal bleeding, and hepatic encephalopathy.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 17.0). Descriptive statistics were computed for all variables, including median ± interquartile range (IQR) and percentiles for continuous variables and frequencies for categorical factors. Mann-Whitney *U* test and χ^2-test were used to compare HCC and non-HCC groups. Kaplan-Meier analysis was performed to estimate the cumulative incidence

Table 1 Baseline clinical characteristics of patients with Budd-Chiari syndrome (*n* = 67)

Characteristics	Budd-Chiari syndrome
Age (yr)	47 ± 16
Gender (male)	34 (50.7)
Obstruction site	
IVC	56 (83.6)
Hepatic vein	5 (7.5)
Combined	6 (9.0)
Alcohol consumption	
None/social/heavy (> 80 g/d)	32 (47.8)/15 (22.3)/20 (29.9)
Positive viral marker	
HBsAg/anti-HCV	3 (4.5)/0 (0)
Liver cirrhosis at diagnosis	54 (80.6)
Child-Pugh A/B/C	25/23/6 (37.3/34.3/9.0)
MELD score	11 ± 6
Decompensate LC symptoms	23 (34.3)
Length of obstruction (cm)	2.0 ± 4.0
HVPG (mmHg)	15 ± 10
Laboratory data	
ALT (IU/L)	19 ± 17
Bilirubin (mg/dL)	1.4 ± 1.3
Albumin (g/dL)	3.8 ± 0.7
Hemoglobin (g/dL)	12.3 ± 2.6
Platelet count (k/μL)	109 ± 78
Creatinine (mg/dL)	0.9 ± 0.2
PT (%)	73 ± 27
Treatment modality	
Angioplasty	27 (40.3)
Shunt operation	4 (5.9)
TIPS	3 (4.5)
Thrombolysis	1 (1.5)
Symptomatic medical treatment	32 (47.7)
Median follow up period (mo)	103 ± 156

Data were expressed as median ± IQR or *n* (%). IVC: Inferior vena cava; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; MELD: Model for End-Stage Liver Disease; LC: Liver cirrhosis; HVPG: Hepatic venous pressure gradient; ALT: Alanine aminotransferase; PT: Prothrombin time; TIPS: Transjugular intrahepatic portosystemic shunt; IQR: Interquartile range.

of HCC from the time of BCS diagnosis and survival of all BCS patients and those who developed HCC. To evaluate the factors associated with the development of HCC in patients with BCS, multivariate Cox regression analysis was used. A two-sided P value < 0.05 was considered to indicate a significant difference.

RESULTS

Baseline clinical characteristics of the patients

The baseline characteristics of the 67 patients are shown in Table 1. The site of obstruction was IVC in 56 patients (83.6%), hepatic vein in five patients (7.4%), and both IVC and hepatic vein sites in six patients who were classified as "combined". There was no patient with history of chronic use of medication such as oral contraceptive, herbal medication, and steroid. Patients with heavy alcohol consumption defined as over 80 g/d were 20 (29.9%). HBsAg was positive in 3 patients (4.5%) and there was no patient with positive for anti-HCV antibody. Fifty-four patients had underlying liver cirrhosis at the time of BCS diagnosis. Twenty-five of them (37.3%) were classified as Child-Pugh class A, 23 (34.3%) as class B, and six (9.0%) as class C. The median MELD score was 11 ± 6 (IQR). Twenty three patients (34.3%) had decompensated liver cirrhosis symptoms such as variceal bleeding, uncontrolled ascites or hepatic encephalopathy higher than grade 3. Twenty-seven (40.3%) patients were treated with percutaneous angioplasty, four (5.9%) with shunt operation, three (4.5%) with transjugular intrahepatic portosystemic shunt (TIPS), and one (1.5%) with thrombolysis. Thirty-two (47.7%) patients received only symptomatic medical treatment. The median follow up period was 103 ± 156 (IQR) mo.

Table 2 Characteristics of the patients with hepatocellular carcinoma at the time point of diagnosis (*n* = 17)

Variables	HCC
Age (yr)	53 ± 12
Time period from BCS to HCC (mo)	51 ± 115
Child-Pugh class	
A/B/C	6 (35.3)/8 (47.1)/3 (17.6)
Tumor stage (AJCC 6th)[1]	
Ⅰ/Ⅱ/Ⅲ/Ⅳ	8 (47.1)/6 (35.3)/3 (17.6)/0 (0)
Treatment modality	
TACE/TACI	9 (52.9)
Intra-arterial chemotherapy	3 (17.6)
Conservative management	3 (17.6)
Operation	2 (11.9)
Prognosis	
Alive	12 (70.5)
Death	3 (17.6)
F/U loss	2 (11.9)

Data were expressed as median ± IQR or *n* (%). BCS: Budd-Chiari syndrome; HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; TACI: Transarterial chemo-infusion; F/U: Follow up; IQR: Interquartile range. [1]AJCC 6th, American Joint Committee on Cancer staging system, 6th edition.

Development of HCC in the patients with BCS

During follow-up periods, HCC was occurred in 17 patients. At the time of diagnosis of HCC, the median age of the patients was 53 ± 12 (IQR) years, and time period between diagnoses of BCS and HCC was 51 ± 115 (IQR) mo (Table 2). HCC was histologically confirmed in 2 patients with hepatic resection. According to the Kaplan-Meier analysis, as shown in Figure 1, the cumulative probability was 18.5% at 5 years, 30.3% at 10 years, and 42.6% at 15 years. The annual occurrence of HCC in BCS patients was 2.8%.

We compared the baseline characteristics (at the time of diagnosis with BCS) of HCC group (n = 17) with non-HCC group (n = 50). The differences between the two groups are shown in Table 3. There were no significant differences in the comparison of age, gender, obstruction site, alcohol consumption and presence of viral markers between two groups. HVPG was significantly higher in HCC group [21 ± 12 (IQR) mmHg] than in non-HCC

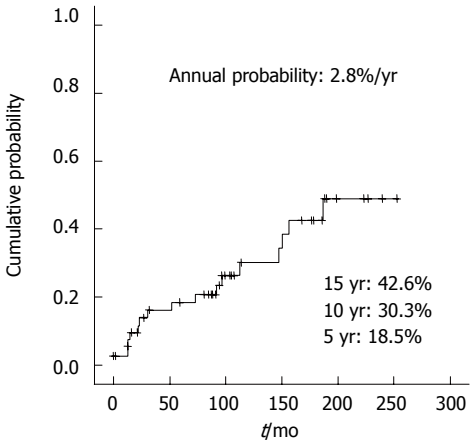

Figure 1 Cumulative probability of hepatocellular carcinoma in patients with Budd-Chiari syndrome (*n* = 17).

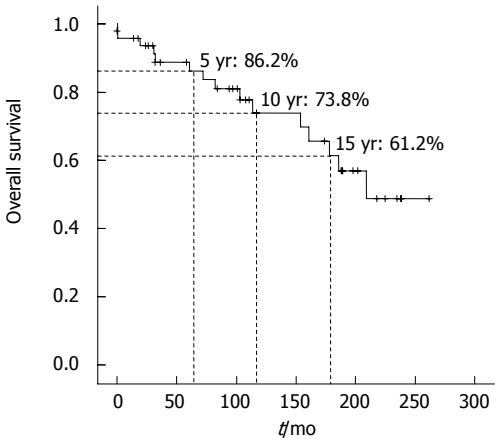

Figure 2 Overall survival of patients with Budd-Chiari syndrome.

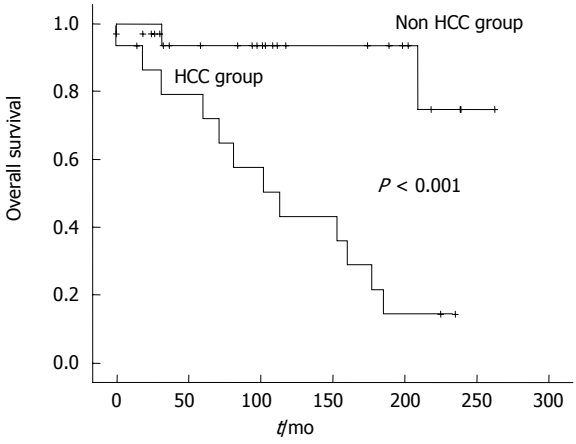

Figure 3 Comparison of survival between hepatocellular carcinoma Group (*n* = 17) and non-hepatocellular carcinoma group (*n* = 50). HCC: Hepatocellular carcinoma.

group [14 ± 7 (IQR) mmHg] (P = 0.019). However, in the multivariate Cox regression analysis, HVPG showed no significant difference between in HCC group and in non-HCC group (P = 0.452).

Table 3 Comparison of baseline clinical characteristics between hepatocellular carcinoma group and non hepatocellular carcinoma group

Variables	HCC (*n* = 17)	Non HCC (*n* = 50)	*P* value
Age (yr)	47 ± 11	47 ± 18	0.863
Gender (male)	10 (58.8)	24 (48)	0.441
Obstructive site			0.264
IVC	15 (88.2)	41 (82)	
Hepatic vein	2 (11.8)	3 (6)	
Combined	0 (0)	6 (12)	
Alcohol consumption			0.329
None	6 (35.3)	26 (52.0)	
Social	4 (23.5)	11 (22.0)	
Heavy (> 80 g/d)	7 (41.2)	13 (26.0)	
Positive for HBsAg	0 (0)	3 (6.0)	0.554
LC at diagnosis	14 (82.4)	40 (80.0)	0.832
Decompensate LC symptoms	8 (47.1)	15 (28.0)	0.148
Child Pugh A/B/C	5/7/2	20/16/4	0.647
MELD score	11 ± 6	11 ± 6	0.778
Follow up period (mo)	103 ± 146	103 ± 160	0.648
Length of obstruction (cm)	1.0 ± 2.5	2.0 ± 4.4	0.144
HVPG (mmHg)	21 ± 12	14 ± 7	0.019
Laboratory data			
ALT (IU/L)	21 ± 21	18 ± 14	0.160
Bilirubin (mg/dL)	1.5 ± 1.9	1.3 ± 1.3	0.521
Albumin (g/dL)	3.6 ± 0.85	3.8 ± 0.72	0.245
Hemoglobin (g/dL)	12.5 ± 4.1	12.3 ± 2.3	0.897
Platelet count (k/μL)	97 ± 59.5	113 ± 80.7	0.559
Creatinine (mg/dL)	0.9 ± 0.14	0.85 ± 0..24	0.798
PT (%)	71 ± 31	74 ± 22	0.756
Survival (%)			< 0.001
5 yr	79	93.4	
10 yr	43.1		
15 yr	21.5	74.7	

Data were expressed as median ± IQR or *n* (%). HCC: Hepatocellular carcinoma; IVC: Inferior vena cava; LC: Liver cirrhosis; MELD: Model for End-Stage Liver Disease; HVPG: Hepatic venous pressure gradient; ALT: Alanine aminotransferase; PT: Prothrombin time; IQR: Interquartile range.

Prognosis and survival of patients with BCS and patients who were diagnosed with HCC

We estimated the overall survival rates of all 67 BCS patients using the Kaplan-Mayer method. The overall survival rate was 86.2% at 5 years, 73.8% at 10 years, and 61.2% at 15 years (Figure 2). During the follow-up periods, three patients among 17 who were diagnosed with HCC died, and two of them died from hepatic failure and the other from massive variceal bleeding. In HCC group, the 5-year survival rate was 79%, 10-year rate 43.1%, and 15-year rate 21.5% (Figure 3). Meanwhile, non-HCC group (n = 50) showed significantly higher survival rates than HCC group: 93.4% for 5 years and 74.7% for 15 years (P < 0.001).

DISCUSSION

BCS is caused by obstruction of hepatic venous outflow at any level from the small hepatic veins to the junction of the IVC with the right atrium. There are two forms of BCS according to the obstruction site: primary hepatic vein obstruction (classical BCS) and obstruction of the

Table 4 Prevalence and characteristics of hepatocellular carcinoma in patients with Budd-Chiari syndrome from the literature

Ref.	Matsui *et al*[3]	Shin *et al*[24]	Moucari *et al*[7]	Gwon *et al*[6]
Year, country	2000, Japan	2004, South Korea	2008, France	2010, South Korea
Study design	Retrospective	Retrospective	Retrospective	Retrospective
Study period	Apr 1968-Feb 1999	Mar 1989-Aug 2001	1987-2005	Mar 1990-Nov 2008
No. of patient with BCS	12	73	97	98
HCC (%)	3 (25)	15 (20.5)	11 (11.3)	23 (23)
Cumulative incidence of HCC	-	-	4 yr 3% 7 yr 6% 14 yr 12%	1 yr 7.3% 5 yr 13.5% 10 yr 31.8%
Tx. for HCC	Resection (1) TAE (1) iA chemotherapy (1)	TACE (11) Resection (2) Conservative tx. (2)	TACE (7) LT (3) Conservative tx. (1)	TACE (20) TACE + LT (3)
Survival rate of HCC in patients with BCS				
Median survival period (mo)	-	58 (range, 3-59)	-	-
Cumulative survival	-	1 yr 93% 2 yr 84% 3 yr 72%	-	1 yr 90% 2 yr 85% 3 yr 61% 4 yr 61% 5 yr 46%
Risk factors for HCC in patients with BCS				
Risk factors	Chronic congestion in the liver, caused by an outflow block of hepatic veins	-	Male gender, Coagulopathy[1], IVC obstruction	Female gender[2]
Analysis method	No statistical analysis (mere presumption)	-	Univariate analysis	Multivariate analysis

HCC: Hepatocellular carcinoma; BCS: Budd-Chiari syndrome; Tx.: Treatment; TAE: Transarterial embolization; TACE: Transarterial chemoembolization; iA: Intra-arterial; LT: Liver transplantation. [1]Coagulopathy harbored factor V Leiden; [2]Female gender showed an odds ratio of 6.02 with $P < 0.001$ in Cox regression analysis; IVC: Inferior vena cava.

hepatic portion of the inferior vena cava (IVCO). The IVCO form is common in Asia and Africa but rarely reported in Western countries[9,15]. Most of our patients (83.6%) had the IVCO form, which is similar to previous studies which reported the predominance of IVCO form in Asia. The major difference between classical BCS and IVCO is that the former is rarely associated with HCC, while the latter is frequently complicated by HCC[3,8]. In our study, HCC was developed in 17 of 67 patients, and the annual incidence was 2.8%, similar to the incidence in patients with other etiologic cirrhosis in South Korea[16,17].

Until now, the accurate pathogenesis of HCC in BCS has not been elucidated yet. Gwon *et al*[6] suggested that chronic liver injuries and congestion caused by obstruction of hepatic venous outflow might contribute to a fibrotic process and development of nodular type of HCC. Prolonged congestion can lead to hepatocyte necrosis, and its replacement with fibrous tissue results in fibrosis, which is assumed to be the mechanism of cirrhosis and HCC development[18-20]. This hypothesis is supported by frequent findings of liver parenchymal cirrhotic change adjacent to HCC in BCS context[7].

The HVPG was significantly higher in HCC group than non-HCC group in our study. HVPG has been accepted as the gold standard for assessing the severity of portal hypertension[21]. With the pathogenesis proposed above, the higher pressure gradient means a greater degree of portal hypertension and hepatic congestion; this might have contributed to a higher pressure gradient in HCC group in our study. Our study that showed significantly high HVPG in HCC group is worthy, which supports the hypothesis of development of HCC in patients with BCS. Until now, there were several published reports to analyze the risk factors for HCC in patients with BCS. Although Moucari *et al*[7] showed that BCS patients with HCC compared with those without HCC presented with IVC obstruction more frequently, there was no report that showed direct differences of pressure gradient as our data presented. For comparison, other published data concerning the prevalence and characteristics of HCC in patients with BCS were reviewed in Table 4.

Varying survival results of BCS have been reported, and the 5-year survival rate ranges from 69% to 87%[22,23]. In our data, the overall survival rate was 86.2% for 5 years, 73.8% for 10 years, and 61.2% for 15 years. In patients with BCS and HCC, the survival rate was 79% for 5 years, 43.1% for 10 years, and 21.5% for 15 years in our study. This is comparable with the results of other published reports[6,24]. Improvement in availability and techniques of diagnostic tools and development of treatment modalities may allow earlier diagnosis of BCS patients and better prognosis[18-20].

Although our study has an advantage of long-term follow up data of patients with BCS, there are still some limitations. First, this was retrospective and therefore has the limitations of such an investigational design. Another limitation was that the values of HPVG obtained during venography were not checked in all patients. Because of invasiveness of venography, it could not be performed on all patients for HVPG measurement. These days,

Doppler US is used to non-invasively assess the HPVG with portal vein velocity. Considering the low incidence of BCS, a multicenter study should be performed to overcome the limitations of patient number and insufficient information. Despite these limitations, this study is worthy to analyze the incidence and prognosis of HCC in BCS with long-term follow-up periods in South Korea.

In conclusion, the annual incidence of HCC in patients with BCS was similar to the incidence in patients with other etiologic cirrhosis in South Korea. Furthermore, the HVPG can be a possible predictive factor of BCS-associated HCC development. Thus, BCS patients who are expected to have high pressure gradient should be actively managed as a high risk group for HCC development. An intervention to decrease the pressure gradient in BCS patients may be helpful to reduce the incidence of HCC. A large-scale study will be necessary to further investigate whether the treatment of congestion decreases the incidence of HCC.

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COMMENTS

Background

Budd-Chiari syndrome (BCS) is a rare disease caused by obstruction of the hepatic venous outflow. BCS induces chronic liver congestion so that it causes hepatomegaly, ascites, leg edema, collateral venous dilatation in the body trunk, and portal hypertension. Several studies have suggested that hepatic congestion caused by obstruction of hepatic venous outflow can lead to cirrhosis and hepatocellular carcinoma (HCC). However, the incidence of HCC in patients with BCS has varied according to regions and investigators and there has been a lack of reports for long-term prognosis of HCC in patients with BCS.

Research frontiers

Although BCS is a relatively rare disease as contrasted with other viral liver disease that can lead to advanced liver disease such as liver cirrhosis or HCC, several studies have reported the association between BCS and HCC. Long-term follow-up data of BCS may help to understand not only the prognosis of BCS but also the process of HCC in patients with BCS.

Innovations and breakthroughs

This study showed that the hepatic venous pressure gradient (HVPG) was significantly higher in HCC group than non-HCC group. Although the accurate pathogenesis of HCC in BCS has not been elucidated, there were several suggestions that chronic liver injuries and congestion caused by obstruction of hepatic venous outflow might contribute to a fibrotic process and development of HCC. The study is worthy because it supports this hypothesis about the development of HCC in patients with BCS.

Applications

From the study, it was suggested that the HVPG can be a possible predictive factor of BCS-associated HCC development. Thus, BCS patients who are expected to have a high pressure gradient should be actively managed as a high risk group for HCC development. An intervention to decrease the pressure gradient in BCS patients may be helpful to reduce the incidence of HCC.

Peer review

This study provides basic and essential data for the clinical care of the patients with BCS.

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BRIEF ARTICLE

Transparent-cap-fitted colonoscopy shows higher performance with cecal intubation time in difficult cases

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Abstract

AIM: To investigate the efficacy of cap-fitted colonoscopy (CFC) with regard to cecal intubation time.

METHODS: Two hundred and ninety-five patients undergoing screening colonoscopy at Gospel Hospital, Kosin University College of Medicine were enrolled in this randomized controlled trial between January and December 2010. Colonoscopies were conducted by a single endoscopist. Patient characteristics including age, sex, body mass index, history of abdominal surgery, quality of preparation, and the presence of diverticulosis were recorded.

RESULTS: One hundred and fifty patients were allocated into a CFC group and 145 into a non-CFC (NCF) group. Cecal intubations were achieved in all patients. Cecal intubation time in the CFC group was significantly shorter than in the NCF group for specific conditions: age ⩾ 60 years, prior abdominal surgery, and poor bowel preparation. The number of detected adenomas was higher in the CFC group than in the NCF group (P = 0.040).

CONCLUSION: CFC facilitated shortening of the cecal intubation time in difficult cases, and was more sensitive for detecting adenomas than was NCF.

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Key words: Colonoscopy; Cap-fitted colonoscopy; Cecal intubation

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INTRODUCTION

Colorectal cancer is a major cause of cancer-related mortality and morbidity, and it is evident that this fatality rate has led to an increase in colonoscopy preventative treatment[1-3]. A multinational and multicenter survey performed in Asia has shown that the overall prevalence of advanced colorectal neoplasm in asymptomatic individuals is comparable with that in the West[4]. Removal of colonic adenomas by screening colonoscopy could reduce colorectal cancer incidence and mortality rate.

Colonoscopy, however, can be a complicated procedure and requires a skillful endoscopist[5-7]. The anatomical factors of difficult cecal intubation can usually be categorized into one of two problems: (1) an angulated and/or narrowed sigmoid colon; and (2) a redundant colon[8]. These anatomical difficulties are commonly observed in specific cases, such as female patients, older age, previous gynecologic surgery, and the presence of diver-

ticulosis[9-17]. Published studies have suggested the use of a narrower instrument shaft or one with both a narrower shaft and a shorter bending section for use in angulated or narrowed sigmoid colons, and a stiffened shaft with simultaneous application of abdominal pressure for overcoming the problems associated with redundant colons[8,18,19]. However, these maneuvers are not always successful and may require the endoscopist to change instruments during the procedure.

Several studies have evaluated the efficacy of transparent cap-fitted colonoscopy (CFC) compared to that of non CFC (NCF), and have found no difference in cecal intubation time between CFC and NCF[20-22]. The one established advantage of CFC is that it is more sensitive to polyp detection than is NCF[20,21]. However, these results are not consistent with our daily experience, in that CFC requires a shorter time for cecal intubation than does NCF.

Short cecal intubation time is important for several reasons: less anesthetic medication is required; colonic inflation results in less discomfort; and sufficient withdrawal time for accurate examination. The purpose of this study was to evaluate whether CFC could result in shorter cecal intubation time compared with NCF. Additionally, we compared the detection rate of colonic adenomas in this study.

MATERIALS AND METHODS

Patients

From January to December 2010, 300 consecutive patients scheduled for their first ever colonoscopy as a routine health check at Gospel Hospital, Kosin University College of Medicine were included in the study. Exclusion criteria were as follows: age < 18 years; hospitalization due to other diseases undergoing colonoscopy investigation; evidence of acute or chronic renal failure; cardiovascular diseases including recent myocardial infarction, congestive heart failure, unstable angina, and cardiac arrhythmias; ascites; electrolyte imbalance; active inflammatory bowel disease, ileus and/or suspected bowel obstruction; pregnant or breast feeding; or childbearing potential without adequate contraception. Patient medical history, demographic data, and body weight were recorded. For all patients, clinical hemodynamic, hematological, and biochemical measurements, including whole blood count, blood sugar, blood urea nitrogen, creatinine, and serum electrolyte (sodium, potassium, chloride, phosphorus, ionized calcium and magnesium) levels were measured. After initial evaluations, patients who had no exclusion criteria were randomized to receive CFC or NCF by one physician who was blinded to the results of previous colonoscopies. This study was approved by the Institutional Review Board of Kosin University College of Medicine, Busan, South Korea.

CFC and NCF

After providing informed consent, patients in both groups were encouraged to adhere to a clear liquid diet from 06:00 h to midnight on the day before colonoscopy, and further oral intake was not allowed after midnight. All patients drank 4 L of polyethylene glycol electrolyte lavage solution, starting 7 h before colonoscopy at a rate of 250 mL every 15 min until all of the solution had been consumed, as recommended by the manufacturer (Olympus Optical Corp, Tokyo, Japan). Before colonoscopy, a physical examination and clinical hemodynamic measurements were repeated. The transparent plastic cap (D-14304; Olympus Optical Corp., Toyko, Japan) used for CFC was 14 mm in outer diameter, 10 mm in length, and had a 1 mm wall thickness. This cap can be fitted and fixed to the tip of the colonoscope (CIF H260; Olympus Optical Corp.). To ensure consistency in the evaluations, all colonoscopies were performed by the same attending endoscopist using the standard technique of negotiating the colon with as little air insufflation as possible. The principal examination was carried out during withdrawal. Ileal intubation was attempted when it was relevant.

Variables

For evaluating the efficacy of CFC against NCF, the duration time of insertion up to the cecum was compared between the CFC and NCF groups. Additionally, the number of adenomas detected during colonoscopy was calculated. Factors presumed to influence cecal intubation time were sex, age, body mass index, and history of abdominal surgery; all of which were evaluated before colonoscopy. The quality of preparation was classified as follows: grade 0, percentage of visible mucosa > 90%, excellent visibility (small volume of clear liquid requiring minimal suctioning for adequate visualization), and no intestinal bubbles; grade 1, percentage of visible mucosa > 90%, good visibility (large volume of clear liquid or small amount of fecal residue, not preventing a reliable examination), and small number of intestinal bubbles; grade 2, percentage of visible mucosa > 90%, fair visibility (some semi-solid stool that could be suctioned or washed away, preventing a reliable examination), and moderate number of intestinal bubbles; and grade 3, percentage of visible mucosa < 90%, poor visibility (large amount of semi-solid stool that could not be suctioned or washed away, not allowing a complete examination to be done), and large number of intestinal bubbles. The number of adenomas was calculated during colonoscopy. Discomfort of each patient was recorded using a four-point scale (1: easy; 2: tolerable; 3: some pain; 4: severe pain).

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS, Chicago, IL, United States). For normally distributed continuous variables, Student's *t* test was used to assess differences between the two groups. For categorical variables, Fisher's exact test was used. Cox multivariate regression analysis was performed to produce statistically significant variables in the present study. Two-sided *P* values < 0.05 were considered statistically significant.

Table 1 Baseline characteristics *n* (%)

	CFC (*n* = 150)	NCF (*n* = 145)	*P* value
Gender			
Male	94 (62.7)	87 (62.6)	1.000
Female	56 (37.3)	58 (37.4)	
Age (yr) mean ± SD	65.4 ± 15.3	66.1 ± 14.8	0.736
Body mass index (kg/m^2), mean ± SD	26.2 ± 10.6	27.4 ± 9.6	0.489
History of abdominal surgery	43 (28.6)	32 (22.1)	0.181
Cesarean section	20 (13.3)	15 (10.3)	0.474
Appendectomy	16 (10.6)	10 (6.9)	0.306
Distal gastrectomy due to peptic ulcer	7 (4.7)	7 (4.8)	1.000
Diverticulosis	51 (34.0)	42 (30.0)	0.382
Preparation score 2 or 3	30 (20.0)	31 (21.3)	0.776

CFC: Cap-fitted colonoscopy; NCF: Non-cap-fitted colonoscopy.

Table 2 Comparison of two groups with regard to cecal intubation time and number of detected colonic adenomas (mean ± SD)

	CFC (*n* = 150)	NCF (*n* = 145)	*P* value
Cecal intubation time (s)	262 ± 154	281 ± 138	0.057
Number of detected adenomas	2.0 ± 2.5	1.2 ± 1.6	0.040
Size of adenoma (cm)	2.0 ± 3.1	2.6 ± 2.9	0.061
Sessile type, *n*	1.8 ± 1.9	1.0 ± 0.9	0.039
Pedunculated type, *n*	0.4 ± 0.8	0.3 ± 0.7	0.557
Patient discomfort, scores	2.3 ± 1.0	2.3 ± 0.8	0.741

CFC: Cap-fitted colonoscopy; NCF: Non-cap-fitted colonoscopy.

RESULTS

Initially, 150 patients volunteered for each group. However, five patients in the conventional endoscopy group withdrew their consent after finishing all examinations; therefore, 150 patients for cap-assisted colonoscopy and 145 patients for conventional colonoscopy were enrolled. There were no significant differences in the backgrounds between the group with CFC and that with NCF (Table 1).

Cecal intubation was achieved in all cases regardless of method. The average time for insertion from anus to cecum was shorter in the CFC group than in the NCF group, but the difference was not statistically significant (262 ± 154 s *vs* 281 ± 138 s, $P = 0.057$). CFC showed greater adenoma detection than did NCF (2.0 ± 2.5 *vs* 1.2 ± 1.6; $P = 0.040$), especially for sessile adenomas (1.8 ± 1.9 *vs* 1.0 ± 0.9; $P = 0.039$). There was no significant difference between the two groups regarding patient discomfort scores during colonoscopy (2.3 ± 1.0 *vs* 2.3 ± 0.8, $P = 0.741$). These results are shown in Table 2.

Multivariate analyses revealed that cecal intubation time was significantly longer in patients aged > 60 years, with a history of abdominal surgery, and two or three points in quality of bowel preparation as described in Table 3.

Multivariate analyses revealed that CFC required a significantly shorter cecal intubation time than did NCF in specific patients, including older patients (244 ± 123 s *vs* 330 ± 213 s; $P = 0.009$), those with history of abdominal surgery (240 ± 106 s *vs* 351 ± 219 s; $P = 0.012$), and those with bowel preparation score 2 or 3 (224 ± 96 s *vs* 302 ± 176 s; $P = 0.006$), as shown in Table 4.

Table 3 Cecal intubation time in all patients (mean ± SD)

	Cecal intubation time	*P* value
Gender		0.881
Male	254 ± 145	
Female	257 ± 135	
Age (yr)		0.012
< 60	244 ± 114	
⩾ 60	322 ± 113	
Body mass index (kg/m^2)		0.047
< 23	243 ± 114	
⩾ 23	277 ± 125	
History of abdominal surgery		0.044
Yes	387 ± 173	
No	221 ± 117	
Diverticulosis		0.747
Yes	251 ± 146	
No	256 ± 142	
Quality of preparation		0.045
0 or 1	278 ± 155	
2 or 3	364 ± 183	

Table 4 Multivariate analysis for influencing factors on cecal intubation time(s) between cap-fitted colonoscopy and non-cap-fitted colonoscopy (mean ± SD)

	CFC	NCF	*P* value
Gender			
Male	234 ± 109	257 ± 137	0.053
Female	276 ± 173	257 ± 134	0.997
Age (yr)			
< 60	249 ± 130	233 ± 106	0.784
⩾ 60	244 ± 123	330 ± 213	0.009
Body mass index (kg/m^2)			
< 23	246 ± 125	240 ± 118	0.067
⩾ 23	296 ± 170	248 ± 148	0.674
History of abdominal surgery	240 ± 106	351 ± 219	0.012
Diverticulosis	218 ± 66	279 ± 189	0.169
Quality of preparation			
0 or 1	255 ± 133	251 ± 147	0.861
2 or 3	224 ± 96	302 ± 176	0.006

CFC: Cap-fitted colonoscopy; NCF: Non-cap-fitted colonoscopy.

DISCUSSION

Colonoscopy is a common endoscopic procedure. It is widely used for the investigation of lower gastrointestinal tract disorders and screening for colorectal adenomas[23]. However, failure to reach the cecum occurs in up to 10% of cases[10,24]. A transparent cap was initially designed for mucosectomy and was later used during colonoscopy to enhance colonic polyp detection[22]. CFC is an effective rescue method for patients who fail to achieve cecal intubation[25]. This benefit is more apparent for inexperienced colonoscopists[26]. Moreover, it has been shown that such a device can shorten cecal intubation time among experienced colonoscopists[27]. However, the present study

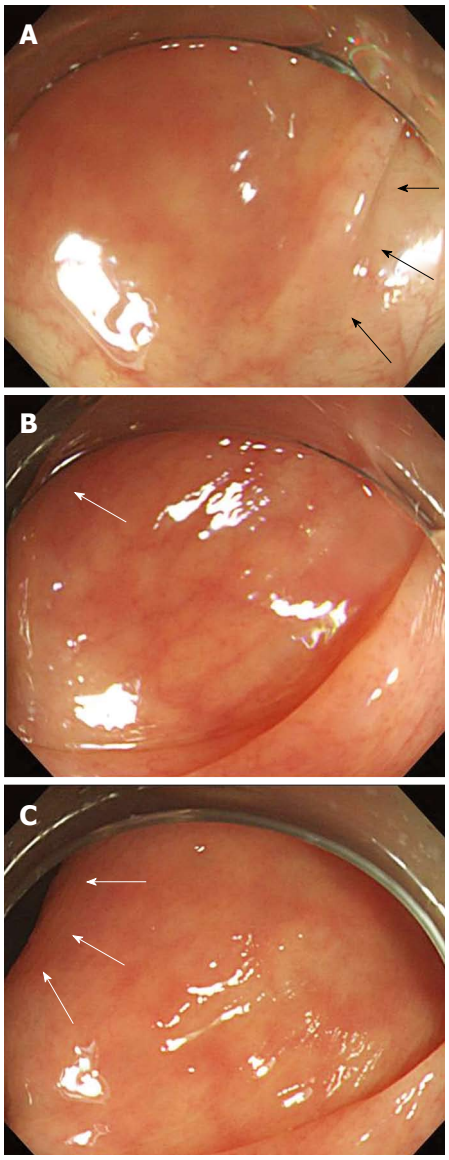

Figure 1 Advantage of cap-fitted colonoscopy for preventing red-out. A: Although the precise direction could not be judged, colonoscopy showed a slight fold (white arrows) without red-out; B: A subtle movement showed a dark area at the 11 o'clock position (white arrow); C: Following the dark are at the 11 o'clock position enabled the colonoscopist to find the direction of insertion (black arrows).

showed that there was no significant difference between CFC and NCF in cecal intubation time, although the average cecal intubation time of CFC was shorter than that for NCF.

Although there was no significant difference in cecal intubation time between the two groups, CFC showed a shorter time than did NCF in several specific situations: age ⩾ 60 years, history of abdominal surgery, and poor bowel preparation. In previous studies, predictive factors for incomplete colonoscopy were female sex, older age, previous gynecologic surgery, and the presence of diverticulosis[9-12]. In addition, female sex and older age are well known factors responsible for longer cecal intubation times[13-17]. Consistent with these previous results, CFC in the current study displayed a shorter cecal intubation

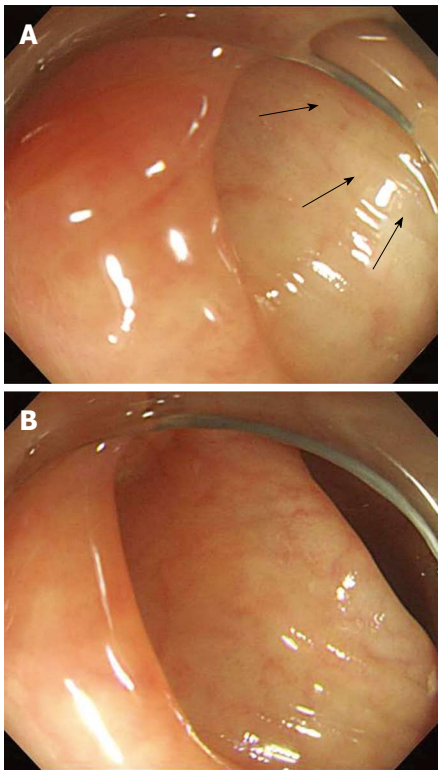

Figure 2 Advantage of cap-fitted colonoscopy for observing lateral side. A: The colonoscopist easily noticed the route of insertion because the transparent cap showed the small dark lumen at the 1 o'clock position through its lateral wall (black arrows); B: Following the route shown through the lateral wall of the transparent cap, a wide lumen was found easily.

time in difficult cases, such as in older patients, and those with a history of abdominal surgery, and poor bowel preparation. In our study, a single experienced endoscopist performed all procedures; this might be the reason why there was no difference between the two groups in cecal intubation time.

A possible explanation for this difference is less air insufflation during CFC than with NCF. A recent study revealed that the limited use of low-air insufflation in the rectum and sigmoid colon shortened the cecal intubation time and decreased post-procedural abdominal bloating[28]. Low-air insufflation causes less bowel inflation and produces less angulations of the bowel, thus enhancing cecal intubation. The use of CFC requires extremely low air insufflations. Experts in CFC can advance a cap-fitted colonoscope by pushing and pulling using meticulous lever manipulation without air insufflation, especially in the rectum and sigmoid colon. Extremely low air insufflation can be achieved in CFC because the cap prevents the mucosa from touching the lens directly and enables continuous lumen observation (Figure 1)[21]. Another important CFC characteristic is that the lateral side can be observed through the transparent wall of the cap (Figure 2)[21]. In the hepatic and splenic flexures, observing the lateral side through the transparent wall of the cap can help endoscopists determine the next step of the colonoscopy.

Another advantage of CFC in cecal intubation was that more adenomas were observed in the CFC group

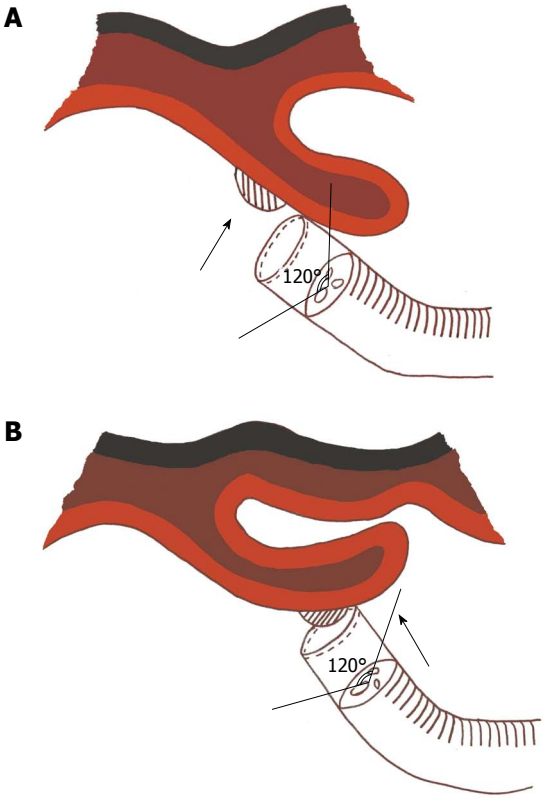

Figure 3 The opposite, blind side of the folds could be observed with fewer problems during colonoscopy. A: Compressing the tip of a fold straightened the entire fold and improved the view; B: Bending the tip of the endoscope allowed a front view of the lesion at the blind side of the fold.

during withdrawal compared to those in the NCF group. Cap usage greatly facilitates the identification of small adenomas. During insertion and withdrawal of the cap-fitted colonoscope, the lumen of the colon can always be seen clearly because the mucosa never directly touches the lens[21]. The opposite, blind side of the folds can easily be observed and treated with fewer problems in CFC because they can be straightened to improve the view (Figure 3)[20], and the lateral side can be observed through the transparent wall of the cap[21]. Fecal matter may stick to the inside of the cap in cases of poor bowel preparation, thereby impairing the view. Using the water insufflation button or simple flushing through the biopsy channel can lead to ineffective cap clearing. The cap, however, was easily cleaned in CFC by pressing the whole circumference of the cap against the mucosal surface and then flushing the biopsy channel[20]. Moreover, CFC enhanced cecal intubation compared to that of NCF in cases of poor bowel preparation (scores of 2 and 3).

In conclusion, CFC has advantages in overcoming the problems associated with angulated and/or narrowed sigmoid and redundant colon, thereby resulting in significantly higher performance in cecal intubation time in difficult cases such as old age, prior abdominal operation, and poor bowel preparation. Furthermore, CFC displayed a higher sensitivity in detecting colonic adenomas than did NCF.

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COMMENTS

Background

Colorectal cancer is a major cause of cancer-related mortality and morbidity, and it is evident that this fatality rate has led to an increase in colonoscopy preventative treatment. Colonoscopy, however, can be a complicated procedure and requires a skillful endoscopist. The anatomical factors of difficult cecal intubation can usually be categorized into one of two problems: (1) an angulated and/or narrowed sigmoid colon; and (2) a redundant colon. These anatomical difficulties are commonly observed in specific cases, such as female patients, older age, previous gynecological surgery, and the presence of diverticulosis. Published studies have suggested the use of a narrower instrument shaft or one with both a narrower shaft and a shorter bending section for use in angulated or narrowed sigmoid colons, and a stiffened shaft with simultaneous application of abdominal pressure for overcoming the problems associated with redundant colons.

Research frontiers

Several studies have evaluated the efficacy of a transparent cap-fitted colonoscopy (CFC) compared to that of non-CFC (NCF) and found that there was no difference in cecal intubation time between CFC and NCF. The one established advantage of CFC is that it is more sensitive to polyp detection than is NCF.

Innovations and breakthroughs

The transparent plastic cap is made by Olympus Optical Corp., Tokyo, Japan. It is 17 mm in outer diameter, with a 2 mm wall thickness and 10 mm in length, and can be fitted and fixed to the tip of the colonoscope. This can cause less air insufflation during CFC than with NCF. Low-air insufflation causes less bowel inflation and produces less angulations of the bowel, thus enhancing cecal intubation. The present study aimed to evaluate whether CFC could result in shorter cecal intubation time compared with NCF. Additionally, the study compared the detection rate of colonic adenomas.

Applications

CFC has advantages in overcoming the problems associated with angulated and/or narrowed sigmoid and redundant colon, thereby resulting in significantly higher performance in cecal intubation time in difficult cases, such as elderly patients, and those with prior abdominal operation, and poor bowel preparation. Furthermore, CFC displayed a higher sensitivity in detecting colonic adenomas than did NCF.

Terminology

Transparent CFC: a colonoscopic procedure with a transparent cap at the front view of the colonoscope.

Peer review

The present study showed that CFC had shorter cecal intubation time in difficult cases. This is an interesting and good study.

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BRIEF ARTICLE

Laparoscopic distal pancreatectomy is as safe and feasible as open procedure: A meta-analysis

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Abstract

AIM: To evaluate the feasibility and safety of laparoscopic distal pancreatectomy (LDP) compared with open distal pancreatectomy (ODP).

METHODS: Meta-analysis was performed using the databases, including PubMed, the Cochrane Central Register of Controlled Trials, Web of Science and BIOSIS Previews. Articles should contain quantitative data of the comparison of LDP and ODP. Each article was reviewed by two authors. Indices of operative time, spleen-preserving rate, time to fluid intake, ratio of malignant tumors, postoperative hospital stay, incidence rate of pancreatic fistula and overall morbidity rate were analyzed.

RESULTS: Nine articles with 1341 patients who underwent pancreatectomy met the inclusion criteria. LDP was performed in 501 (37.4%) patients, while ODP was performed in 840 (62.6%) patients. There were significant differences in the operative time, time to fluid intake, postoperative hospital stay and spleen-preserving rate between LDP and ODP. There was no difference between the two groups in pancreatic fistula rate [random effects model, risk ratio (RR) 0.996 (0.663, 1.494), $P = 0.983$, $I^2 = 28.4\%$] and overall morbidity rate [random effects model, RR 0.81 (0.596, 1.101), $P = 0.178$, $I^2 = 55.6\%$].

CONCLUSION: LDP has the advantages of shorter hospital stay and operative time, more rapid recovery and higher spleen-preserving rate as compared with ODP.

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Key words: Laparoscopy; Distal pancreatectomy; Pancreatic fistula; Spleen-preserving; Morbidity

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Xie K, Zhu YP, Xu XW, Chen K, Yan JF, Mou YP. Laparoscopic distal pancreatectomy is as safe and feasible as open procedure: A meta-analysis. *World J Gastroenterol* 2012; 18(16): 1959-1967 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i16/1959.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i16.1959

INTRODUCTION

With improvement of advanced surgical techniques and endoscopic instrument, laparoscopic distal pancreatectomy (LDP) is becoming a primary modality for the treatment of benign or borderline tumors of distal pancreas[1-3].

Recently, several researches have shown the advantages of LDP of shorter hospital stay and operative time and less intraoperative blood loss[4-5]. But the efficacy of LDP compared with open distal pancreatectomy (ODP) required further assessment, especially the incidence of pancreatic fistula (PF) which may lead to further com-

plications such as an intra-abdominal abscess, sepsis or lethal bleeding[6]. With a better understanding of the anatomy and immune function of spleen, especially the increased risks of overwhelming post splenectomy infection (OPSI) and long-term lung thrombosis[7], laparoscopic spleen preserving distal pancreatectomy (LSPDP) was performed first by Kimura *et al*[8] and Warshaw[9]. However, the role of "laparoscopy" in the spleen preservation is still unclear.

All the published studies we retrieved were based on a small number of patients and no randomized trials were available. Therefore, we strictly established the inclusion criteria and conducted a comprehensive meta-analysis to evaluate more systematically the feasibility and safety of LDP.

MATERIALS AND METHODS

Search strategy

We searched databases of PubMed, The Cochrane Central Register of Controlled Trials, Web of Science and BIOSIS Previews for the literatures comparing LDP and ODP published between January 1995 and June 2011. The language of the publications was confined to English. Two investigators reviewed the titles and abstracts, and assessed the full text to establish the eligibility. The search strategies were as follows (Table 1).

Inclusion criteria

All clinical studies should meet the following criteria for the meta-analysis: (1) published in English with data comparing ODP and LDP; (2) with clear case selection criteria, containing at least the following information: the number of cases, surgical methods and perioperative data; (3) continuous variables (e.g., operative time and hospital stay) expressed in mean ± SD. Dichotomous variables (e.g., incidence of PF and number of death) such as odds ratio (OR) and 95% confidence interval (CI); and (4) if there was overlap between authors, centers, or patient cohorts, the higher quality or recent literatures were selected.

Exclusion criteria

The papers containing any of the followings were excluded: (1) intra-operative conversion of LDP to an open laparotomy, which was classified into the laparoscopic group; (2) single surgical procedure; and (3) laparoscopy-assisted DP or hand-assisted LDP.

Data extraction and quality assessment

Two authors independently extracted the data using a unified datasheet, and decided the controversial issues through discussion. Extracted data included: first author, study period, the number of cases, operative time, spleen preservation, hospital stay, cases of malignant tumors, incidence of post-operative complications, and PF. Selected documents were rated according to the Grading of the Centre of Evidence-Based Medicine (Oxford, United Kingdom; www.cebm.net).

Table 1 Database and search strategy

Database	Search strategy
PubMed	"laparoscopy" (MeSH terms) or "laparoscopy" (all fields) or "laparoscopic" (all fields) or (minimally (all fields) and invasive (all fields) and ("pancreas" (MeSH terms) or "pancreas" (all fields) or "pancreatic" (all fields) and "humans" (MeSH terms) and English (lang) and "1995/1/1" (PDAT): "2011/06/30" (PDAT)
Web of Science	"pancreas" or "pancreatic" or "pancreatectomy" and "laparoscopy" or "laparoscopic" (limited year: 1995-2011)
Cochrane Library	"pancreas" or "pancreatic" or "pancreatectomy" and "laparoscopy" or "laparoscopic" (limited year: 1995-2011)
BIOSIS Previews	"pancreas" or "pancreatic" or "pancreatectomy" and "laparoscopy" or "laparoscopic" (limited year: 1995-2011) (related term and limited English and human and year: 1995-2011)

PDAT: Pulication date; MeSH: Medical subject headings.

Statistical analysis

This meta-analysis was performed according to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) and the Quality of Reporting of Meta-analyses (QUORUM) as a guideline[10,11]. Weighted mean differences (WMD) were used for continuous variables, and relative risk for dichotomous variables. P values < 0.05 indicated statistically significant difference between the two groups. When heterogeneity test showed no significant differences ($P > 0.05$), we used fixed effects model to calculate the summary statistics. When the heterogeneity test showed statistically significant differences ($P < 0.05$), we used random effects model based on DerSimonian and Laird method. If the heterogeneity was high or extracted data were less than three sets, we performed descriptive analysis. The potential publication bias was determined by the Begg's test and funnel plots based on the dichotomous variables. All data were analyzed using Stata SE11.0 software.

RESULTS

We retrieved 1663 papers in English. After the titles and abstracts were reviewed, papers without comparison of LDP and ODP were excluded. As a result, a total of 20 studies[12-31] were collected, of which 11 studies were excluded because of intraoperative conversion and using "assisted" approach. However, we preserved them for the analysis as "conversion to open". Finally, 9 studies[23-31] were included and extracted for detailed data. A flow chart of search strategies is illustrated in Figure 1.

Totally, 1341 patients (sample sizes ranging from 44 to 310) entered into this meta-analysis, including 501 (37.4%) cases of LDP and 840 (62.6%) cases of ODP. The detailed study design and surgical techniques in 10 trials are summarized in Table 2.

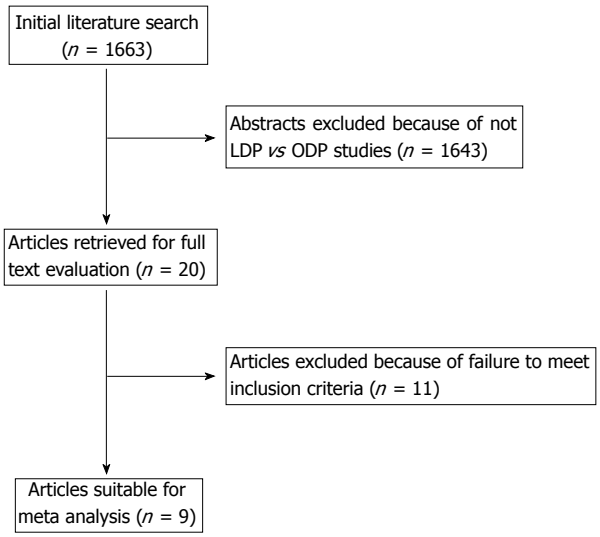

Figure 1 A flow chart of search strategies. The initial search strategy retrieved 1663 papers in English. Finally 9 studies were included and extracted for detailed data. LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy.

Intraoperative effects

The operative time was reported in four articles[23,24,26,28]. Meta-analysis of the pooled data showed that the operative time of ODP was significantly shorter than LDP [random effects model, WMD 44.947 (13.857, 76.037), P = 0.005] (Figure 2A).

In the included articles, five studies[23,25,27,29,30] covered the spleen-preserving DP, 95 cases (29.6%) of LSPDP were conducted among 321 cases of LDP as compared with 76 cases (13.3%) of SPDP among 571 cases of ODP. The pooled data showed that the spleen-preserving rate in LDP was significantly higher than in ODP [random effects model, RR 2.380 (1.177, 4.812), P = 0.016, I^2 = 73.2%], (Figure 3A). Although there was moderate heterogeneity, spleen-preservation occurred more often in LDP. Most authors tended to use the "Kimura method", and "Warshaw method" was used in a few LDPs and in cases with severe adhesion or vessels involved in tumors. Technical details of spleen-preservation are listed in Table 3.

Among 714 cases of LDP, 100 cases (14.0%) converted to open surgery and 6 cases converted to the hand-assisted approach as shown in 20 articles[12-31] because of severe bleeding, abdominal adhesions, large tumor, organ injury, and difficult anatomy.

The techniques of pancreatic stump closure in the included studies are summarized in Table 4. Because the pooled data was derived from 9 institutions, no single technique was used for both procedures, but similar principles were applied. In LDP, the gland was divided by staplers and in ODP, stapler or scalpel + suture was used. In some cases, bio-sealant was attached to the stump reported by Baker, DiNorcia, Kim and Aly[23,24,27,30].

Postoperative outcome

Three studies[23,26,30] contained information about time to post-operative fluid intake. Meta-analysis of the pooled data showed that time to fluid intake was shorter in LDP than in ODP [random effects model, WMD -0.948 (-1.863, 0.032), P = 0.042] (Figure 2B). Another three studies[23,26,28] reported the postoperative hospital stay. The pooled data showed that postoperative hospital stay was significantly shorter in LDP than in ODP [random effects model, WMD -2.713 (-3.799, 1.628), P = 0.00] (Figure 3B).

The proportion of malignant tumors reported by four articles[24,26,28,31] was 20% (36/180) in LDP and 20.1% (54/269) in ODP, and most of them were adenocarcinomas. The proportion of malignant tumors in LDP was not significantly different as compared with ODP [fixed effects model, RR 1.036 (0.708, 1.516), P = 0.000, I^2 = 0%] (Figure 3C). There was no difference in patient selection between the two groups.

All studies illustrated the criteria for PF. Seven articles followed the criteria by International Study Group for Pancreatic Fistula (ISGPF): any measurable volume of fluid on or after postoperative day 3 with an amylase level > 3 times that of normal serum amylase level. Eom *et al*[28] used the following PF criteria: drainage exceeding 30 mL with an amylase level > 600 U/dL on or after postoperative week 1. Kim *et al*[30] chose the PF criteria: a level of drain amylase five times greater than the serum level and drainage of more than 30 mL 5 d or longer after the operation.

One study was excluded[31] due to no available data, 8 studies reported 50 (12.5%) PF cases in 401 LDP (12.5%) and 99 (13.4%) PF cases in ODP. The pooled data showed no significant difference between the two groups [random effects model, RR 0.996 (0.663, 1.494), P = 0.983, I^2 = 28.4%] (Figure 2C) and no publication bias was found by Begg's test (Figure 4). Similarly, there was no significant difference in the overall morbidity between LDP and ODP [random effects model, RR 0.81 (0.596, 1.101), P = 0.178, I^2 = 55.6%] (Figure 5), although there was moderate heterogeneity.

DISCUSSION

Since Cuschieri and Gagner[32,33] documented the earliest attempts at LDP in humans, there have been an increasing number of reports indicating the advantages of LDP of minimal trauma, rapid recovery, and shorter hospital stay. But due to the high postoperative morbidity and a high level of laparoscopic technical requirements and extensive experiences in open pancreatic surgery, the progression of LDP was considerably restricted. In particular, the randomized clinical trials (RCT), which are the ideal objects of meta-analysis, have been extremely difficult to achieve. The published articles comparing LDP and ODP were all retrospective studies with common defects such as long-term research, small number of cases and incomplete data. With the development of surgical technology, potential bias and inappropriate results could be produced from the recent literature analyzed with the earlier clinical data. Furthermore, due to a relatively low incidence of left pancreas diseases, there are few LDP

Table 2 Characteristics of the literatures

Ref.	Study year	Nation	Case number		Study type	Pancreatic transection		Spleen persevation		Total morbidity		PF		Mortality %		Level of evidence
			LDP	ODP		LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	
Aly *et al*[23]	1998-2009	Japan	40	35	Retro	Stapler	Stapler/ scalpel + suture	13	3	8	11	5	6	0	0	4
Baker *et al*[24]	2003-2008	USA	27	85	Pros	Stapler/ scalpel + micro sealer device	Scalpel + suture	NA		10	30	6	12	0	0	2b
Butturini *et al*[25]	1999-2006	Italy	43	73	Retro	Stapler	Scalpel + suture	19	8	21	33	12	10	0	0	2b
Casadei *et al*[26]	2000-2010	Italy	22	22	Case control	Stapler	Stapler	NA		6	6	2	4	0	0	2b
DiNorcia *et al*[27]	1991-2009	USA	71	192	Retro	Stapler	Stapler/ Scalpel + suture	11	30	20	84	8	27	0	2	4
Eom *et al*[28]	1995-2006	Korea	31	62	Retro	Stapler	Scalpel + suture	13	NA	11	15	3	4	0	0	4
Jayaraman *et al*[29]	2003-2009	USA	74	236	Retro	Stapler/ scalpel + suture	Stapler/ scalpel + suture	14	33	11	94	6	31	NA		4
Kim *et al*[30]	NA	Korea	93	35	Retro	Stapler	Stapler/ Scalpel + suture	38	2	23	11	8	5	NA		4
Vijan *et al*[31]	2004-2009	USA	100	100	Retro	Stapler	NA	25	NA	34	29	17	17	3	1	4

Retro: Retrospective observational study; Pros: Prospective observational study; LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy; PF: Pancreatic fistula; NA: Not available.

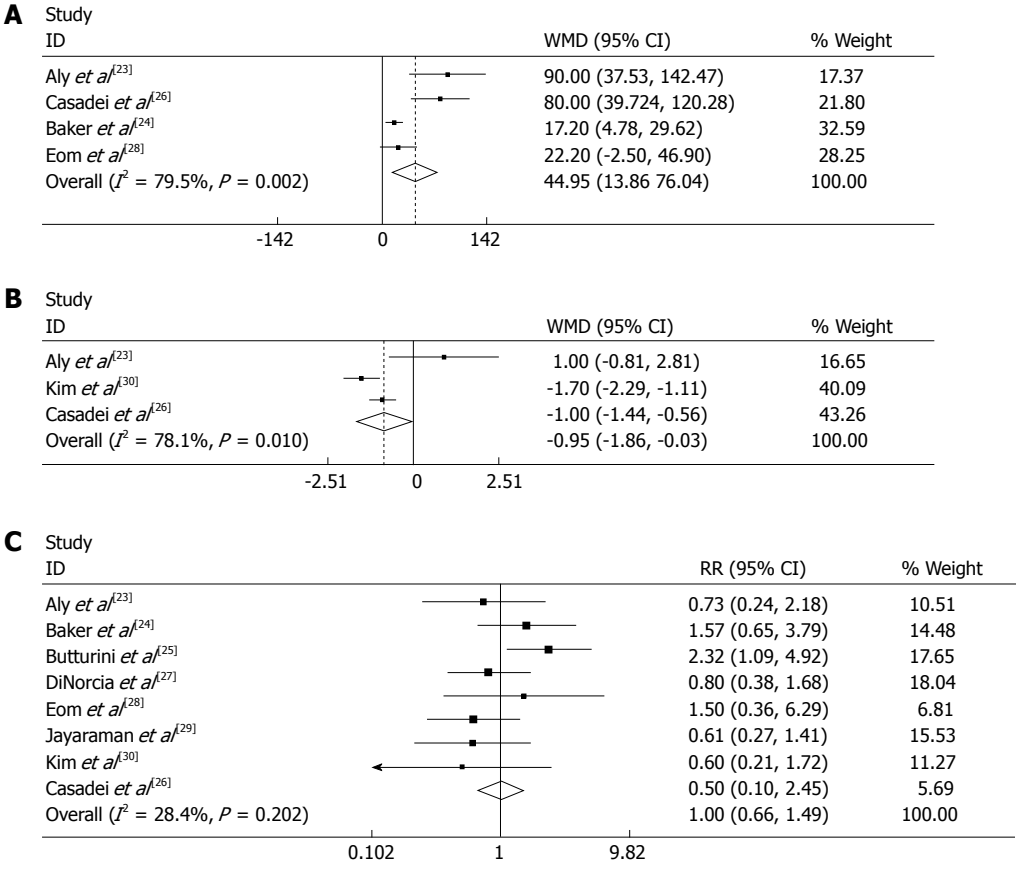

Figure 2 Meta-analysis of the pooled data. A: operative time was significant ly shorter in open distal pancreatectomy (ODP) than in laparoscopic distal pancreatectomy (LDP) [random effects model, WMD 44.947 (13.857, 76.037), P = 0.005]; B: Time for fluid intake was shorter in LDP than in ODP [random effects model, WMD -0.948 (-1.863, 0.032), P = 0.042]; C: Pancreatic fistula occurrence has no significant difference between LDP and ODP [random effects model, RR 0.996 (0.663, 1.494), P = 0.983, I^2 = 28.4%]. Weights are from random effects analysis. CI: Confidence interval; RR: Risk ratio; WMD: Weighted mean differences.

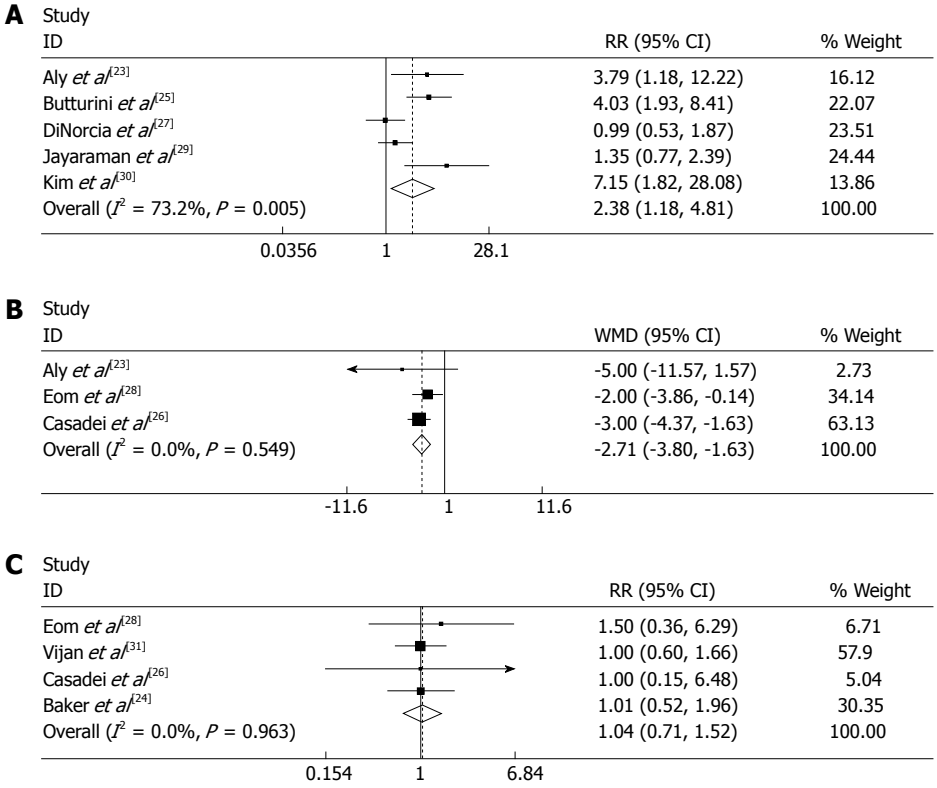

Figure 3 The pooled data. A: The spleen-preserving rate of laparoscopic distal pancreatectomy (LDP) was significantly higher than open distal pancreatectomy (ODP) [random effects model, RR 2.380 (1.177, 4.812), P = 0.016, I^2 = 73.2%]; B: Postoperative hospital stay was significantly shorter in LDP than in ODP [random effects model, WMD -2.713 (-3.799, 1.628), P = 0.00]; C: The proportion of malignant tumors showed no significant difference between LDP and ODP [fixed effects model, RR 1.036 (0.708, 1.516), P = 0.000, I^2 = 0%]. Weights are from random effects analysis. CI: Confidence interval; RR: Risk ratio; WMD: Weighted mean differences.

Table 3 Technical details of spleen-preservation

Ref.	Spleen preserving %		Technical details
	LDP	ODP	
Aly *et al*[23]	32.5	8.6	Both procedures, spleen vessel ligation were performed, leaving the short gastric vessels to supply the spleen (Warshaw)
Baker *et al*[24]	NA		In ODP, the benign and premalignant pathology, the spleen was routinely saved by means of the splenic vein and artery preserved In LDP, splenic salvage by means of Warshaw: ligating the splenic artery and vein but preserve the short gastric vessel
Butturini *et al*[25]	44.2	11.0	Both procedures, exposing the splenic vein up to the splenic hilum; the distal pancreas was detached from the splenic artery in the opposite direction by tractioning the parenchyma
Casadei *et al*[26]	NA		Mobilization of the distal pancreas from retroperitoneum and splenic vessels
DiNorcia *et al*[27]	15.5	15.6	For spleen preserving distal pancreatectomy, an attempt to spare the splenic artery and vein was made in all patients
Eom *et al*[28]	41.9	NA	For spleen preserving distal pancreatectomy, both the splenic artery and vein were preserved
Jayaraman *et al*[29]	18.9	14.0	When splenic preservation was performed, the splenic vein and artery were isolated
Kim *et al*[30]	40.9	5.70	In spleen preserving distal pancreatectomy, both the splenic artery and vein were preserved. In one case, the splenic artery was ligated with preservation of splenic vein. In the other case, both the splenic artery and vein were ligated, with preservation of short gastric vessels (Warshaw)
Vijan *et al*[31]	25	NA	If splenic preservation is indicated, the pancreas is dissected off the splenic vessels

LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy; NA: Not available.

studies with large sample sizes. Therefore, with strictly defined inclusion and exclusion criteria, we performed a comprehensive analysis to assess the current status of LDP *vs* ODP.

The proportions of malignant tumors were 20% in both LDP and ODP, which showed no difference in the patient selection between the two groups. DP was most frequently used in the treatment of benign or borderline tumors, which was in agreement with previous studies[34,35] using either "laparoscopy" or "open".

The most important indicators to represent the operative effect were shorter operative time, time to fluid

Table 4 Technique of pancreatic stump closure

Ref.		Technique description
Aly *et al*[23]	LDP	The pancreatic parenchyma was transected using a laparoscopic linear stapler
	ODP	The pancreatic parenchyma was transected using a scalpel, and the main pancreatic duct was ligated using nonabsorbable sutures. The pancreatic stump was closed with fish-mouth sutures. A linear stapler was used to transect the pancreatic parenchyma
Baker *et al*[24]	LDP	The gland was divided by one of 3 mechanisms: vascular stapler, harmonic scalpel, or harmonic scalpel following ablation at the pancreatic resection margin with the Habib 4*3 microsealer device
	ODP	Directly ligate the pancreatic duct when visible with a monofilament absorbable suture. The neck of the gland was oversewn with nonabsorbable monofilament suture
Butturini *et al*[25]	LDP	The pancreatic body was transected by a linear endostapler
	ODP	Pancreatic parenchyma was sharply transected. The main pancreatic duct was closed with nonabsorbable sutures (polypropylene 4/0). Subsequently the pancreatic stump was oversewn with interrupted mattress nonabsorbable sutures or closed using a linear stapler
Casadei *et al*[26]	LDP	The pancreas was divided at the neck using an endo-GIA instrument
	ODP	The pancreas was divided using GIA 55
DiNorcia *et al*[27]	LDP	Sutures, staples, sutures and staples combined, or staples with bioabsorbable staple-line reinforcement
	ODP	
Eom *et al*[28]	LDP	The pancreas was transected using the 48- or 35-mm vascular endoscopic linear stapler
	ODP	The pancreatic parenchyma was divided using a blade and electrocautery. The main pancreatic duct was ligated with nonabsorbable sutures, and the transected pancreas was occluded with interlocking interrupted mattress sutures of 4-0 black silk and reinforced with 4-0 polypropylene
Jayaraman *et al*[29]	LDP	The pancreas was stapled using a vascular stapler with or without a Seamguard attachment
	ODP	Ligate pancreas with staples, or via suture ligation, or a combination of techniques
Kim *et al*[30]	LDP	For pancreatic transaction, straight endoscopic linear staplers of various sizes (staple height, 3.5-4.2 mm) were used according to the thickness or hardness of the pancreas. Four or five small titanium clips were applied along the stapling line
	ODP	The pancreatic stump underwent main duct ligation, multiple suture ligation of the branch duct exposed at the resection margin, and reinforcement of the mattress suture to the pancreas stump
Vijan *et al*[31]	LDP	The pancreatic parenchyma is divided with the harmonic scalpel (preferred) or with an Endo GIA stapler
	ODP	NA

LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy; NA: Not available; GIA: Gastrointestinal incision anastomose.

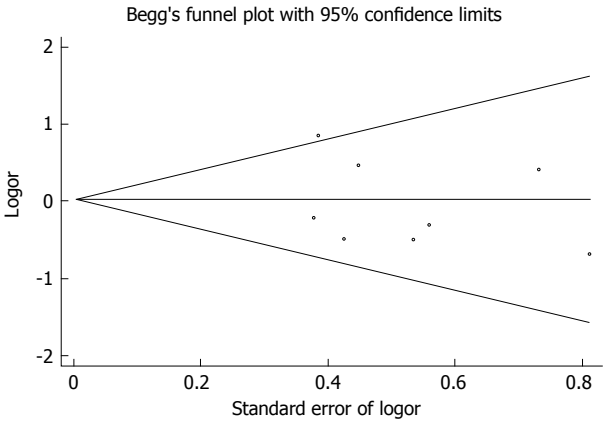

Figure 4 Begg's test showing no publication bias of pancreatic fistula occurrence.

intake and hospital stay, and less blood loss, low conversion rate and high spleen-preservating rate. These indicators have demonstrated the safety and feasibility of the laparoscopic procedures. In this meta-analysis, the operative time of LDP was longer than ODP (WMD 44.947, $P = 0.005$), but a recent research showed that operative time is becoming shorter with the improved expertise of surgeons[17]. And the time to fluid intake and postoperative hospital stay were also shorter in LDP than in ODP (WMD -0.948, $P = 0.042$) and (WMD -2.713, $P = 0.00$). Blood loss estimate was not conducted in this study because of different numeric types. The results of the included articles showed that blood loss was less in LDP than in ODP, which were similar with other literatures[18,19]. In addition, conversion rate of LDP from the pooled data showed a low level of 14% in 714 LDPs because of severe bleeding and abdominal adhesion[14].

The rate of spleen-preservation ranged from 15.5% to 44.2% in LDP and from 5.7% to 15.6% in ODP as shown in Table 3. Kimura method was more frequently used as compared with Warshaw method used when intraoperative bleeding, adhesion, and blood vessels embedded by the tumors occurred. Effects of the two surgical methods have long been a concern. Rodriguez *et al*[36] retrospectively reported Kimura method used in 185 cases compared with Warshaw method in 74 cases of LDP from 1994 to 2004; the two groups had no statistically significant difference in occurrence of ascites (9% *vs* 8%), intra-abdominal abscess (14% *vs* 8%), pancreatic leakage (33% *vs* 36%) and incision complications (10% *vs* 8%). Although Warshaw method was proved to be sufficiently safe[37,38], due to individual differences of the short gastric vessels, spleen relied entirely on the short gastric blood vessels which inevitably brought some uncertainties. In the event of severe splenic infarction, reoperation was often required. So spleen-preservation by Kimura method was widely accepted in LDP, but under some special conditions, such as bleeding, adherent tumor and difficult anatomy, Warshaw method could elevate the spleen-preserving rate. In this meta-

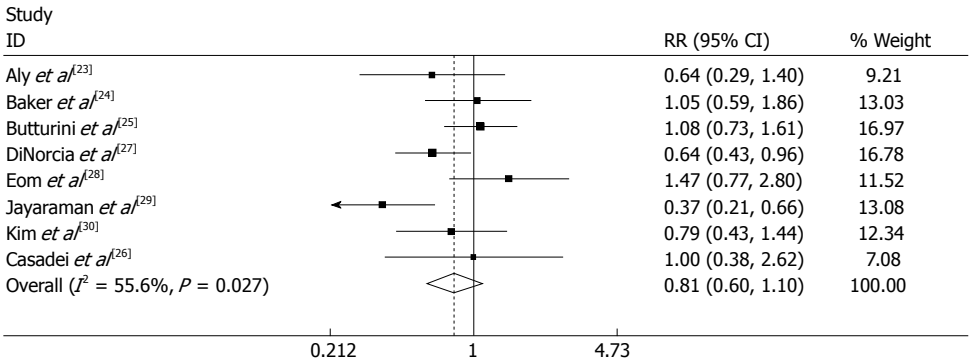

Figure 5 There was no significant difference in overall morbidity between laparoscopic distal pancreatectomy and open distal pancreatectomy [random effects model, risk ratio 0.81 (0.596, 1.101), P = 0.178, I^2 = 55.6%] and there was moderate heterogeneity. Weights are from random effects analysis. CI: Confidence interval; RR: Risk ratio.

analysis, five articles described the spleen-preserving DP, the pooled data showed that the spleen-preserving rate of LDP was significantly higher than that of ODP (RR 2.380, P = 0.016, I^2 = 73.2%). Although there was moderate heterogeneity, spleen-preservation occurred more often in LDP. The reasons for the high spleen-preserving rate in LDP may be as follows: (1) surgeons in different stages of learning curve may achieve different clinical outcomes. In the early period, because of the immature LDP technique, especially laparoscopic vascular treatment, fewer cases of LSPVP were performed; and (2) many cases of ODP without spleen-preservation were included in each study, leading to a low spleen-preservation rate of ODP.

PF was the most important complications after DP which resulted in serious consequences such as extended hospital stay, poor quality of life, even intra-abdominal bleeding, and infection. Although at some high-volume centers, PF after DP has declined over the past decade, the incidence of PF still kept from 5% to 30%[39-41]. In this study, a large variation in the PF rate was recorded, ranging from 8.1% to 27.9% in LDP and 6.5% to 18.2% in ODP. The major reason for the variability may be lacking uniform criteria for PF. The diagnostic criteria of PF were generally based on clinical signs and laboratory indicators, including the occurrence time, the daily amount of leakage, leakage amylase, the duration, *etc*. The ISGPF criterion[42] was most frequently used, but it failed to explain whether the drainage amount was related to the diagnosis of PF. Because of the lack of different quantitative indicators, other criteria were also questioned[43,44].

The original disease, pancreatic transection, pancreas texture, blood supply, and stump closure are factors affecting the incidence of PF. Recently, body mass index > 25 kg/m^2 was also reported contributing to the increased incidence of PF after DP[45]. However, the treatment of pancreatic stump is a unique controllable factor for preventing PF. In order to reduce the incidence of PF, a variety of stump closure techniques were applied or used in combination, but the coexistence of methods may reflect the lacking of a widely accepted and effective method. In this study, stapler was used in LDP while both stapler/scalpel + suture were used in ODP. The surgeons could choose different staplers according to the pancreatic texture and size in LDP. And in some groups, small titanium clips were applied along the stapling line[30] and fibrin glue was splashed over the pancreatic stump in an attempt to prevent PF and postoperative bleeding[23,24,27,30]. Subset analysis could not be accomplished as no detailed data was available. A published meta-review analyzed 16 articles with 2286 patients who underwent DP and compared the preventive effect for PF between 671 cases with stapler closure and 1615 cases with suture closure. The results showed no significant differences between suture and stapler closure of the pancreatic remnant with respect to the PF or intra-abdominal abscess[46]. Likewise, the pooled data of this study showed no significant difference both in the incidence of PF (RR 0.996, P = 0.983) and overall morbidity between LDP and ODP (RR 0.81, P = 0.178).

The authentication strength of this study may be affected by the following factors: (1) publication bias: some gray literatures which contained negative results were difficult to obtain because most authors tended to show positive results; (2) grouping bias: notwithstanding the literatures dealing with significantly different diseases and surgical methods have been excluded in this study, in practice, patients should be grouped inevitably according to the disease condition and surgeons' choices; and (3) observation bias: due to the varied measurement methods used by different authors, significantly different results were almost inevitable in the non-RCT or non-blind RCT studies.

In summary, LDP has shown the advantages of intraoperative effects, rapid recovery and spleen-preservation for benign and borderline tumors. But the superiority has not been displayed in preventing the overall morbidity and occurrence of PF. Thus, the RCT studies with a large sample size should be conducted and new surgical techniques should be introduced in future studies.

COMMENTS

Background

Laparoscopic distal pancreatectomy (LDP) is becoming a primary treatment

modality for benign or borderline tumors of distal pancreas. But due to the high postoperative morbidity and a high level of laparoscopic technical requirements and extensive experiences in open pancreatic surgery, the progression of LDP was considerably restricted.

Research frontiers

Recently, several studies have shown shorter hospital stay and operative time and less intraoperative blood loss in LDP. But the efficacy of LDP compared with open distal pancreatectomy (ODP) required further assessment.

Innovations and breakthroughs

Pancreatic fistula (PF) and spleen-preservation in LDP have been the major concern after the surgery. What the role of "laparoscopy" in the spleen preservation and PF prevention is unclear. In this meta-analysis, the authors pointed out that LDP has the advantages of shorter hospital stay and operative time, more rapid recovery and higher spleen-preserving rate compared with ODP.

Applications

Due to a relatively low incidence of left pancreas diseases, fewer LDP studies with a large sample size have been published. Especially the RCT clinical study, which is the ideal object of meta-analysis, is extremely difficult to accomplish. This meta-analysis assessed the safety and feasibility of LDP compared with ODP based on the review of the literature published over the past 15 years.

Peer review

This paper is a systematic review of the laparoscopic distal pancreatectomy. The summary of LDP experiences and results is interesting.

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BRIEF ARTICLE

A prospective randomized trial of transnasal ileus tube *vs* nasogastric tube for adhesive small bowel obstruction

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Abstract

AIM: To study the therapeutic efficacy of a new transnasal ileus tube advanced endoscopically for adhesive small bowel obstruction.

METHODS: A total of 186 patients with adhesive small bowel obstruction treated from September 2007 to February 2011 were enrolled into this prospective randomized controlled study. The endoscopically advanced new ileus tube was used for gastrointestinal decompression in 96 patients and ordinary nasogastric tube (NGT) was used in 90 patients. The therapeutic efficacy was compared between the two groups.

RESULTS: Compared with the NGT group, the ileus tube group experienced significantly shorter time for relief of clinical symptoms and improvement in the findings of abdominal radiograph (4.1 ± 2.3 d *vs* 8.5 ± 5.0 d) and laboratory tests ($P < 0.01$). The overall effectiveness rate was up to 89.6% in the ileus tube group and 46.7% in the NGT group ($P < 0.01$). And 10.4% of the patients in the ileus tube group and 53.3% of the NGT group underwent surgery. For recurrent adhesive bowel obstruction, ileus tube was also significantly more effective than NGT (95.8% *vs* 31.6%). In the ileus tube group, the drainage output on the first day and the length of hospital stay were significantly different depending on the treatment success or failure ($P < 0.05$). The abdominal radiographic improvement was correlated with whether or not the patient underwent surgery.

CONCLUSION: Ileus tube can be used for adhesive small bowel obstruction. Endoscopic placement of the ileus tube is convenient and worthy to be promoted despite the potential risks.

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Key words: Adhesive; Small bowel obstruction; Ileus tube; Nasogastric intubation; Gastrointestinal decompression

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INTRODUCTION

Gastrointestinal decompression is the most effective approach to treat the patients with acute bowel obstruction without any indications of strangulation[1,2]. The traditional nasogastric tube (NGT) is not long enough for suctioning the distal intestine and its decompression efficacy is relatively low. Since the 1930s, the concept of nasointestinal decompression and use of nasointestinal tubes have been developed and applied in clinical practice. Some studies have verified the efficacy of long nasointestinal tubes in treating adhesive small bowel obstructions (SBO)[3-7]. However, a prospective randomized trial demonstrated no significant differences with regard to the decompression achieved, the success of non-surgical treatment, or the morbidity rate after surgical intervention as compared with the use of short NGT[8]. In 2003, a new hydrophilic silicon triple-lumen ileus tube was first introduced and used in Japan for nasointestinal decompression. It could be advanced through the gastroscope in shorter time with a higher tolerance[9]. Up till now, there has been no randomized controlled study about the efficacy of the ileus tube. This randomized controlled trial attempted to investigate and compare the decompression efficacy between the new ileus tube and the traditional NGT for patients with adhesive SBO.

MATERIALS AND METHODS

Patients

Approved by the hospital's ethics committee, a total of 186 patients with acute adhesive SBO who were admitted to the Gastroenterology and Colorectal Surgery wards of the First Affiliated Hospital, College of Medicine, Zhejiang University and its Ningbo Branch Hospital from September 2007 to February 2011 were enrolled into this study. The entry criteria were as follows: (1) clinical symptoms and physical signs arising from acute bowel obstruction; (2) a diagnosis of adhesive SBO based on abdominal plain films and computed tomography (CT) scans confirmed by at least two attending radiologists; and (3) admission to the hospital within 12 h after bowel obstruction onset. All patients who presented with symptoms of fever, vomiting or hematemesis, hematochezia, severe or sudden abdominal pain, and the signs of tachycardia, leukocytosis, abdominal tenderness, peritoneal irritation, asymmetric abdominal distension or isolated swelling bowel loops and even shock, should be suspected of strangulation obstruction, which needed immediate operation. Besides, patients who had contraindications for endoscopy, or with postoperative adynamic obstruction or malignancy, or who had been treated in other hospitals before admission were excluded. Patients were randomized into two groups by the sealed envelope method: the ileus tube group and the NGT group; an opaque box containing an equal number of envelopes that indicated either ileus tube or NGT, was used for randomization. Written informed consents were obtained from all the patients before enrollment. This trial conformed to the provisions of the World Medical Association Declaration of Helsinki. Ileus tube was used in 96 patients (56 men and 40 women) for gastrointestinal decompression; their ages ranged from 21 to 86 years (mean, 58 years). Among the 96 patients, 25 had a history of prior adhesion, and 89 patients received prior abdominal surgery. Ninety patients (56 men and 34 women) treated with NGT served as the control group; their ages ranged from 19 to 86 years (mean, 54 years). Among the 90 patients, 38 had a history of prior adhesion, and 86 had a history of abdominal surgery.

Patient preparation

On admission, all patients underwent abdominal plain film radiography and CT scanning to confirm acute bowel obstruction. Patients requiring emergency surgery were excluded. Performed by the same technicians, the ileus tube was advanced endoscopically and the traditional NGT was inserted for gastrointestinal decompression at a similar negative pressure level for constant suction. For all patients, routine laboratory blood tests were performed, and C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR) were determined.

Instrument and procedures

The CLINY Ileus Tube suite (Create Medic, Tokyo, Japan) and the ordinary NGT (Terumo Medic, Hangzhou, China) were used. The ileus tube is 300 cm in length and 16 Fr with three channels (suction channel, injection channel and balloon channel) and two balloons (anterior balloon and posterior balloon). Beside the tip hole, there are side holes in the distal end of the tube. Compared with other long tubes, this tube has weighted tip which consists of several metal balls for easier advancing. The posterior balloon is intended for contrast radiography. Water and contrast medium can be injected into the tube for lavage and imaging. Under some instances, the tube can directly remove the obstruction by its weighted tip. The guidewire is 350 cm long and 1.24 mm in diameter. The NGT is 110 cm in length and 16 Fr. All patients received gastrointestinal decompression within 12 h after admission. In the ileus tube group, the tube was pre-set through the nasal cavity to the stomach. The guidewire was inserted into the main channel to the tips. After endoscopic suction of stomach contents, the wire was moved into the descending duodenum by forceps, and the tube was inserted into the duodenum while the wire was kept fixed (Figure 1). Then the anterior balloon was inflated with 20 mL distilled water. The gastroscope was withdrawn after the long tube was fixed to the cheek. The tube was propelled by bowel peristalsis and its weighted tip, and the outside terminal of the tube was connected to a spontaneous negative pressure bag. Intermittent lavage (twice a day) through the long tube was performed from the second day after intubation, and the length of the advanced tube was carefully measured. In the control group, the NGT was inserted from nose to stomach to

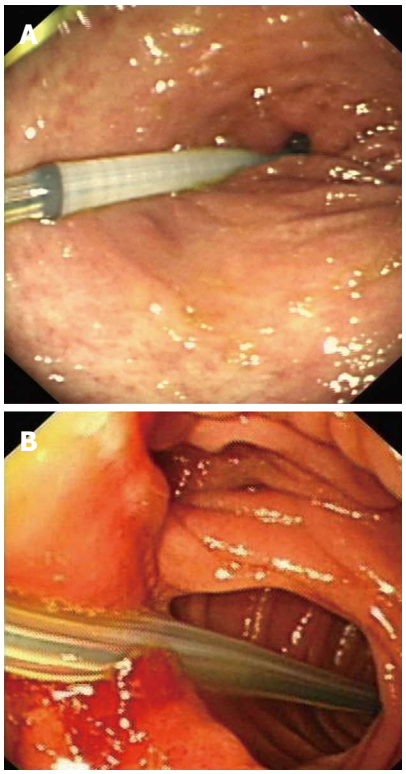

Figure 1 Endoscopic placement of ileus tube. A: An ileus tube is passed through the pylorus under gastroscopy in a patient with postoperative adhesive small bowel obstruction; B: An ileus tube led by a guidewire is endoscopically advanced into the efferent loop in a patient who had distal partial gastrectomy.

a depth of 45-55 cm. All patients were supported with total parenteral nutrition and received nothing by mouth. Emergency surgery was performed when the patient was suspected to have developed bowel ischemia. The potential risks for endoscopic placement of the ileus tube were throat injury, upper gastrointestinal perforation and bleeding, aspiration pneumonia and cardiovascular events.

Outcome measurement

We compared the clinical and laboratory variables before intubation, including age, sex, type of prior surgery, symptoms and physical signs, and laboratory indexes on admission between the two groups. Physical examination (every 2 h), laboratory test (once a day) and abdominal imaging (days 2-7) were conducted frequently after intubation. The clinical and laboratory findings included: the time for relief of abdominal symptoms; time for abdominal radiographic improvement and recovery of white blood cell (WBC) counts, CRP level and ESR; drainage volume on the first day; surgery rate, the overall efficacy and the different therapeutic responses to the two kinds of tubes. The therapeutic effectiveness in the ileus tube group was defined as clinical or radiological improvement, relief of abdominal symptoms, decreased drainage volume, disappearance of air-fluid levels or reduced gas and fluid in bowel loops. Oral feeding was then administered gradually and the tube was removed. If the patient presented no improvement 72 h after decompression, or even progressed into strangulation, surgery is recommended[4]. In the NGT group, if a fairly prompt response occurs within 48 h, especially within the first 8-12 h after nasogastric decompression and resuscitation, the obstruction will probably be resolved without surgery[10,11]. Surgery was recommended if patients showed no response 72 h after non-surgical treatment, which was defined as treatment failure[12], otherwise, conservative treatment was continued.

Sample size calculation

Determination of the sample size was based on the previous studies[4,9]. The effectiveness rate was 51% in the short tube decompression while 85.7% in the long nasointestinal tube. If the effectiveness rate of the NGT and the ileus tube was defined as 50% and 80%, respectively, a study with 48 patients per group would have a 90% power to detect a difference at a two-sided significance level of 0.05. We extended our sample size to account for potential dropouts

Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc, Chicago). Results were expressed as mean ± SD. The χ^2 test was used to identify differences in the effectiveness rate between the two groups. The Student's *t* test was used for unpaired data to determine differences in means between the two groups. Odds ratios (ORs) were determined by logistic regression analysis. Two-tailed *P* value of < 0.05 was considered statistically significant.

Study limitations

This study was designed as a randomized controlled trial (RCT), but it was not double-blinded. No standard criteria are available for the treatment success by this long tube in the literature. The two kinds of tubes were placed by different methods, while the patients in ileus tube group suffered more during intubation.

RESULTS

Patient clinical characteristics

Of the 186 patients, 96 were treated with ileus tube, and 90 were randomized into NGT group. There was no significant difference between the two groups with regard to clinical characteristics and laboratory variables documented on admission, including age, sex, abdominal symptoms, and laboratory indexes such as WBC counts, CRP, and ESR ($P > 0.05$). The type of prior surgery and obstruction also did not differ significantly ($P > 0.05$). In this study, the ileus tube or NGT was successfully placed in all the patients, without any obvious complications. Patient characteristics of the two groups are shown in Table 1.

Therapeutic efficacies

The time for improvement in abdominal symptoms, ra-

Table 1 Clinical characteristics, difference decompression responses and therapeutic efficacies of ileus tube group and nasogastric tube group

Clinical characteristics	Ileus tube group (n = 96)	NGT group (n = 90)	P value	OR (95% CI)
Mean age (yr)	58	54	0.07	-
Male/female	56/40	56/34	0.59	-
Past laparotomies (n)	89	86	0.41	0.59 (0.17–2.09)
Surgery type				
Colorectal surgery	35	28	0.35	1.34 (0.72–2.50)
Small-bowel resection	15	14	0.92	1.04 (0.47–2.31)
Gastroduodenal surgery	8	12	0.3	0.61 (0.24–1.57)
Appendectomy	8	6	0.62	1.32 (0.44–3.97)
Gallbladder and pancreas surgery	4	4	1	0.97 (0.23–3.99)
Splenectomy	3	4	0.96	0.72 (0.16–3.29)
Bladder or kidney surgery	2	2	1	0.97 (0.13–7.01)
Gynecologic surgery	14	16	0.61	0.82 (0.37–1.80)
Symptoms on admission (n)				-
Abdominal pain	85	82	0.56	0.75 (0.29–1.97)
Distention	93	85	0.65	1.82 (0.42–7.86)
Nausea or vomiting	58	65	0.09	0.59 (0.32–1.09)
Disappearance of flatus and defecation	84	72	0.17	1.75 (0.29–3.88)
Laboratory data before intubation (n)				
Elevated WBC count	70	67	0.81	0.92 (0.48–1.78)
Elevated CRP level	37	42	0.26	0.72 (0.40–1.29)
Elevated ESR level	31	27	0.74	1.11 (0.60–2.07)
Therapeutic efficacies (%)				
Rate of abdominal pain or distention relieved within 48 h	95.8 (92/96)	46.7 (42/90)	< 0.01 (χ^2 = 55.75)	26.29 (8.90–77.66)
Surgery rate	10.4 (10/96)	53.3 (48/90)	< 0.01 (χ^2 = 39.87)	0.10 (0.05–0.22)
Effectiveness rate for recurrent adhesive small bowel obstruction	95.8 (24/25)	31.6 (12/38)	< 0.01 (χ^2 = 25.55)	52.00 (6.28–430.67)
Total effectiveness rate	89.6 (86/96)	46.7 (42/90)	< 0.01 (χ^2 = 39.87)	9.83 (4.53–21.33)
Differences in decompression responses by ileus tube and NGT (mean ± SD)				t
Time for relief of abdominal pain or distention (h)	23.8 ± 10.9	59.1 ± 30.1	< 0.01	-10.4
Appearance of flatus and defecation (d)	2.4 ± 1.7	6.5 ± 3.2	< 0.01	-10.4
Time to abdominal radiographic improvement (d)	4.1 ± 2.3	8.5 ± 5.0	< 0.01	-6.9
WBC recovery (d)	4.0 ± 2.4	7.0 ± 4.8	< 0.01	-4.6
CRP recovery (d)	5.5 ± 2.5	8.8 ± 3.9	< 0.01	-4.4
ESR recovery (d)	5.8 ± 2.4	8.7 ± 3.9	< 0.01	-4.1
Drainage volume on the first day (mL)	698 ± 428	280 ± 167	< 0.01	8.9

CI: Confidence interval; CRP: C-reaction protein; ESR: Erythrocyte sedimentation rate; NGT: Nasogastric tube; OR: Odds ratio; SD: Standard deviation; WBC: White blood cells.

diographic findings, and laboratory variables was significantly shorter ($P < 0.01$) in the ileus tube group as compared with the NGT group. In addition, more patients had relief from abdominal pain or distention within 48 h in the ileus tube group ($P < 0.01$). The drainage volume on the first day after intubation was 698 ± 428 mL in the ileus tube group and 280 ± 167 mL in the NGT group, with a significant difference ($P < 0.01$, $t = 8.9$). After ileus tube decompression, 86 patients presented clinical or radiographic relief (Figure 2), the tube was removed one week after oral feeding was started, with an effectiveness rate up to 89.6% (86/96). The other 10 patients defined as treatment failure by gastroenterography underwent operation to determine the site of the obstruction (Figure 3). In follow-up study, 6 patients still had recurrent adhesive SBO confirmed by surgery, the intervals varied from one month to seven months. In the control group, the total effectiveness rate was 46.7% (42/90); the other 48 patients defined as treatment failure were managed with surgery. However, 16 patients had recurrent adhesive SBO after successful treatment, the recurrence peak occurred between 3 mo and 5 mo (Table 1).

Therapeutic outcome in ileus tube group treated with or without surgery

In the ileus tube group, 10 patients without initial relief underwent surgery to remove the obstruction. Significant differences were found in the drainage output on the first day and the length of hospital stay ($P < 0.05$). Besides, no patient showed abdominal radiographic improvement within 72 h in decompression in the surgical group as compared to 51.2% (44/86) in the non-surgical group. These results are summarized in Table 2.

DISCUSSION

Conservative treatment is usually administered to the patients with acute bowel obstruction when ischemic bowel is excluded. Surgeons are inclined to choose conservative treatment for adhesive bowel obstruction because of the risk of recurrence along with surgery[13]. Since the 1930s, various types of tubes have been devised and used for nasointestinal decompression[14,15]. A tube was designed specifically for endoscopic placement and the ileus tube has been developed with three channels and two bal-

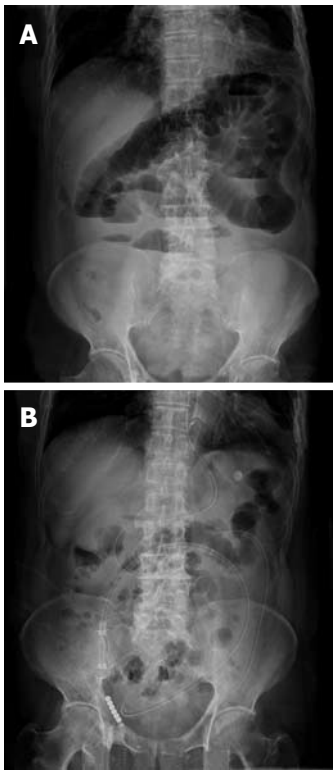

Figure 2 Radiographs of ileus tube decompression. Plain abdominal radiographs (A) and (B) reviewed 3 d after ileus tube decompression compared with scans on admission in a patient with postoperative adhesive small bowel obstruction. A: The diffuse distended loops of small bowel that was filled with gas and fluid before intubation; air-fluid levels were seen in the enteric cavity; B: Reviewed 3 d after intubation; the previous gas-filled or fluid-filled small bowel loops showed no evidence of distention, the air-fluid levels disappeared, and the long tube had moved downward while the tip reached the distal jejunum.

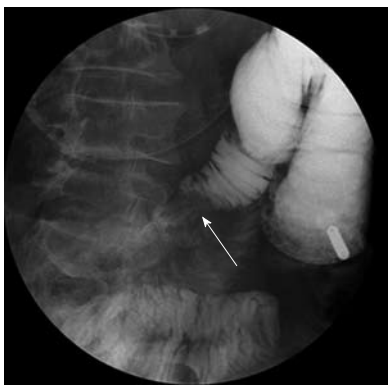

Figure 3 Diagnostic radiographic enteroclysis. Gastroenterography displayed on the 5th day of ileus tube decompression in a patient with postoperative adhesive small bowel obstruction. The tip of the tube had reached to the distal jejunum. After the anterior balloon was inflated with gas, angiografin was injected into the tube for gastrointestinal imaging to locate the lesion or stenosis in the bowel. Stenosis was found (arrow) in the small intestine with a filling defect, but none was developed in the distal bowel.

loons. A study confirmed an efficacy rate of ileus tube of up to 85.7% for intraluminal decompression in the bowel[9]. Intubation methods then changed from fluoroscopy to direct placement under endoscopy and afforded safety and high success rates[16-21]. However, if there is any side effect along with the endoscopic placement procedure, the tube should be pulled out and endoscopic treatment for gastrointestinal bleeding or perforation should be given if possible. If patients have aspiration pneumonia, antibiotics and mechanical ventilation should be considered. Intensive care and emergent therapy are needed for any cardiovascular event. Our clinical practice testified the safety and flexibility of the endoscopic placement of the ileus tube, and the procedure and the instruments we used are available in most hospitals. However, the long tube and endoscopy cost more than ￥4000 RMB, that is 20 times more than an ordinary NGT, even though it is much lower than surgery. The cost as well as the discomfort caused by endoscopy may limit the promotion of the use of ileus tube.

Table 2 Therapeutic outcomes in ileus tube group treated with or without surgery (mean ± SD)

Variables	Surgical treatment	Non-surgical treatment	*P* value	*t*
Cases (*n*)	10	86	-	-
Time for relief of distention (h)	27.6 ± 16.9	23.4 ± 10.2	0.51	0.69
Appearance of flatus and defecation (d)	3.2 ± 2.4	2.4 ± 1.7	0.27	1.12
Radiographic improvement within 72 h (*n*)	0	44	-	-
WBC recovery (d)	5.2 ± 1.9	4.0 ± 2.4	0.26	1.14
CRP recovery (d)	8.3 ± 5.0	5.3 ± 2.1	0.40	1.04
ESR recovery (d)	5.0 ± 2.8	5.9 ± 2.3	0.61	-0.51
Drainage volume on the first day (mL)	390 ± 287	734 ± 428	0.02[a]	-2.47
Length of hospital stay (d)	33.0 ± 13.7	21.5 ± 10.4	< 0.01[a]	3.19

[a]$P < 0.05$ *vs* therapeutic outcomes in ileus tube group treated with or without surgery. CRP: C-reaction protein; ESR: Erythrocyte sedimentation rate; SD: Standard deviation; WBC: White blood cells.

From the data of this study, we found a great comparability between our two groups with regard to sex, age, past laparotomies, symptoms, and radiographic signs as well as laboratory findings on admission. It is known that delayed visit to hospital leads to a higher failure in conservative measures, so we selected the patients admitted within 12 h after obstruction onset to enforce the rigidity of the study. With bowel peristalsis and the weighted tip, the ileus tube passes downward to the small bowel and decompression can be achieved. As the tip can reach to the site of obstruction, thorough suctioning can be performed, leading to a rapid relief of the symptoms, as shown in our results. In addition, the recovery time for laboratory variables of inflammatory markers was shorter in ileus tube group, probably because of the improvement in the blood supply to the bowel wall, which can reduce the local inflammatory response and bacterial multiplication. A previous study demonstrated no significant differences in therapeutic efficacy between the long and short tube decompression[8]. However, we found that in the ileus tube group the effectiveness rate was significantly higher and the surgery rate was lower than that in the control group. We attribute it to the ad-

vanced technique of the tube and the endoscopic placement method that can avoid the delay by passing beyond the pylorus. Another study confirmed that a previous episode of adhesive bowel obstruction and the duration of the tips not advancing (> 72 h) were highly correlated with a recurrence of obstruction. If patients fail to respond 3 d after decompression or have indications of ischemic bowel or the drainage volume is > 500 mL on the third day, surgery is recommended[22,23]. For NGT decompression, after 48 h of non-operative management, the risk of complications increases substantially, and the probability for resolving the obstruction diminishes. Surgery is required if a patient's condition has deteriorated or has not significantly improved within 72 h. In the ileus tube group, intermittent lavage was performed from the second day after intubation so that we could record the drainage volume on the first 24 h to compare the decompression responses with the NGT group.

We admitted relatively a large number of patients with adhesive SBO for this study. Our results showed that for adhesive SBO, the ileus tube had the decompression efficacy that was significantly superior to the NGT, especially for patients with recurrent adhesive SBO. Although a tendency toward recurrence can not be avoided after successful treatment using the ileus tube in patients with past episodes, it is still superior to the traditional NGT treatment and should be therefore recommended in clinical practice. The ileus tube has many advantages in addition to thorough decompression. It can remove the kinks in the obstructed bowel loops when the tip progresses downward, and the long tube itself can perform through a straddle mechanism to arrange the bowel and reduce the adhesion recurrence rate. Diagnostic radiographic enteroclysis studies are facilitated, which are helpful to surgeons for preoperative preparation[24,25].

Previous studies have confirmed the efficacy of nasointestinal decompression through a long tube for SBO, especially for adhesive SBO[3-7]. The approach of endoscopic placement of the long tube was also advised[15,26]. According to our clinical application, the ileus tube has a prospective therapeutic efficacy for adhesive SBO. However, surgical intervention can easily be undertaken when NGT decompression failed, because it is thought to be the most immediate modality for remission. Another focus is that water-soluble contrast agent (WSCA) is helpful in the diagnosis and treatment of adhesive SBO according to a recent meta-analysis[27], appearance of contrast in the colon within 4-24 h after administration had a sensitivity of 96% and a specificity of 98% in predicting resolution of SBO. The WSCA can draw fluid from intravascular and extracellular spaces into bowel lumen because of its high osmolarity, thus promoting proximal bowel distension and peristalsis, and avoiding the operative interference. However, as there are potential risks of renal failure and anaphylaxis, these agents still can not take the place of gastrointestinal decompression, which is thought to be the key to the treatment of bowel obstruction. Based on our results, we highly recommend this triple-lumen tube for patients with adhesive SBO. The endoscopic placement of the tube is convenient, and with close monitoring and intermittent lavage, surgeries can be avoided.

We also tried to find certain indications for surgical interventions in the patients treated with ileus tube. Compared with patients who underwent surgery in the ileus tube group, those who were successfully managed without surgery had a significantly shorter hospital stay and a larger drainage output on the first day after intubation. Up to 51.2% of the patients showed abdominal radiographic improvement within the first 3 d in the non-surgical group while no patient achieved relief in the surgical group. This indicates that the drainage output on the first day and radiographic improvement could be two independent factors for evaluating the therapeutic efficacy of nasointestinal decompression. They may also be indications for surgery. Further studies should be performed to identify the clinical value of the ileus tube within the parameters of indications for surgery.

In summary, we believe that endoscopic placement of transnasal ileus tube is safe, effective, and convenient and is worth being promoted in clinical practice. The ileus tube can quickly relieve the clinical symptoms and reduce the rate of surgical indications. It is greatly superior to the NGT in treating patients with adhesive SBO. However, the potential risks and extra costs should be taken into consideration when selecting patients. For patients with recurrent adhesive SBO, the use of triple-lumen ileus tube is the optimal choice. Close observation of drainage output and abdominal radiographic changes during decompression can help provide some clues for indications of surgery.

COMMENTS

Background

Adhesive small bowel obstruction is a worldwide problem characterized by a high incidence rate and repeated episodes. Gastrointestinal decompression is one of the important approaches in conservative therapy. However, surgical intervention can easily be undertaken when nasogastric tube decompression failed. Long intestinal tube for nasointestinal decompression is a new method for adhesive small bowel obstruction and has been successfully applied in clinical practice and the therapeutic efficacies were satisfactory.

Research frontiers

A new long tube named ileus tube was first introduced in Japan in 2003, and later studies have confirmed its therapeutic value in adhesive small bowel obstruction. However, there had been no randomized controlled study about the efficacy of the ileus tube. This randomized controlled trial attempted to investigate and compare the decompression efficacy between the new ileus tube and the traditional nasogastric tube (NGT) for patients with adhesive small bowel obstruction.

Innovations and breakthroughs

The previous studies of long intestinal tube for gastrointestinal decompression were mainly described retrospectively. This research compared the therapeutic efficacies between the new long tube and the ordinary NGT in a large number of patients. The authors confirmed the superiority of the new ileus tube to the ordinary NGT through a series of statistical analysis. The authors also introduced the detailed procedure of the endoscopic placement of this long tube.

Applications

The study indicated the therapeutic value of the new ileus tube. The application

of the ileus tube can significantly reduce the surgery rate and the hospital-stay cost, and the endoscopic placement method introduced by the authors can be applied in almost all the hospitals. Thus, the use of ileus tube is worthy to be promoted.

Peer review

This is an important area and the work represents a significant advance in clinical therapy. As it is possible not only to increase efficacy on a single intervention but also reduce the need for subsequent intervention, the technology provides the possibility to lower costs and reduce intervention to the patient.

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BRIEF ARTICLE

Analysis of infections in the first 3-month after living donor liver transplantation

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Abstract

AIM: To identify factors related to serious postoperative bacterial and fungal infections in the first 3 mo after living donor liver transplantation (LDLT).

METHODS: In the present study, the data of 207 patients from 2004 to 2011 were reviewed. The pre-, intra- and post-operative factors were statistically analyzed. All transplantations were approved by the ethics committee of West China Hospital, Sichuan University. Patients with definitely preoperative infections and infections within 48 h after transplantation were excluded from current study. All potential risk factors were analyzed using univariate analyses. Factors significant at a $P < 0.10$ in the univariate analyses were involved in the multivariate analyses. The diagnostic accuracy of the identified risk factors was evaluated using receiver operating curve.

RESULTS: The serious bacterial and fungal infection rates were 14.01% and 4.35% respectively. *Enterococcus faecium* was the predominant bacterial pathogen, whereas *Candida albicans* was the most common fungal pathogen. Lung was the most common infection site for both bacterial and fungal infections. Recipient age older than 45 years, preoperative hyponatremia, intensive care unit stay longer than 9 d, postoperative bile leak and severe hyperglycemia were independent risk factors for postoperative bacterial infection. Massive red blood cells transfusion and postoperative bacterial infection may be related to postoperative fungal infection.

CONCLUSION: Predictive risk factors for bacterial and fungal infections were indentified in current study. Pre-, intra- and post-operative factors can cause postoperative bacterial and fungal infections after LDLT.

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Key words: Bacterial infection; Fungal infection; Living donor liver transplantation

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INTRODUCTION

Despite the major advances in immunosuppressant regimens, perioperative management and medical care have contributed to improvements in the survival rate of solid organ transplant recipient, infection continues to be a leading cause of postoperative mortality and morbidity resulting from the poor preoperative condition, immunosuppressive therapy and exposure to nosocomial pathogens[1,2]. Liver transplantation has one of the highest rates

of postoperative infection among all sold organ transplant procedures[3]. It has been reported that the postoperative bacterial infection rate may up to more than 60% and accounted for an in-hospital mortality rate of 30%-50%[4]. Previous studies have reported that the incidence of postoperative fungal infection ranged from 5% to 40%, and the mortality associated with fungal infection was between 25% and 69%[5]. Moreover, the mortality of patient with *aspergillus* has been found to approach 100% if untreated[6]. Accordingly, to identify which factors may cause postoperative bacterial and fungal infections is important to transplant surgeon. However, this issue is still not well established until now. In current study, we used a large cohort to identify the pattern and risk factors associated with postoperative bacterial and fungal infections that occurred within the first 3 postoperative months after living donor liver transplantation.

MATERIALS AND METHODS

Study group

Patients who received adult-to-adult living donor liver transplantation (LDLT) from 2004 to 2011 at our center were considered in present study. All transplantations were approved by the ethics committee of West China Hospital, Sichuan University. Patients with definitely preoperative infections and infections within 48 h after transplantation were excluded from current study.

Donor selection

Donors must be healthy close relatives with compatible ABO blood types. Serological testing for viral hepatitis and human immunodeficiency virus antibodies as well as testing for other acute or chronic diseases was negative. Volumetric computed tomography with contrast was performed to evaluate the hepatic volume of the donors. Right hepatic lobe without middle hepatic vein of the donors need to be at least 0.8% of the recipient's standard weight and the remnants must be at least 40% of the donor's liver volume. Magnetic resonance cholangiopancreatography was performed to assess the anatomy of the biliary tree[7,8].

Immunosuppression

The postoperative immunosuppression consisted of tacrolimus, mycophenolate mofetil and steroids. Steroids were withdrawn as soon as possible. Acute rejection episodes were confirmed by pathology. Steroid pulse therapy was conducted to patient with rejection[9]. OKT3 monoclonal antibody was administrated to patients with persistent rejection or steroid-resistant rejection. When necessary, these treatments were repeated[10].

Infection prophylaxis

Antimicrobial prophylaxis consisted of Cefoperazone and Sulbactam for three to five days. Fluconazole was administrated to patients with risk factors of fungal infection for one to two weeks after liver transplantation.

Definitions

Serious infection was defined as the culture-positive bacterial or fungal infection in blood, sputum, urine, or ascetic fluid which was obtained on the basis of clinical suspicion of an infection[11,12]. Preoperative renal dysfunction was defined as the level of serum creatinine greater than 1.5 mg/dL[13]. Bile leak was defined as bilirubin concentration in the drainage greater than the plasma level[14]. Model for end-stage liver disease (MELD) scores were calculated according to the formula: MELD score = 9.57 × Ln creatinine (mg/dL) + 11.2 × (Ln INR) + 3.78 × Ln bilirubin (mg/dL) + 6.43[15]. Massive red blood cells (RBCs) transfusion was defined as transfusion not less than 6 packed RBCs in the first 24 h of surgery[16]. Severe hyperglycemia was defined as glucose concentrations more than or equal to 20 mg/dL[17]. Hyponatremia was defined as a serum sodium concentration of less than 130 mEq/L[18].

Statistical analysis

All continuous variables were presented as mean ± SD and compared using one way analysis of variance. χ^2 test or Fisher's exact test was used for comparing categorical variables. Independent risk factors were identified by Cox regression. Factors significant at a $P < 0.10$ in the univariate analyses were involved in the multivariate analyses. The diagnostic accuracy of the identified risk factors was evaluated using receiver operating curve (ROC). All analyses were performed using SPSS 16.0. We considered a P value of less than 0.05 to be significant.

RESULTS

Patient characteristics

A total of 207 patients were included in current study. The mean age was 42.93 ± 8.77 years for the recipients, whereas the mean age was 34.83 ± 9.99 years for the donors. Twenty-seven patients were female, whereas eighty-one donors were female. Nine patients had pretransplant diabetes mellitus. Sixteen patients suffered from preoperative renal dysfunction. The mean MELD score was 16.40 ± 9.84. The mean graft to recipient weight ratio (GRWR) was 0.94% ± 0.18%. The causes for transplantation were hepatitis B (n = 116), hepatocellular carcinoma (n = 75), hepatolithiasis (n = 3), hepatitis C (n = 3), alcoholic cirrhosis (n = 3), polycystic liver (n = 1), primary biliary cirrhosis (n = 2), hepatic hydatidosis (n = 1), huge hepatic hemangioma (n = 1), trauma (n = 1), autoimmune hepatitis (n = 1).

Pattern of infection

During the first 3 postoperative months, serious bacterial infection was observed in 29 recipients, whereas serious fungal infection was found in 9 patients. Among the 9 patients with fungal infection, 6 patients were combined with or secondary to bacterial infection. Only 3 patients infected fungal infection alone. Four patients had two kinds of bacterial infection. One patient suffered from three kinds of bacterial infection. *Enterococcus faecium* (n = 8) was the most common pathogen in patients with bacterial infec-

Table 1 Univariate analysis for risk factors for postoperative bacterial infection

Variables	Infected	No infected	*P* value
Donor variables			
Age (yr)	34.45 ± 8.78	34.89 ± 10.19	0.825
Gender (female)	10	71	0.580
BMI (kg/m^2)	22.80 ± 2.69	22.99 ± 2.53	0.711
Recipient variables			
Age (yr)	46.72 ± 8.27	42.31 ± 8.63	0.012
Gender (female)	2	25	0.384
BMI (kg/m^2)	21.77 ± 4.21	22.63 ± 2.93	0.172
MELD score	17.24 ± 7.40	16.26 ± 10.18	0.621
Renal dysfunction	1	15	0.705
Diabetes mellitus	1	8	0.798
Starting albumin level < 2.8 g/dL	10	32	0.040
Starting TB level ≥ 20 mg/dL	3	24	0.774
Starting INR level	1.62 ± 0.54	1.71 ± 1.50	0.744
Hyponatremia	7	12	0.008
Graft variables			
GRWR (%)	0.99 ± 0.23	0.93 ± 0.17	0.111
Intraoperative variables			
Massive RBCs transfusion	8	35	0.335
FFP transfusion > 10 units	7	42	0.949
Postoperative variables			
Bile leak	10	6	0.000
Rejection	2	3	0.090
Hyperglycemia	5	10	0.042
ICU stay	17.38 ± 11.11	12.28 ± 12.48	0.040

BMI: Body mass index; MELD: Model for end-stage liver disease; TB: Total bilirubin; INR: International normalized ratio; GRWR: Graft to recipient weight ratio; RBC: Red blood cell; FFP: Fresh frozen plasma; ICU: Intensive care unit.

tion, followed by *Escherichia coli* (*n* = 6), *Staphylococcus aureus* (*n* = 4), *Bowman acinetobacter* (*n* = 4), *Hemolytic streptococcus* (*n* = 3), *Pseudomonas aeruginosa* (*n* = 2), *Burkholderia cepacia* (*n* = 1), *Acinetobacter lwoffii* (*n* = 1), *Xanthomonas maltophilia* (*n* = 1), *Haemophilus influenza* (*n* = 1), *Klebsiella pneumonia* (*n* = 1), *Enterobacter cloacae* (*n* = 1), *Leuconostoc pseudomesenteroides* (*n* = 1). The most common bacterial infective site was lung (*n* = 15), followed by abdominal cavity (*n* = 12), blood (*n* = 3). *Candida albicans* (*n* = 6) was the most common pathogen in patients with fungal infection, follow by *Saccharomycetes* (*n* = 2), *Aspergillus* (*n* = 1). Fungal infections were observed in lung in 7 patients and in gastrointestinal tract in 2 recipients.

Risk factors related to bacterial infection

As shown in Table 1, recipient age, starting albumin level < 2.8 g/dL, preoperative hyponatremia, postoperative bile leak, severe hyperglycemia, rejection and longer intensive care unit (ICU) stay were potential risk factors in univariate analysis. ROC curve analysis showed the best cut-off values for recipient age and ICU stay were 45 years and 9 d respectively (Figures 1 and 2). The corresponding are under the ROC were 0.641 and 0.680 respectively (Figures 1 and 2). When we analyzed the potential risk factors using Cox regression, only recipient age > 45 years, preoperative hyponatremia, postoperative bile leak, severe hyperglucemia and length of ICU stay > 9 d were independent risk factors for bacterial infection (Table 2).

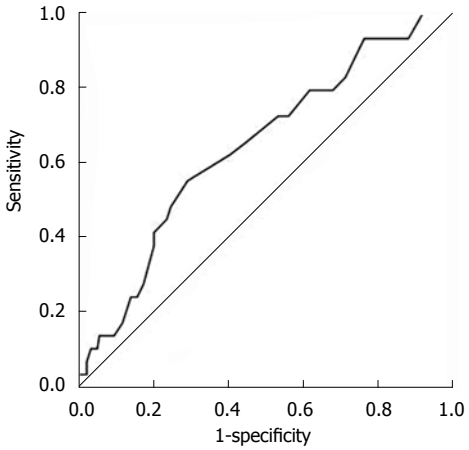

Figure 1 Receiver operating curve curve for recipient age.

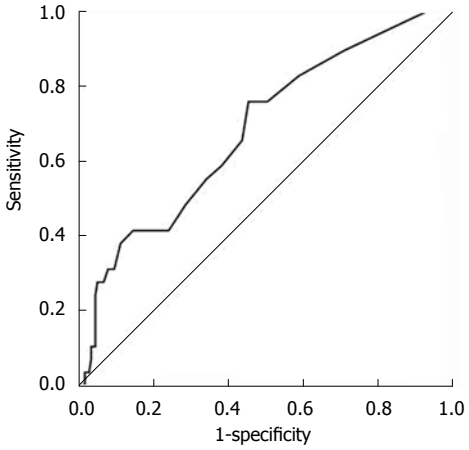

Figure 2 Receiver operating curve curve for the length of intensive care unit stay.

Risk factors related to fungal infection

As listed in Table 3, we studied the factors may be related to fungal infection using univariate analysis. Correlation testing shoed preoperative hyponatremia, massive intraoperative RBCs transfusion and postoperative bacterial infection may be contributed to postoperative fungal infection. These potential risk factors were further examined with Cox regression analysis. Only bacterial infection and massive intraoperative RBCs transfusion showed prognostic power in multivariate analysis (Table 4).

DISCUSSION

Postoperative infection is one of the most common complications in liver transplant recipients. In our current study, the incidences of bacterial and fungal infections were 14.01% and 4.35%, which were lower than previous reports[19-21]. We suggested this difference may be related to the different definition of infection. In current study, only culture-positive infections were included. Consistent with previous studies, gram-negative bacteria, especially *Enterococcus faecium* and *Escherichia coli*, were the predomi-

Table 2 Multivariate analysis for risk factors for postoperative bacterial infection

Variables	B	SE	Wald	*P* value	Exp (B)	95% CI	
						Lower	Upper
Bile leak	1.890	0.421	20.156	0.000	6.622	2.901	15.116
Hyponatremia	1.512	0.487	9.649	0.002	4.535	1.747	11.770
Hyperglycemia	1.171	0.508	5.308	0.021	3.226	1.191	8.737
ICU stay > 9 d	0.932	0.458	4.145	0.042	2.540	1.035	6.230
Recipient age > 45 yr	1.253	0.408	9.440	0.002	3.501	1.574	7.785

SE: Standard error; CI: Confidence interval; ICU: Intensive care unit.

Table 3 Univariate analysis for risk factors for postoperative fungal infection

Variables	Infected	No infected	*P* value
Donor variables			
Age (yr)	37.00 ± 7.76	34.73 ± 10.08	0.507
Gender (female)	4	74	0.739
BMI (kg/m^2)	21.64 ± 2.27	23.02 ± 2.54	0.110
Recipient variables			
Age > 45 yr	5	62	0.153
Gender (female)	1	26	0.860
BMI (kg/m^2)	21.44 ± 2.80	22.56 ± 3.16	0.295
MELD score	20.11 ± 9.35	16.23 ± 9.85	0.248
Renal dysfunction	1	15	0.698
Diabetes mellitus	0	9	0.513
Starting albumin level < 2.8 g/dL	3	39	0.391
Starting total bilirubin level	2	25	0.332
Starting INR level	1.91 ± 0.55	1.69 ± 1.43	0.650
Hyponatremia	3	16	0.010
Graft variables			
GRWR (%)	0.93 ± 0.21	0.94 ± 0.18	0.886
Intraoperative variables			
Massive RBCs transfusion	6	37	0.003
FFP transfusion > 10 units	4	45	0.220
Postoperative variables			
Bile leak	1	15	0.698
Rejection	0	5	0.629
Hyperglycemia	2	13	0.076
ICU stay > 9 d	5	98	0.748
Postoperative bacterial infection	6	23	0.000

BMI: Body mass index; MELD: Model for end-stage liver disease; INR: International normalized ratio; GRWR: Graft to recipient weight ratio; RBC: Red blood cell; FFP: Fresh frozen plasma; ICU: Intensive care unit.

nant bacterial pathogens, whereas *Candida albicans* was the most common fungal pathogen[22].

Postoperative bile leak was an independent risk factor for bacterial infection of LDLT in current study. This risk factor was not considered in some studies following deceased donor liver transplantation (DDLT)[23]. This difference was related to the low incidence of postoperative bile leak in patients underwent DDLT. However, bile leak was one of the most common complications in LDLT recipients. This factor should not be ignored in LDLT. Patients with postoperative bile leak suffered from longer abdominal drainage which may increase the risk of intraabdominal and wound infection[24]. Additionally, bile leak can cause biloma that often progress to an infected abscess[25].

Table 4 Multivariate analysis for risk factors for postoperative fungal infection

Variables	B	SE	Wald	*P* value	Exp (B)	95% CI	
						Lower	Upper
Massive RBCs transfusion	1.887	0.710	7.062	0.008	6.599	1.641	26.542
Bacterial infection	2.429	0.711	11.686	0.001	11.346	2.819	45.673

SE: Standard error; CI: Confidence interval; RBC: Red blood cell.

It was interesting that patient more than 45 years old was a risk factor related to postoperative bacterial infection. We acknowledge the cut-off value of recipient age was so young in our study. The mean recipient age in current study was 42.93 ± 8.77 years. This was a potential explanation. Similar to our results, Nayaranan *et al*[26] suggested patient's age greater than 42 years old was significantly associated with a poor long-term survival. This finding suggested the incidence of postoperative bacterial infection may be increased with the increasing of recipient age.

Preoperative diabetes mellitus didn't increase the risk of postoperative infection in our study. However, John *et al*[27] suggested pretransplant diabetes was associated with increased postoperative morbidity and mortality. Recently, Ling *et al*[28] confirmed preexisting diabetes was not a contraindication for liver transplantation. Well controlled pretransplant diabetes will not increase the risk of postoperative complication. In our practice, the nine patients with pretransplant diabetes had normal blood sugar level at the time of transplantation. Contrary to pretransplant diabetes mellitus, severe postoperative hyperglycemia was an independent risk factor for bacterial infection in current study. However, after transplantation, the administration of immunosuppressive agents, including cyclosporine, steroids and tacrolimus, may cause postoperative hyperglycemia. Ata *et al*[29] confirmed postoperative hyperglycemia was the most important risk factor for surgical site infection in general surgery patients. Rueda *et al*[30] reported hyperglycemia will increase the risk for and severity of pneumonia among non-diabetic patients.

It was easy to understand prolonged ICU stay and hyponatremia were associated with postoperative bacterial infection. Mnatzaganian *et al*[31] confirmed the incidences of bloodstream and urinary infections of patients in ICU were higher than those in regular ward. Suljagic *et al*[32] confirmed the incidence of nosocomial bloodstream infection of ICU patients was higher than non-ICU patients. Stormont *et al*[33] confirmed hyponatremia was associated with pneumonia. Zilberberg *et al*[34] suggested hyponatremia was associated with worsened clinical outcomes among patients with pneumonia.

Although the relationship between massive RBCs transfusion and bacterial infection was well established in previous studies, there were little information of the correlation of massive RBCs transfusion and fungal in-

fection. Current study suggested massive RBCs transfusion will increase the risk of fungal infection after liver transplantation. Blood transfusion can cause transfusion-related immunomodulation which will suppress the recipient's immune function[35]. However, it remains unclear why massive transfusion was not a risk factor for bacterial infection in current study.

Postoperative bacterial infection showed significantly prognostic power for fungal infection in current study. Antibiotics, especially broad-spectrum antibiotics, were administrated to patients with bacterial infection in the case of lacking culture results. Broad-spectrum antibiotics might lead to dysbacteriosis and increase fungal infection[36]. However, a recent study which was performed by Nafady-Hego *et al*[37] suggested bacterial infection was not a risk factor for fungal infection after pediatric LDLT. Younger recipient age, lower dosages of immunosuppressive agents and different infection prophylaxis might be the potential explanation for this difference.

In conclusion, preoperative hyponatremia, recipient age > 45 years, longer ICU stay, postoperative bile leak and severe hyperglycemia may be related to postoperative bacterial infection, whereas massive intraoperative RBCs transfusion and postoperative bacterial infection may lead to postoperative fungal infection. Current finding suggested postoperative bacterial and fungal infections were associated with pre-, intra- and post-operative factors.

COMMENTS

Background

Infection is a leading cause of postoperative mortality and morbidity after liver transplantation. To identify the pattern and risk factors related to postoperative bacterial and fungal infections is important to transplant surgeon.

Research frontiers

The pattern and risk factors of postoperative infections following living donor liver transplantation are not well established. This study was performed to identify the pattern and risk factors related to bacterial and fungal infections in the first 3-mo after living donor liver transplantation.

Innovations and breakthroughs

This study oultlines a comprehensive experience of 207 patients over an eight year period of infections in the first 3-mo following living donor liver transplantation. It documents the site of infection, organisms involved and the predictive risk factors.

Applications

This study could guide the clinical management of early bacterial and fungal infections after living donor liver transplantation.

Terminology

Serious infection was defined as the culture-positive bacterial or fungal infection in the blood, sputum, urine, or ascetic fluid which was obtained on the basis of clinical suspicion of an infection.

Peer review

The article is well written. The analysis of the data is sound and the conclusions reached appear valid. Overall, it significantly contributes to a relevant issue in this field and should be considered for publication.

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BRIEF ARTICLE

Individualized peri-operative fluid therapy facilitating early-phase recovery after liver transplantation

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Abstract

AIM: To investigate the correlation between peri-operative fluid therapy and early-phase recovery after liver transplantation (LT) by retrospectively reviewing 102 consecutive recipients.

METHODS: Based on whether or not the patients had pulmonary complications, the patients were categorized into non-pulmonary and pulmonary groups. Twenty-eight peri-operative variables were analyzed in both groups to screen for the factors related to the occurrence of early pulmonary complications.

RESULTS: The starting hemoglobin (Hb) value, an intra-operative transfusion > 100 mL/kg, and a fluid balance ⩽ -14 mL/kg on the first day and the second or third day post-operatively were significant factors for early pulmonary complications. The extubation time, time to initial passage of flatus, or intensive care unit length of stay were significantly prolonged in patients who had not received an intra-operative transfusion ⩽ 100 mL/kg or a fluid balance ⩽ -14 mL/kg on the first day and the second or the third day post-operatively. Moreover, these patients had poorer results in arterial blood gas analysis.

CONCLUSION: It is important to offer a precise and individualized fluid therapy during the peri-operative period to the patients undergoing LT for cirrhosis-associated hepatocellular carcinoma.

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Key words: Fluid therapy; Liver transplantation; Early-phase recovery; Pulmonary complications; Hemoglobin

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INTRODUCTION

Liver transplantation (LT) is the optimal therapy for end-stage liver diseases. Although LT has undergone a rapid progress, early pulmonary complications are common and known to contribute significantly to the morbidity and mortality of the patients[1-4]. Post-operative pulmonary complications may be caused by many factors during the patient's recovery, and fluid therapy is an important

factor[5,6]. Individualized fluid therapy during the peri-operative period may be a significant strategy to achieve a better early-phase recovery after LT. In this study, early-phase refers to the first month after LT.

In a previous study, we investigated and assessed the use of fluid therapy in all the patients undergoing LT[7]. This study focused on the patients with cirrhosis-associated hepatocellular carcinoma (HCC), and fluid transfusion was administered based on the body weight of the patients per kg.

The purpose of this study was to investigate the clinical significance of the correlation between peri-operative fluid therapy and early-phase recovery after LT in an attempt to establish a precise and individualized fluid therapeutic strategy in the peri-operative period of LT.

MATERIALS AND METHODS

The medical records of all consecutive patients with cirrhosis-associated HCC who underwent orthotopic LT at the First Affiliated Hospital of Guangxi Medial University between July 1996 and July 2009 were retrospectively reviewed. Patients aged from 23-72 years with a mean ± SD of 45.26 ± 9.54 years. This series represents the first 102 consecutive LT recipients with cirrhosis-associated HCC in our cohort study.

All LT procedures were performed using the piggyback technique without venovenous bypass. In addition to fluid transfusion given intra-operatively, small amounts of vasopressors (dopamine and noradrenaline) and shallow anesthesia were used to maintain hemodynamic stability. All patients were admitted to the intensive care unit (ICU) immediately after surgery and extubated as soon as they met standard criteria for termination of mechanical ventilation, such as the presence of adequate gas exchange function, hemodynamic stability, and ability to protect the airway. Antibiotics and anti-fungals for infection were used prophylactically.

Radiographic analysis was standardized by assessment of eight separate observations designed to determine the presence of pulmonary edema[8].Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) were defined according to the American-European Consensus Conference[9], and pneumonia was defined according to a Joint Committee of the American Thoracic Society and Infectious Diseases Society of America[10].

All variables

Twenty-five peri-operative variables affecting post-operative early-phase recovery were as follows: age; body weight index; hemoglobin (Hb); hematocrit (HCT); serum creatinine (CRE); blood urea nitrogen (BUN); serum uric acid (UA); American Society of Anesthesiologists (ASA) grade; Child-Pugh score; acute physiology and chronic health evaluation (APACHE) Ⅱ score; model for end-stage liver disease (MELD) score; warm ischemia time; cold ischemia time; anhepatic phase; operative time; diabetes; lung function; volume of intra-operative blood transfusion; volume of intra-operative packed red blood cell (RBC) transfusion; volume of intra-operative plasma transfusion; volume of intra-operative fluid transfusion; volume of intra-operative bleeding; intra-operative fluid balance; transfused volume and fluid balance on the first post-operative day. Bivariate correlation analysis for the relationship between these intra-operative variables and occurrence of pulmonary complications showed the following significant variables: blood transfusion > 30 mL/kg ($P = 0.046$); packed RBC transfusion > 0.05 U/kg ($P = 0.041$); plasma transfusion > 25 mL/kg ($P = 0.042$); fluid transfusion > 100 mL/kg ($P = 0.014$); bleeding > 10 mL/kg ($P = 0.018$); fluid balance > 64 mL/kg ($P = 0.037$); fluid transfusion volume on the first post-operative day ⩽ 44 mL/kg ($P = 0.010$); and fluid balance on the first post-operative day ⩽ -14 mL/kg ($P = 0.018$). The threshold values of these variables were obtained by bivariate correlation analysis between the volume of intra-operative transfusion and post-operative pulmonary complications. The statistically significant threshold value was 100 mL/kg. If no statistically significant threshold value was obtained, the lowest P value was recorded. The critical value of other fluid variables was determined by the same analysis.

Because of the special importance of the first 3 post-operative days for patients' recovery, the following three variables were analyzed: fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after operation ($P = 0.010$); fluid balance ⩽ -14 mL/kg on post-operative ⩾ 1 d ($P = 0.612$); and fluid balance ⩽ -14 mL/kg on post-operative ⩾ 2 d ($P = 0.014$).

Other variables analyzed included the worst outcome of arterial blood gas analysis in the first 7 post-operative days. The outcome variables reflecting post-operative recovery included: extubation time; time to initial passage of flatus; and ICU length of stay.

In this study, fluid balance in the surgery did not include the "third space" loss, evaporative loss, and insensible loss. So far, no method has provided estimated losses for LT.

Statistical analysis

Data were presented as the mean ± SD, median/range, or percentage (%). Group means were compared using Student's t test or the Mann-Whitney U test as appropriate. The χ^2 test was used to compare percentages. Bivariate correlation was used to determine the significant threshold value of peri-operative fluid variables. Multivariate regression analysis was performed with stepwise elimination of non-significant variables. A $P < 0.050$ was considered significant. All analyses were performed with SPSS 12.0 software (SPSS, Chicago, IL, United States).

RESULTS

Of the 102 patients (89 males and 13 females), 47 patients (46.08%) had pulmonary complications after LT. No hypoxia was found preoperatively. Pulmonary edema (PE; $n = 8$, 17.02%), acute lung injury (ALI; $n = 12$,

Table 1 Comparison of 25 variables between the groups with and without pulmonary complications

Variables	Non-pulmonary complication group (n = 55)	Pulmonary complication group (n = 47)	P value
Age (yr)	46.29 ± 9.14	44.06 ± 9.95	0.242
Weight index (kg/m^2)	22.78 ± 3.35	22.36 ± 3.29	0.531
Hb (g/L)	122.79 ± 21.12	109.54 ± 25.25	0.005
HCT (%)	36.21 ± 6.42	32.53 ± 7.53	0.009
CRE (mmol/L)	83.75 ± 21.35	85.96 ± 23.88	0.623
BUN (mmol/L)	4.70 ± 1.92	5.22 ± 3.09	0.323
UA (mmol/L)	303.76 ± 113.27	272.43 ± 117.94	0.175
ASA grade	2.76 ± 0.61	3.0 ± 0.75	0.082
Child-Pugh score	7.29 ± 2.40	8.38 ± 2.88	0.039
APACHE Ⅱ score	3.95 ± 2.47	4.79 ± 3.53	0.174
MELD score	14.22 ± 7.34	16.68 ± 9.39	0.140
Warm ischemia time (min)	5.80 ± 1.52	5.53 ± 1.63	0.392
Cold ischemia time (min)	710.13 ± 184.99	654.21 ± 208.25	0.154
Anhepatic phase (min)	123.04 ± 34.46	126.00 ± 47.00	0.715
Operative time (min)	423.22 ± 84.92	489.38 ± 150.00	0.009
Diabetes (%)	9.09	8.51	1.000
Lung dysfunction (%)	12.73	19.15	0.374
Volume of intra-operative blood transfusion > 30 mL/kg (%)	65.45	82.98	0.046
Volume of intra-operative packed RBC transfusion > 0.05 U/kg (%)	76.36	91.49	0.041
Volume of intra-operative plasma transfusion > 25 mL/kg (%)	43.64	63.83	0.042
Volume of intra-operative fluid transfusion > 100 mL/kg (%)	58.18	80.85	0.014
Volume of intra-operative bleeding > 10 mL/kg (%)	67.27	87.23	0.018
Intra-operative fluid balance > 64 mL/kg (%)	32.73	53.19	0.037
a (%)	21.82	4.26	0.010
b (%)	38.18	17.02	0.018
c (%)	25.45	6.38	0.010
d (%)	72.73	68.09	0.612
e (%)	36.36	14.89	0.014

Hb: Hemoglobin; HCT: Hematocrit; CRE: Creatinine; BUN: Blood urea nitrogen; UA: Serum uric acid; ASA: American Society of Anesthesiologists; APACHE: Acute physiology and chronic health evaluation; MELD: Model for end-stage liver disease. a, transfused volume on the first post-operative day ⩽ 44 mL/kg; b, fluid balance on the first post-operative day ⩽ -14 mL/kg; c, fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after operation; d, fluid balance ⩽ -14 mL/kg on ⩾ 1 d of the first 3 d after operation; e, fluid balance ⩽ -14 mL/kg on ⩾ 2 d after operation. ASA grade I was assigned a score of 1, grade Ⅱ was assigned a score of 2, grade Ⅲ was assigned a score of 3, and grade IV was assigned a score of 4. Mean ± SD was used for continuous variables, otherwise percentage was used (%).

25.53%), adult respiratory distress syndrome (ARDS; n = 6, 12.77%), and pneumonia (n = 21, 44.68%) occurred after surgery. Four patients survived no more than one month after LT.

Table 1 shows the comparison of 28 variables between the non-pulmonary and pulmonary complication groups. The following variables were found significant: Hb; HCT; Child-Pugh score; operative time; intra-operative blood transfusion > 30 mL/kg; intra-operative packed RBC transfusion > 0.05 U/kg; intra-operative plasma transfusion > 25 mL/kg; intra-operative fluid transfusion > 100 mL/kg; intra-operative bleeding > 10 mL/kg; intra-operative fluid balance > 64 mL/kg; fluid transfusion volume on the first post-operative day ⩽ 44 mL/kg; fluid balance ⩽ -14 mL/kg on the first post-operative day; fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after operation; and fluid balance ⩽ -14 mL/kg ⩾ 2 d after operation.

Table 2 Summary of the logistic regression model and odds ratios

Variables	Beta	SE	Sig	Exp (B)	95% CI
Hb	-0.025	0.010	0.11	0.975	0.956-0.994
Volume of intra-operative transfusion	1.097	0.496	0.27	2.995	1.132-7.922
c	2.037	0.722	0.05	7.670	1.862-31.603

SE: Standard error; Sig: Statistical significance; CI: Confidence interval; Hb: Hemoglobin. c, fluid balance ⩽ -14 mL/kg on the first day and the second or the third after operation. When the volume of intra-operative transfusion ⩽ 100 mL/kg, the variable was assigned a score of 0, and when the variable > 100 g/L, the variable was assigned a score of 1.

Table 3 Comparison of outcome variables reflecting post-operative recovery between groups A and B

	Group A (n = 32)	Group B (n = 70)	Z or t	P value
Extubation time (h)	12/8-99	16.5/5-504	-2.779	0.005
Time to initial passage of flatus (h)	78.66 ± 21.05	90.51 ± 45.28	-1.411	0.161
ICU length of stay (h)	36.5/8-144	62/14-600	-4.173	0.000

Values are given as the mean ± SD (median/range). ICU: Intensive care unit.

The statistically significant variables were regarded as independent variables, and post-operative pulmonary complications were regarded as dependent variables. Multivariate regression analysis was performed to screen out the variables related to early pulmonary complications. Table 2 shows the statistically significant variables: Hb; intra-operative transfusion > 100 mL/kg; and fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after operation.

The 32 patients who received an intra-operative transfusion ⩽ 100 mL/kg were referred to as group A and the other 70 patients who received an intra-operative transfusion > 100 mL/kg were as group B. Table 3 shows the comparison of outcome variables reflecting post-operative recovery between the two groups. As expected, both the extubation time and ICU length of stay in group A were shorter than in group B (P < 0.01). A comparison of the worst outcome of arterial blood gas in the first 7 post-operative days between the two groups showed that both PaO_2 and arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ratio in group A were higher than in group B (110.422 ± 28.305 *vs* 90.641 ± 31.169, P < 0.01 for PaO_2; and 272.355 ±

Table 4 Comparison of outcome variables reflecting post-operative recovery between groups A and B

	Group A (n = 17)	Group B (n = 85)	Z or t	P value
Extubation time (h)	12/7-32	15/5-504	-2.708	0.007
Time to initial passage of flatus (h)	59.71 ± 12.17	92.21 ± 40.96	-6.094	0.000
ICU length of stay (h)	40/13-219	60/8-600	-1.590	0.112

Values are given as the mean ± SD (median/range). ICU: Intensive care unit.

Table 5 Comparison of the worst outcome of arterial blood gas in the first 7 post-operative days between groups A and B

	Group A (n = 17)	Group B (n = 85)	t	P value
PH	7.436 ± 0.041	7.437 ± 0.055	-0.108	0.914
PaO_2	117.471 ± 31.283	92.722 ± 30.105	3.075	0.003
$PaCO_2$	44.094 ± 7.168	44.787 ± 12.038	-0.229	0.819
BE	4.459 ± 4.207	4.789 ± 4.062	-0.305	0.761
PaO_2/FiO_2	295.891 ± 92.741	224.243 ± 81.820	3.223	0.002

Values are given as the mean ± SD. PH: Power of hydrogen; PaO_2: Arterial partial pressure of oxygen; $PaCO_2$: Arterial pressure of carbon dioxide; BE: Base excess.

79.486 *vs* 219.649 ± 86.462, $P < 0.01$ for PaO_2/FiO_2).

The 17 patients who received a fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after operation served as group A and the other 85 patients who did not receive a fluid balance ⩽ -14 mL/kg served as group B. Table 4 shows a comparison of outcome variables reflecting post-operative recovery between the two groups. As expected, both the extubation time and the time to initial passage of flatus in group A were shorter than in group B ($P < 0.01$). Table 5 shows the comparison of the worst outcome of arterial blood gas in the first 7 post-operative days between the two groups. Both PaO_2 and PaO_2/FiO_2 in group A were higher than in group B ($P < 0.01$).

DISCUSSION

The LT recipients in this study presented the following characteristics: (1) All had chronic liver cirrhosis with water-sodium retention to some degree; (2) Because of the operative injury for LT, the capillary leak syndrome (CLS) created a "third space" effect; (3) Intra-operative fluid overload was common in order to maintain stable hemodynamics; and (4) A large-dose methylprednisolone was used to avoid reject reaction during and after surgery. All these factors contributed to aggravated water-sodium retention. Therefore, a precise and individualized fluid therapy is strongly recommended.

Collective studies demonstrated the importance of intra-operative fluid management to maintain body fluid equilibrium by restricting the volume of fluid infusion. Excessive fluid administration was associated with a higher risk of post-operative complications[11-14]. Aduen *et al*[15]reported that pulmonary complications interfered with the peri-operative course of patients undergoing LT and portended a worse outcome. Meanwhile, the time for extubation, ICU stay and hospital stay were significantly prolonged.

The restrictive intra-operative fluid management may be advantageous because it reduces morbidity and mortality and shortens mechanical ventilation, the time to initial passage of flatus, intensive care, and hospital stay[16-21].

In this study, binary logistic regression revealed that an intra-operative transfusion > 100 mL/kg (Table 2) was an independent risk factor for post-operative pulmonary complications. The incidence of post-operative pulmonary complications in group A was significantly lower than in group B (28.13% *vs* 54.29%, $P = 0.014$). The extubation time and ICU length of stay were also significantly shorter than in group B (Table 3). Group A had higher PaO_2 and PaO_2/FiO_2 than group B. These outcomes demonstrated that intra-operative fluid therapy was very important, which was associated with the incidence of pulmonary complications and the post-operative recovery.

Post-operative fluid overload is an independent risk factor for post-operative pulmonary complications after LT. Alsous *et al*[22]demonstrated that a fluid balance ⩽ -500 mL on ⩾ 1 d of the first 3 d of septic shock was associated with fewer pulmonary complications and better recovery. Our results demonstrated that it is a significant means to keep a fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after LT (Table 2). The incidence of post-operative early pulmonary complications in group A was lower than in group B (17.65% *vs* 51.76%, $P = 0.01$). This fluid therapy contributed to a better recovery (Tables 4 and 5) possibly because a fluid balance at ⩽ -14 mL/kg within 3 d after LT could prevent edema, thus improving the blood supply and promoting recovery.

Of the four variables (fluid balance on the first post-operative day ⩽ -14 mL/kg, fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after surgery, fluid balance ⩽ -14 mL/kg on ⩾ 1 d after surgery, and fluid balance ⩽ -14 mL/kg on ⩾ 2 d after surgery), only the fluid balance ⩽ -14 mL/kg on the first day and the second or the third day after operation was included in the logistic regression analysis of outcome. It suggested that precise and individualized fluid balance in the peri-operative period should be accomplished as early as possible. In contrast, a negative fluid balance in the peri-operative period should be achieved to some extent.

The first 3 d after operation comprise the stress phase. Vascular recovery time varied from 36 h to 72 h after surgery. The transition points from positive to negative fluid balance also occurred. LT recipients need a large-dose methylprednisolone after operation, especially during the first few days. However, methylprednisolone would cause and aggravate water-sodium retention which may appear post-operatively, so the transition points would be postponed. Fluid balance during the first 3 d after surgery is crucial for recovery. If a positive fluid balance lasts too long, it is difficult for patients to recover. Thus, fluid bal-

ance should be properly managed as early as possible, and the quantity and time of negative fluid balance should be assessed individually.

Under the circumstances of stable hemodynamics, we should extrude the sequestered fluid from the peripheral circulation and the "third space" back to the central circulation. Whether or not a negative fluid balance is needed, the quantity and time of a negative fluid balance should be assessed by the following measurement: blood pressure, pulse, central venous pressure, HCT, the estimated volume of transfusion on the second day, and liquid intake and output volume of the preceding day. The fluid balance on the second post-operative day could be accomplished by adjusting the urine volume per hour. With administration of diuretics and colloid, urine volume could be controlled as needed. If hemodynamics were unstable, blood volume should be supplemented and inotropic drugs, such as dopamine, should be properly used.

We also found that Hb was an independent risk factor for post-operative pulmonary complications, which may be a significant factor for early recovery. When the patients with hepatic cirrhosis developed hypersplenism, which was always parallel with a reduction of hemocytes, their immune function became obviously lower and their hepatic function was poorer than other patients without a reduction of hemocytes. A low level of Hb may result in oxygen deficiency.

It is important to keep a fluid balance during the peri-operative period of LT. The precise and individualized fluid administration at the first 3 d after surgery significantly decreased the incidence of early pulmonary complications after LT. The strategies of an intra-operative transfusion $\leqslant$ 100 mL/kg and a fluid balance $\leqslant$ -14 mL/kg on the first day and the second or the third day after LT should be recommended. This study was limited by its small sample size. The shortcomings may limit the extrapolation of these results to all LT recipients. The hypothesis should be re-examined and verified in a much larger cohort before it is used for improving the prognosis and patient management. Nonetheless, if validated by future prospective studies, the fluid therapy used in this study would provide a simple and inexpensive method of augmenting the current prognostic indicators, and would be of obvious benefit for the anxious family members and care providers as well.

COMMENTS

Background

Liver transplantation (LT) is the optimal therapy for end-stage liver disease. Early-phase complications after LT are common and known to contribute significantly to morbidity and mortality of the patients. Individualized fluid therapy during the peri-operative period may be a significant strategy to achieve a better early-phase recovery after LT.

Research frontiers

Recently, more attention has been paid to peri-operative fluid therapy. Fluid therapy is an important factor related to pulmonary complications and patient recovery.

Innovations and breakthroughs

This research focused on the LT recipients with cirrhosis-associated hepatocellular carcinoma, and fluid was administrated based on the body weight in kilograms so as to guarantee a precise and individualized fluid therapy peri-operatively.

Applications

It is important to offer a precise and individualized fluid therapy during the peri-operative period to the patients undergoing LT for cirrhosis-associated hepatocellular carcinoma. With an intra-operative transfusion > 100 mL/kg and a fluid balance $\leqslant$ 14 mL/kg on the first day and the second or the third day after LT can significantly improve the early-phase recovery after LT.

Terminology

The capillary leak syndrome is a rare condition characterized by recurrent episodes of generalized edema and severe hypotension associated with hypoproteinemia. A shift of fluid and protein from the intravascular to the interstitial space results in hypovolaemia. Attacks vary in frequency, severity, and duration, and can be fatal.

Peer review

Authors have investigated the clinical significance of the correlation between peri-operative fluid therapy and early-phase recovery after LT. The topic is interesting and offers points of reflection. The manuscript is original and may be useful to clinicians.

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CASE REPORT

Acute chylous peritonitis due to acute pancreatitis

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Abstract

We report a case of acute chylous ascites formation presenting as peritonitis (acute chylous peritonitis) in a patient suffering from acute pancreatitis due to hypertriglyceridemia and alcohol abuse. The development of chylous ascites is usually a chronic process mostly involving malignancy, trauma or surgery, and symptoms arise as a result of progressive abdominal distention. However, when accumulation of "chyle" occurs rapidly, the patient may present with signs of peritonitis. Preoperative diagnosis is difficult since the clinical picture usually suggests hollow organ perforation, appendicitis or visceral ischemia. Less than 100 cases of acute chylous peritonitis have been reported. Pancreatitis is a rare cause of chyloperitoneum and in almost all of the cases chylous ascites is discovered some days (or even weeks) after the onset of symptoms of pancreatitis. This is the second case in the literature where the patient presented with acute chylous peritonitis due to acute pancreatitis, and the presence of chyle within the abdominal cavity was discovered simultaneously with the establishment of the diagnosis of pancreatitis. The patient underwent an exploratory laparotomy for suspected perforated duodenal ulcer, since, due to hypertriglyceridemia, serum amylase values appeared within the normal range. Moreover, abdominal computed tomography imaging was not diagnostic for pancreatitis. Following abdominal lavage and drainage, the patient was successfully treated with total parenteral nutrition and octreotide.

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Key words: Chylous ascites; Chyloperitoneum; Chyle; Peritonitis; Pancreatitis

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Georgiou GK, Harissis H, Mitsis M, Batsis H, Fatouros M. Acute chylous peritonitis due to acute pancreatitis. *World J Gastroenterol* 2012; 18(16): 1987-1990 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i16/1987.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i16.1987

INTRODUCTION

Accumulation of lymph within the peritoneal cavity is a rare pathological entity described as chylous ascites. Most cases occur progressively and the patient suffers from abdominal distention, nausea, vomiting, fatigue or low grade fever. A small number of cases have reported an acute development which may present as a surgical urgency mimicking appendicitis, hollow organ perforation and generalized peritonitis (acute chylous peritonitis). We hereby document a rare case of acute chylous peritonitis in a 46-year-old man with a history of alcoholism presenting with hypertriglyceridemia and acute pancreatitis.

CASE REPORT

A 46-year-old man presented to the emergency department with an 8-h history of abdominal pain localized to the right abdomen. The pain was of acute onset and was primarily felt at the epigastrium. The patient also men-

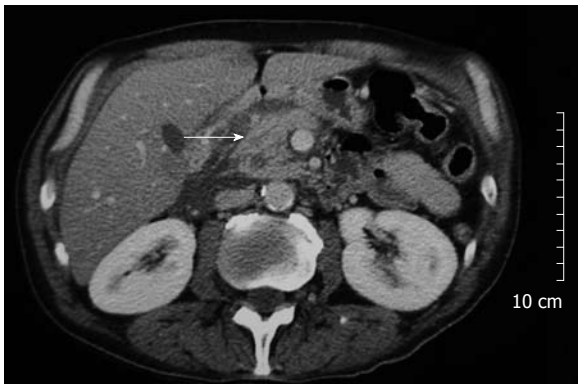

Figure 1 Preoperative abdominal computed tomography image showing inflammatory changes surrounding the head of the pancreas (arrow).

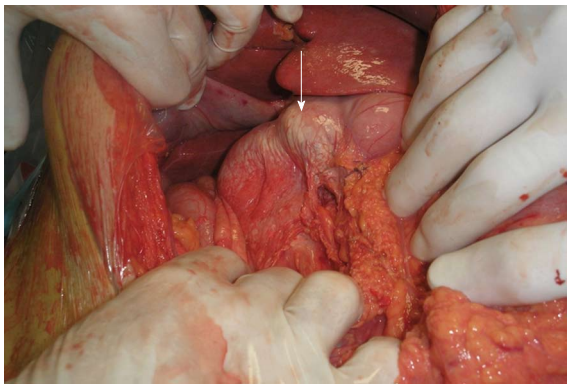

Figure 2 Intraoperative photo showing the milky-like appearance of the wall of the distal portion of the stomach and the duodenum due to congestion of the intestinal lymphatic drainage (arrow).

tioned nausea and one episode of vomiting but no fever or diarrhea. Apart from a history of ankylosing spondylitis he also admitted systematical alcohol consumption over a period of several years (> 120 mg/d).

The patient's overall health condition was not gravely affected, showing a temperature of 37.2 ℃, heart rate at 100 bpm and arterial pressure of 125/75 mmHg. On examination, no bowel sounds could be heard and right upper and lower abdominal quadrants appeared tender at palpation with rebound tenderness. Rectal digital examination did not reveal blood or tenderness.

Laboratory investigation showed no elevation of white blood cell count (7.740/mL with 57% polymorphonuclear leukocytes) while C-reactive protein values were only slightly affected (14 mg/L). The patient's biochemistry showed a mild elevation of hepatic enzymes (aspartate aminotransferase: 107 IU/L, alanine aminotransferase: 45 IU/L, alkaline phosphatase: 165 IU/L and total bilirubin: 1.6 mg/dL). Amylase values were normal both in blood (55 IU/L) and urine samples (404 IU/L). The laboratory notes mentioned that the blood sample of the patient was not totally appropriate for analysis because of a high concentration of lipids. Plain chest and abdominal X-rays(supine and elevated position) revealed no significant pathology such as free air or ileus. The computed tomography (CT) scan showed the presence of free fluid in the abdominal cavity, mainly at the subhepatic space and the right paracolic space, with mild inflammatory changes around the pancreatic head (Figure 1). No atrophy of the pancreas or calcification of the main pancreatic duct was demonstrated.

The patient was subsequently taken to the operating room for an exploratory laparotomy. In the peritoneal cavity a great amount of "milky" peritoneal fluid was discovered. Specimens were taken for biochemistry and microbiology. Careful examination of the abdomen revealed a bulky and rigid pancreatic head. Moreover, the surface of the distal stomach, duodenum and upper segment of posterior peritoneum had a white, milky-like appearance (Figure 2). A laceration in the peritoneum (at the root of the transverse mesocolon) was discovered, through which the milky fluid, apparently of retroperitoneal origin, entered the peritoneal cavity. Attempts to obtain a biopsy specimen from the pancreas (with a biopsy needle) were unsuccessful because of hemorrhage after the first attempt due to the pancreatic edema. After a thorough peritoneal lavage and the insertion of 3 drain tubes the midline incision was closed.

During the initial postoperative days, serum samples from the patient appeared to be of high lipidemic concentration, with normal serum amylase values. Only after serial dilutions with the assay buffer were we able to obtain a proper sample for analysis, showing serum amylase values of 870 IU/L. Total cholesterol reached as high as 618 IU/L (normal values up to 200) and triglycerides had a concentration greater than 1.000 mg/dL (normal values up to 175). No elevation of white blood cell count was documented at any time. Analysis of samples from free peritoneal fluid that was collected intraoperatively showed 337 mg/dL cholesterol and 2800 mg/dL triglyceride thus establishing the diagnosis of chylous ascites. Laboratory investigation of the fluid from the drain tubes showed values of amylase to be as high as 60 000 IU/L, decreasing to normal at postoperative day 7, at which time all of the tubes had been removed. Cancer markers α-fetoprotein, carcinoembryonic antigen and carbonic anhydrase 19-9 were within normal values.

The patient received nothing po for 10 d and then was restored gradually to a full (fat-free) diet. Total fat-free parenteral nutrition was given intravenously from postoperative day 4 and for the subsequent 2 wk. Broad spectrum antibiotics were administered for 1 wk postoperatively and were not stopped because the patient suffered outbursts of fever during the end of the first week. Twenty five days after the first operation a second procedure was undertaken, this time due to persisting ileus. The laparotomy revealed adhesions in the peritoneal cavity. Octreotide (0.1 mg) was administered every 8 h from the day of the first surgery until the time of discharge, which came to be after 33 d of hospitalization.

DISCUSSION

Under normal circumstances, lymph from the lower parts of the body as well as from the viscera is circulated

through lymphatic vessels that follow a retroperitoneal course before emptying in the cisterna chyli and finally the thoracic duct and the venous system. This fluid consists of converted long-chain triglycerides at high concentrations that originate from the gut during ingestion. In cases wherea disruption to this normal flow occurs, the peritoneal cavity may be filled with a high-density, milky-like fluid that is called "chyle". Although there are no unequivocal diagnostic criteria, it is generally agreed that a high concentration of lipids is indicative[1]. A sample of ascites fluid (either acquired by paracentesis or during laparotomy) showing values of triglycerides 2 to 8 times that of plasma is characterized as chyle and the situation "chylous ascites" or "chyloperitoneum". Some authors have set absolute indicators such as a peritoneal lipid content greater than 200 mg/dL[2]. Other characteristics of chyle are a protein concentration ratio > 0.5 compared to that of plasma, a low cholesterol level (lower than plasma) and elevated amylase values in case of pancreatitis[3].

The formation of chylous ascites is usually a chronic procedure, and the patient typically mentions symptoms of progressive and painless distention of the abdomen for some time before the diagnosis is established. Multiple causes for this relatively rare pathological entity have been described. Aalami *et al*[1] present a detailed classification of them in a comprehensive review published in 2000. Whereas congenital abnormalities in the formation of the lymphatic vessels (lymphatic hypoplasia or lymphangiectasia) are a frequent cause in infants, surgery and malignancy (especially lymphoma) are among the most common causes in adults[4]. In particular, surgery involving the thoracic cavity or the aorta and the retroperitoneal space has often been associated with the pathogenesis of chylous ascites by means of interrupting the normal lymphatic drainage[5]. Chyloperitoneum may also be the result of trauma to the intestines or mesentery. Idiopathic retroperitoneal fibrosis, sarcoidosis and abdominal or pelvic radiation therapy have also been mentioned in the literature. Infectious diseases are another possible cause, such as filariasis in tropical countries, and tuberculosis mostly in countries with low social and economical level[1].

However, in rare cases the accumulation of chyle within the peritoneal cavity may occur rapidly and the patient may present with symptoms and signs of acute abdomen[6]. Vettoretto *et al*[6] found less than a 100 cases of acute chylous peritonitis in their review of 2008. The pain appears to be diffuse, possibly due to peritoneal distention and irritation of the root of the mesentery as the retroperitoneal space expands, since the fluid itself is not irritating to the peritoneum. During clinical examination, rebound tenderness and guarding may be documented, which is often localized at the right iliac fossa and this can possibly be explained by pooling of chyle at the right paracolic gutter. Thus the clinical picture may be misleading, with appendicitis, hollow organ perforation and visceral ischemia being the most commonly suspected diagnosis preoperatively[3,7-9]. Chyloperitoneum is usually discovered during exploratory laparotomy, and in some cases this is the only intraoperative finding. Negative laparotomies have been reported[3,6,10,11], in which the underlying cause was never discovered[11].

Pancreatitis is a rare cause of chylous ascites formation[12]. It is believed that either lymph may actually leak through destroyed lymphatics due to pancreatic enzyme erosion or that chylous accumulation is the result of exudation of chyle, caused by the obstruction of lymphatic channel flow secondary to severe inflammatory changes that take place in the retroperitoneal space surrounding the pancreas[1]. Most cases involve chronic pancreatitis[13], though acute pancreatitis has also been recognized as the causative reason, with the first such report dating back to 1984[12]. Since then, only a few cases of chylous ascites secondary to acute pancreatitis have been documented[8,13-15]. In almost all of them, the presence of chyle into the peritoneal cavity was discovered at some time after the episode of pancreatitis, usually days or weeks[13-15]. However, Khan *et al*[16]reported a case of acute hyperlipidemic pancreatitis-with normal serum amylase as in our case-that presented with acute chylous peritonitis and was treated conservatively. Smith *et al*[3] operated on a patient with relapsing pancreatitis and acute chylous ascites formation, due to a clinical resemblance with appendicitis.

Therapeutic choices may vary in accordance with the underlying pathology. Thorough lavage of the abdomen and adequate drainage has proven to be an excellent treatment modality for acute chylous peritonitis, since resolution of chylous ascites usually occurs within the next few days. However, successful conservative treatment has also been reported[13,16-18]. This requires proper preoperative diagnosis, which is often difficult due to the exceptional rarity of this pathological condition and its resemblance to other surgical urgencies that call for immediate laparotomy. Long-term fasting supported by total parenteral nutrition offers resolution in many cases. Alternatively, a highprotein and low fat diet has proven to be efficacious in reducing the amount of chyle produced. Administration of octreotide remains controversial[1,13].

In our case, the localization of the pain at the epigastrium and its later course mimicking peritonitis, together with the mild inflammatory changes of the pancreatic head demonstrated on CT imaging, as well as normal amylase values, drew the attention towards a possible duodenal perforation, thus leading the patient to the operating room. Only in the subsequent day and after serial dilutions of the serum samples with the assay buffer were we able to detect an abnormally high level of serum amylase. The interference of excessive serum triglyceride concentrations in the measurement of serum amylase is a known reason for false-negative results and this was also the case with our patient[16]. Moreover, routine serum lipase measurement is not available in our hospital, which could otherwise serve as an adjunct in the diagnosis of acute pancreatitis. However, it should be noted that serum lipase levels too can appear normal in patients with triglyceride-rich serum[16]. In our patient, peritoneal lavage and adequate drainage offered sufficient treatment, while

the patient was gradually restored to a full fat-free diet in order to deal with the pancreatitis. Finally, the patient was encouraged to cease alcohol use.

In conclusion, acute abdominal pain due to sudden accumulation of chyle in the peritoneal cavity is a rare situation that the clinician should be aware of in cases of acute abdomen.

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CASE REPORT

Paroxysmal drastic abdominal pain with tardive cutaneous lesions presenting in Henoch-Schönlein purpura

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Abstract

Henoch-Schönlein purpura (HSP) is a small-vessel vasculitis mediated by IgA-immune complex deposition. It is characterized by the clinical tetrad of non-thrombocytopenic palpable purpura, abdominal pain, arthritis and renal involvement. The diagnosis of HSP is difficult, especially when abdominal symptoms precede cutaneous lesions. We report a rare case of paroxysmal drastic abdominal pain with gastrointestinal bleeding presented in HSP. The diagnosis was verified by renal damage and the occurrence of purpura.

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Key words: Henoch-Schönlein purpura; Abdominal pain; Gastrointestinal bleeding

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INTRODUCTION

Henoch-Schönlein purpura (HSP) is the most common systemic vasculitis in children. The diagnostic criteria include palpable purpura with at least one other manifestation including abdominal pain, IgA deposition, arthritis or arthralgia, and renal involvement[1,2]. Immune complex deposits induce necrosis of the walls of small- and medium-sized arteries with infiltration of the tissue by neutrophils and the deposition of nuclear fragments, a process called leukocytoclastic vasculitis[3]. It is often associated with infection, certain medications, or tumors. It may coexist with or mimic Crohn's disease[4]. Periumbilical and epigastric pain worsen with meals due to bowel angina. Bleeding is usually occult or, less commonly, associated with melena. Intussusception is the most common surgical complication. Perforations, usually ileal, may occur spontaneously or be associated with intussusception. An ultrasound, recommended as the first diagnostic test, and computed tomography (CT) scans may reveal intussusception and asymmetric bowel wall thickening mainly involving the jejunum and ileum. There are a range of

possible endoscopic findings including gastritis, duodenitis, ulceration, and purpura, with the second portion of the duodenum characteristically being involved more than the bulb. Intestinal biopsies show IgA deposition and leukocytoclastic vasculitis in the submucosal vessels[3]. Superficial biopsies may show inflammation, ulceration, edema, hemorrhage, and vascular congestion, presumably due to vasculitis-induced mucosal ischemia[5]. The efficacy of corticosteroids in preventing severe complications or relapses is controversial. The majority of patients, however, improve spontaneously.

CASE REPORT

A 15-year-old boy was referred from another hospital to our institution in November 2010. He was previously healthy without any remarkable past medical history and denied any recent non-steroidal anti-inflammatory drug use or illicit drug use. He complained of progressive epigastrium and periumbilical pain, nausea and mild diarrhea with melena that had lasted for 3 wk. No rash was noticed on the skin. On physical examination, his vital signs were normal, except for epigastric pressing pain and suspicious rebound pain. Importantly, no skin rash was observed.

Laboratory blood examinations showed the following indexes (normal range in parentheses): hemoglobin, 109 g/L (120-140 g/L); peripheral white cell count, 20.09 $\times 10^9$/L (5-10 $\times 10^9$/L); neutrophils, 83.7% (40%-60%); peripheral red cell count, 5.43 $\times 10^{12}$/L (4.0-4.5 $\times 10^{12}$/L); platelet count, 267 $\times 10^9$/L (100-300 $\times 10^9$/L); C-reactive protein, 68.0 mg/L (0-6.0 mg/L); erythrocyte sedimentation rate, 14 mm/h (0-20 mm/h); albumin, 24.8 mg/L (36-51 mg/L); and total immunoglobulin (Ig), 17.6 mg/L (25.0-35.0 mg/L); IgA, 2.292 g/L (0.7-3.3 g/L); total bilirubin, 6.3 μmol/L (4-23.9 μmol/L); alkaline phosphatase, 38 U/L (35-125 U/L); c-glutamyl transpeptidase, 12 U/L (7-50 U/L); aspartate aminotransferase, 15 U/L (14-40 U/L); alanine aminotransferase, 21 U/L (5-35 U/L); prothrombin time, 13.8 s (11.0-14.5 s); creatinine, 453 μmol/L (31.8-91.0 μmol/L), and blood urine nitrogen, 33 g/L (31.8-91.0 g/L). A routine urine test did not reveal any RBCs or proteins on the first day of hospitalization. Autoimmune-related indicators and tuberculosis (TB)-related antibodies were not found in the blood, and the TB-purified protein derivative (PPD) skin test was negative. Hepatitis B and C markers were also negative.

Plain abdominal radiography revealed incomplete small-intestine obstruction. Gastroscopy and colonoscopy revealed some mucosal swelling, erosion, and active ulcers with hemorrhage in the duodenum and terminal ileum (Figure 1), but not in the stomach or colon. The histopathology of duodenal mucosa and ileal mucosa showed a chronic mucosal inflammation with necrosis (data not shown). Ultrasound endoscopy (EUS) and abdominal CT examinations revealed the presence of thickened intestinal mucosa with submucosal hemorrhage and enlarged mesenteric and retroperitoneal lymph nodes (Figure 2). EUS-guided fine-needle aspiration (EUS-FNA) biopsy of the enlarged lymph nodes revealed an inflammatory reaction without lymphoma (data not shown). Positron emission tomography and computed tomography also indicated that the enlarged lymph nodes were a sign of inflammation, not of a malignant tumor (data not shown).

During the first two weeks of hospitalization, the patient accepted treatment with antibiotics and a proton pump inhibitor (pantoprazole 40 mg, twice daily), but his abdominal pain was not alleviated, and the paroxysmal abdominal pain worsened progressively. After two weeks of hospitalization, the patient's urine test revealed significant albuminuria. Levels of urine albumin increased to 1170 mg/L (0-20 mg/L), and 24-h total urine protein increased to 4.08 g (0-0.12 g). Further, the urine protein analysis showed a remarkable increase in kappa and lambda light chain levels to 62.5 mg/L (0-7.1 mg/L) and 28.8 mg/L (0-3.9 mg/L), respectively. The level of urine IgA increased to 102 mg/L (0-17.5 mg/L). Therefore, the diagnoses were considered to be an autoimmune-related disorder such as Crohn's disease and multiple vasculitis or lymphoma. The patient was treated with corticosteroid therapy in the form of intravenous methylprednisolone (40 mg/d). By the next day, the drastic abdominal pain was rapidly alleviated, and the melena also gradually disappeared. After one week of methylprednisolone therapy, the treatment was changed to the oral administration of prednisolone therapy (30 mg/d). During the fourth week of corticosteroid therapy, palpable purpura appeared over the patient's lower extremities. A diagnosis of HSP was ultimately established. After four weeks of corticosteroid treatment, endoscopy and EUS showed that the patient's mucosal damage had improved significantly, and the mucosal edema was observably mitigated (Figure 3). A percutaneous renal biopsy revealed focal segmental endocapillary crescent-like proliferation (Figure 4). Positive immunofluorescence staining for mesangial IgA was also observed (data not shown). The histopathology of renal biopsies supported the diagnosis of anaphylactoid purpura nephritis (APN), which was classified as ISKDC Ⅳ according to the classification criteria of the International Study of Kidney Disease in Children[6].

DISCUSSION

HSP is a form of blood vessel inflammation or vasculitis, also referred to as anaphylactoid purpura. Vasculitis is involved in many conditions[1]. Each of the forms of vasculitis tends to involve certain characteristic blood vessels. HSP is a multisystem disorder predominantly affecting the skin, joints, gastrointestinal tract and kidneys, but other organs can be affected as well. Neurological, pulmonary, cardiac and genitourinary complications rarely occur. HSP results in a skin rash (most prominent over the buttocks and behind the lower extremities) associated with joint inflammation (arthritis) and sometimes cramping pain in the abdomen. The condition primarily affects children (over 90% of cases); occurrences in adults have

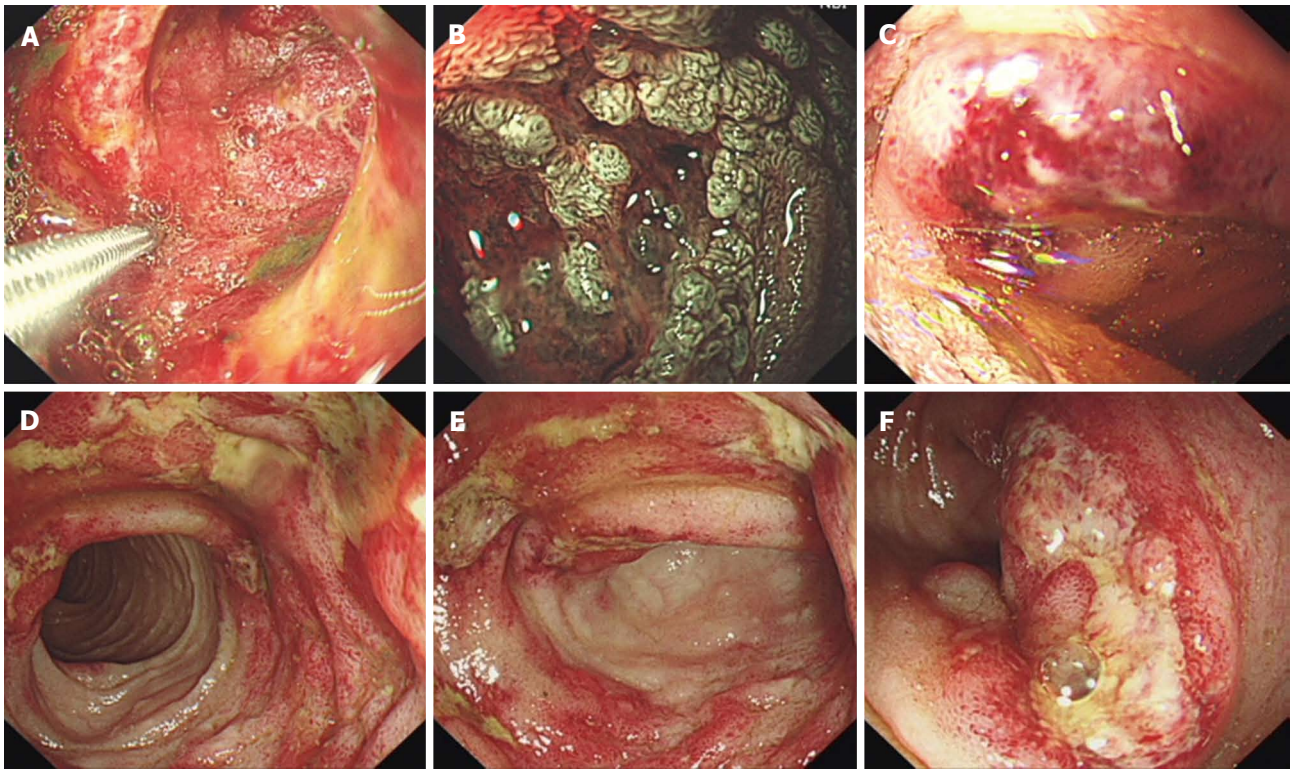

Figure 1 The endoscopy examination revealed intestinal damage. A-C are gastroscopic images, which show significant damage to the duodenum. A: Diffuse redness, swelling, hemorrhage and petechiae in the mucosa; B: Distortion and proliferation of the duplicature (narrow-band image); C: Ulcer; D-F: Colonoscopic images and demonstrate significant damage to the terminal ileum; D: Mucosal hemorrhage and petechiae; E, F: Ulcers.

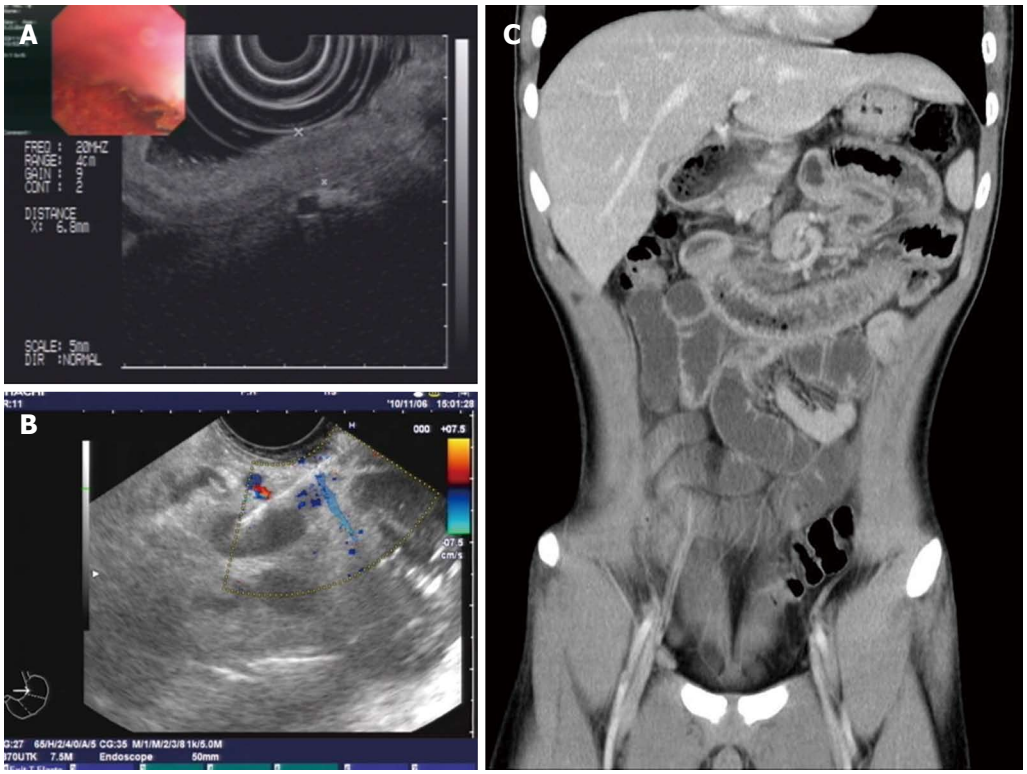

Figure 2 The ultrasound endoscopy and the computed tomography scan indicated the presence of thickened intestinal mucosa and enlarged abdominal lymph nodes. A, B: Ultrasound endoscopy scan images; C: Computed tomography scan images.

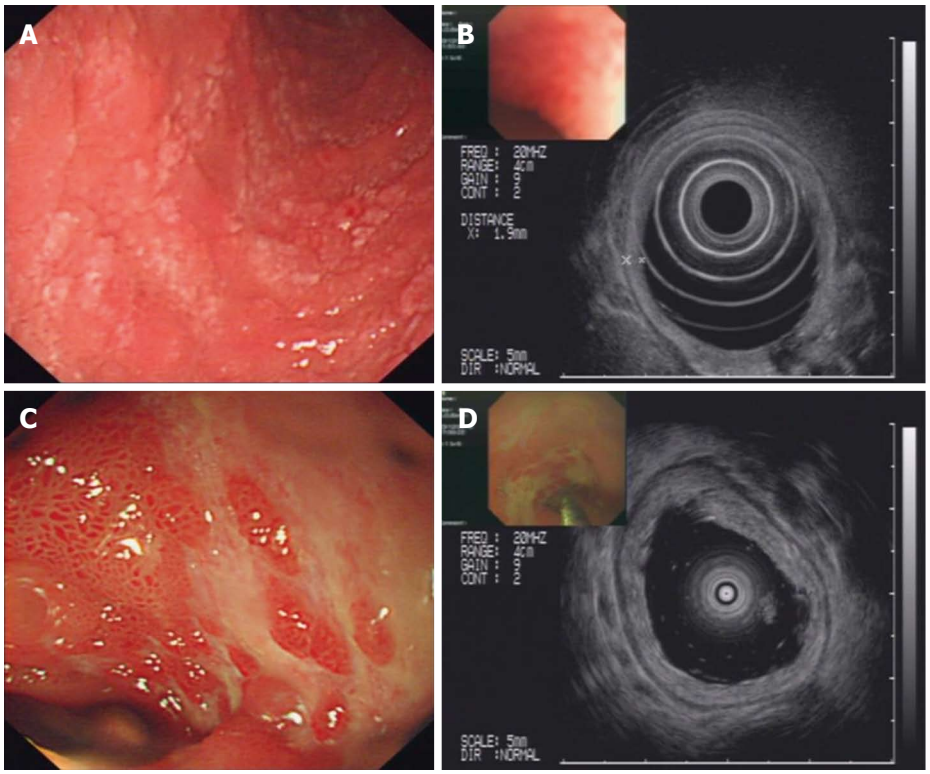

Figure 3 The extent of damage to the intestinal mucosa improved after treatment. A: Gastroscopic image of the duodenum; B: Ultrasound endoscopy (EUS) image of the duodenum; C: Colonoscopic image of the terminal ileum; D: EUS image of the terminal ileum.

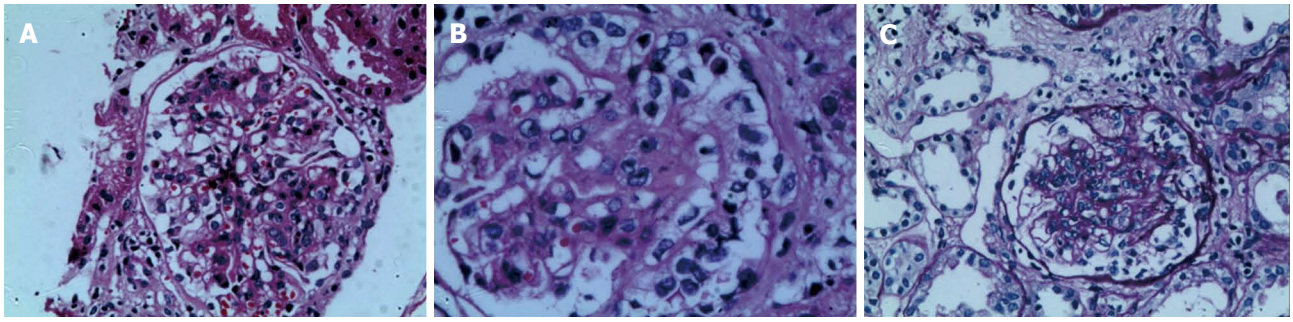

Figure 4 A percutaneous renal biopsy revealed focal segmental endocapillary crescent-like proliferation. A: HE staining, 200 ×; B: HE staining, 400 ×; C: Periodic acid-Schiff staining, 200 ×. HE: Hematoxylin and eosin.

rarely been reported (3.4 to 14.3 cases per million)[3]. HSP occurs most often in the spring and frequently follows an infection of the throat or breathing passages. HSP seems to represent an unusual reaction of the body's immune system in response to this infection (either bacterial or viral). In addition to infection, drugs can trigger the condition. HSP occurs most commonly in children, but people of all age groups can be affected.

HSP is usually diagnosed based on skin, joint, and kidney findings. Throat culture, urinalysis, and blood tests for inflammation and kidney function are used to determine the diagnosis. A biopsy of the skin, and less commonly, the kidneys, can be used to demonstrate the presence of vasculitis. Special staining techniques (direct immunofluorescence) of the biopsy specimen can be used to document antibody deposits of IgA in the blood vessels of involved tissue. Renal involvement is rarely severe and is observed in approximately 50% of patients[1,2].

According to the diagnostic criteria of the European League against Rheumatism and the Paediatric Rheumatology European Society published in 2006, palpable purpura often presents with one of the following: diffuse abdominal pain, a biopsy showing predominant IgA expression, acute arthritis/arthralgia, or renal involvement defined as any hematuria or proteinuria. However, when gastrointestinal manifestations occur alone or precede dermatological or renal disease, the diagnosis is difficult[7,8]. Typically, palpable purpura will not precede renal involvement[9,10]. However, in this case, purpura occurred during the seventh week of onset, and renal involvement occurred during the fourth week of onset, which delayed the diagnosis of HSP. HPS may coexist with Crohn's

disease or mimic its symptoms (e.g., ileitis or ulcerative colitis)[4,11,12]. The clinical manifestations of HSP in the gastrointestinal tract are similar to gastrointestinal tuberculosis, lymphoma, inflammatory bowel disease, and other autoimmune disorders, which renders the diagnosis of HSP difficult. In this case, paroxysmal drastic abdominal pain with gastrointestinal bleeding was presented as the princeps clinical situation. The characteristic endoscopic findings included diffuse mucosal redness, small ring-like petechiae, hemorrhagic erosions and ulcers. These manifestations created confusion until the diagnosis of HSP was verified by the presence of renal damage and purpura. Mucosal lesions develop anywhere within the gastrointestinal tract. The small intestine is considered to be the most frequently affected site, and duodenal involvement was more prominent in the second part of the duodenum than in the bulb and stomach or colon.

Gastrointestinal involvement is frequent in HSP. The diagnosis of HSP may be difficult, especially when abdominal symptoms precede the characteristic palpable purpura. Typical endoscopic findings may alert gastroenterologists to the need to consider this diagnosis early in treatment and thus avoid unnecessary laparotomy.

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CASE REPORT

Melena-associated regional portal hypertension caused by splenic arteriovenous fistula

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Abstract

Regional portal hypertension is a rare cause of upper gastrointestinal bleeding. We reported an extremely rare case in which regional portal hypertension was associated with both the splenic arteriovenous fistula and chronic pancreatitis. In June 2010, our patient, a 41-year-old man, was admitted to a local hospital due to a sudden melena and dizziness without haematemesis and jaundice. The splenic arteriovenous fistula in this patient was successfully occluded through transcatheter arterial embolization. At the 12-mo follow-up, our patient was in good condition.

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Key words: Regional portal hypertension; Splenic arteriovenous fistulas; Pancreatitis; Transcatheter arterial embolization; Upper gastrointestinal bleeding

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INTRODUCTION

Regional portal hypertension is a rare cause of upper gastrointestinal bleeding, with pancreatitis disease being the most frequently reported cause in the literature[1,2]. Splenic arteriovenous fistulas are also rare. Until now, there are approximately 126 reported cases of splenic arteriovenous fistula in the database of PubMed. Herein, we reported an extremely rare case in which regional portal hypertension was associated with both the splenic arteriovenous fistula and chronic pancreatitis.

CASE REPORT

A 41-year-old man was admitted to a local hospital in June 2010 due to a sudden melena and dizziness without haematemesis and jaundice. He was managed conservatively, with fluid support and blood transfusion. No melena occurred again, and the symptom of dizziness was reduced. With the melena of unknown causes, he was referred to our institution for further diagnosis and treatment. On admission, his temperature was 36.7 ℃, pulse was 85 times/min, respiration was 19 times/min, and blood pressure was 124/76 mmHg. Splenomegaly was found in physical examination. The liver was not palpable and no signs of jaundice were observed. The patient had a history of alcohol abuse, but no history of liver disease, trauma and surgery. Five years ago, the patient had an acute pancreatitis which recurred for five times. The details of laboratory tests were as follows: red blood

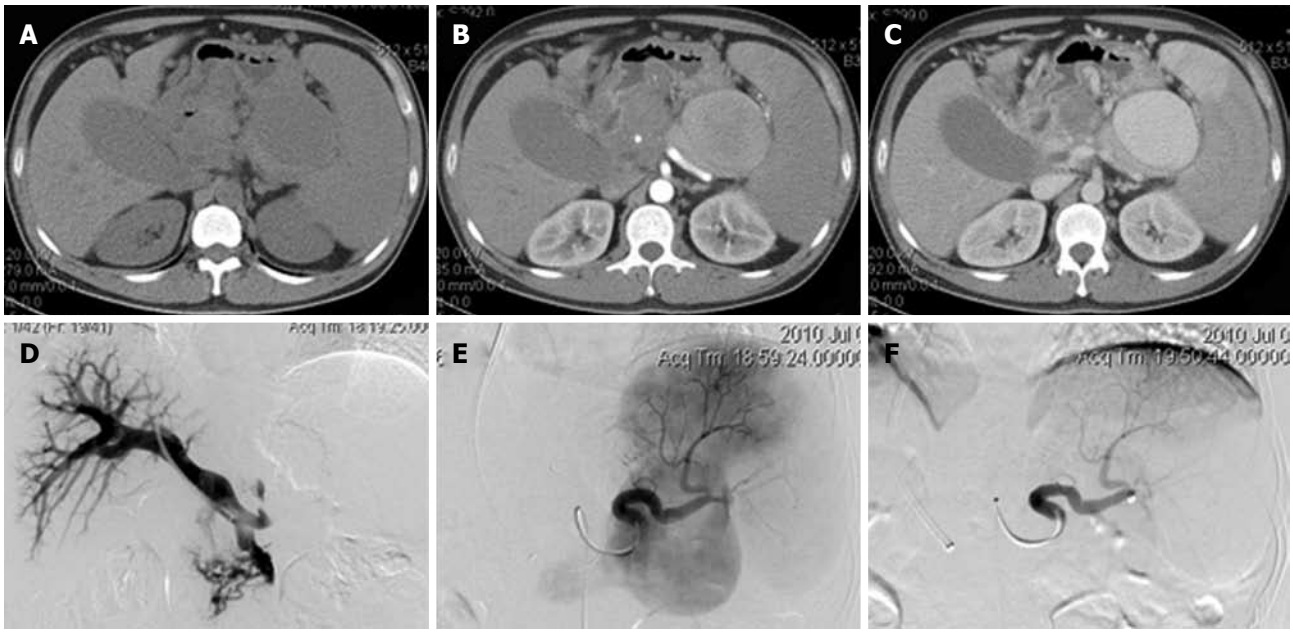

Figure 1 The main foundings of enhanced computed tomography and angiography. A: An oval cystic low-density lesion located in the area of splenic hilum; B: The lesion was enhanced significantly on the arterial phase; C: Significantly enhanced on the portal phase of contrast-enhanced scan; D: A normal portal pressure and an increased pressure of splenic vein of 23.5 mmHg; E: Splenic arteriovenous fistula manifested by angiography; F: Splenic arteriovenous fistula being totally occluded after embolization.

cell 4.13 × 1012/L, hemoglobin 119 g/L, platelet 246 × 109/L, white blood cell 6.90 × 109/L, total bilirubin 13.3 μmol/L, albumin 42.9 g/L, creatinine (CREA) 572.9 μmol /L, glucose (GLU) 0 9.53 mmol/L, GLU120 21.75 mmol/L, prothrombin time 12.0 s, and alpfa-fetoprotein 1.36 ng/mL. Tests for HBsAg, HBeAg, anti-HBe, anti-HBc, anti-HCV and anti-HBs were all negative, except for anti-HBs. Gastroscopy revealed severe gastric varices and non-atrophic gastritis with bile reflux. Unenhanced computed tomography (CT) scanning of upper abdomen showed an oval cystic low-density lesion located in the area of splenic hilum, which was easily misdiagnosed as pancreatic pseudocyst on unenhanced CT (Figure 1A) and as pseudoaneurysm on contrast-enhanced scan. The lesion was significantly enhanced on the arterial phase of contrast-enhanced scan (Figure 1B). And on the portal phase of contrast-enhanced CT scan, this lesion was further enhanced and collateral vessels could be shown at the fundus of the stomach (Figure 1C). Additionally, thrombosis in the splenic vein was also found on the portal phase of contrast-enhanced CT scan. Biopsy of the liver was not performed. The current diagnosis was regional portal hypertension considered to be related to chronic pancreatitis. Under local anesthesia, a percutaneous transjugular approach was used to estimate the portal venous pressure; the portal pressure was normal and an increased pressure of splenic vein was 23.5 mmHg (Figure 1D). Through a percutaneous femoral approach, splenic artery angiography showed, on the arterial phase, a smaller splenic artery, splenic vein aneurysmal expansion, and esophageal and gastric varices, suggesting the formation of splenic arteriovenous fistula (Figure 1E). With splenic artery being extremely tortuous, it was difficult for conventional catheter to track the orifice of the fistula through target vessels, therefore embolization of the fistula was performed using a micro-catheter. Postoperative angiography revealed that splenic arteriovenous fistula had been totally occluded (Figure 1F).

DISCUSSION

Regional portal hypertension, also known as sinistral or left-sided portal hypertension, is a rare cause of upper gastrointestinal bleeding, with pancreatitis disease being the most frequently reported cause in the literature[1,2]. Splenic arteriovenous fistula is a rare but potentially curable cause of portal hypertension. The fistula may be congenital or acquired. It is thought that they arise from rupture of a splenic artery aneurysm or after abdominal trauma, surgery or pancreatitis[3]. To our best knowledge, splenic arteriovenous fistulas related to pancreatitis have been rarely reported and its pathogenesis remains unclear[4]. In this case, the fistula arose from pancreatitis, and regional portal hypertension was caused by splenic arteriovenous fistula and splenic vein thrombosis, with the former playing a major role. As for regional portal hypertension, the key point is to make the correct diagnosis as soon as possible, because it is curable. It should be considered in the presence of gastrointestinal bleeding with normal liver function tests and splenomegaly. Surgical excision of splenic arteriovenous fistula is technically difficult, and is sometimes unsuccessful because of the remote location of the lesion, presence of numerous portal collaterals, and adhesion. Interventional radiologic technique, such as transcatheter arterial embolization, has

been demonstrated to be a safe and effective alternative to surgery[5]. The splenic arteriovenous fistula in our patient was successfully occluded through transcatheter arterial embolization. At the 12-mo follow-up, this patient was found in good condition.

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Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

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

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
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Hollywood, FL 33028, United States

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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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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