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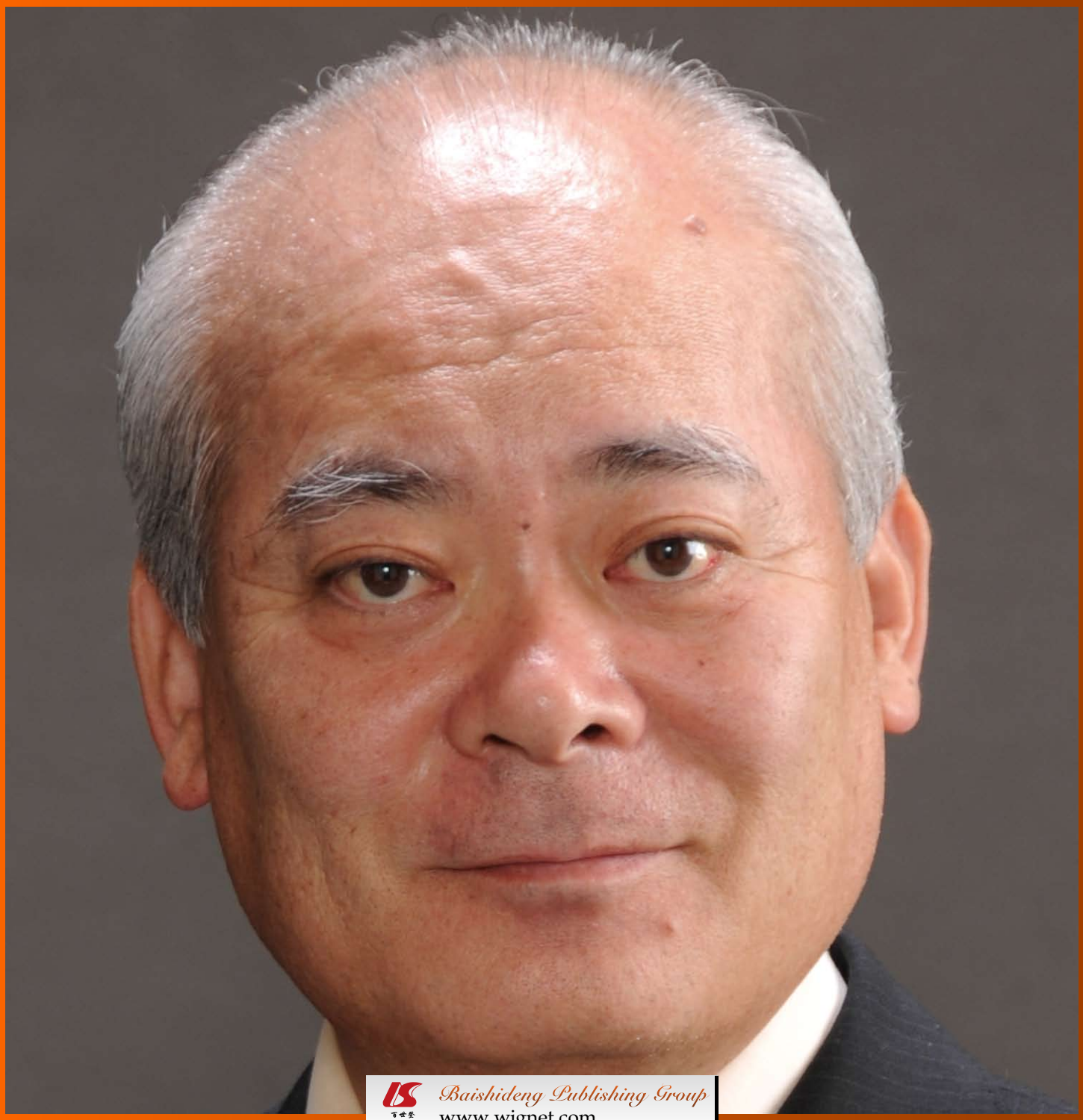

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EDITORIAL

Biliary cysts: Etiology, diagnosis and management

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Abstract

Biliary cysts (BC) are rare dilatations of different parts of a biliary tract. They account for approximately 1% of all benign biliary diseases. BC occur the most frequently in Asian and female populations. They are an important problem for pediatricians, gastroenterologists, radiologists and surgeons. Clinical presentation and management depend on the BC type. Cholangiocarcinoma is the most serious and dangerous BC complication. The other complications associated with BC involve cholelithiasis and hepatolithiasis, cholangitis, acute and chronic pancreatitis, portal hypertension, liver fibrosis and secondary liver cirrhosis and spontaneous cyst perforation. Different BC classifications have been described in the literature. Todani classification dividing BC into five types is the most useful in clinical practice. The early diagnosis and proper treatment are very important, because BC are associated with a risk of carcinogenesis. A malignancy risk increases with the age. Radiological investigations (ultrasonography, computed tomography, endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography) play an important role in BC diagnostics. Currently, prenatal diagnosis using ultrasonography is possible. It allows to differentiate biliary disorders in fetals and to perform the early surgical treatment that improves results. In most patients, total cyst excision with Roux-Y hepaticojejunostomy is the treatment of choice. Surgical treatment of BC is associated with high success rate and low morbidity and mortality. The early treatment is associated with a lower number of complications. Patients following BC surgery require permanent and careful postoperative observation using laboratory and imaging investigations because of possibility of biliary anastomosis stricture and biliary cancer in tissue remnant.

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Key words: Biliary cysts; Biliary drainage; Hepaticojejunostomy

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INTRODUCTION

Biliary cysts (BC) are rare dilatations of different parts of a biliary tract. They account for approximately 1% of all benign biliary diseases. BC occur the most commonly in Asian populations and in females (with a female:male ratio of 3-4:1). Incidence of this pathology in Asian populations is 1:1000 and it is lower in western countries (1:100 000 to 1:150 000)[1-6]. They are usually a surgical problem of infancy or childhood; however in approximately 20% of cases they are recognized in adults[2]. The early diagnosis and proper treatment are very important, because BC are associated with a risk of carcinogenesis. According to the literature, biliary tract malignancy occurs in 2.5%-28% of patients with BC[3,4]. The risk of biliary cancer increases with the age. It is the lowest in the childhood in the first decade (< 1%)[6]. In the third decade the cancer

risk is > 10%[7,8]. According to the literature, cholangiocarcinoma is reported the most frequently at the age of 32 years in patients with BC (about 20 years earlier than in general population)[7]. There are reports of cholangiocarcinoma developing in adult many years after radical BC resection in infant[9]. Cholangiocarcinoma is the most serious and dangerous BC complication. The other complications associated with BC involve cholelithiasis and hepatolithiasis, cholangitis, acute and chronic pancreatitis, portal hypertension, liver fibrosis and secondary liver cirrhosis and spontaneous cyst perforation[8-12]. Nicholl *et al*[9] noted a higher number of complications in adults compared to children. Therefore, BC constitute an important problem for pediatricians, gastroenterologists, radiologists and surgeons. The role of pediatricians and gastroenterologists is a proper early diagnosis. Radiological investigations play an important role in BC diagnostics. Currently, the prenatal BC diagnosis using ultrasonography (USG) is possible[6,13,14].

Surgery is the treatment of BC. The goal of surgical treatment is to remove a cyst and to reconstruct the proper bile flow to the alimentary tract. In order to achieve this goal, different techniques are used. Roux-Y hepaticojejunostomy is the method of choice in most patients with BC[5-22].

ETIOLOGY AND PATHOGENESIS OF BC

Many theories explain the etiology and pathogenesis of BC[2]. In 1936, Yotsuyanagi supposed that BC arouse from inequality in the cellular proliferation of the biliary tract during the early fetal life[23]. Babbitts's theory of the "common channel" (1969) is the most widely accepted in the literature. According to this theory, the common cannel is formed by abnormal pancreaticobiliary junction (APBJ) of the pancreatic and bile ducts outside the ampulla of Vater. This condition leads to pancreaticobiliary reflux and mixing of the pancreatic and biliary juices and activations of pancreatic enzymes, because the maximal pressure within the pancreatic duct is two to three timed higher than within a biliary tract. Activated pancreatic enzymes cause inflammation and deterioration of the bile duct wall that leads to biliary dilatation[24]. A number of studies analyzed the levels of amylase within the bile duct. The higher amylase concentrations have been observed in patients with BC compared to the control groups. Also, association between the amylase level, earlier presentation and dysplasia grade in patients with BC has been reported in the literature[25,26]. Therefore, there is a theory, that the pancreaticobiliary reflux leads to inflammation and dysplasia in patients with BC[2,26-29]. APBJ occurs only in 50%-80% patients with BC. Another counterargument to this hypothesis is a fact, that immature neonatal acini do not produce sufficient pancreatic enzymes to explain antenatal BC[2,30,31]. The counterargument supporting Babbitt's theory is arbitral definition of the long common channel that depends on imaging modality and angles. Therefore, a defined length of a long common channel has been described from 10 mm to 45 mm in the literature. Therefore, APBJ can occur in more patients than radiological investigations show[2]. According to Okada *et al*[32], long common channel as any pancreaticobiliary junction is defined, if it lies outside of the duodenal wall and thus could result in pancreaticobiliary reflux and mixing. APBJ has been divided into three types by Komi *et al*[33]: (1) A right-angled union without an accessory pancreatic duct; (2) An acute-angled union without an accessory pancreatic duct; and (3) A right- or acute-angled union with an accessory pancreatic duct.

Table 1 Types of an anomalous pancreaticobiliary junctions (Komi classification)

Type name	Type description
Type Ⅰ	A narrowed common bile duct joins the pancreatic duct at a right angle
ⅠA	A slender common channel
ⅠB	A short or long ectatic common channel
Type Ⅱ	A pancreatic duct joins the common bile duct at an acute angle
ⅡA	A slender common channel
ⅡB	A short or long ectatic common channel
Type Ⅲ	It is complicated by a patent accessory pancreatic
ⅢA	A classic pancreas divisum
ⅢB	A pancreas divisum without a pancreatic duct
ⅢC	A patent accessory pancreatic duct with an intricate network
ⅢC1	A tiny communicating duct between the main and accessory ducts
ⅢC2	A communicating duct with the same caliber as the main and accessory ducts
ⅢC3	A total or partial dilatation of the ductal system

These types are subdivided into subtypes according to the shape of the common channel. The Komi's classification is presented in Table 1. The type of BC and clinical presentation can depend on the type of an angle of the anomalous ductal junction. A right-angled union is associated with cystic dilatation of the common bile duct. In patients with this type of union a palpable mass or jaundice as the main sign of disease are observed. An acute-angled union is associated with fusiform dilatation of the common bile duct. Patients with such dilatation usually complain on abdominal pain, and an erroneous diagnosis of acute pancreatitis is occasionally made. Type ⅠB, ⅡB, and Ⅲ junctions are associated with a dilated common channel and an accessory pancreatic duct. They are frequently complicated by relapsing pancreatitis leading to chronic pancreatitis[34]. Therefore, it's important to describe the type of APBJ. The type of APBJ is based on a cholangiopancreatographical picture[33-35]. The other theories regarding BC pathogenesis are less popular. According to Singham *et al*[2], biliary dilation can be a result of embryologic overproliferation of epithelial cells within solid bile ducts during the fetal life. According to Davenport and Basu, dilations are due to a lower number of neurons and ganglions in patients with BC that have been recognized pathologically. They suggested that round cysts were congenital with distal obstruction due to agan-

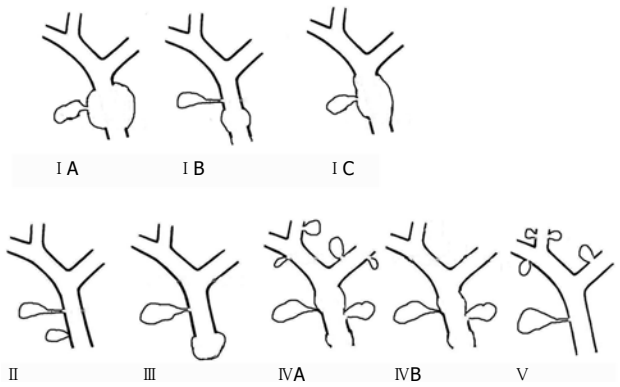

Figure 1 Types of biliary cysts according to Todani classification. I: Solitary extrahepatic cyst (40%-85%): I A: Common type; I B: Segmental extrahepatic duct dilatation; I C: Diffuse extrahepatic dilatation; II: Extrahepatic diverticulum (2%-3%); III: Choledochocele (1.4%-5.6%); IVA: Multiple extra- and intrahepatic cysts; IVB: Multiple extrahepatic cysts (18%-20%); V: Multiple intrahepatic cysts (Caroli's disease) (rare).

glionosis and proximal dilation similarly to Hirschprung's disease. In this theory, chronic inflammation and clinical manifestation are caused by bile stasis within dilated bile ducts[2,31,36].

CLASSIFICATIONS OF BC

The first classification system for BC was proposed by Alonso-LEJ *et al*[37] in 1959. This classification involves three BC types: congenital cystic dilatation (the most common), congenital diverticulum of the common bile duct, and congenital choledochocele[37]. In 1977, Todani *et al*[38,39]modified BC classifications distinguishing five types (Figure 1). The most frequent (more than 90%) is type I cyst. The type I A cyst involves dilatation of the common bile duct, with marked dilatation of part or all of the extrahepatic bile ducts. The type I B cyst involves segmental dilatation of the common bile duct, usually of its most distal part. The type I C cyst involves fusiform dilatation of the common bile duct, along with diffuse, cylindric dilatation of the common hepatic duct and common bile duct. In 2011, Michaelides *et al*[40] reported and proposed a new BC variant and called it the I D type. In this new variant, apart from the dilatation of the common hepatic and the common bile duct, dilatation of the central portion of the cystic duct was also observed, giving a bicornal configuration to the cyst. The type II cyst is a diverticulum of the common bile duct usually arising laterally but may arise in the pancreatic portion. The type III cyst (which is called a choledochocele) is a cystic dilatation of the intraduodenal portion of the common bile duct. According to some authors, choledochocele is different from other types of BC. In 2010, Ziegler *et al*[41], compared choledochocele with other types of BC (types I, II, IV and V according to Todani classification). Based on study including 146 patients with BC (46 patients with choledochoceles), they concluded that classifications of choledochal cysts should not include choledochoceles. They reported that patients with choledochoceles differed from patients with other choledochal cysts with respect to age, gender, presentation, pancreatic ductal anatomy, and their management. Patients with cholechoceles were the most frequently male, older, complained with acute pancreatitis rather than jaundice or cholangitis, and were managed with endoscopic therapy. A pancreas divisum and a low risk of malignancy compared to other BC were observed with patients with choledochoceles. The type IV cyst involves dilatation of the intrahepatic and extrahepatic bile ducts. In the type IVA cyst, both the intrahepatic and extrahepatic bile ducts are dilated. The type IVB cyst involves dilatation of multiple segments of the extrahepatic bile ducts. The type V cyst (Caroli's disease) involves dilatation of one or several segments of the intrahepatic bile ducts. First, it was described by Todd in 1818, but precise definition of this disorder was constructed by Jaques Caroli in 1958[41-45]. There are two descriptions (Caroli's disease and Caroli's syndrome) in the literature. Caroli's syndrome is the more serious condition compared to Caroli's disease. In Caroli's disease, cholangitis due to bile stasis within dilated intrahepatic bile ducts, is observed. In Caroli's syndrome, recurrent bouts of cholangitis due to bile stasis, hepatolithiasis, gallbladder stones lead to liver fibrosis with portal hypertension and liver failure. Caroli's syndrome can be associated with autosomal recessive polycystic kidney disease caused by a mutation of the *PKHD1* gene[46,47].

BC can be associated with the biliary atresia. Three biliary atresia (BA) types have been distinguished in the literature: 1 BA (atresia of the common bile duct), 2 BA (atresia of the common hepatic duct), and 3 BA (atresia of the porta hepatis)[18]. Muise *et al*[18], concluded that BA associated with BC formed a distinct subtype of BA, characterized by a preponderance of type 1 BA, a relatively good clinical outcome after surgery, and an absence of associated congenital anomalies. In 2004, Visser *et al*[48] proposed modified classification of BC and distinguished the following BC types: choledochal cyst, choledochal diverticulum, choledochocele, and Caroli's disease. All BC classifications are presented in Table 2.

CLINICAL PRESENTATION OF BC

The classic triad of clinical symptoms was described by Alonso-LEJ *et al*[37] and it involves right hypochondriac pain, palpable abdominal mass and jaundice. All clinical signs are observed only in 20%-30% patients[49]. Two of three clinical symptoms occur in 2/3 patients. The most frequently (in 80% of patients) clinical presentation appears before the age of 10 years[44]. Clinical presentation is different and it depends on the patient's age. According to the age, patients are divided into two groups: neonatal patients (under 12 mo) and adults (above 13 mo). Prenatally, BC are diagnosed incidentally during prenatal ultrasonography visualized as an intra-abdominal cystic mass[6,13,14]. In neonatal patients, obstructive jaundice

Table 2 Classifications of biliary cysts

Ref.	Classification
Alonso *et al*[37]	Congenital cystic dilatation
	Congenital diverticulum of the common bile duct
	Congenital choledochocele
Todani *et al*[38,39]	Ⅰ A Common type
	Ⅰ B Segmental extrahepatic duct dilatation
	Ⅰ C Diffuse extrahepatic duct dilatation
	Ⅱ Diverticulum type in the whole extrahepatic duct
	Ⅲ Choledochocele
	ⅣA Multiple cysts of the extra- and intra-hepatic ducts
	ⅣB Multiple cysts of the extrahepatic ducts
	Ⅴ Caroli's disease (intrahepatic bile duct cysts)
Vesser *et al*[48]	Choledochal cyst
	Choledochal diverticulum
	Caroli's disease

and abdominal mass are present the most frequently. Pain, fever and nausea are the most common for adult patients[44]. According to Lopez *et al*[21], pain is the commonest clinical sign in adults. Recurrent cholangitis and pancreatitis are complications of BC due to pancreaticobiliary reflux[49,50]. The other complications observed commonly in adults are the following: cholangiocarcinoma, cholecystolithiasis and cholecystitis, and liver cirrhosis[5]. Cholecystolithiasis and cholecystitis occur due to bile stasis in patients with BC. The secondary liver cirrhosis leads to different complications associated with portal hypertension such as upper gastrointestinal bleeding, splenomegaly and pancytopenia[49]. Portal hypertension can also occur without liver cirrhosis due to mechanical obstruction of the portal vein by the cyst[48]. An spontaneous BC rupture occurs in about 1%-12% of patients. It is presented as abdominal pain, peritonitis signs and sepsis[49,51]. Patients with choledochocele are usually asymptomatic. The Ⅲ BC type can be also presented by gastric outlet obstruction symptoms caused by direct obstruction of the duodenal lumen or intussusceptions[44]. It should be highlighted that BC are premalignant disorder. The overall reported risk of cancer is 10%-30% and it increases with age (from 0% in patients aged < 10 years to 75% in patients aged 70-80 years). Nicholl *et al*[9] reported direct correlation between the patient age and cancer risk: 0 year to 30 years (0%), 31 years to 50 years (19%), and 51 years to 70 years (50%). The histopathological types of cancer are the following: *adenocarcinoma* (73%-84%), *anaplastic carcinoma* (10%), *undifferentiated cancer* (5%-7%), *squamous cell carcinoma* (5%), and others (1.5%). The locations of cancer are the following: the extrahepatic bile duct (50%-62%), gallbladder (38%-46%), intrahepatic bile ducts (2.5%), the liver (0.7%), and the pancreas (0.7%)[49]. Todani *et al*[52] reported that 68% of cancers were associated with type-Ⅰ, 5% type-Ⅱ, 1.6% type-Ⅲ, 21% type-Ⅳ and 6% type-Ⅴ BC. Cholangiocarcinoma occurs as a result of chronic inflammation, cell regeneration and DNA breaks, leading to dysplasia[49,52]. The following molecular lesions have been reported in BC during carcinogenesis: microsatellite instability, k-ras mutations, expression of COX-2 and bcl-2, and increased telomerase activity that occur early and involvement of cyclin D1, beta-catenin, DPC-4/Smad4 and p53 that occur later[7].

DIAGNOSTICS OF BC

Laboratory investigations

Laboratory investigations may demonstrate mildly abnormal liver function and cholestasis tests (serum bilirubin, alkaline phosphatase, γ-glutamyltranspeptidase, alanine and aspartate aminotransferases) or amylase values, but these findings are not specific[34,49-56].

Radiological investigations

Imaging investigations are the most useful in BC diagnosis. The following radiological examinations are performed in order to visualize biliary dilatation: ultrasonography (USG) of the abdominal cavity, computed tomography (CT) of the abdominal cavity, and cholangiography[34,49-56].

USG of abdominal cavity

USG is the initial and easy to perform examination. It allows imaging of intrahepatic and extrahepatic bile ducts with measurement of the diameter of common bile duct or common hepatic duct and BC. It demonstrates (with the exception of type-Ⅲ and type-Ⅴ cysts) a cystic mass in the right upper quadrant (usually at the porta hepatis) separately from the gallbladder[49].

Use of USG in prenatal diagnosis

USG is very useful in prenatal diagnosis of BC. USG shows BC as a round intra-abdominal cystic mass located in the upper abdominal quadrant. Color Doppler USG shows no significant flow within the mass. Differential diagnosis of the cystic mass in prenatal USG should involve simple hepatic cysts, biliary atresia, ovarian, omental or mesenteric cysts, duodenal or gall bladder duplications, adrenal cysts, renal cysts, dilated loop of bowel, hydronephrotic renal pelvis and situs inversus. Proper early differential diagnosis between BC and another biliary disorder such as BA is very important, because BA needs an immediate surgery[6,13,14]. When the differential diagnosis of a cystic mass in the right upper quadrant is difficult to make by conventional USG alone, ultrasound-guided BC aspiration may serve as an alternative to the prenatal diagnosis of BC in the fetus[57,58]. Tanaka *et al*[14] compared imaging (USG and CT) and laboratory investigations in BC and BA in order to differentiate these two pathologies. They concluded that patients with biliary disorder smaller than 21 mm, direct bilirubin level higher than 2.5 mg/dL, and total bile acid level higher than 111 μmol/L in the neonatal period were more likely to have BA than BC and needed a surgery as soon as possible before irreversible liver cirrhosis. Okada *et al*[17] described the differential diagnosis between BC and BA by immunohistological examination using liver biopsy specimens.

They indicated that CD56-positive biliary duct cells were present in prenatally diagnosed type-1 cystic BA.

Cholangiography

Proper diagnosis requires demonstration of continuity of the cyst with the biliary tract, because it allows to differentiate BC from other intrabdominal cysts such as pancreatic pseudocysts, echinococcal cysts or biliary cystadenomas[49]. In order to show it, cholangiography is performed. There are the following methods of cholangiography: endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance-cholangiopancreatography (MRCP), percutaneous transhepatic cholangiography (PTC) and intraoperative cholangiography. These investigations allow to demonstrate the anatomy of biliary tree and define the BC type[49].

ERCP

ERCP is invasive investigation, but it has got therapeutic possibility. It accurately reveals the presence of any associated intraductal pathology or an APBJ. In the a type Ⅲ BC, it allows to perform a therapeutic papillotomy simultaneously[49].

MRCP

MRCP is a non-invasive procedure compared to ERCP. It is a favoured alternative to ERCP, but it has a lower accuracy in the detection of APBDJ and lacks the therapeutic possibility in case of the type Ⅲ BC. MRCP, as a non-invasive procedure, is investigation of choice in preoperative imaging of biliary tree[5,49,54-56]. A gadoxetic acid-enhanced MRCP in order to diagnose the biliopancreatic bile reflux and pancreatico-biliary reflux of pancreatic secretions in patients with anomalous union of the pancreatico-biliary duct has been described in the literature. Gadoxetic acid-enhanced MRCP can visualize the physiology of bile excretion, in contrast to conventional T2-weighted MRC which can visualize fluid filled space by heavily T2-weighted and fat-suppressed images. It is associated with specific properties of the gadoxetic acid which is uptaken by hepatocytes. Gadoxetic acid is excreted into the bile ducts that allows visualization of the bile ducts on hepatobiliary phase T1-weighted images[59].

PTC

Preoperative PTC allows to define the proximal extent of biliary dilatation and to use this information in preoperative resection planning[49]. According to Lipsett *et al*[53], the preoperative placement of a percutaneous ring catheter after cholangiography is useful for the surgeon during the operative procedure and can be used as a stent for the biliary reconstruction.

A technetium-99 HIDA investigation

A technetium-99 HIDA investigation is recommended to visualize the continuity BC with bile ducts. This investigation shows an initial area of photopenia within BC, with subsequent filling and then delayed emptying into the bowel[49].

CT of abdominal cavity

CT is useful in showing continuity of the cyst with the biliary tree, its relation to surrounding structures and the presence and staging of associated malignancy. It is better than USG in visualization of the intrahepatic bile ducts, distal bile duct and pancreatic head. In patients with type-ⅣA cysts and Caroli's disease, it is useful to describe the intrahepatic dilations and the extent of disease (diffuse hepatic or localized segmental involvement). It is important for a surgeon before operation, because localized type-ⅣA BC or Caroli's disease can be treated with segmental lobectomy[49]. Computed tomography cholangiography (CTC) after infusion of meglumine iodoxamate with subsequent 3-dimensional rendering has been described in the literature. Fumino *et al*[60] compared CTC cholangiography with MRCP in visualizing the pancreaticobiliary maljunction in 53 children. They noted superior visualization of the intrahepatic duct and the pancreatic system by MRCP compared with CTC. But the great advantage of CTC was its ability to produce high-quality images without respiratory artifacts in young infants in whom performing a good-quality MRCP is very difficult.

MANAGEMENT IN BC

BC require surgical intervention in order to avoid complications associated with pancreaticobiliary reflux. Management depends on the BC type. Currently, complete cyst excision with cholecystectomy followed by biliary reconstruction using a Roux-en-Y hepatico-jejunostomy is the treatment of choice[53,61,62].

Internal drainage (cystenterostomy) of BC

Currently, this method of treatment has been abandoned due to a high risk of morbidity and malignancy up to 50% following internal biliary drainage. This surgical procedure involved incision of BC and anastomosis it to the duodenum or jejunum depending to its location. Despite relief of clinical symptoms in operated patients, it was associated with a high postoperative mortality rate due to biliary reflux. Reflux of the enteric juice into the retained cyst and biliary tract leaded to recurrent ascending cholangitis. The another complication of this procedure was biliary anastomosis stricture. The most important complication of this treatment method was malignant transformation within the wall of retained BC[62-64]. Therefore, internal drainage is currently not considered to perform and all patients previously operated in this way should be reoperated in order to totally remove the cyst[62,63,65,66].

Total excision of BC with hepaticoenterostomy

Currently, total BC excision is recommended in order to avoid above mentioned complications. Total BC excision

with hepaticoenterostomy separates the biliary tree from the pancreatic duct that ends mixing pancreatic juice with bile. In situations where the intensity of fibrosis precludes safe periductal or pericystic dissection, Lilly's technique is useful. In this technique, the most densely adherent portion of the cyst wall is retained on the hepatoduodenal ligament, removing only the less adherent portion. The mucosal lining of the retained cyst wall should be ablated by diathermy or be stripped or destroyed by abrasion and iodine or alcohol application, because 57% of the bile duct cancer in BC arises from the posterior wall of the cyst[54,63-70].

There are two possible methods of hepaticoenterostomy: hepaticoduodenostomy and Roux-Y hepaticojejunostomy. According to the literature, the success rate of Roux-Y hepaticojejunostomy is 92%, with complication rate of 7%, compared with a complication rate of 42% following hepaticoduodenostomy. Some authors have compared these two reconstructions[63]. Shimotakahara *et al*[71] did not recommend hepaticoduodenostomy for reconstruction after BC excision due to a higher number of complications (33.3%) such as bilious gastritis due to duodenogastric bile reflux and adhesive bowel obstruction and cholangitis. Contrary to this report, Mukhopadhyay *et al*[72] recommended hepaticoduodenostomy as a simple and quick procedure with preservation of normal anatomy and physiology and minimum complications.

Some technical aspects of Roux-Y hepaticojejunostomy should be discussed. According to the literature, the anastomosis should be wide in order to prevent the postoperative stricture and Roux-Y jejunal loop should be 40-50 cm long[66]. Conventionally, an adult's standard of a 40-cm loop is adopted in Roux-Y hepatojejunostomy) also in children, irrespective of patient size. Diao *et al*[73] compared two patients groups according to Roux-loop length: (1) conventional group (n = 108) where a standard 35-40 cm Roux-loop length was used regardless of the child's size, and (2) short Roux-loop group (n = 110) in which the Roux-loop length was based on the distance between hepatic hilum and umbilicus. The mean Roux-loop length in patients in the second group was significantly shorter than in the conventional group. There was no significant difference between both groups in age, operative blood loss, operative time, postoperative hospital stay, and duration of drainage. In the conventional group, 2 of (1.8%) 108 patients developed Roux-loop obstruction was observed in 1.8% patients in 1st group and did not appear in 2nd group. Mild reflux was reported in 2 patients in 1st group and 1 patient of 2nd group. Authors concluded that shorter Roux-Y loop according to patient's size can be safe and effective alternative for biliary reconstruction in children with BC.

Another technical aspect of BC surgery is management with the distal bile duct stump, after cyst excision, which is usually ligated. Diao *et al*[74] compared two patients groups in order to investigate the feasibility of selectively leaving distal stump unligated. In this study, patients were divided into two groups: (1) non-ligation group (n = 207), where the distal stump was stenotic and was left unligated, and (2) ligation group (n = 63), where the distal stump was not stenotic and was ligated. The pancreatic juice leakage rates were compared. Authors concluded that not ligating distal stump is a feasible approach in patients with the stenotic distal common bile duct. In authors' opinion, this management simplifies the operative procedure and may minimize pancreatic duct injury.

Different treatment depending on BC type

Total cyst excision with Roux-Y hepatojejunostomy has been recommended by most authors in the treatment of I and Ⅳ types of BC[62]. The risk of malignancy in type Ⅱ and Ⅲ BC is low. Therefore, complete cyst excision is not necessary. Simple excision of type Ⅱ BC is sufficient. Choledochoceles often just require endoscopic sphincterotomy in order to allow free duodenal drainage of bile and stones. Large choledochoceles should be treated surgically and excised *via* duodenostomy because of biliary, duodenal or gastric outlet obstruction. In some cases of small cysts, endoscopic excision is possible. Type ⅣA BC is treated by surgical cyst excision and a wide hilar hepaticoenterostomy, but clinical symptoms are frequently observed in patients following operation that is caused by the intrahepatic involvement of disease. If the intrahepatic involvement is localized, a segmental hepatectomy can be performed. In case of diffuse disease, a percutaneous hepaticojejunostomy, surgical or endoscopic unroofing of some intrahepatic cysts can be made. Unilobar Caroli's disease can be treated by hepatic lobectomy. Diffuse Caroli's disease with recurrent cholangitis, liver failure and cirrhosis and portal hypertension or malignant disease requires orthotopic liver transplantation[61]. There are a lot reports regarding laparoscopic excision of BC with extracorporeal biliary reconstruction. Total intracorporeal biliary reconstruction has been also described in the literature. This minimally invasive treatment method is safe and effective, and it is associated with earlier postoperative feeding and discharge from hospital[75,76].

The timing of BC surgery

Early surgical treatment in order to prevent further complications is recommended. It has been observed that early diagnosis and surgical treatment was associated with a lower morbidity compared with later diagnosis and treatment. Therefore, prenatal diagnosis is such important[77,78]. Foo *et al*[77] evaluated the clinical outcomes of patients with prenatally diagnosed choledochal cysts compared with those diagnosed after birth and the optimal timing of definitive treatment. The mean age at operation for the prenatally diagnosed group was 4.4 mo. For the postnatal diagnosed group, the mean age at operation was 5.7 years. Based on analysis of 45 patients, they concluded that prenatal diagnosis of BC resulted in earlier

definitive surgery. In patients operated in older age, more adverse complications were observed[77]. Tsai *et al*[79] compared results of surgical treatment according to patients age at the time of surgery. They compared three patients groups that had been operated: < 1 year old (group Ⅰ, n = 26), 1-16 years old (group Ⅱ, n = 48), and > 16 years old (group Ⅲ, n = 33). Group Ⅰ suffered significantly fewer surgical complications and less severe liver fibrosis compared with groups Ⅱ and Ⅲ. Therefore, authors recommended early surgical treatment. Germani *et al*[80] recommended the surgical treatment after 3 mo of life to increase the success according to the size of the anatomic structure. They recommended the early treatment before 3 mo of life, only in case of severe symptoms.

RESULTS OF THE SURGICAL TREATMENT OF BC

Short-term results and early complications

Surgical treatment of BC is successful in more than 90%. It is associated with a low postoperative morbidity (2.5%-27%) and mortality (0%-6%) rate according to the surgical center and patient's age. The following early complications have been reported in the literature: biliary-enteric anastomosis dehiscence with biliary fistula, intra-abdominal bile collection (biloma) or abscess and acute peritonitis, pancreatic leakage and pancreatic fistula due to injury to the pancreatic duct, acute pancreatitis, acute cholangitis, ileus caused by bowel obstruction due to intussusception or bowel kinking due to manipulation or adhesions, wound infection and wound dehiscence, gastrointestinal bleeding, hepatic failure, and multiple system organ failure[49,54,64,65,67,68,78,81-87].

Long-term results

Late postoperative complications occur in up to 25% patients and they are the following: biliary-enteric anastomosis stricture, peptic ulcer disease, cholangitis, biliary and intrahepatic stones, pancreatitis, liver failure and biliary cancer. Authors recommend to perform a wide bilio-enteric anastomosis in order to prevent its stricture[49,54,64,67,68,77,80-88]. According to the literature, 0%-6% incidence of malignancy occurs following surgery. It is caused by remnant cyst tissue or subclinical malignant disease which had not been detected before surgery. Therefore, some authors recommend intraoperative endoscopic ultrasonography and histopathological investigation of frozen sections to rule out dysplasia, hyperplasia and malignant disease. All patients with BC require long-term follow-up for bile duct cancer, using ultrasonography and laboratory investigations including liver parameters and cancer markers [carcinoembryonic antigen (CEA), CA 19-9, CA-125][49,87-89]. CA 19-9 is the mosts significant, because it is elevated in up to 85% of patients with cholangiocarcinoma. CEA is raised in about 30% of patients and CA-125 in 40%-50% of patients with cholangiocarcinoma. These markers are not specific for bile duct cancer, because their levels are also raised in other neoplasms and inflammatory diseases[90]. Lee *et al*[91] analyzed factors predicting cholangiocarcinoma in patients operated for BC. They pointed the following factors: age > 40 years, the absence of a gallstone, elevated CEA or cancer antigen 19-9 serum level, and the presence of anomalous pancreaticobiliary ductal union in univariate analysis, and an elevated cancer antigen 19-9 level in multivariate analysis. Therefore, laboratory investigations of CEA and CA 19-9 in patients following surgery for BC are useful.

Cytology of bile and bile ducts specimens taken during ERCP ora PTC by brush or needle biopsy play an additional role in cholangiocarcinoma diagnosis. Negative cytology from brushing does not exclude malignancy. Combined brush and biopsy cytology specimens increase sensitivity to 40%-70%[90].

Frequency of follow-up in patients following BC resection has not been clearly established in the literature. In my opinion, laboratory investigations including liver parameters and cancer markers (CA 19-9 and CEA) and ultrasonography should be performed as a screening every 6-12 mo.

In cases of the intrahepatic lithiasis and Caroli's disease, the use of ursodeoxycholic acid (UDCA) in the treatment has been described in the literature. Ros *et al*[92] UDCA observed that therapy with UDCA caused clinical remission, return to normal liver function, and dissolution of intrahepatic stones on USG in all patients. Therefore, litholytic therapy was indicated by authors for intrahepatic stones in Caroli's syndrome. Successful treatment of patients with the hepatilithiasis and Caroli's disease was also described by Guma *et al*[93].

In conclusion, clinical presentation and management of BC depend on the cyst type according to Todani classification. The early and proper treatment allows to avoid serious complications (including bile duct cancer) in patients with BC. The early treatment is also associated with a lower number of complications. In most cases, total cyst excision with Roux-Y hepaticojejunostomy is the treatment of choice. Surgical treatment of BC is associated with low morbidity and mortality. Patients following surgery for BC require permanent and careful postoperative observation because of possibility of biliary anastomosis stricture and biliary cancer in tissue remnant.

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FRONTIER

Quality of ulcer healing in gastrointestinal tract: Its pathophysiology and clinical relevance

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Abstract

In this paper, we review the concept of quality of ulcer healing (QOUH) in the gastrointestinal tract and its role in the ulcer recurrence. In the past, peptic ulcer disease (PUD) has been a chronic disease with a cycle of repeated healing/remission and recurrence. The main etiological factor of PUD is *Helicobacter pylori* (*H. pylori*), which is also the cause of ulcer recurrence. However, *H. pylori*-negative ulcers are present in 12%-20% of patients; they also recur and are on occasion intractable. QOUH focuses on the fact that mucosal and submucosal structures within ulcer scars are incompletely regenerated. Within the scars of healed ulcers, regenerated tissue is immature and with distorted architecture, suggesting poor QOUH. The abnormalities in mucosal regeneration can be the basis for ulcer recurrence. Our studies have shown that persistence of macrophages in the regenerated area plays a key role in ulcer recurrence. Our studies in a rat model of ulcer recurrence have indicated that proinflammatory cytokines trigger activation of macrophages, which in turn produce increased amounts of cytokines and chemokines, which attract neutrophils to the regenerated area. Neutrophils release proteolytic enzymes that destroy the tissue, resulting in ulcer recurrence. Another important factor in poor QOUH can be deficiency of endogenous prostaglandins and a deficiency and/or an imbalance of endogenous growth factors. Topically active mucosal protective and antiulcer drugs promote high QOUH and reduce inflammatory cell infiltration in the ulcer scar. In addition to PUD, the concept of QOUH is likely applicable to inflammatory bowel diseases including Crohn's disease and ulcerative colitis.

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Key words: Quality of ulcer healing; Peptic ulcer disease; Recurrence; Prostaglandin; Cytokines; Growth factors

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BIOGRAPHY

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INTRODUCTION

Prior to the introduction of *Helicobacter pylori* (*H. pylori*) eradication treatment, peptic ulcer disease (PUD) had been a chronic disease affecting 5%-10% of the population with a cycle of repeated healing/remission and recurrence. The treatment aimed at eradication of *H. pylori* has changed the life cycle of this disease and the prevalence rate has dramatically decreased. However, the dictum "no *H. pylori*, no ulcer" is over-rated[1]. *H. pylori*-negative PUD exists, similar to PUD in nonusers of nonsteroidal anti-inflammatory drugs (NSAIDs). This PUD is referred to as non-*H. pylori*, non-NSAIDs ulcer or idiopathic PUD (IPUD)[2]. A prevalence rate of IPUD has been reported as 12%-20%, suggesting that IPUD is not rare[2]. A recent report has shown the increase in the prevalence of IPUD[3], however this remains controversial[4].

The recurrence rate of IPUD is higher than *H. pylori* and/or NSAID-associated PUD[5] and their management is more difficult and more costly compared to other ulcers[5]. The recurrence rate of ulcer may be related to quality of ulcer healing (QOUH). The pathogenesis of poor QOUH may be the underlying cause of the refractoriness and ulcer recurrence. In addition to PUD, the concept of QOUH may be applicable to inflammatory bowel disease (IBD), which is also a chronic and recurrent ulcer disease. It is likely that in order to obtain a permanent ulcer healing and remission, the high QOUH is necessary for any type of ulcer disease.

Figure 1 Tetsuo Arakawa, MD, PhD, Dean, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan.

MYSTERY OF ULCER RECURRENCE: PAST HISTORY OF PATHOPHYSIOLOGY

Ulcer recurrence has long been thought to be an unavoidable feature of PUD, and therefore, maintenance treatment has been necessary to prevent recurrence. *H. pylori* is a major cause of both PUD and its recurrence. The bacteria are, however, not the only cause of ulcer recurrence, and still there are a certain number of patients with PUD not related to *H. pylori* and NSAIDs, referred to as IPUD[2,4]. To prevent recurrence of IPUD, the investigations examining the pathophysiology of ulcer recurrence are important.

Oi *et al*[6] have proposed the double-regulation theory to explain why PUD favors the gastric angle. They examined many stomachs with ulcers that had been operated upon, and found that the ulcer site was usually at the interphase of the distal side of the glandular borderline (mucosal rule) and line of distortion of movement (muscular rule). This may be the point of combination of the mechanical tension and exposure to high concentrations of acid in the pyloric gland area. The latter mucosa has a weaker resistance to acid compared to the fundic gland area. This theory may also explain why the ulcer often recurs at the same or neighboring site of a previous ulcer. This theory, however, does not fit with duodenal or gastric ulcer at other sites. Therefore, it is important to determine how the ulcer recurs.

Pan *et al*[7] have reported that the recurrence of healed duodenal ulcer depends on the histological maturity of regenerated mucosa. They have indicated that the cause of ulcer recurrence may be related to the abnormalities of the scar of healed ulcer, leading to the concept of QOUH[7]. Takagi *et al*[8] have developed a chronic gastric ulcer model in rats by topical application of acetic acid from the serosal side of the gastric glandular borderline. This model closely mimics human PUD both in histol-

Table 1 Definition and criteria of quality of ulcer healing

Definition:
QOUH represents histological maturity of healed peptic ulcer; Evaluation of QOUH should be done to assess functional and endoscopic maturity additionally to histological maturity; For a clinical use an endoscopic evaluation of maturity should be the main method
Criteria:
1: Endoscopic evaluation of maturity
Dye-contrast method, magnified endoscopy, endoscopic ultrasonography
2: Histological maturity
Regenerated mucosa: thickness, width, morphological abnormalities of glands, inflammatory cell infiltration
Granulation tissue: angiogenesis, fibroblasts and fibrosis, regeneration of muscularis mucosae, inflammatory cell infiltration
3: Functional evaluation of maturity
Microcirculation, production of mucin, prostanoids, growth factors, cell proliferation, receptor expression, adhesion molecules

Arakawa *et al*[12]. QOUH: Quality of ulcer healing.

ogy and life cycle of ulcer recurrence. Tarnawski *et al*[9] have demonstrated in this rat model that the subepithelial mucosa of macroscopically healed gastric ulcer displays disorganized restoration of glandular and vascular structures and remains histologically and ultrastructurally abnormal. They have concluded that these abnormalities may interfere with oxygenation, nutrient supply, and with mucosal resistance and defense, and therefore could be the basis for ulcer recurrence. They proposed the concept of QOUH based on an experimental ulcer model in 1990[9-11]. In a previous clinical study by our group, we proposed the concept and definition of QOUH in human gastric ulcer in a Japanese journal, which was published in an English journal 18 mo later[12] (Table 1). A flat or non-flat ulcer scar pattern has been defined with chromoendoscopy using indigo carmine, which was not demonstrated with conventional endoscopy (Figure 2). These patterns are compatible with histological maturity of regenerated mucosa of healed gastric ulcer; the flat pattern represents a good QOUH and non-flat represents poor QOUH[13] (Figure 2). The incidence of ulcer recurrence is much higher in a non-flat scar than in a flat scar pattern[13,14] (Figure 3), suggesting that the endoscopically assessed QOUH may detect scars that are predisposed to future ulcer recurrence.

ULCER HEALING AND PROSTAGLANDINS

Prostaglandins (PGs) and the discovery of the phenomenon of cytoprotection by Robert *et al*[15] sparked an enormous interest in the critical role of PGs in mucosal defense and ulcer healing. In humans, "artificial" ulcers in the gastrointestinal tract produced by endoscopic mucosal resection for neoplasm heal rapidly and do not recur. We have shown that the surrounding mucosa of such artificial ulcers synthesizes increased amount of prostaglandin E2 (PGE2) and prostacyclin[16], which may have a crucial role in producing high QOUH. This observation has led to our contention that PUD may be a PG-deficiency disease[17] and that PG deficiency may cause poor QOUH. We have demonstrated that low-dose indomethacin causing PG-insufficiency forms an experimental chronic gastric ulcer with poor QOUH, and that exogenous PGE2 reverses poor QOUH as assessed by recurrence rate after healing, and reduces inflammatory cell infiltration of the ulcer scar[18] (Figure 4). Therefore, PG derivatives and/or PG-inducing drugs such as mucosal protective compounds may promote high QOUH[19,20].

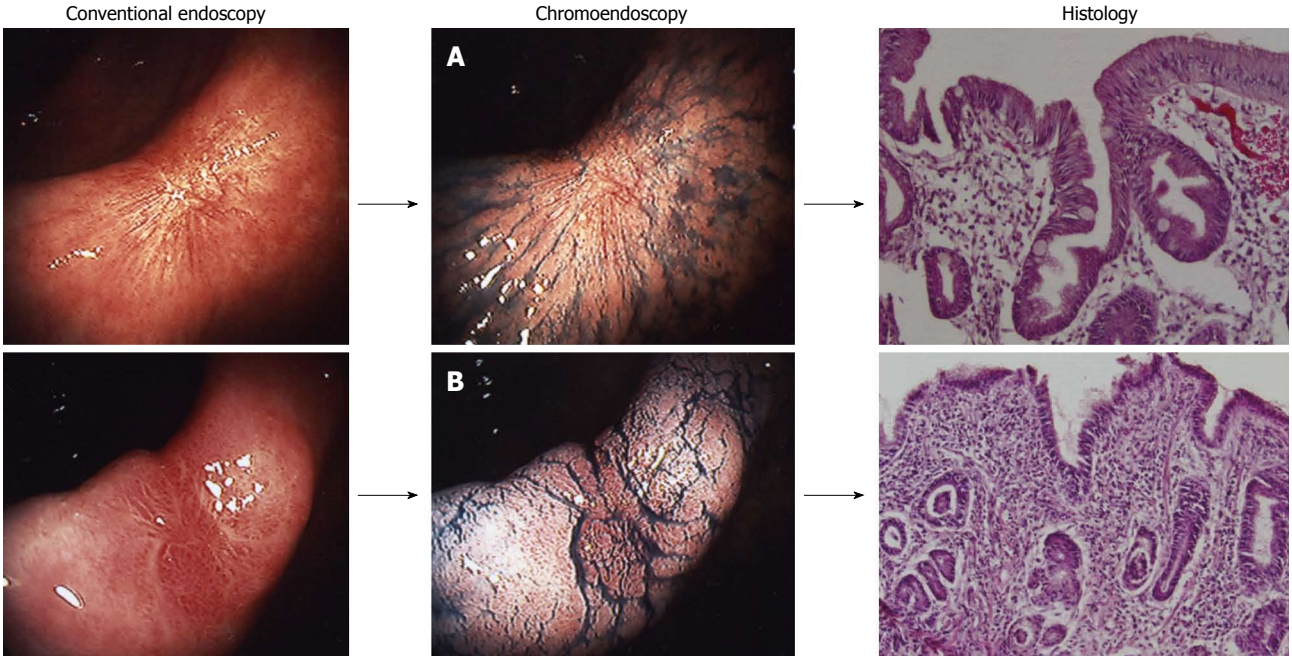
Another important factor in poor QOUH could be a deficiency and/or an imbalance of endogenous growth factors. Jones *et al*[21] have demonstrated that local gene therapy with vascular endothelial growth factor (VEGF) and angiopoietin 1 (Ang1), with limited duration of target gene expression, significantly accelerates experimental gastric ulcer healing in rats. Co-injection of both plasmids encoding rhVEGF 165 and rhAng1 resulted in formation of more mature vessels and more complete restoration of gastric glandular structures within the ulcer scar, reflecting better QOUH. Inhibition of accelerated healing by a neutralizing anti-VEGF antibody indicates an essential role for VEGF and enhanced angiogenesis in ulcer healing.

PATHOPHYSIOLOGY OF QOUH

Histological evaluation of scars of healed gastric ulcers shows increased infiltration of regenerated tissue with numerous neutrophils and macrophages in a non-flat scar compared to a flat scar (Figure 5). The persistence of chronic inflammation may be reflected by the finding of a hypoechoic area beneath the ulcer scar with a non-flat pattern, assessed by endoscopic ultrasonography (Figure 6). The number of macrophages infiltrating scar tissue is five times higher than neutrophils in a non-flat scar, suggesting that these macrophages may play a key role in pathophysiology of QOUH, and hence future ulcer recurrence. These macrophages produce increased amounts of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and monocyte chemotactic protein (MCP)-1 (Figure 7). The proinflammatory cytokines, IL-1β and TNF-α further activate and stimulate macrophages, thus constituting a self-perpetuating circuit. The increased stimulation of production of these cytokines induced by NSAIDs, stress, or *H. pylori* may cause these macrophages to increase cytokine production and/or release, leading in turn to attraction and accumulation of neutrophils. Neutrophils by releasing proteases and active oxygen species damage the scar tissue and induce ulcer recurrence.

MECHANISMS UNDERLYING ULCER RECURRENCE

As mentioned previously, Okabe's rat model of chronic ulcer mimics human peptic ulcer not only morphologi-



Endoscopic pattern	*n*	Regenerated gland index	*P*-value
Flat	10	48.0 ± 2.8	
Non-flat	5	40.2 ± 2.6	< 0.05

Figure 2 Assessment of endoscopic quality of ulcer healing. Flat pattern (A) and non-flat pattern (B) are identified by chromoendoscopy, but not by conventional endoscopy. Flat pattern reflects good quality of healing compared to nodular pattern as assessed by histological maturity (Arakawa *et al*[12]).

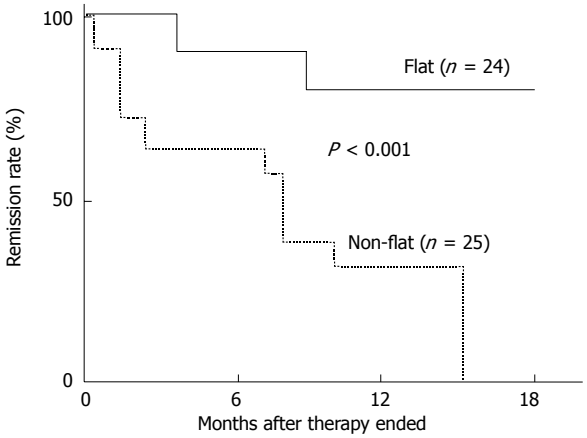


Figure 3 Cumulative remission rates of healed gastric ulcers with flat and non-flat pattern. The incidence of ulcer recurrence is much higher in the ulcer scar with a non-flat than flat pattern (Nebiki *et al*[14]).

cally and histologically but also with regard to the life cycle of spontaneous recurrence. Numerous neutrophils and macrophages persist in and beneath the regenerated epithelium even after ulcer healing. This persistent chronic inflammation may have a key role in causing future ulcer recurrence. Watanabe *et al*[22] have demonstrated that inflammatory cytokines, IL-1β and TNF-α, administered systemically in rats with macroscopically healed gastric ulcer, cause ulcer recurrence at the site of the previous ulcer (Figure 8). In this model of gastric ulcer recurrence that we reported in 1997, we found increased expression of adhesion molecules, intercellular adhesion molecule (ICAM)-1 in endothelial cells and leukocytic β_2 integrins, lymphocyte function-associated antigen (LFA-1; CD11a/CD18) and Mac-1 (CD11b/CD18) in leukocytes, and also of cytokines, IL1β and TNF-α[22], and chemokine, MCP-1[23]. This increase occurred in the regenerated tissue of the healed ulcer site (scar) 12 h after injection of an inflammatory cytokine, and was followed by massive infiltration of macrophages and neutrophils, ultimately resulting in ulcer recurrence. Antineutrophil antiserum prevents ulcer recurrence in this model, suggesting that neutrophils (producing noxious protease and active oxygen species) are the final mediator of tissue injury[22]. This hypothesis is further supported by the demonstration that antibodies against adhesion molecules (ICAM-1 and LFA-1) also inhibit ulcer recurrence[22]. These molecules regulate migration of neutrophils from arterioles into the interstitial space. Antibody against MCP-1 prevents gastric ulcer recurrence in this model[23], suggesting that the overexpression of MCP-1 in resident macrophages accumulated in the interstitial space of the ulcer scar is a first step in the mechanism of ulcer recurrence, because neutrophils and macrophages infiltrate the interstitial space of the ulcer scar only after overexpression of MCP-1[24]. Proton-pump inhibitors (PPIs) prevent gastric ulcer recurrence caused by injection of IL1β[24] and administration of

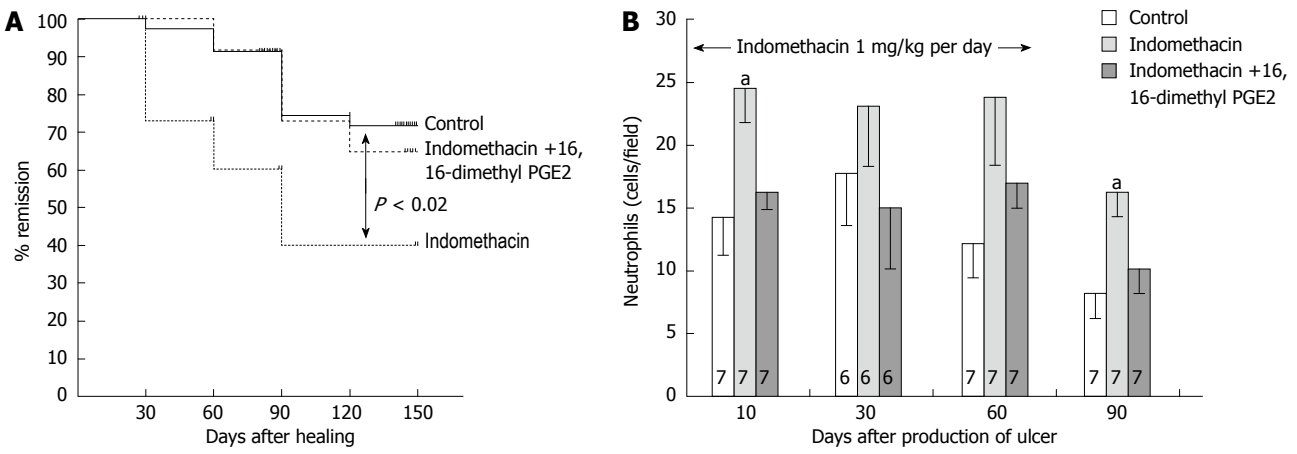


Figure 4 Effect of low-dose indomethacin given during initial period of healing process of gastric ulcer induced by application of acetic acid in rats on cumulative remission rate after healing and on neutrophil infiltration of the interstitial space of the ulcer scar. Indomethacin treatment enhances neutrophil infiltration and this phenomenon persists even after cessation of this drug. Concomitant administration of 16, 16-dimethyl prostaglandin E2 (PGE2) with indomethacin reverses these effects by indomethacin. A: Cumulative remission rate after healing; B: Infiltration with polymorphonuclear cells during and after healing. Control; indomethacin; indomethacin + 16, 16-dimethyl prostaglandin E_2 (Arakawa *et al*[18]). $^aP < 0.05$ *vs* control group.

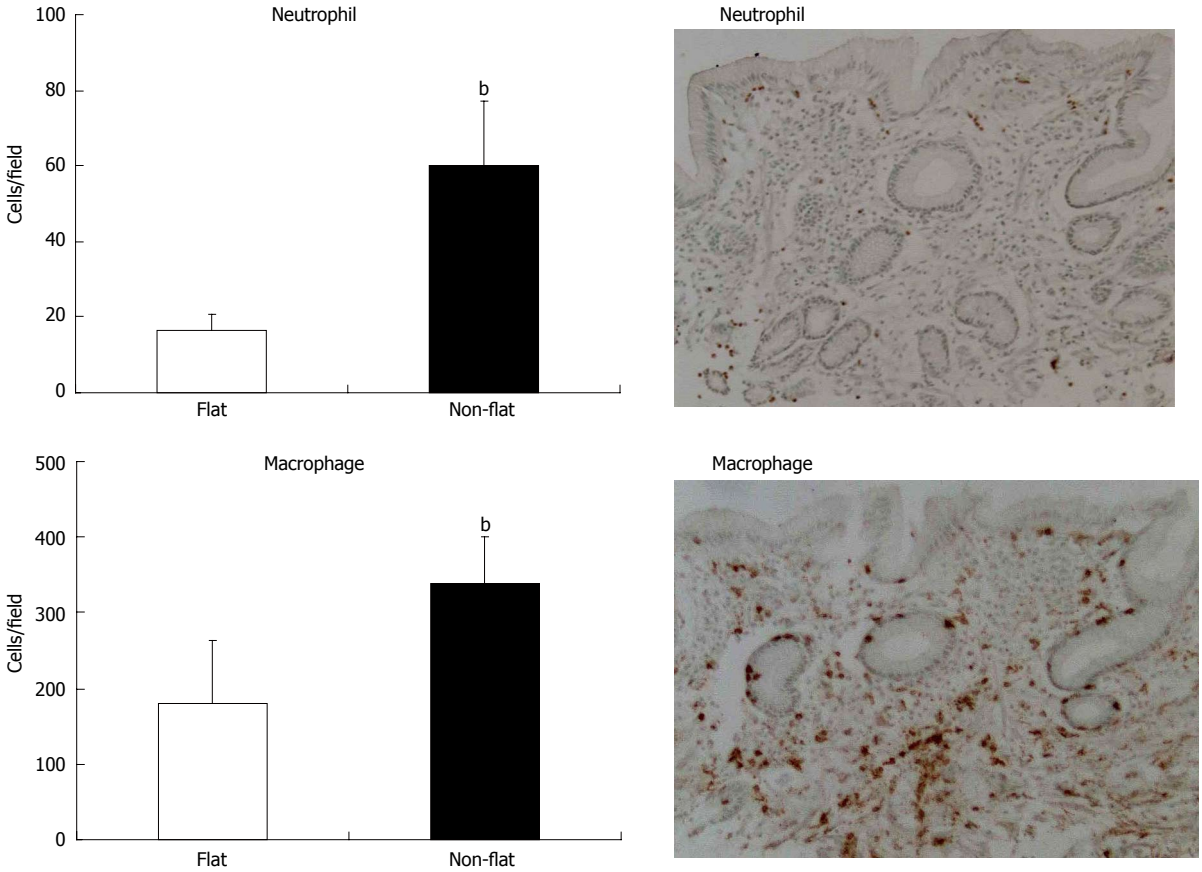
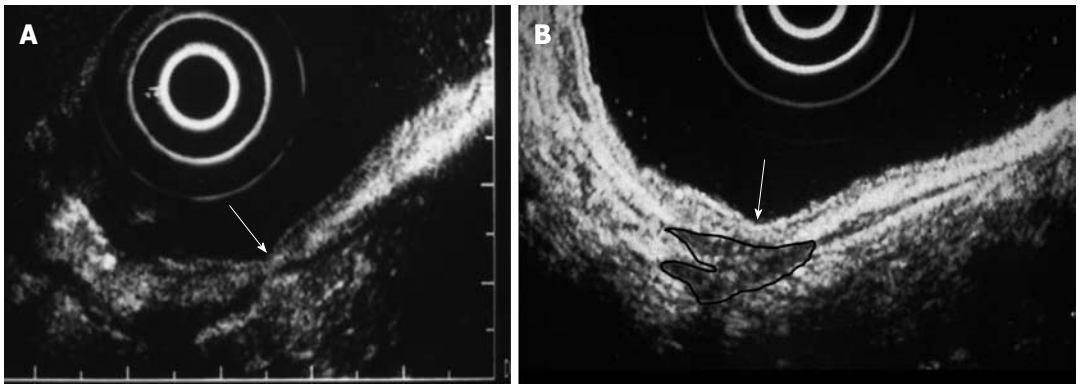


Figure 5 Number of inflammatory cells infiltrating interstitial space of ulcer scar with flat or non-flat pattern assessed by chromoendoscopy in patients with macroscopically healed gastric ulcer. The number of neutrophils and macrophages is much higher in non-flat than in flat pattern. The number of macrophages is increased five times *vs* neutrophils in non-flat scar. $n = 6$, $^bP < 0.01$ *vs* flat group.

exogenous acid reverses the protective action of PPIs on ulcer recurrence, suggesting that presence of acid is necessary to induce ulcer recurrence[24]. Based on these studies, we propose a working hypothesis of gastric ulcer recurrence presented in Figure 9[25].

EFFECT OF ANTIULCER DRUGS ON QOUH

The histological maturity of regenerated mucosa is poor after treatment with H_2-receptor antagonists[26]. This



	Flat	Non-flat	*P*-value
Internal hypoechoic area (+/-)	3/21	16/9	0.0003

Figure 6 Assessment of interstitial space beneath gastric ulcer scar with endoscopic ultrasonography. Hypoechoic area (circle of black line) is clearly seen in non-flat scar (B), but not in flat scar (A). Arrow shows a point of ulcer scar (Nebiki *et al*[14]).

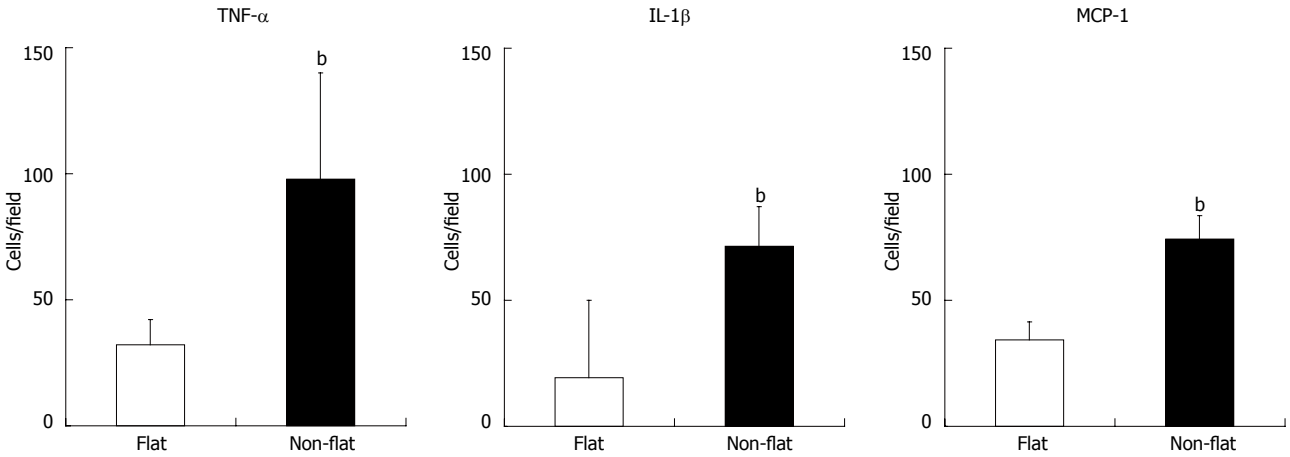


Figure 7 Number of tumor necrosis factor-α-, interleukin-1β-, and monocyte chemotactic protein-1-producing cells in interstitial space of ulcer scar site in patients with gastric ulcer whose ulcer healed with flat *vs* non-flat pattern assessed by chromoendoscopy. $n = 6$, $^bP < 0.01$ *vs* flat group. TNF-α: Tumor necrosis factor α; IL-1β: Interleukin-1β; MCP-1: Monocyte chemotactic protein-1.

may be related to their action to decrease mucosal PG levels[27]. Rebamipide, a mucosal-protective drug that stimulates synthesis of PGs, accelerates ulcer healing and reduces relapse of acetic-acid-induced gastric ulcer in rats. In contrast, ulcer relapse is not prevented by cimetidine[26]. Similar results regarding improving QOUH by rebamipide have been reported by Qi *et al*[28]. In a clinical setting rebamipide has been shown to improve QOUH of human gastric ulcers, as assessed by chromoendoscopy. Moreover, the ulcer recurrence rate after healing with rebamipide was similar to that found in gastric ulcer patients after *H. pylori* eradication with dual therapy[29]. Rebamipide has been shown to exert anti-inflammatory action, accelerate gastric ulcer healing, and promote high QOUH in both experimental and clinical studies[30,31].

Regarding the action of other drugs related to QOUH, antacid hydrotalcite provides better restoration of glandular structures in the gastric ulcer scar in rats compared to omeprazole[32]. In clinical studies, initial treatment with sucralfate is superior to cimetidine in decreasing recurrence rate of healed duodenal ulcer during maintenance therapy with cimetidine[33]. Direct evidence of promoting better QOUH, as assessed by chromoendoscopy, has been reported with lafutidine in a placebo-controlled trial, and ranitidine compared to famotidine in patients with gastric ulcer[34,35]. Lafutidine is a novel H_2-receptor antagonist developed in Japan. It has the unique property of stimulating capsaicin-sensitive nerves, producing NO, a cytoprotective mediator.

RECENT ADVANCES IN EVALUATION OF QOUH

Recent preliminary findings with magnifying endoscopy and narrow band imaging (NBI) may be useful for a more precise evaluation of QOUH. Magnifying endoscopy shows a fine regular glandular pattern in flat scar

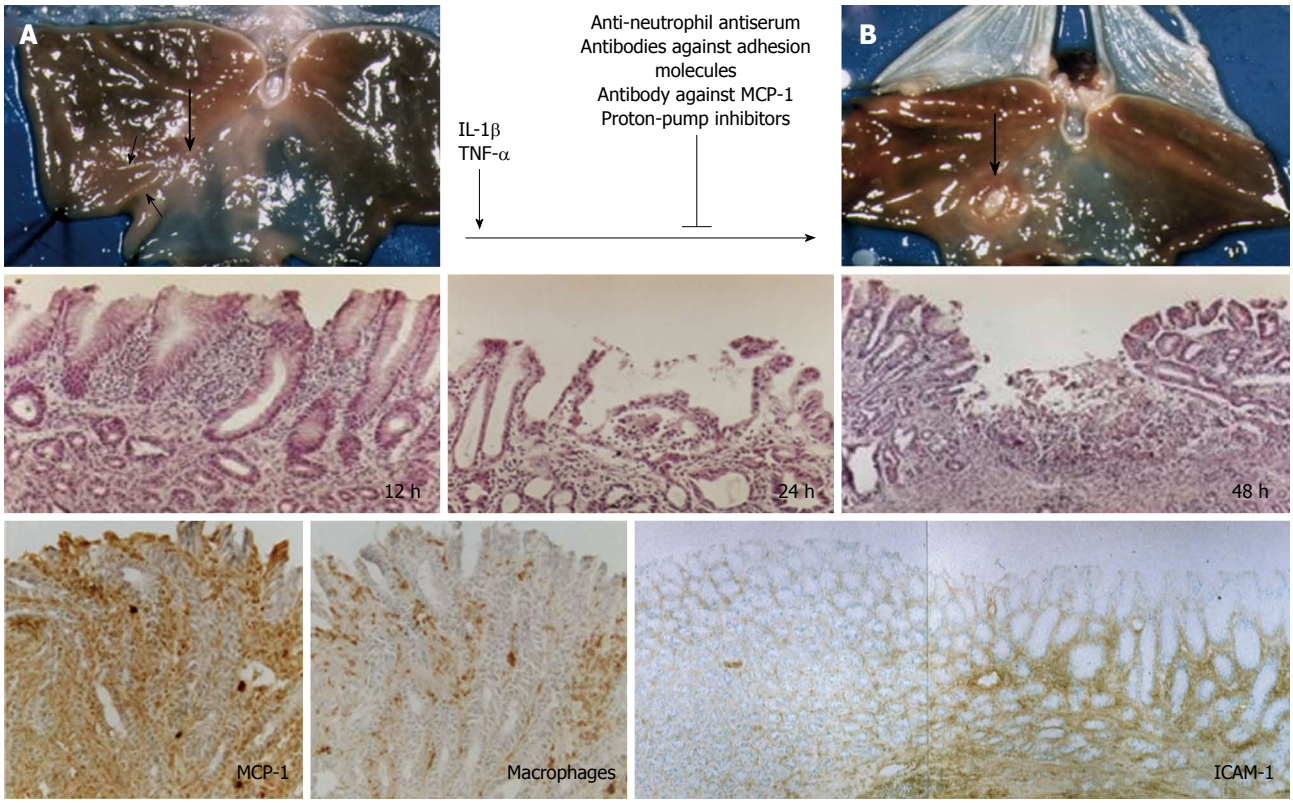


Figure 8 Original rat model of gastric ulcer recurrence. A: Ulcer scar of acetic-acid-induced gastric ulcer healed spontaneously (big arrow) with fold convergence (small arrows); B: Recurrence of ulcer with white coat at the same site as previous ulcer (big arrow). Interleukin-1β (IL-1β) or tumor necrosis factor α (TNF-α) administered systemically causes recurrence of ulcer macroscopically and histologically. Antineutrophil antiserum, antibodies against adhesion molecules, antibody against monocyte chemotactic protein-1 (MCP-1), or proton-pump inhibitors prevent recurrence. MCP-1 is overexpressed in macrophages in the interstitial space of the ulcer scar site at 12 h after administration of IL-1β or TNF-α. Adhesion molecules are overexpressed only at the ulcer scar site (Watanabe *et al*[22-24]). ICAM: Intercellular adhesion molecule.

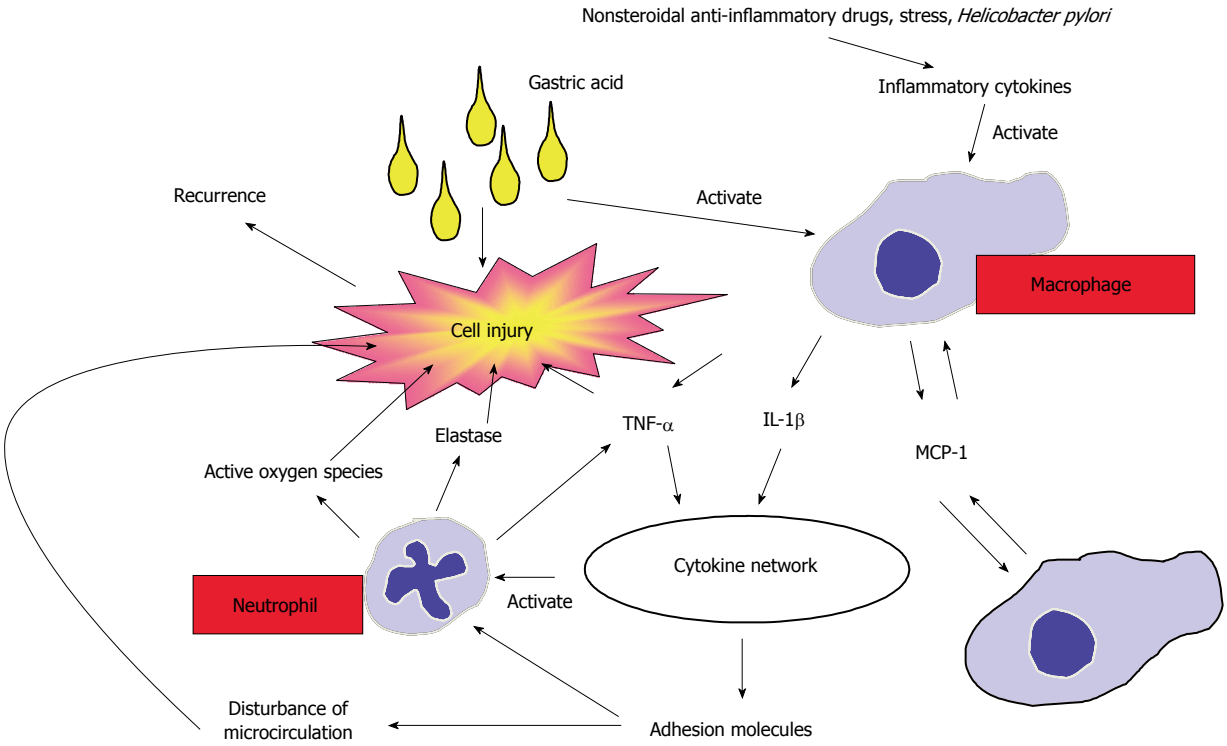


Figure 9 Proposed mechanisms of ulcer recurrence. Ulcerogenic factors such as nonsteroidal anti-inflammatory drugs, stress, and *Helicobacter pylori* stimulate the production of inflammatory cytokines, which activate macrophages under acidic conditions. The activated macrophages produce monocyte chemotactic protein-1 (MCP-1), which accumulates other macrophages. These macrophages all produce a large amount of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), which activate the cytokine network, resulting in activation of adhesion molecules and neutrophils. Activated neutrophils migrate from arterioles to the interstitial space and injure the tissue with noxious substances such as reactive oxygen species and elastase produced by themselves together with gastric acid (Arakawa *et al*[25]).

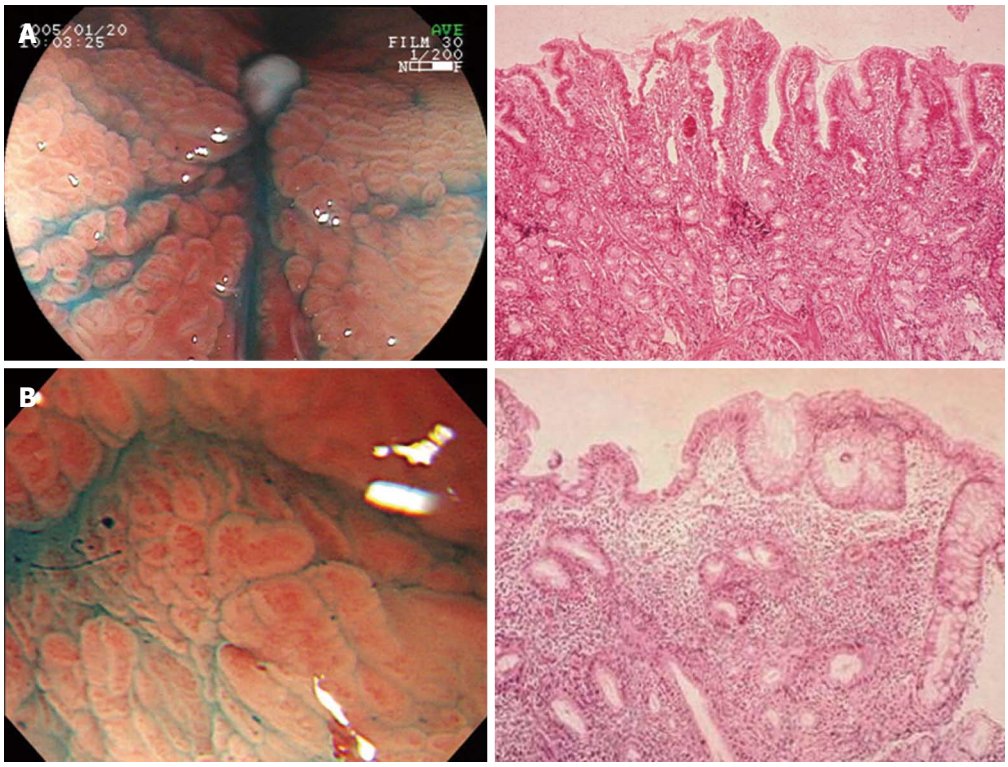


Figure 10 Magnifying chromoendoscopy shows different findings in flat scar as a fine regular pattern (A) and non-flat scar as coarse irregular pattern (B) compatible with histological findings.

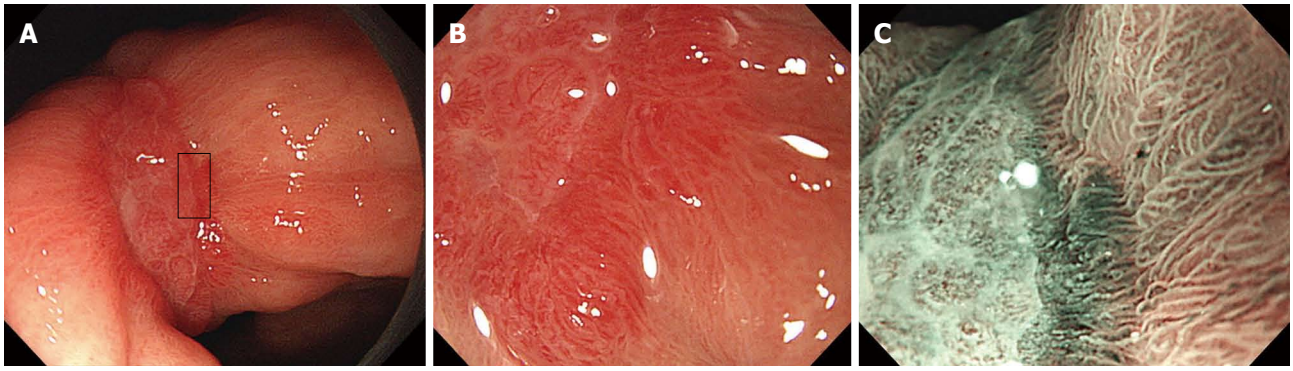


Figure 11 Narrow band imaging with magnification by endoscopy in patients with gastric ulcer shows fine vascular patterns, which may reflect precise quality of ulcer healing. A: Conventional endoscopy; B: Magnifying endoscopy; C: Magnification + narrow band imaging.

and coarse, irregular, and hyperemic glandular pattern in non-flat scar (Figure 10), suggesting that a more precise assessment is possible in the near future. In addition to this procedure, NBI may be the most promising tool to assess QOUH combined with magnification because NBI shows pattern of vessels that may reflect QOUH (Figure 11).

MUCOSAL HEALING IN CROHN'S DISEASE

Biological drugs aimed at TNF-α, such as infliximab (and other related antibodies) as well as thalidomide[36], promote ulcer healing in Crohn's disease resistant to corticosteroids. This action is completely different from the mucosal healing obtained with a PPI for gastric and/or duodenal ulcers, because a PPI inhibits only acid secretion, but may not suppress inflammation. Therefore, PPIs promote rapid healing of acid-related ulcers, but do not improve QOUH, especially in the presence of *H. pylori*. The biological drugs directly suppress inflammation, and thus they may improve QOUH. However, as long as the factor responsible for inflammation is unknown, the efficacy of such drugs may be limited.

Granulomas beneath the regenerated tissue may have an important role in cell-to-cell contact of macrophages with T lymphocytes in Crohn's disease and cause chronic inflammation, and on this background poor QOUH[37,38].

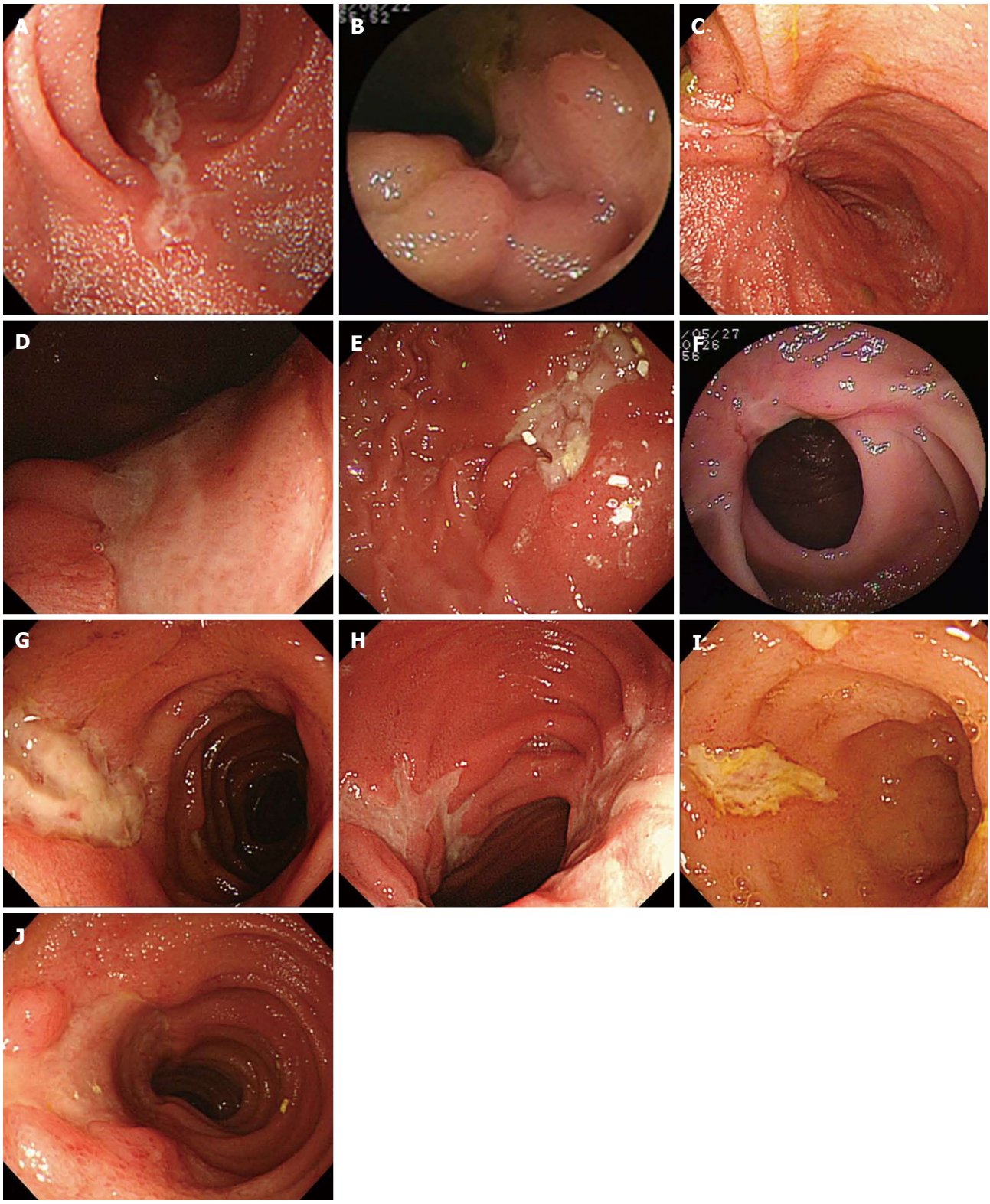


Figure 12 Morphological characteristics of active ulcer in patients with Crohn's disease. Shape of ulcer edge, flat (A) or non-flat (B); fold convergence, negative or positive (C); shape of ulcer base, flat (D) or non-flat (E); stenosis, negative or positive (F); neighboring ulcer, negative (G) or positive (H); width of ulcer, narrow (I) or wide (J).

The inflammation in Crohn's disease in based on Th1 and Th17 reaction[39-41] that is similar to *H. pylori*-associated gastritis[42,43]. Therefore, the inflammation in Crohn's disease may have similar immunopathophysiology to *H. pylori*-associated inflammation, which causes poor QOUH and underlies the ulcer recurrence. The similarity of immunological abnormality in Crohn's disease and *H. pylori*-associated gastritis may also be related to distur-

Table 2 Morphological characteristics of active ulcers in patients with Crohn's disease reflecting possible future mucosal healing after treatment with infliximab

Morphological finding		Mucosal healing		
		Good (n = 10)	Poor (n = 11)	P value[1]
Shape of ulcer edge	Flat	8	3	
	Non-flat	2	8	0.03
Fold convergence	Negative	7	3	
	Positive	3	8	0.08
Shape of ulcer base	Flat	4	3	
	Non-flat	6	8	0.65
Stenosis	Negative	8	9	
	Positive	2	2	0.99
Neighboring ulcer	Negative	9	4	
	Positive	1	7	0.02
Width of ulcer	Narrow	9	4	
	Wide	1	7	0.02

[1]Fisher's exact test. P value indicates the statistical difference in morphological findings between good and poor mucosal healing.

bance of the ghrelin system[44,45].

In our preliminary study of 21 patients with Crohn's disease treated with infliximab, morphological characteristics of active ulcer were defined, namely shape of ulcer edge, flat or non-flat; fold convergence, negative or positive; shape of ulcer base, flat or non-flat; stenosis, negative or positive; neighboring ulcer, negative or positive; width of ulcer, narrow or wide (Figure 12). The incidence of mucosal healing of active ulcer with a flat edge, absence of neighboring ulcer, or a narrow width of ulcer is significantly higher. A negative mucosal fold convergence tends to be more frequent than non-flat ulcer edge, positive fold convergence, positive neighboring ulcer, or wide ulcer (Table 2). The latter type of ulcer may require stronger anti-inflammatory treatment to obtain mucosal healing. These ulcers may need an endoscopic follow-up observation with assessment of mucosal healing after remission. If the ulcer does not recur, the mucosal healing may be high QOUH. Mucosal fold convergence (Figure 12C) may be one of the factors reflecting poor QOUH. Therefore, prevention of the convergence may improve QOUH of Crohn's disease. Stimulation of the local angiotensin Ⅱ system may have a key role in fibrosis, resulting in fold convergence and stricture[46], suggesting that inhibitors on this system such as angiotensin Ⅱ receptor blockers may promote high QOUH in Crohn's disease. However, to identify a causative factor of inflammation in Crohn's disease is the most essential. The responsible antigen needs to be elucidated from candidates such as yeast-like antigen[47], and others. Elimination of the possible antigen may be a powerful tool to obtain high QOUH and permanent remission.

The concept and the paradigm of QOUH may also apply to ulcerative colitis. Tarnawski and coworkers using confocal endomicroscopy and molecular imaging have demonstrated in colonic mucosa of patients with ulcerative colitis in remission impaired crypt regeneration, persistent inflammation, pathological angiogenesis and increased microvascular permeability; all reflecting impaired QOUH. They have found that the underlying mechanisms include dysregulation of survivin, aberrant activation of VEGF gene, and persistent inflammatory cell infiltration[48].

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GUIDELINES FOR CLINICAL PRACTICE

Use of the tumor necrosis factor-blockers for Crohn's disease

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Abstract

The use of anti-tumor necrosis factor-α therapy for inflammatory bowel disease represents the most important advance in the care of these patients since the publication of the National Co-operative Crohn's disease study thirty years ago. The recommendations of numerous consensus groups worldwide are now supported by a wealth of clinical trials and several meta-analyses. In general, it is suggested that tumor necrosis factor-α blockers (TNFBs) are indicated (1) for persons with moderately-severe Crohn's disease or ulcerative colitis (UC) who have failed two or more causes of glucocorticosteroids and an acceptably long cause (8 wk to 12 wk) of an immune modulator such as azathioprine or methotrexate; (2) non-responsive perianal disease; and (3) severe UC not responding to a 3-d to 5-d course of steroids. Once TNFBs have been introduced and the patient is responsive, therapy given by the IV and SC rate must be continued. It remains open to definitive evidence if concomitant immune modulators are required with TNFB maintenance therapy, and when or if TNFB may be weaned and discontinued. The supportive evidence from a single study on the role of early versus later introduction of TNFB in the course of a patient's illness needs to be confirmed. The risk/benefit profile of TNFB appears to be acceptable as long as the patient is immunized and tested for tuberculosis and viral hepatitis before the initiation of TNFB, and as long as the long-term adverse effects on the development of lymphoma and other tumors do not prone to be problematic. Because the rates of benefits to TNFB are modest from a population perspective and the cost of therapy is very high, the ultimate application of use of TNFBs will likely be established by cost/benefit studies.

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Key words: Adalimumab; Adverse effects; Certolizumab pegol; Crohn's disease; Economic evaluation; Infliximab; Secondary lack of response; Ulcerative colitis

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INTRODUCTION

Crohn's disease (CD) affects about 500 000 persons in North America[1], while the prevalence rates in northern Europe are 27-48 per 105[2]. A selected group of these persons will benefit from the efficacy and safety of tumor necrosis factor-α blockers (TNFBs). This use of TNFB has been reviewed by way of meta-analysis of placebo-controlled trials[3], as well as with recent consensus statements [e.g., Canadian Agency of Drugs and

Technologies in Health (CADTH, 2009)]. The overall view is that TNFBs are superior to placebo for the induction and maintenance of luminal CD or ulcerative colitis (UC). There are insufficient head-to-head studies comparing TNFB with mesalamine [5-aminosalicylic acid (5-ASA)], intermittent use of glucocorticosteroids (GCS), azathioprine (AZA), or methotrexate (MTX). In fistulizing CD, TNFBs other than infliximab (IFX) require further investigation. It remains to be established how early in the course of CD that TNFBs should be started, and what are the factors that predict which patient is most likely to require or to respond to this therapy.

MECHANISMS OF ACTION OF TNFB

There are numerous reviews on the molecule, cellular, physiological and pharmacological properties of TNFB[4-9]. Tumor necrosis factor (TNF) (aka "cachectin") has been called the body's sentinel cytokine or "fire alarm"[10], as it initiates the defence response to local injury. As a result of innate and adaptive immune responses, the level of tumor necrosis factor-α (TNFα) is increased in the serum and mucosa of patients with CD[11-13], and is a trigger for and a mediator of positive and negative feedback loops which influence the chronicity of inflammation.

TNF in the membranes (tmTNF) is a protein which undergoes proteolysis by the protease TNFα converting enzyme, forming a soluble protein, sTNF. Reverse signalling by tmTNFα may alter cellular responses to stimuli such as bacterial lipopolysaccharide[14-16]. The sTNF and the precursor tmTNF bind to the two TNF receptors, TNF receptor 1(TNFR1) (aka pSS or DC120a), and TNFR2 (aka p75 or CD120b)[1]. Binding the TNFR1IR2 to the cleft in the TNF subunits causes activation, and initiates the expression of interleukin (IL)-1, IL-6, interferon-gamma (IFN-γ), cell adhesion molecules (e.g., intracellular adhesion molecule-1), as well as a number other inflammatory molecules[17].

TNFR1 is expressed in all cells, and generates the proinflammatory properties of TNF. TNFR2 is present in immune as well as endothelial cells, and initiates the immunoregulatory properties of TNF[7]. If the TNF/TNFR1 clusters in membrane lipid rafts, it becomes antiapoptotic, whereas if TNF/TNFR1 is internalized within the cell, it becomes proapoptotic. Whether TNFR1 activates apoptosis or not depends upon accessory cell proteins, such as TNA receptor-associated factor 2 (TRAF2). TNFB affects both sTNF-mediated mechanisms, as well as tmTNF-mediated mechanisms[18]. TNFB neutralize sTNF with mean inhibitory concentrations in the nM range. TNFB alter tmTNF interactions with TNFR1 and 2, or as agonists that initiate differential induction of cytokine suppression through reverse signalling, involving signal-peptide-peptidase-like 2a (SPPL2a) and SPPL26. This leads to apoptosis, cell activation, cytokine suppression, induction of the adhesion membrane E-selectin, and resistance to endotoxin[18,19].

Recruitment of Fas-associated death domain binds and activates procaspase-8, which then activates caspase-3, and results in apoptosis. In this way, TRAF2 may lead to apoptosis. Alternatively, TRAF2 may recruit c1 activator-protein-1 (AP-1)-1 and c1AP-2. In turn, c1AP-1/-2 cause nuclear factor κ B (NF-κB) and AP-1 to block apoptosis increase anti-inflammatory proteins, and enhance cell proliferation as well as differentiation. In addition, TNF increases IL-2 receptor, *HLA-DR* antigen gene expression, and I5-A costimulator of IL-2-dependent IFN-γ production.

TNF may be immunostimulatory or immunosuppressive, depending on the genetic background of the patient, the timing and concentration of TNF[17,18], as well as depending on whether TRFR1 or TRFR2 are involved[20]. In clinical situations, TNFB do not likely function as immunosuppressants: TNFB may in fact provide immune enhancements[21], and TNFR may down-regulate some immune reactions that are activated in CD.

Reduced apoptosis in CD may cause inflammation[22], and the death domains in TNFR may induce apoptosis[23]. The tmTNF molecule has a cytoplasmic domain which can induce apoptosis by acting as a ligand for TNFRs, or as a receptor that transmits a reverse signal into the tmTNF-bearing cell[14]. In this way, TNFB may block or induce tmTNFB-mediated apoptosis. TNF-expressing cells such as monocytes, macrophages and T-cells are acted upon either by pathogen-associated molecules which express toll-like receptors (TLRs), or by NF-κB transportation factors which have been stimulated by inflammatory cytokines such as IL-1. The response to TNFB IFX in CD is determined by a single nucleotide polymorphism in the FCGR3A gene encoding for FCjR Ⅲa receptors on NK cells and macrophages[24]. TLR and NF-κB act through p38 MAPK and NF-κB to increase TNF mRNA (gene transcription) and TNF protein (translation).

Both IFX and adalimumab (ADA) induce apoptosis in peripheral blood monocytes as well as in lamina propria T-cells[25,26]. Certolizumab (CER) does not produce apoptosis *via* tmTNF, possibly because it cannot form cross-linkages with tmTNF, or because of its different epitope specificity. The fact that CER does not induce apoptosis and yet is clinically effective in CD provides evidence for mechanisms of action in addition to apoptosis being important in the clinical benefit of TNFBs.

For IFX and ADA, the presence of antibodies to the TNF reduces the serum concentrations and effectiveness of the drugs[27-29]. These antidrug antibodies form multivalent immune complexes with the TNFB, leading to their rapid clearance and therefore to reduced clinical response, as well as to the potential for the development of future infusion reactions.

It is not clear why IFX induces antinuclear, anti-ds DNA and anticardiolipid IgA or IgM antibodies. In reviewing this topic, Tracey *et al*[18] speculated that TNFB either dysregulated apoptosis and release autoimmunogenic plasma nucleosomes from the apoptotic cells, or inhibit some cytotoxic T-lymphocyte response that normally suppresses autoreactive B cells[30].

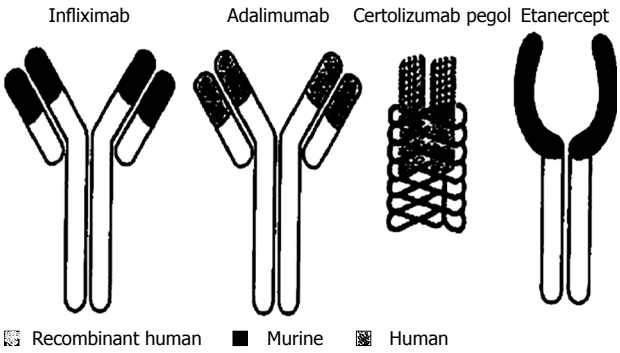

Figure 1 Comparative structures of the anti-tumor necrosis factor agents infliximab, adalimumab, certolizumab pergola and etanercept.

Control of intracellular infections such as Mycobacterium requires macrophages and T-cells in granulomas to come close to bacteria, and then wall them off. Etanercept (ETA) does not show efficacy against granulomutous diseases such as Wegener's granulomatosis and sarcoidosis[18,31]. This is unlikely the main mechanism of clinical benefit of these TNFBs in CD, since ETA is not effective in this disease. Furthermore, ETA, IFX and ADA induce apoptosis, but again ETA is not clinically active in CD. Some other pathway(s) must represent the mechanism of action of IFX, ADA, and CER in CD. IFX, ADA and CER but not ETA almost completely inhibit lipopolysaccharide-stimulated IL-IB release from monocytes, suggesting that inhibition of the production of cytokine may be important in the clinical efficacy of IFX, ADA and CER in CD[32]. Because ETA has not been shown to be clinically effective in the treatment of CD, and will not be discussed further in this paper.

The general comparative structures and individual biological properties of the TNFBs which have been approved in various countries for use in persons with CD are shown in Figure 1, and in Tables 1-3. IFX, ADA and CER are discussed in the order in which they became generally available for clinical use in CD, and the order of presentation does not imply in any way the superiority of one TNFB over another.

Etanercept is a soluble TNF receptor fusion protein (humanized IgG1 Fc fragment fused with two identical humanized p75 TNF receptors) that binds and inactivates soluble, but not membrane-bound TNFα, and although effective for the treatment of rheumatoid arthritis and other forms of inflammatory joint diseases, etanercept failed to demonstrate efficacy at similar doses in a clinical trial for CD.

Biologics other than anti-TNFα agents

Recently, natalizumab, a humanized IgG4 monoclonal antibody that antagonizes integrin heterodimers containing α4-integrin, was approved as a second-line biologic for therapy in the United States for CD patients who failed conventional therapies. Natalizumab inhibits leukocyte trafficking by preventing α4-mediated adhesion of leukocytes to adhesion molecules and transmigration of leukocytes across endothelium into inflamed mucosa[33,34]. Natalizumab is well tolerated, but is associated with an increased risk for infections, acute hypersensitivity reactions, and hepatotoxicity. The primary concern regarding natalizumab therapy has been the reactivation of latent human JC polyomavirus that can lead to a fatal central nervous system infection, progressive multifocal leukoencephalopathy, which has an estimated risk of 1:1000[35]. Therefore, natalizumab use has been restricted to monotherapy, without concomitant immune suppressants, and has a mandated special safety monitoring program and consent that has limited its patient acceptance. Nevertheless, natalizumab remains a viable option for patients who have lost a mechanistic response to anti-TNFα agents.

Table 1 The general biological properties of tumor necrosis factor blockers in clinical practice

Pro- or anti-apoptotic effects[1]
↑ Adhesion molecules on endothelial cells
↑ Epithelial permeability
↑ Antigen-stimulated B-cell proliferation and differentiation
IL-2R production[1]
↑ IL-2 dependent production of IFN-γ
↑ *HLA-DR* gene expression

[1]Represent the data are increased. IFN-γ: Intracellular interferon-γ; IL: Interleukin.

CLINICAL ASSESSMENTS REQUIRED BEFORE THE INITIATION OF TREATMENT WITH TNFBS

Absolute or relative contraindications for use of TNFBs

TNFBs are contraindicated if the CD patient has an abscess, intestinal stricture, a flare of colitis in association with an enteric infection with Clostridium difficile or cytomegalovirus, severe congestive cardiac failure (NYHA Ⅲ or Ⅳ), uncontrolled HIV disease, endemic mycosis, multiple sclerosis, or evidence of tuberculosis (TB) (positive interferon-gamma assay and/or chest X-ray prior to a four weeks course of isoniazid). TNFBs should also be used with caution and balanced consideration in CD persons with neurological disease, chronic liver disease, a history of malignancy (especially lymphoma), and in the woman who is pregnant or plans to begin parenthood (see below).

TFNBs and pregnancy: Lactation and contraception

Because the peak prevalence of inflammatory bowel disease (IBD) is between approximately 20 years to 40 years of age, for the couple where one or both partners have CD or UC, the issues of pregnancy, lactation and contraception are important. These issues need to be discussed proactively and in detail with the patient and their sexual partner.

The American Food and Drug Administration (FDA) classifies IFX and ADA as pregnancy class B agents. So,

Table 2 Individual biological properties of tumor necrosis factor blockers

IFX
High sensitivity and specificity for binding to both sTNF and tmTNF
2-3 IFX molecules bind to each TNF trimer, preventing TNF binding to cellular receptors, and reversing the actions of TNF
The TNF trimer complexes are biologically inactive and are thought to be cleared by the hepatic reticuloendothelial system
Most IFX is in the vascular compartment
No systemic accumulation
Clearance of IFN is slowed by MTX and is ATI
The VOD at steady state is independent of dose
VOD and clearance are not affected by patient age or weight. The effect of liver or kidney disease is unknown
Does not produce a generalized suppression of the body's immune system
Response to IFX is affected by CRP polymorphisms
Linear relationship between dose and serum concentration of IFX
Serum concentration is correlated with clinical response (in at least persons with rheumatoid arthritis)
Reductions in IL-10 levels also correlate with improvement in clinical activity, and FGF and improvement in perianal disease
For the 5 mg/kg dose, the median terminal half-life (*t*) is 10.9 d[1]
↓ Cells expressing TNF, IL-10, IFN-γ
↓ Cells staining for CD4, CD5, CD6, MMP-9
↓ TNF levels (that are ↑ in serum and diseased mucosa in CD)
↓ ICAM-1
↓ Lamina propria T-lymphocytes and peripheral blood monocytes
↓ Growth hormone resistance
↓ Markers of bone reabsorption, ↑ markers of bone formation
ADA
Forms high molecular weight complexes with human TNF (600-5000 kDa)
Linear relation between dose and serum concentration
Serum concentration is correlated with clinical response
Mean serum *t* is 10-20 d
Clearance is slowed by MTX, and is accelerated by ATA
Certolizumab pegol
Lacks IgG Fc domain, and thus does not fix lysed cells or complement
The Fab' component of CER contains a free cysteine residue to the hinge region, which provides site-specific attachment of the PEG to the Fab' at a site well removed from the antigen binding site
The retention time of protein conjugates in the blood is increased by pegylation, and immunogenicity is reduced. Pegylation increases in the half-life ($t_{1/2}$) of the antibody fragment, so dosing may be less frequent
The half-life of CER in healthy volunteers is 313 h
Site-specific pegylation of the Fab' fragment of CER is directed to a site well away from the antigen-binding region. In this way, the conjugate has the same high affinity of the TNF as the tmTNF and sTNF without the pegylation
Does not cause apoptosis of lymphocytes and monocytes, and antibodies to IFX (ATI) do not cross-react with CER
Accumulates preferentially in inflamed rather than in non-inflamed tissue
Has a greater affinity for sTNFs than do IFX or ADA
Has twice the neutralizing potency as IFX or ADA for sTNFα
Has the same neutralizing potency as IFX and ADA for tnTNFα, and occurs through p55/p75 TNFR
Anti-CER antibodies levels are low, and do not affect the efficacy of CER
Does not cross-react with ATI (antibodies to IFX)
Has a linear pharmacokinetic profile
Bioavailability of subcutaneously administered CER is almost 100%
Does not lyse cells, cause compliment-dependant cytotoxicity, antibody-dependant cell-mediated cytotoxicity, apoptosis of activated monocytes or lymphocytes or necrosis of neutrophils

[1]Represent the counts or concentration is decreased. IFX: Infliximab; TNF: Tumor necrosis factor; MTX: Methotrexate; ATI: Accelerated by antibodies to IFX; VOD: Volume of distribution; CRP: C-reactive protein; FGF: Fibroblast growth factor; IFN-γ: Intracellular interferon-γ; IL: Interleukin; MMP-9: Metalloproteinases; ADA: Adalimumab; ATA: Antibodies to ADA; ICAM-1: Intracellular adhesion molecule-1; PEG: Polyethylene glycol; CER: Certolizumab; CD: Crohn's disease; TNFR: TNF receptor.

IFX and ADA may be used during the first two trimesters of pregnancy if clinically indicated, and absolutely necessary[36-38]. There is no detailed published data on the safety of CER in pregnancy. Because clinically significant levels of IFX have been described in the serum of a baby 6 wk after delivery[39], it is recommended for the pregnant IBD mother to stop IFX in the third trimester of her pregnancy. There is no transfer of IFX to mother's breast milk, so the IFX may be restarted shortly after the child has been delivered.

Vaccination

Recommendations have been made for the regular scheduled monitoring of persons on TNFB[40]. When the diagnosis of IBD is initially established, the patient's vaccination status should be reviewed. Unfortunately, the assessment of the IBD patient for vaccine-preventable diseases is not well considered by many healthcare providers[41]. If the vaccination program is not current, then it must be updated before the use of TNFBs. Ideally, vaccinations should be updated before the use of immu-

Table 3 Comparison of *in vitro* properties of different tumor necrosis factor-α blockers in clinical practice for inflammatory bowel disease

Agent	Binds	Mediates		Increases proportion of apoptotic cells	Inhibits cytokine production	Effective in IBD patients
	TNF	CDC	ADCC			
Infliximab	Yes	Yes	Yes	Yes	Yes	Yes
Etanercept	Yes	Yes	Yes	Yes	No	No
Adalimumab	Yes	Yes	Yes	Yes	Yes	Yes
Certolizumab pegol	Yes	No	No	No	Yes	Yes

TNF: Tumor necrosis factor; ADCC: Antibody-dependent cell-mediated cytotoxicity; CDC: Complement-dependent cytotoxicity; IBD: Inflammatory bowel disease.

Table 4 Management of hepatitis B virus infection in inflammatory bowel disease patient being considered for tumor necrosis factor-α blockers therapy

Serology	Management
HBsAg+, LE ↑	Treat HBV
HbsAg+, normal LE	Vaccinate for HBV; monitor LE and HBV-DNA
HbsAg-	Vaccinate for HBV
Consider giving a combined vaccination for HAV-HBV	Measure anti-HBs-Ab titer 1 mo after third injection to determine adequacy of response; additional doses may be necessary

LE: Liver enzymes; HBV: Hepatitis B virus; HAV: Hepatitis A virus; HBsAg: Hepatitis B surface antigen.

nosuppressants such as AZA or MTX, or GCS.

Under no circumstance should the patient on TNFB be given live vaccines. To stress, these include vaccines for measles-mumps-rubella, varicella (oral), yellow fever vaccine, or oral polio vaccine. The injectable (non-oral) vaccines for polio and for typhoid are considered to be safe. Live vaccines may be given safely three weeks before or three months after stopping AZA or metronidazole (MTZ), or given at any time to persons on GCS, as long as the dose is below prednisone 20 mg/d. There are no guidelines for the ideal time to vaccinate before or after the use of higher doses of GCS.

In the IBD patient who is hepatitis B virus (HBV) positive (regardless of negative hepatitis B surface antigen, and regardless of elevated or normal liver enzyme test), the HBV must be treated before starting TNFB (Table 4).

The regular travel precautions are taken for malaria. As with all travellers, ciprofloxacin and/or metronidazole should be available to the traveler, in the event that they develop symptoms suggestive of traveller's diarrhea. It is useful if the traveler has a typed copy of their medical records, a copy of their consultant physicians' most recent letter, documentation for custom officials of their prescribed medications, purchase of traveller's insurance without exclusion for pre-existing conditions (i.e., IBD), contact names and numbers of physicians in foreign countries with IBD experience, and contact numbers for care-givers at home. IBD patients with an ileostomy should be aware of the importance of their not becoming dehydrated.

APPROVED INDICATIONS FOR TNFBS IN CD

The approved indications for the use of TNFB vary from country to country. According to the Canadian Association of Gastroenterology, as well as the Canadian Expert Drug Advisory Committee[42,43], IFX is recommended: (1) For patients with moderately to severely active CD who have continuing symptoms despite the use of conventional therapies (5-ASA, antibiotics, GCS, AZA, MTX); (2) For patients with CD who cannot tolerate conventional therapy; (3) For fistulizing CD; and (4) For pediatric CD with inadequate response to conventional therapy.

When to avoid TNFB

There are several situations when TNFB should not be used, or used after full consideration of the consequences and patient awareness and patient awareness and consent.

Narrowing of bowel and TNFB use

Narrowing of bowel and TNFB use: (1) Inflammatory-yes (no pre-stenotic dilation); and (2) Fibrostenotic-no.

Latent infection

Latent infection: (1) TB; (2) HBV; (3) HIV; and (4) Live vaccines within last 3 mo.

Active infection

Active infection: Perianal or intra-abdominal abscess must be drained.

Present malignancy or disorder

Present malignancy or disorder: Present/previous malignancy (other than non-melanoma skin cancer) or lymphoprolifer-ative disorder.

Cautious use in children

Cautious use in children: Because of 48 cases of malignancy, 88% (almost 9 out of 10) of which were while the patient was on both TNFB plus immunomodulators, and half developed lymphoma.

Efficacy of TNFBs

General considerations: There are several goals of therapy in CD (Table 5). It is important to consider a

Table 5 Goals of therapy in Crohn's disease

Induce and maintain remission off steroids
Improve patient's quality of life
Reduced risk of need for surgery or hospitalization
Reduce risk of development of adenocarcinoma/lymphoma
Maintain mucosal healing as a possible predictor or surrogate marker of better future disease outcome
Acceptable efficacy/safety balance

number of factors pertaining to the clinical trials which have been used in meta-analyses to provide therapeutic recommendations for the use of TNFBs for induction and maintenance of CD. What proportion of CD patients will have an initial non-response to IFX (primary non-response), a continued response or remission, and a secondary non-response? The definition of response and remission may vary between studies, the time of assessment of these outcomes may vary, and most studies exclude the primary non-responders at 2 wk. Also, the placebo response rates vary widely, and therefore so too does the therapeutic gain [therapeutic gain (TG); therapeutic minus placebo response]. Some patients may have a short or a long duration of CD before IFX for example was initiated used, and some trial participants were previously or concurrently on GCS, AZA, or MTX, and the dose and duration of use of GCS, AZA and MTX may have varied widely.

About a quarter of persons treated with TNFB will be primary non-responders, i.e., will not respond to an initial dose of TNFB. Because primary non-responders are often excluded from analysis, the response and remission rates in the intention-to-treat group are much lower than the per-protocol group[44]. Patients and physicians must be made aware that some of the published rates of TNFB response (about 2/3 of TNFB users) and remission (about 1/3) are in those persons who responded initially, and do not include the percentages based on all users (i.e., intention-to-treat). The overall desired outcome of TNFB therapy is prolonged steroid-free remission, mucosal healing, good quality of life, and normalization of laboratory markers of inflammation [e.g., C-reactive protein (CRP), erythrocyte sedimentation rate], an acceptably low risk/benefit ratio, and acceptable cost to ensure widespread availability.

There are different ways in which a response to therapy may be defined, and these definitions vary from study to study. In clinical trials of CD, the CD activity index (CDAI) is often used as one of the outcome measures. The CDAI is based on the patient's symptoms, the presence of an abdominal mass, anemia (reduced hematocrit), weight loss, and use of an anti-diarrheal agent. CD is arbitrarily said to be "active" if the CDAI is above 150 points. Clinical response in CD may be a decrease in CDAI of ⩾ 70 points, or a decrease in CDAI of 70 to 100 points plus a 25% improvement from baseline[45]. Clinical remission might be variably defined as a CDAI of < 150, or CDAI < 150 plus a decrease by 50 to 100 points from baseline. Because of these variations in definition, the results from one study cannot be directly compared with another study. Comparing the placebo responses gives only a very rough approximation of the comparison of the population of patients examined in two studies, because of the very large heterogeneity of CD sufferers. The only totally valid method of comparison of one TNFB to another is a head-to-head randomized controlled trial (RCT). Note that there are no head-to-head comparisons of IFX, ADA, CER. Taking the largest trials into account, and considering all of these previously mentioned cautionary points, it is suggested that there are numerically similar post-induction success rates of clinical remission and fistula closure for IFX, ADA and CER.

Table 6 Predictors of good response to tumor necrosis factor-α blockers

Luminal
Short history of CD
Less severe disease
Isolated colonic disease
Inflammatory CD[46-49]
No previous abdominal surgery
Use shortly after ileocolonic resection
CRP > 5 mg/L (76% *vs* 46% response to TNFB), or ↑ CRP returning to normal detectable through serum TNFB (IFX) levels. CRP concentrations are inversely correlated with the rates of placebo response[50]
Non-smoking
Fistulizing
Perhaps for response with rectovaginal fistula

TNFB: Tumor necrosis factor-α blocker; CD: Crohn's disease; CRP: C-reactive protein; IFX: Infliximab.

Specific considerations: Induction of remission, and primary non-response: Patient selection characteristics and the time in the course of the patients' disease will influence outcomes with TNFB therapy, so there may be large variations in the placebo response in one study *vs* another, and therefore gain achieved with TNFB (Table 6)[46-50]. Careful consideration must be given as when to start TNFB therapy: When to start TNFB depends upon consideration of (1) clinical characteristics (steroid-refractory and steroid-dependent); (2) previous response to treatment; (3) complications and comorbidity; (4) cost/availability; and (5) patient preference and IFX. The 2 wk induction response rates for IFX ranges from 52% to 73% (Table 7). However, the therapeutic gain for single dose IFX for induction of response and remission in CD varies with the time of assessment and dose, and is quite modest, for example, only a 10% to 22% remission rate at week 12. Quoting from the CADTH review[51], "a total of 47 citations reporting 20 RCTs and 17 observational and uncontrolled studies were included. All the RCTs had a parallel group design. Four studies were open-label, single-arm trials. Of the remaining 13 cohort studies, seven studies used a prospective design, and the remaining six studies used a retrospective design. Five cohort studies and one RCT were published as abstracts only. The remaining studies had at least one full peer-

Table 7 Therapeutic gain for single dose infliximab for induction Crohn's disease: Response and remission (%)

Assessment week	IFX (mg/kg)			PL	Therapeutic gain
	5	10	20		
Response					
2	73	52	53	17	56-35-36
4	81	50	65	17	64-33-48
12	48	29	46	12	36-17-34
Remission					
2	40	22	22	4	36-18-18
4	50	26	26	4	46-22-22
12	30	18	25	8	22-10-17

PL: Placebo; IFX: Infliximab.

reviewed publication. IFX was used as the treatment for patients with CD in eight RCTs (total number of participants 1091; range: 36-335) and 10 observational and uncontrolled studies (total number of participants 1402; range: 12-614), and for patients with UC in six RCTs (total number of participants 1477; range: 299-499) and five observational and uncontrolled studies (total number of participants 157; range: 15-36). One cohort study (10 participants) reported the use of ADA in UC. One RCT of 43 participants evaluated the effects of ENT in patients with CD. Of six which included placebo-controlled trials assessing the effectiveness of IFX in the treatment of CD, three studies used short-term regimens of IFX to induce disease remission, and three RCTs evaluated the effectiveness of long-term IFX in maintenance of remission"[51].

For the treatment of fistulizing CD, reporting on 776 patients in 10 studies, after open-label induction, maintenance trials showed a mean difference of 16% (95%CI: 8%-25%, $P < 0.001$)[3]. Thus, only 11% had induction of remission as compared to placebo, and only 8% maintained their remission for about half a year.

The TG is the treatment response minus placebo response. The TG for significant response at weeks 4, 12, 24, and 30 and 54, a response will be seen in 50% and 37%, 30, 52, and 54 is 48%, 29%, 28%, 23%, 18%, and 22% with TG for remission of 16% and 13%.

In IFX primary non-responders, there is no proven benefit in using ADA. Of those who do respond initially at 2 wk, then after 4 wk of IFX about two-thirds respond (CDAI > 70 point reduction), and about one-third are in remission (CDAI < 150 points)[52]. At 24 wk, 57% will still show a response, but will not be in remission [IFX = placebo (PL), $P = 0.31$][52,53]. At 30 wk and 54 wk, 39% and 28% were in remission[54].

Adalimumab

The two major studies evaluating ADA in CD need to be evaluated separately due to heterogeneity in terms of whether the CD patients were naive to TNFB[51]. Similar to IFX, about 30% of persons with CD given ADA will be primary non-responders. Of those who do respond initially at week 1, about 52% will have responded at 4 wk and 21% will be in remission. The secondary non-response rate to ADA is 23%-48% at 24-26 wk and 23%-59% at 56 wk. The TG (active agent-placebo response) at weeks 4, 26, and 56 was 14%, 23%-25% and 23%-24% (Table 8)[54-56].

Table 8 Comparison of tumor necrosis factor-α blockers for Crohn's disease remission and fistula closure

	IFX (mg/kg)	ADA (mg)	CER (mg)
Remission (26-30 wk)	42	43	48
Fistula closure	36[78]	32	54

CER: Certolizumab; IFX: Infliximab; ADA: Adalimumab.

The Community Economic Development Assistance Corporation recommends ADA for patients with moderate to severely active CD who are "refractory to or who experience contraindication to an adequate course of 5-ASA and corticosteroids and other immunosuppressive therapy"[42]. It is not defined how long the patient must have been on conventional therapies before declaring that they have continuing symptoms, or how minor these continuing symptoms may be before considering adding TNFB therapy. It is not clear whether patient preference represents a "contraindication" to an adequate course of conventional therapy.

The mean percentage of loss of response to ADA among primary responders was 18.2% and the annual risk was 20.3% per patient-year. The mean percentage of patients who required dose intensification among primary responders to ADA was 37% and the annual risk was 24.8% per patient-year. When considering initial responders and patients with primary non-response, the mean percentage of patients who needed an ADA dose escalation was 21.4% and the annual risk was 24.4% per patient-year. Pooled analysis showed that dose escalation permitted response to be regained in 71.4% and remission in 39.9% of patients. Predictors for loss of response or dose escalation were male gender, current/former smoker status, family history of inflammatory bowel disease, isolated colonic disease, extra-intestinal manifestations, 80/40 mg induction therapy, longer disease duration, greater baseline Crohn's Disease Activity Index (Table 9), concomitant corticosteroid use, no deep remission at week 12, low serum trough concentrations of ADA, previous IFX non-response and being previously treated with an anti-tumor necrosis factor agent.

Certolizumab pegol

The Precise 2 trial with CER in CD over 26 wk showed clear superiority to placebo in terms of clinical response and remission[57].

Meta-analysis

There have been five systematic reviews and meta-analyses published on the efficacy of TNFB in inducing remission of active luminal CD[3,58-61], as well as Cochrane review of the efficacy of natalizumab in the same clini-

Table 9 Therapeutic gain for adalimumab induction of response and remission (%)

Study	Assessment week	ADA[1]	ADA[2]	ADA[3]	PL	Therapeutic gain
Gain	Response (CDAI ↓ ≥ 100 pts)					
	1	-	-	20	12	8
	2	-	-	37	18	19
	4	-	-	38	25	13
	Remission					
	1	-	-	6	4	2
	2	-	-	21	6	15
	4	-	-	21	7	14
(Classic Ⅰ)	Response (CDAI ↓ ≥ 100 pts)					
	1	23	25	21	16	7-9-5
	2	31	37	31	15	16-22-16
	4	34	40	50	25	9-15-25
	Remission					
	1	16	13	16	7	9-6-9
	2	14	20	24	14	0-6-10
	4	18	24	36	12	6-12-24
(Classic Ⅰ)	Response (CDAI ↓ ≥ 70 pts)					
	1	-	-	6	4	2
	2	-	-	21	6	15
	4	-	-	21	7	14
	Response					
	1	37	40	32	24	13-16-8
	2	44	55	45	30	14-25-15
	4	54	59	59	37	17-22-22

[1]40 mg at week 0, 20 mg at week 2; [2]80 mg at week 0, 40 mg at week 2; [3]160 mg at week 0, 80 mg at week 2. PL: Placebo; CDAI: Crohn's disease activity index; ADA: Adalimumab.

Table 10 Meta-analysis of efficacy and safety of anti-tumor necrosis factor α antibodies *vs* placebo (4 wk to 12 wk) in inducing remission in active luminal Crohn's disease

Treatment	Outcome (RR of remission not achieving)
All biological *vs* PL	RR = 0.87; 95%CI: 0.80-0.94; NNT = 8 (95%CI: 6-17)
IFX *vs* PL[1]	RR = 0.68; 95%CI: 0.52-0.90, I^2 = 78%, P = 0.01; NNT = 4 (95%CI: 3-7)
ADA *vs* PL	RR = 0.85; 95%CI: 0.79-0.91, I^2 = 0%, P = 0.99; NNT = 7 (95%CI: 5-12.5)
CER *vs* PL	RR = 0.95; 95%CI: 0.90-1.01, I^2 = 0%, P = 0.62
Natalizumab *vs* PL	RR = 0.88; 95%CI: 0.83-0.94, I^2 = 0%, P = 0.72
Adverse effects	
	No statistically significant difference in the incidence of adverse events was detected with TNFB *vs* placebo (RR of experiencing any adverse event, including infusion or injection site reactions = 0.99; 95%CI: 0.90-1.08 With anti–α4–integrin antibodies (natalizumab) *vs* PL, significantly more patients reported headache (compared with placebo: RR = 1.23; 95%CI: 1.03 to 1.47, I^2 = 0%) With NAT there were trends towards more infusion reactions (RR = 1.41; 95%CI: 0.94 to 2.10, I^2 = 0%) and infections (RR = 1.12; 95%CI: 0.97 to 1.30, I^2 = 0%) Number needed to harm with NAT = 17 (95%CI: 9-71)

Independent data extraction was undertaken by two reviewers, and discrepancies checked and resolved intention-to-treat. Analysis and pooled data with a random effects model was used, including the reporting of the search strategy, inclusion criteria, and data extraction processes. [1]Note that when the Sonic trail was excluded from analysis, there was no statistically significant difference between IFX *vs* placebo. RR: Risk ratio; IFX: Infliximab; ADA: Adalimumab; CER: Certolizumab; PL: Placebo; CI: Confidence interval; TNFB: Tumor necrosis factor-α blockers; NAT: Natalizumab; NNT: Needed to treat.

cal setting[62]. The most recent meta-analyses on the therapeutic management of IBD have appeared in the "Red Journal" (Table 10)[61]. Funnel plot asymmetry was considered regarding evidence of publication bias and small study effects, and Cochran Q values for heterogeneity. The criteria for inclusion of studies was stringent, using rigorous and conservative methodologies and these data should be used to better guide and care for patients suffering from CD and UC.

In summary, in persons with active luminal CD who have failed treatment with first and second-line agents or who are corticosteroid dependent, for induction of remission of active luminal CD, "IFX, natalizumab, and ADA appear to have the most evidence for their use, although the latter two therapies performed only moderately in this setting"[61].

Primary non-response

The clinical definition of primary non-response is lack of improvement of clinical signs and symptoms with induction therapy. Definitions and time-frames for the assessment of response and non-response have varied amongst clinical trials for different biological agents; in the original "Targan" study of IFX in refractory CD, response was defined as a 70-point reduction in the CDAI after 4 wk[52].

To a shift in the dominant mechanism of inflammation (loss of the pharmacodynamic effect), increasing symptoms or signs not related to IBD activity (for example, irritable bowel syndrome, concomitant infection, bacterial overgrowth, and so on), or failure to wean off corticosteroids. Loss of response can also imply.

A "relative" term for patients who have shorter durations of response (for example, less than 4 wk for IFX, 1 wk for ADA, or 2 wk for certolizumab) or require dose escalation, which may become economically impractical.

In the Accent I study of IFX, which assessed the maintenance benefits of IFX, the initial response was defined as a 70-point reduction in the CDAI at 2 wk[54]. Most clinicians will consider lack of response after two consecutive infusions of IFX of at least 5 mg/kg body weight and will assess treatment failure at 4 wk[50].

Recent data suggest that patients who initially respond may, more gradually, accrue remissions over time[55].

All three currently approved anti-TNFα biological agents are administered differently and have different pharmacokinetics and dosing intervals, and a reasonable assessment of lack of response to IFX would be after 2 wk, and to ADA and certolizumab pegol would be made after completion of induction therapy at 4 wk and 6 wk.

In approximate terms, 70% of IFX-treated CD patients show a response after 2 wk of treatment. In these primary responders, the secondary non-response rate (i.e.,

Table 11 Mechanisms which are speculated to be the cause of primary non-response

Lack of response may be due to different immunoinflammatory mechanism(s)[63]
A differential role of TNFα in certain stages of disease
Individual differences in drug metabolism and elimination, drug binding in serum or tissues based on disease activity level[27,148]
The presence of innate anti-TNFα antibodies that may exhibit greater neutralizing activity in non-responders[175]
Absence of inflammation accounting for clinical symptoms
Unidentified, genetic or pharmacogenetic or serological backgrounds of individual patients[24,176-179]
Individual differences in bioavailability and pharmacokinetics, leading to inadequate concentrations of a biologic secondary to immunogenicity (neutralizing or non-neutralizing antibodies, or unmeasured or unknown antibody) or other factors that increase drug clearance (decreased circulation half -life and possible high consumption in severe disease)[27,128]

TNFα: Tumor necrosis factor-α.

Table 12 Secondary failure rates

Maintenance of response month (%)	Loss of response (%)	Loss of response per month (%)
13 (64)	36	2.8
55 (63)	37	0.7
28 (78)	22	0.8

In another long-term maintenance study, the percentage of Crohn's disease patients who maintained response declined over time: 30 wk, 83%; 54 wk, 64%; 108 wk, 45%.

Table 13 Therapeutic options for tumor necrosis factor-α blockers secondary non-response

Continue the same TNFB, but use a higher dose at the same time interval
Continue the same TNFB and the same starting dose, but use a shorter interval between doses
Re-induction therapy with another TNFB
Use another therapeutic option (e.g., tacrolimus or cyclophosphamide)
Surgery

TNFBs: Tumor necrosis factor-α blockers.

those who initially respond, but then lose their response) is 40%-50% at 24-30 wk, and 60%-63% at 52-54 wk. At one year, about 30% of the initial responders will still be responding, and about 20% will be in remission.

About 30% of persons given TNFB therapy do not initially respond (primary non-response). The explanation of this is unknown, but it may be speculated that this may be done to the use of too low a dose of IFX (the serum through concentration of TNFB generally correlates with CD response), or the inflammatory process may be independent of TNFα. Of the initial primary non-response responders, about 70% respond and 35% go into remission. Including all persons with active CD, and taking into consideration the initial non-responders, out of 100 CD patients with active disease treated with TNFBs, approximately (100 × 70% = 70, 70 × 70% = 49) will respond and (70 × 30% = 21) will go into remission. These figures are lower than historically reported clinical response and remission rate with conventional (i.e., standard-of-care) management with GCS or, AZA/MTX, however it must be stressed again that because of large differences in study populations and study designs, the clinician must be very caution about accepting comparisons in anything other than direct head-to-lead studies.

There are numerous possible reasons for lack of response (Table 11)[63].

MAINTENANCE OF REMISSION AND SECONDARY FAILURES

Once the CD patient has gone into remission, we wish to prevent relapse. In CD, we lack benefit from 5-ASA/Sulfasalazine (SASP). Antibiotic maintenance has been shown to be significantly better than placebo, but there are insufficient studies for most clinicians to use long-term antibiotics as suppressive therapy. Few clinicians are sufficiently adapt at complex statistical methodology to understand the subtle aspects of whether to believe results reported as risk ratios (RRs) or odds ratio (ORs); immunosuppressives will likely continue to be used for maintenance therapy in CD, at least in the foreseeable future.

About 43% of patients with CD require GCS again within one year of starting GCS. The lifetime need to use GCS is 44%[64]. At 14 wk of use of full therapeutic doses of GCS for moderately active CD, 84% respond and 58% are in remission. At one year 32% are in remission, 28% are still on GCS (steroid-dependent), and 40% have required surgery. It may be speculated that the use of GCS may identify a group of patients who have more aggressive disease, and in whom immunosuppression or TNFB might need to be started early in the course of the disease[65]. AZA/MTX reduce steroid-dependence and maintain disease remission, but do not reduce the need for surgery[66].

Using meta-analysis, Akobeng[67,68] demonstrated that MTX, IFX, ADA, and CER were more effective than placebo for maintaining remission in CD, with number needed to treat (NNT) of 4-6. GCS, 5-ASA, budesonide, cyclosporine, antimicrobials and probiotics did not differ from placebo for maintaining remission at 6-24 mo. Enteral nutrition (EN) was more effective than no EN. Over 50% of CD patients become steroid-dependent or have a surgical resection within one year of starting GCS[64]. The monthly rate of loss of response to TNFB is higher in the shorter than in the longer durations of follow-up, ranging from 2.8% loss of response per month in a 13-mo study, to 0.7%-0.8% loss of response per month for 28- to 55-mo long study (Table 12).

Table 14 Therapeutic gain of infliximab for Crohn's disease maintenance of response and remission (%)

Assessment week	Treatment	IFX (mg/kg)			PL	Therapeutic gain
		5	10	20		
Remission	0, 2, 6 wk					
12		75[1]	-	-	38	37[1]
24		57[1]	-	-	29	28[1]
52		40[1]	-	-	22	18[1]
Response	2, 6, 8 wk					
30		50[1]	58[1]	-	27	23-31[1]
54		37[1]	47[1]	-	15	22-32[1]
Remission	2, 6, 8 wk					
30		39[1]	39	-	21	18[1]-18
54		28[1]	38	-	14	14[1]-24
Response	q8 wk					
54	until week 54	36[1]	-	-	6	30[1]
Response	q8 wk (4 infusions)					
36		-	72[1]	-	44	28[1]
44			62[1]	-	37	25[1]
Remission	q8 wk (4 infusions)	-				
12		-	14[1]		44	-30[1]
36			60[1]		35	25[1]
44			53[1]		20	33[1]

[1]Significant difference. PL: Placebo; IFX: Infliximab.

Those who have initially responded, but then subsequently relapse, are said to be secondary non-responders. In those persons who initially respond to TNFB, and then lose the benefit (CDAI increases, representing active disease; secondary non-response), there are several therapeutic options (Table 13).

IFX

Secondary failures to IFX may be as common as 48% in the first year of anti-TNF therapy, and 66% in the second year[69]. In secondary IFX non-responders, switching to ADA results in a response in 59%-83%, and remission in 29%-50%[70-72].

Secondary non-responders may respond to an increase in IFX dose, or a decrease in dosing interval, or both[73]. In secondary non-responders to IFX, 80%-90% responded to treatment intensification with IFX[54,74]. After two and a half years after an initial response to IFX, about half (54%) of patients require dose intensification[51,75]. Other studies have confirmed the use of dose escalation[76,77]. Dose acceleration helps maintain response: for luminal disease, 90%[74], for fistulising disease, 60%[78]. At 40 wk, 65% were still responding after the last dose intensification over 52 wk[72,73]. The therapeutic gain for IFX maintenance of remission was low, ranging from 14% to 33% at one year (Table 14).

In the Accent I Study[54], dose escalation was needed for persons who initially responded but then worsened at some time during the 54 wk study in 49% of persons on episodic treatment, 30% on 5 mg/kg IFX scheduled treatment, and 26% on 10 mg/kg scheduled treatments (secondary, non-response). Response was re-established with higher doses in 90% of the 5 mg/kg and 80% of the 10 mg/kg scheduled treatment groups treated with higher doses. Most of the cross-overs to higher doses occurred at week 14. In a 40 wk study, 83% of secondary IFX non-responders did respond after the first intensification of IFX, and 65% of patients were still responding after the last intensification dose.

Patients who lost their response to IFX were switched to ADA, and then continued with it for 52 wk. Secondary non-response to ADA was seen in 25%, and therefore, patients required a dose escalation[72]. These response rates are even higher in persons treated with ADA for the first time (i.e., IFX- and ADA-naive CD patients). Two RCTs and four observational and uncontrolled studies assessed the clinical effects of ADA after a loss of response or intolerance to IFX[51]. Re-induction therapy with ADA has a 60% clinical response[79]. Similarly, the Precise Ⅱ study[57] showed a benefit of reinduction in clinical response and remission of CD with CER in those patients who had received IFX in the past .

Long-term maintenance with IFX 3 mg/kg maintains mucosal integrity and avoids CD recurrence one year after surgery[80]. Another study evaluated endoscopic recurrence of CD post-operatively and found the rates to be lower with IFX at one year *vs* placebo (9.1% *vs* 85%)[81]. However, clinical remission was not significant (80% IFX *vs* 53.8% placebo, $P = 0.38$). A recent review of the literature also shows that there is statistical benefit with IFX use within 4 wk of surgery on mucosal healing. But this effect was not statistically significant in reduction of CDAI.

Adalimumab

There are two RCTs[55,56] and four observational and uncontrolled studies[70-72,82,83] assessing the clinical effects of ADA after a loss of response or intolerance to IFX. Blinded clinical trials show that ADA is effective first-line therapy for TNFB-naive CD patients, and is an option for IFX-refractory or-intolerant persons[84]. In the Gain study[55], secondary non-responders to IFX responded better when switched to ADA than when switched to placebo (IFX→ADA *vs* IFX→PL). In an uncontrolled open-label study, the switch from IFX to ADA (IFX→ADA) was associated at week 12 with a 59% clinical response and 29% remission rate[70]. A 50% or greater decrease in the number of draining fistulas was seen in 56%, while 33% had complete absence of fistula. In a second study[82], switching from IFX to ADA (IFX→ADA) gave a partial response in 31% and a complete response in 54%. In a single-arm study[72], 50% of IFX→ADA had a clinical remission at week 52. Four weeks after switching to ADA, 83% had a clinical response and 42% were in remission[72].

In the Crohn's trial of the fully human antibody adalimumab for remission maintenance (CHARM) study[56], there was no statistically significant response to ADA in IFX non-responders (IFX→ADA) or in those who were IFX-naive. When switching from IFX to ADA[56], the response at 26 and 56 wk in the TNFB-naive patients was 47% and 42%, compared to 32% and 31% in the non-responder group. Overall the ADA

Table 15 Therapeutic gain of adalimumab maintenance of response and remission (%)

Study	Assessment week	ADA[2]	ADA[3]	PL	Therapeutic gain
	Maintenance				
CHARM	Response (CDAI ↓ ≥ 100 pts)				
	26	52[1]	52[1]	27	25[1]-25[1]
	56	41[1]	48[1]	17	24[1]-31[1]
	Response (CDAI ↓ ≥ 70 pts)				
	26	54[1]	56[1]	28	26[1]-28[1]
	52	43[1]	49[1]	18	25[1]-31[1]
(Classic Ⅱ)	Response (CDAI ↓ ≥ 100 pts)				
	24	84	94[1]	61	23-33[1]
	56	79	89[1]	56	23-33[1]
	Remission (CDAI ↓ ≥ 100 pts)				
	26	40[1]	46[1]	17	23[1]-29[1]
	56	36[1]	41[1]	12	24[1]-19[1]
	Remission (CDAI ↓ ≥ 70 pts)				
	24	95	94	83	12-11
	56	79	89	72	7-17
	Remission				
	4	95	100[1]	89	6-11[1]
	12	90[1]	89[1]	56	44[1]-33[1]
	24	84[1]	94[1]	50	34[1]-44[1]
	32	84[1]	100[1]	39	45[1]-61[1]
	48	74	94	44	30-50
	56	79	83	44	35-39

[1]Significant difference; [2]40 mg at week 0, 20 mg at week 1; [3]80 mg at week 0, 40 mg at week 2. CDAI: Crohn's disease activity index; PL: Placebo; pts: Points; ADA: Adalimumab; CHARM: Crohn's trial of the fully human antibody adalimumab for remission maintenance.

treated group did better than the placebo group. The ADA group had a higher rate of staying free of steroids for > 90 d and achieved clinical remission at week 26 (19% for 40 mg every other weeks *vs* 15% for 40 mg every week *vs* 3% for placebo, $P < 0.001$). The therapeutic gain of ADA for maintenance of remission ranged from only 25% to 39% (Table 15).

The proportion of ADA-treated patients who required dose escalation and the results of treatment were reported in two RCTs and one small before-after study[51]. In secondary non-responders to ADA, 76% responded to dose escalation and 45% went into remission[55,72,85].

There is no data available to assess the effectiveness of using IFX for secondary non-responders of ADA. In the CHARM study[55], 46% of the persons randomized to receive ADA were secondary non-responders. Of those who received dose escalation of ADA, 76% had a no-point response rate and 45% went into clinical remission. In the Classic Ⅱ study[55] (following from the Classic Ⅰ study), only 5% of patients who completed the 56 wk of treatment needed dose escalation.

Certolizumab pegol

In CD patients who responded to CER induction therapy (Precise 2), and then relapsed during continuous or interrupted maintenance therapy, recapture therapy with CER was provided. The response rates at week 4 were 63%-65%, and 55%-59% at 1 year (Precise 4)[86]. Using Certolizumab pegol maintenance therapy for persons

Table 16 Therapeutic gain of certolizumab Induction of response and remission (%)

Week	Response		TG (CER-PL)
	CER	PL	
CRP < 10 mg/L			
6	37[1]	26	11[1]
26	22	12	10
Overall population			
6	35[2]	27	8[1]
26	23[2]	16	7[1]
Remission			
6	17	22	-
6 + 26	10	14	-
Maintenance with CER			
Week 26 clinical response	62[1]	34	28[1]
Overall	63[1]	36	27[1]
Group			

[1]Significant difference; [2]The secondary failure rates for CRP were 72% at 26 wk (approximately 10% per month). Precise Ⅰ study (Pegylated Antibody Fragment Evaluation in Crohn's Disease: Safety and Efficacy Ⅰ). PL: Placebo; CRP: C-reactive protein; CER: Certolizumab; TG: Therapeutic gain.

with CD, there was clinically meaningful improvement with the Inflammatory Bowel Disease Questionnaire score (60% *vs* 40%, $P < 0.001$), Short Form 36-Hem Health Survey (SF-36) physical (51% *vs* 34%, $P < 0.001$) and mental component summary responses (44% *vs* 32%, $P = 0.016$), and the proportion of persons living a normal life (21.4% *vs* 12.4%, $P = 0.019$), (57% *vs* 35%, $P = 0.001$). The therapeutic gain of CER for maintenance of remission was at 28% at week 26 (Table 16), similar to the 24 wk and 30 wk result for IFX and ADA. The therapeutic gain of CER varied little between weeks 2 and 12, and with doses of CER 100 mg, 200 mg and 400 mg (Table 17).

In the Precise 2 trial, the 26 wk therapeutic gain in response rates of therapeutic gain with CER were lower in those who had first failed IFX, than in those starting CER and never previously having been on IFX (19% *vs* 29%)[87]. This was also the case with IFX in the Precise 2 study: the response in the IFX-naive group was 69%, compared with 44% ($P < 0.001$) in the non-naive group. Thus, there may be benefit switching from IFX to ADA in the IFX-failure patient. In patients who were treated with IFX and experienced secondary failure, and then were treated with open-label CER, 62% enjoyed a response and 39% remission rate at week 6[88]. When extended to 26 wk, the response and remission rates were 40% and 37%, respectively. CER also improved work productivity during the induction and maintenance components of the Precise study[89].

Also from the Precise 2 study, 58 CD patients with draining fistulas (mostly perianal fistula) who responded initially to CER were continued on CER or placebo[90]. At week 26, 36% on CER had fistula obscure compared with 17% receiving placebo (TG, 19%). Using the definition of ≥ 50% closure at two consecutive post-baseline visits ≥ 3 wk apart, there was only a trend for achieving this end point (54% *vs* 43%, $P = 0.069$).

Table 17 Therapeutic gain of varying doses of certolizumab

Week	Response				Therapeutic gain		
	PL	CER (mg)			CER (mg)		
		100	200	400	100	200	400
2	18	33	19	28	15	1	10
4	19	37[1]	26	33	18[1]	7	11
6	18	36[1]	31	39[1]	18[1]	13	21[1]
8	22	36	29	39[1]	14	7	17[1]
10	27	44	36	44[1]	17	9	17[1]
12	23	38	24	39[1]	15	1	16[1]

[1]Significant difference. PL: Placebo; CER: Certolizumab.

Table 18 Meta-analysis of efficacy and safety of biological therapies (26 wk to 60 wk) *vs* placebo in preventing relapse of disease activity in quiescent luminal Crohn's disease

Treatment	Outcome (RR in preventing relapse)
IFX, ADA, CER	RR = 0.71 (95%CI: 0.65-0.76, I^2 = 5%, P = 0.38); NNT = 4 (95%CI: 3-5)
IFX, CER	Superior to PL
ADA	No statistically significant difference between ADA *vs* PL in preventing relapse
NAT (only one trial)	RR = 0.71; 95%CI: 0.61-0.84
Adverse events	
Any event	RR = 0.93; 95%CI: 0.84–1.03
Infusion or injection site reactions	RR = 0.64; 95%CI: 0.06-6.66; (note that these reactions were actually fewer in those on anti-TNFα therapy, but the difference was not statistically significant)

PL: Placebo; CER: Certolizumab; IFX: Infliximab; ADA: Adalimumab; TNF: Tumor necrosis factor; RR: Risk ratio; CI: Confidence interval; NAT: Natalizumab.

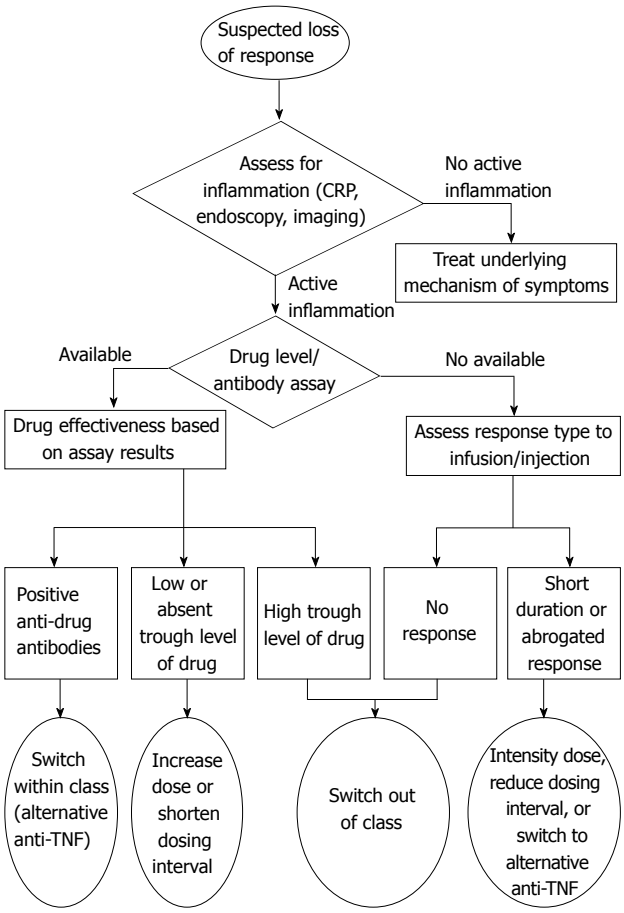

Figure 2 Suggested clinical approach to loss of response to anti-tumor necrosis factor-α therapy. CRP: C-reactive protein; TNF: Tumor necrosis factor.

The maintenance response rate was inversely related to the duration of the patient's history of CD: maintenance of response to CER was present in 90% of patients with a CD diagnosis less than one year, compared with 57% continued response in those with a CD diagnosis ≥ 5 years[91]. Corresponding remission rates were 68% *vs* 44%. This suggests that there may be improved efficacy of TNFB maintenance therapy when initiated "early" as compared "late" in the history of the patient's disease[92].

Meta-analysis

The results of the most recent vigorous meta-analysis of biological therapies in preventing relapse of disease activity in quiescent luminal CD is shown in Table 18. These authors suggest that the most marked effect of biological therapies was in preventing relapse of luminal CD once remission had been achieved, with a NNT of only 4. This benefit was observes for both IFX and certolizumab, but not for adalimumab[61].

Secondary non-response

About a third of patients treated with anti-TNFα therapy, who meet the criteria for an initial clinical response, eventually lose response, with the magnitude depending on the duration of follow-up on maintenance therapy. Loss of response, also referred to as secondary non-response, is defined as recurrence of disease activity during maintenance therapy after achieving an appropriate induction response. In maintenance trials with IFX and ADA, significant percentages of patients required increased doses (IFX) and or decreased treatment intervals (adalimumab) in order to restore response after interval symptoms developed between dosing[54,56,78,93]. Even after scheduled maintenance therapy and dose adjustments, substantial numbers of patients will still have a poor response[44]. Of the initial responders, 65% need dose escalation, which appears to be successful in approximately 70%, and the probability for dose escalation throughout time is approximately 80% at 120 wk[94].

From the Figure 1, it is apparent that approximately 10% (56%/5 years.) of patients treated with TNFB lose their response each year. If patients who are doing well on IFX + AZA, and the IFX is stopped, 39% relapse within one year[95]. Persons who are likely to relapse when IFX is stopped are those with objective signs of continued inflammatory activity (↑CRP, Crohn's disease endoscopic index of severity ≥ 2) or low IFX though level. Fortunately, almost all (> 95%) of those who recurred when IFX was stopped respond to IFX retreatment. Presumably a greater proportion relapse as this

interval is followed out beyond one year; it is unknown if those longer term relapsers will continue to have such a favorable response to retreatment with the originally successful TNFB.

How is this loss of response to TNFB managed? A useful suggested clinical approach to loss of response to TNFB therapy has been published (Figure 2). First determine if the patients symptoms might be due to something other than active CA. For example, exclude enteric infection (including *Clostridium difficile*) or small intestinal bacterial overgrowth, bile salt wastage, or adverse reaction to other drugs. What are the options if the CD is active[96]? The initially chosen TNFB is increased in dose, decreased in dosing interval, or both! If there is no response, switch to another TNFB[96].

Suggested clinical approach to loss of response to TNFB (Figure 2), to quote the approach suggested by Yanai *et al*[63]: (1) Confirm that intestinal inflammation is the cause of the "loss of response"; (2) Measure IFX levels at 4 wk after an infusion (therapeutic ⩾ 12 μg/mL); (3) Measure antibodies to IFX (ATI) measurements not available for ADA or CER. The Mayo Clinic group has demonstrated the utility of combining IFX levels and determination of ATI in the clinical management of patients who lose response to IFX[97]; (4) "Patients who lose response to a first agent are more likely to lose response to a second and, similarly, those with toxicity to a first agent are more likely to develop toxicity from a second anti-TNFα agent. This diminishing outcome may, in part, be due to a class effect"; (5) "Patients who lose response to IFX associated with anti-IFX antibodies respond well to switching to adalimumab or certolizumab pegol"; (6) "In the majority of patients, response can be restored by dose and interval adjustments"[44,94]; (7) "Switching between biologics is an important strategy for patients who lose response because of immunogenicity followed by rapid drug clearance or when a significant alternative pathogenic role is suspected. Switching can be within class or with an alternative class"; and (8) "Efficacy of successive agents is decreased compared with the primary agent".

In this situation where there has developed secondary failure, 75% to 78% regain a durable response after dose intensification[73,98,99]. It is recognized that over time after a surgical resection "cure", most CD patients recur endoscopically, and fewer recur symptomatically. In the study by Regueiro *et al*[81], the endoscopic recurrence rate of 85% was reduced to 9% with continued IFX treatment.

TERTIARY FAILURES

If two TNFBs fail, there may be value in switching to a third TNFB[100], with 6 wk response seen in 58%. This, however, needs to be supported by further data[101]. In a European prospective cohort study of 67 adult CD patients who either lost response or developed intolerance to two TNFBs (usually IFX and ADA), treatment with a third TNFB resulted in a 6-wk response and remission rate of 61% and 22%, respectively[100]. "Concomitant therapy with corticosteroids or immunosuppressants order of anti-TNF administration, and prior history of response (i.e., primary non-response, secondary loss response or intolerance) to anti-TNFs did not affect patient's response to the third anti-TNF".

Table 19 Comparative efficacy of infliximab, adalimumab and certolizumab (%)

Induction	Response[1]	Remission[2]		Therapeutic gain
		TNFBs	PL	
Accent I	58	39	21	18
CHARM	58	46	17	29
Precise 2	64	48	29	19

[1]Weeks 2 to 6; [2]Weeks 26 to 30. Remission is often defined by the Crohn's disease activity index, but may also be defined using the Inflammatory Bowel Disease Questionnaire. PL: Placebo; TNFBs: Tumor necrosis factor-α blockers; CHARM: Crohn's trial of the fully human antibody adalimumab for remission maintenance.

The NNT for induction of remission was 9. In maintenance studies, when initial non-responders were excluded, NNT was 4 among responders, but NNT was 9 when including both responders and non-responders[3]. Peyrin-Biroulet *et al*[3] performed a meta-analysis of 14 placebo-controlled trials enrolling 3995 CD patients, of TNFB efficacy and safety in CD. The TNFBs included IFX, ADA, CER, ETA. Quoting directly from their report, "In overall analysis, anti-TNF therapy was effective for induction of remission at week 4 (mean difference, 11%; 95%CI: 6%-16%, $P < 0.001$) and maintenance of remission at weeks 20-30 in patients who responded to induction therapy and in patients randomized before induction (mean difference, 23%; 95%CI: 18%-28% and mean difference, 8%; 95%CI: 3%-12%, respectively; $P < 0.001$, for all comparisons)".

In adult CD patients who either lost response or became intolerant of two TNFBs given at optimal doses, when switched to a third TNFB, 22% achieved full remission and 51% continuing a clinical response to week 20[100].

How long to use TNFB

How long to treat with TNFB? Five observational studies have shown the response to IFX or ADA ranges from 73% to 100% at year one, 65% to 90% at year two, 61% to 68% at year three, and 52% to 58% at year five[102-105]. RCT data is not available yet for more than one year. Clear evidence based guidelines have not been developed as to when to stop TNF therapy, but reasonable empirical suggestions would be with loss of response or severe adverse effects to TNFB. Perhaps after one year TNFB treatment, when the patient is in clinical remission, there are no signs of active inflammation, and perhaps when mucosal healing is achieved, stopping TNFB therapy may be considered and discussed with the patient.

Comparative efficacy of IFX, ADA and CER

There are no head-to-head trials of one TNFB against another. There is heterogeneity in the CD patients recruited for the clinical studies, but interestingly the therapeutic gain for remission at weeks 26 to 30 were close to 18% to 23% (Table 19). It is unfortunate that these rates are so low.

Patient-reported outcome (PRO) instruments are used to provide information about health-related quality of life. The minimal clinically important difference (MCID) and minimal important difference (MID). PRO score changes have been determined from two TNFB clinical trials[106]. The MCID is the smallest difference in a PRO score that is associated with a clinically relevant difference or change[1,107]. The MID is the smallest difference in a PRO score that a patient can perceive as beneficial and would require a change in their treatment. The MCID and MID units are meaningful measures to assess alterations in PRO scores, rather than changes which albeit are statistically significant, are not necessarily useful in a practical sense.

When considering possible advantages of different TNFB, for example, ADA *vs* IFX, ADA has greater ease of administration (subcutaneous, self-administered *vs* infusion facility), no infusion reactions, higher remission rates among secondary non-responders to IFX[72,108], possibly reduced need for dose escalation over time [fewer secondary failures[109], higher later rates of response and remission (maintenance), lower cost [$ incremental cost-utility ratios (ICUR) per quality-adjusted life-year (QALY)][51], and lower risk of developing neutralizing antibodies. Again it must be stressed that there is no head-to-head direct data to allow for a definitive statement to be made about any comparison, including cost and patient preference. These conclusions are based on observations.

In contrast, the possible advantages of IFX *vs* ADA include no injection site reactions or infections, fewer numbers of individual adverse effects, higher early rates (induction) of response and remission, and high rate of fistula improvement or remission with both short- and long-term therapy. IFX also gave higher initial response and remission rates as compared to ADA. Comparatively, the use of ADA led to lower relapse rates, indicating that patients retained longer benefit from ADA *vs* IFX[51].

Continuation of immunosuppression with TNFB therapy

Most guidelines suggest that TNFB therapy be started when immunosuppression alone has failed. The used of TNFB plus immunosuppression is known as concomitant immunosuppression (CI). To understand the subtle aspects of the question about CI use with TNFBs in CD, we need to reflect upon several important studies.

Scheduled maintenance IFX treatment is more effective than episodic treatment (Accent I)[74]; Scheduled maintenance therapy is superior to episodic treatment in terms of the use of steroids or the use of hospitalization and surgery[44]. IFX induction plus azathioprine/6-mercaptopurine (6-MP) is more effective than azathioprine/6-MP alone in AZA-naive patients who are steroid-dependent (GETAID)[53]. Early treatment with IFX in combination with an antimetabolite is more effective than conventional therapy (step-up top-down)[110]. IFX plus azathioprine is significantly better for inducing steroid-free remission and mucosal healing than azathioprine alone in azathioprine-naive patients [study of biologic and immunomodulator naive patients in Crohn's disease (SONIC)][102]. In initial IFX responders, the maintenance of response falls from 83% at 30 wk, to 64% at 54 wk, and to 45% at 108 wk[111].

Table 20 Concurrent immunosuppression with azathioprine plus infliximab *vs* azathioprine alone in Crohn's disease[53] (%)

	Week		
	12	24	52
AZA	38	29	22
AZA + IFX x3	75[1]	57[1]	40
Therapeutic gain	37[1]	28[1]	18

[1]Significant difference. AZA: Azathioprine; IFX: Infliximab.

Scheduled maintenance IFX is more effective than episodic treatment[74]. The standard of care is to use TNFB on a regular and scheduled basis. The benefit of scheduled *vs* episodic use of IFX is related to there being lower levels of antibodies to IFX (ATI), fewer infusion reactions, and fewer hospitalizations and major surgery[54,112,113]. IFX induction plus AZA/6-MP is more effective than AZA/6-MP alone in AZA-naive patients who are steroid-dependent[53]. Early treatment with IFX in combination with an immunosuppressant is more effective than conventional therapy[110]. IFX plus AZA is significantly better for inducing steroid-free remission and mucosal healing, than is AZA alone in AZA-naive patients[114].

There is no additional benefit of adding MTX in the steroid-treated CD patient who is also on IFX[115]. It remains unknown if the continuation of MTX might be useful if the person were just on MTX + TNFB, rather than just being on GCS.

Most current guidelines recommend that AZA/MTX must have been used and be considered to have failed before TNFBs are introduced what should be. The duration of previous use of this immunosuppression and should AZA be continued TNFB once has been started is not known. Some data suggested that continuation of AZA after the introduction of IFX offers no additional benefit beyond 6 mo of combined therapy, as long as the TNFB is used on the basis of a regular schedule, rather than being given episodically. The authors of one trial suggested that "continuation of immunosuppression for more than 6 mo offers no clear benefit over scheduled IFX monotherapy"[116]. IFX monotherapy is superior to IFX plus immunosuppressive therapy after CD induction with IFX. In one study, four times more relapses occur with AZA maintenance after IFX induc-

tion, as compared with IFX induction and placebo maintenance[53]. In a second study, maintenance of remission of CD was no different when AZA plus IFX were given for maintenance, *vs* IFX alone[117].

In the study by Van Assche *et al*[116], at 6 mo in person on IFX in whom AZA had been withdrawn (IFX plus AZA D/C *vs* IFX + AZA), the CRP levels were higher, and IFX levels were significantly lower. Perhaps if the patients had been followed for longer than 6 mo, the AZA discontinuation group might have begun to experience more recurrences. The RAND appropriateness methodology, through a modified Delphi panel approach based on expert interpretation of the available literature, has been used with a "globally diverse panel of 13 gastroenterologists clinically experienced in inflammatory bowel disease"[118]. Using a total of 134 clinical scenarios, concomitant use of immunomodulatory was generally rated inappropriate for young males, and in some scenarios involving uncomplicated disease. However, CI were appropriate for those with extensive disease, shorter duration of disease, perianal involvement, prior surgery, females, and older patients (> 26 years)". In the GETAID study, the IFX + AZA (CI) gave a significant therapeutic gain of clinical remission off steroids, as compared with AZA alone, of 37% and 28% at weeks 12 and 24 respectively. But the benefit was lost at week 18 (Table 20).

Several important aspects to consider when attempting to answer the question of the benefit of using CI with IFX are the duration of follow up, whether the patient is naive to the immunosuppressants or the TNFB, whether the activity of the disease is high as suggested by an elevated surrogate marker such as CRP or mucosal lesions, what is using an endpoint of immunogeneity (such as antibody to TNFB or a disease activity index or QoL score), and whether the question is focused on the CD patient in whom you are trying to achieve induction or maintenance of remission. CI is clearly beneficial when added to IFX regimens which are episodic[112,119], but the controversy about CI comes from studies using regularly scheduled TNFB, the method which is new to the standard of care.

In the SONIC study, CD patients who were naive to both immunosuppression and to TNFB, the rate of clinical remission at week 26 was higher in those given IFX + AZA as compared with IFX alone or AZA alone (57%, 44%, and 31%, respectively)[120]. In a subgroup of patients who initially had both an elevated CRP and mucosal lesions at baseline, the IFX plus AZA + IFX alone values were even higher (69% and 57%, respectively). Since the difference between IFX + AZA was statistically greater than IFX alone, the data may be interpreted to indicate that CI is advisable, at least in the relatively short period of 26 wk, for steroid-free remission. However, the benefit of CI appears to fades after 6 mo to 12 mo[116,121].

In the infliximab maintenance immunosuppression discontinuation study (Van Assche), in CD patients who had been on AZA/MTX before starting IFX, stopping the immunosuppression at 6 mo did not have an adverse effect at 2 years to shorten the interval between IFX infusions, or the time to stopping IFX dosing. The prevalence of antibodies to IFX were similarly low in each group, and CI with IFX + AZA had no different effect in terms of mucosal healing.

The same message comes from the 2-year long COMMIT trial of steroid-requiring CD patients on IFX or IFX + MTX (Feagan 2010): CI does not provide a medium term (2-year) advantage. On the other hand, CI may increase the risk of adverse effects such as non-Hodgkin's lymphoma in adults[122], hepatosplenic lymphomas in children[123], as well as other concerns in children[124]. As well, CI did not benefit the medium term risk of surgery[125]. These individual studies suggest that in many CD patients, CI is "bridge" therapy for 6 mo while the full effect of TNFB is being achieved.

In an observational serial observation of 88 CD patients in remission and on IFX, half had their CI with AZA stopped. The IFX failure free status was 85% at 12 mo, and 41% at 24 mo and 32 mo[126]. This failure rate of IFX maintenance may have been due to the withdrawal of CI with AZA. Failure of CI on AZA-withdrawal was more likely to occur if the initial duration of IFX-AZA therapy was less than 27 mo, if the CRP was greater than 5 mg/L or the platelet count higher than 298 109/L. Until further data becomes available, we can be encouraged by "expert opinion" from the BRIDGE group analysis[118], suggesting that bridging CI may not be necessary such as in young males especially with limited disease, or some case scenarios including uncomplicated CD.

CI[127]treatment also reduces antibody to IFX (ATI) formation [24% *vs* 63%, on CI *vs* not on CI, $P = 0.007$; 43% *vs* 75%, $P < 0.01$]. Former researchers have proposed a time-structural treatment algorithm for moderate CD. This may of course need to be altered in future if "top down" (IFX→AZA→GCS) approaches to CD are shown to be beneficial.

The major role of CI may be in the patient who is on episodic IFX: in these persons, the incidence of ATI was 0% on CI *vs* 60%, not on CI, $P = 0.018$[128]. Comparatively, CI had no relationship on ATIs with scheduled IFX infusions. Combined immunomodulator with TNFB provides small therapeutic gain for 24 wk. Then, it is likely that CI can be abandoned without any detrimental effect of clinical response, and possibly a lower risk of adverse events. In fair balance for the issue of AZA discontinuation in the TNFB-treated patient, the "jury is still out", or as the Scots would say, the case is "not prove". Clearly, more data is needed.

In summary of Combination therapy: (1) In AZA and TNFB naive persons, with ↑ CRP or mucosal lesions on colonoscopy, remission and one year mucosal healing response to combination therapy (SONIC) A AZA = TNFB; (2) Unknown what to do after one year: for IFX alone after successful induction, maintenance for 54 wk gave a therapeutic gain of (28%-14%) 14% and 24% (38%-14%) for 5 mg/kg and 10 mg/kg IFX (Ac-

Table 21 Infliximab for fistulizing Crohn's disease: Induction and maintenance (%)

Study	Week of assessment	Outcome	IFX (mg/kg)		PL	TG
			5	10		
Present *et al*[75]	18	Response	68[1]	56[1]	26	42-30
		Closure	55[1]	38[1]	13	42-25
(ACCENT Ⅱ)	54	Response	46[1]		23	23
		Closure	36[1]		19	17

[1]Complete closure of draining fistulas; *P* value for each IFX group *vs* placebo group. For IFX 5 mg/kg, the TG for fistula closure ranged from 42% at 18 wk to 23% to 54 wk. Adapted from CADTH ('09). These differences are statistically significant. See Tables 3 and 4 of the CADTH report for *P*-values, number of participants and events. IFX: Infliximab; PL: Placebo; TG: Therapeutic gain.

Table 22 Adalimumab for fistulizing Crohn's disease induction, response and closure (%)

Study	Assessment week	ADA[1]	ADA[2]	ADA[3]	PL	TG
GAIN	Response					
	4	-	-	15	20	(-5)
	Closure					
	4	-	-	5	8	(-3)
Classic Ⅰ	Response					
	4	75	20	8	33	(42, -13, -25)
	Closure					
	4	75	0	0	17	(58, -17, -17)

[1]40 mg at week 0, 20 mg at week 2; [2]80 mg at week 0, 40 mg at week 2; [3]160 mg at week 0, 80 mg at week 2. ADA: Adalimumab; PL: Placebo; TG: Therapeutic gain.

cent Ⅰ). It is unknown what is best to do after one year treatment with IFX; (3) In post hoc analysis of combination therapy of immunomodulators plus IFX/ADA/certolizumab pegol (CZP) *vs* monotherapy with TNFB, there is no benefit in terms of induction synergy; (4) Because of study design, it is not possible to comment whether MTX plus TNFB is superior to TNFB alone; and (5) Not possible yet to predict which patient can be induced with TNFB and maintained on AZA (TNFB as a "bridge").

FISTULIZING DISEASE

Because between 20% to 40% of CD patients will develop fistulas[75,129], and because of the seriousness of perianal fistulas[78,129,130], the improved healing of fistulizing CD with TNFB would be anticipated to reduce the patient's lifetime need for major surgery. The presence of fistulas increases the risk of the need for colectomy[69,82]. Anti-TNF therapy is indicated for use in fistulizing CD. What is the evidence for IFX or ADA in this clinical setting? In the IFX infusion study by Present *et al*[75], IFX was given at 0 wk, 2 wk and 6 wk, and fistula response and complete closure of draining fistulas were assessed at 18 wk. As compared with placebo, IFX 5 mg/kg and 10 mg/kg gave 18-wk fistula response rates of 68% (P = 0.002) and 56% (P = 0.02) *vs* 26%, respectively. The corresponding closure rates were 55% (P = 0.001), 38%

Table 23 Adalimumab for fistulizing Crohn's disease maintenance (%)

Study	Assessment week	Combined ADA group[1]	PL	TG
CHARM	Closure			
	26	30	13	17
	56	33	13	20

[1]Statistically significant. Note that the two higher dose regimes of ADA do poorly for induction of fistulizing CD. The TG for IFX and ADA for fistula closure at 54-56 wk is a similar low value of 17%. PL: Placebo; TG: Therapeutic gain; ADA: Adalimumab; CHARM: Crohn's trial of the fully human antibody adalimumab for remission maintenance; IFX: Infliximab; CD: Crohn's disease. 140 mg q0w and 40 mg qw.

Table 24 Meta-analysis of efficacy and safety of biological therapies *vs* placebo (4 wk to 26 wk) in healing of fistulizing Crohn's disease

Treatment	Outcome (RR of fistulas remaining unhealed)
IFX, ADA, CER *vs* PL (4 wk to 26 wk)	RR = 0.88; 95%CI: 0.73-1.05; I^2 = 67%, P = 0.01 (considerable heterogeneity between studies, with no statistically significant difference in fistula healing at 4 wk to 26 wk, compared to placebo, when considering the 3 TNFB)
IFX *vs* PL (one trial)	RR = 0.62; 95%CI: 0.48-0.81
IFX, ADA, CER *vs* PL (excluding 2 trials reporting fistula healing up to only 4 wk)	RR = 0.80; 95%CI: 0.65-0.98; I^2 = 56%, P = 0.08 (heterogeneity between studies remained)
IFX (only 1 trial) *vs* > PL (followed until 54 wk)	RR = 0.81; 95%CI: 0.68-0.96

Adverse events could not be determined from data. CER: Certolizumab; IFX: Infliximab; ADA: Adalimumab; PL: Placebo; RR: Risk ratio; TNFB: TNF-α blockers; CI: Confidence interval.

(P = 0.040), and 13% at 18 wk. For the 5 mg/kg dose of IFX for fistulising CD, this gave a therapeutic gain of 42% for both fistula response and closure.

In the IFX maintenance study by Sands *et al*[130] (Accent Ⅱ), IFX was infused every 8 wk until week 54. As compared with placebo, IFX 5 mg/kg gave 54 wk response rates of 46% *vs* 23% (P = 0.001); and the complete closure rates were 36% *vs* 19% (P = 0.009). This gave a 54 wk therapeutic gain of 23% and 17% for response and closure, respectively. Note the need to caution your CD patient with fistulizing CD: while early TG for response and closure was an encouraging 42%, at one year half this benefit was lost (falling to 23% and 17% at 54 wk)[75,78,129].

The TG in fistula response and closure for IFX (5 mg/kg) at 19 wk was 42% and 42%, respectively, with curiously lower rates of response for the 10 mg/kg IFX dose. At 54 wk, the TG for fistula response and closure was 23% and 17%, respectively. These are low values, but clearly IFX is of considerable benefit to individual patients who may have failed conventional therapy.

The 4-wk fistula response and closure rates for ADA (160 mg at week 0 and 80 mg at week 2) was not statistically different from placebo, but at weeks 26 and 56, the TGs were 17% and 20%, respectively. In the CHARM study, complete healing of fistulas was achieved in 30%

of those receiving ADA either weekly or every other week by week 26. This compared to 13% in the placebo group (P = 0.043). Recall that with fistulizing CD, the early response and closure with IFX was statistically significant, but was not so with ADA. At one year, the fistula response and closure rates were numerically similar for IFX and ADA, but the TGs were small for both TNFBs (Tables 21-23). Therefore, at this time, it is best to use IFX as 1st line to treat fistulising disease. There is insufficient data to recommend the same for CER. Meta-analyses have been performed for biological therapies in healing fistulising CD (Table 24). These authors suggested that "In preventing recrudescence of fistula, there was only one RCT reporting on this end point, which demonstrated that IFX was superior to placebo (Table 24). Although concerns about adverse events arising from the long-term use of these drugs are understandable, the data from the RCTs included in this meta-analysis did not demonstrate any increase in overall adverse events, or serious adverse events, with use for up to 1 year[61]."

In summary of the use of TNFB in fistulising CD "…. there is less evidence for the use of biological therapies, though there was a clear beneficial effect when only IFX was studied, although in one trial, and when only RCTs with follow-up in excess of 4 wk were considered in the analysis. There was also a benefit in favour of biological therapies over placebo when only studies that reported fistula healing in the longer term were included in the analysis."

THERAPEUTIC GAINS

Induction and maintenance, response and remission

At 2 wk and 4 wk after a single dose of IFX (5 mg/kg), the therapeutic gain (TG; IFX response minus the PL response) for clinical response was 56% and 64%, respectively (Table 7), and then fell to 36% at 12 wk[52]. The TG for remission was 36% and 46%, respectively, for weeks 2 and 4. When IFX (5 mg/kg) is given 2, 6 and every 8 wk until week 48[54] or 54[69,82], the week 54 TG for response ranged from 22%[54] to 30%[69,82], and for remission, the TG was 14% for IFX 5 mg/kg and 24% for 10 mg/kg. IFX doses of 10 mg/kg or 20 mg/kg did not give higher therapeutic gains in terms of response.

Thus, after excluding the approximately 30% initial IFX primary non-responders, about the two-thirds will respond and about one-third will go into remission at 4 wk (therapeutic gain). With continued 8-weekly IFX infusion for approximately one year, the TG for response is about one in four, and for remission one in eight. These represent relatively small gains which are not necessarily higher than reported historical figures for conventional therapy induction or maintenance therapy in CD. In children with CD, Kaplan-Meier analysis has shown that the cumulative probability of losing response to IFX after 1 year, 3 years and 5 years is 13%, 40% and 50%[131]. In adults with CD who chose to stop their IFX after achieving remission, 50% relapsed within 477 d after IFX discontinuation[132].

For the currently recommended dose of ADA of 160 mg at weeks 0 and 80 mg at week 2, TGs for response range from 15% to 19% at week 2, and 13% to 25% at week 4 (Sandborn *et al*[93]). The corresponding TGs for remission at 2 wk and 4 wk range from 10% and 14%-24%. The TGs for response at 56 wk range from 7%-25% when ADA is given as 40 mg q every other week, to 15%-33% when ADA is given as 40 mg q weekly[55,56,85,127,133]. The corresponding values for TG for remission at 56 wk range from a low of 4% to a high of 39%[55,56,85,127,133]. The values for the initial TGs in response and remission for induction therapy are numerically slightly lower for ADA; it is slightly higher for ADA for maintenance of response and remission. After two years of maintenance therapy with ADA every other week, 42% were in remission compared to 38% on placebo[134], and 50% with weekly ADA. This study also showed an improvement in IBD QoL questionnaires, suggesting clinical response. There is insufficient evidence at this time to recommend one type of ADA therapy over another.

In addition to therapeutic gains in terms of response and remission rates, other important clinical outcomes include improvements in quality of life, hospitalization and surgery. In patients with RA, combinations of TNFB with low-dose MTX are generally more efficacious than either drug used alone[135], the mechanism of anti-inflammatory properties of this combination is not totally clarified and it is not known if TNFB plus MTX have a synergistic mechanism[136], especially in CD.

Health utilization

Hospitalization: In Canada, about 20% of persons with CD have IBD-associated hospitalizations per year[2]. The proportion of patients who needed hospitalization for IBD were reported in three IFX trials and one ADA trial. Two IFX trials and one ADA trial reported the patients who needed surgery[51]. Overall, CD patients who received IFX maintenance therapy had an approximately 50% reduction in the rate and duration of hospitalization[74,129]. Lower hospitalization rates were also seen with ADA[93]. Infusing IFX on a scheduled *vs* an episodic basis reduces the rate of all-cause hospitalization, as well as IBD-related hospitalization. For example, in Accent I [74,137], the rate of IBD-related hospitalization was 28% in the episodic treatment group *vs* 23% in the IFX 5 mg/kg group (P = 0.047) and 24% in the 10 mg/kg group (P = 0.023). Furthermore, the number and duration of hospitalizations were lower in the scheduled *vs* episodic group (P = 0.018). In Accent II [129], the hospitalization rates were lower with IFX than placebo (14% *vs* 31%, P < 0.01), and 11% *vs* 33% in the initial IFX responders *vs* placebo (P < 0.05). Also, the mean duration of days in hospital was less with IFX *vs* placebo (0.5 d *vs* 2.5 d, P < 0.05).

In a population based study from the University of Manitoba of health care resource use among IFX us-

Table 25 Reduction (%) of health service utilization in Crohn's disease patients treated with Infliximab

	All CD patients[180]	CD fistula patients	
		(Rubenstein *et al*[180])	(Harrison *et al*[181])
ER visits	66	64	372
All surgery	38	66	
GI surgery	18	59	
Hospitalizations		59	37
Endoscopies	43		52
Radiographs	12	40	58-147
Outpatient visits	16	27	22-33

CD: Crohn's disease; ER: Emergency Room; GI: Gastrointestinal.

Table 26 Natural history of Crohn's disease and ulcerative colitis %

Natural history			
UC		CD[1]	
Remission at any point in time	50	Relapse	10-30
Overall intermittent course	90	Low activity	15-20
Remission: 3-7 yr after diagnosis	25	Remission	55-65
Yearly relapse	18	Chronic active	13-20
Intermittent relapse	51	Chronic intermittent	67-73
		Remission for 10 yr	13
10 yr colectomy rate	10	Surgery by 20 yr	> 75
Change in site of UC over 25 yr		Change in behavior	
LC→ C	50	NSNF → S	27
C → LC	75	NSNF → F	29

[1]Course 1 year after diagnosis. C: Entire colon; LC: Left colon; S: Structuring; F: Fistulising; NSNF: Non-stricturing, non-fistulizing; UC: Ulcerative colitis; CD: Crohn's disease.

ers, hospitalizations were higher in the IFX cohort until 18-24 mo after the first IFX prescription, at which point the rate of hospitalization in the IFX group fell to the level of the CD patients treated with AZA or GCS[138]. The likelihood of surgery was similar in IFX and GCS, which was higher than in AZA; even at 3 years post-closing. Overall physician visits were similar between IFX, GCS and AZA. Interestingly, ADA reduced hospitalization rates at 12 mo from 14% with placebo *vs* 10% and 3% with maintenance ADA given every other week and every week ($P = 0.12$ and $P < 0.01$, respectively)[115]. Hospitalizations account for more than 50% of the direct costs of CD[115]. Thus, in responders, TNFB reduced the rate of hospitalization, and this reduction in hospital days carries a major economic impact, which has to be countered against the high cost of maintenance TNFB requiring long-term use of drug, and the cost of management of adverse effects.

Surgery

CD-related surgery was more likely in the episodic *vs* the scheduled treatment groups (9% *vs* 3%, $P = 0.01$)[74], and the mean number of surgeries was higher (118 *vs* 60, $P < 0.01$). As compared with the placebo group, the mean number of surgeries in the IFX group was 13 *vs* 2 per 100 in all patients, and from 11 to 11 per 100 in the IFX initial responders[78]. With ADA maintenance, the 12-mo risk of major surgeries was 0.6% *vs* 3.8% in the placebo group ($P = 0.0005$)[115]. Data to date shows that TNFB reduce serious health utilization in persons with IBD.

Table 27 Meta-analysis of efficacy and safety of biological therapies *vs* placebo in inducing remission in moderately to severely active ulcerative colitis[61]

Treatment	Outcome (RR of remission not being achieved)
IFX *vs* PL	RR = 0.72; 95%CI: 0.57-0.91 NNT = 4 (95%CI: 3-8)
Adverse event	
Any adverse event was no higher in IFX *vs* PL, and risk of serious adverse events was lower	RR of serious adverse event = 0.64; 95%CI: 0.41-1.00, $P = 0.05$ NNH = 13 (95%CI: 8-50)

IFX: Infliximab; PL: Placebo; RR: Risk ratio; CI: Confidence interval; UC: Ulcerative colitis; NNT: Needed to treat.

TNFBS AND THE NATURAL HISTORY OF CD

It is not yet certain whether TNFB will achieve disease modification, or sustained mucosal healing[139]. CD is very heterogenic, and we need much more data on the possibility of patient risk-stratification based on environmental factors, disease phenotype, genotype and disease-initiating and-perpetuating factors (Table 25)[140].

A brief consideration of the use of TNFB in ulcerative colitis

There are many differences between CD and UC (Table 26). Only for purposes of comparison, a very brief overview is provided on IFX use in persons with UC, bearing in mind that these are separate and distinct conditions. About 20% of UC patients on 5-ASA maintenance therapy will have an acute attack each year, and about 10% of those with more severe symptoms will require hospitalization, usually for intravenous (IV) steroid therapy, cyclosporine or TNFB therapy; or surgery. About a third of these will not respond to IV steroids, and may be candidates for colectomy, cyclosporine or IFX.

In the Accent I and 2 studies, approximately half as many of those in the combined IFX group than placebo-treated UC patients required surgery (74% *vs* 8%), and the number of hospitalizations were also lower (18/102 patients *vs* 9/102 patients, $P < 0.01$)[74,115,129]. The colectomy rate in the placebo group is also approximately twice as high as in the IFX group: at 90 d, 67% *vs* 29% ($P = 0.017$)[141], and at 2 years, 76% *vs* 46% ($P < 0.05$)[142]. When IV GCS fail in patients with severe UC admitted to hospital for IFX[143], colectomy rates were as follows: in hospital colectomy, 24%; later colectomy, 14%; no colectomy, 62%. It is not clear how many will eventually require colectomy, whether they will need to

Table 28 Associations of mucosal healing in Crohn's disease

Fewer symptoms	
No previous surgery	
Previous surgery	
Less inflammation at 5 yr requiring steroid use	
Less use of steroids	
Steroid-free remission and no flare at years 3 and 4	Yes (TG, 44%)
Steroid-free remission, no flare and no TNFB at years 3 and 4	Yes (TG, 44%)
No new or persistently active fistulas	No

How do these associations with mucosal healing (MH) in CD compare with UC? About 90% of patients remain asymptomatic after achieving MH on once-a-day 5-aminosalicylic acid (Mezavant®), and after MH, 40% of UC patients remain asymptomatic (TG, 22%). TG: Therapeutic gain; CD: Crohn's disease; UC: Ulcerative colitis; TNFB: Tumor necrosis factor-α blockers (response in active treatment group minus response in placebo group; differences statistically significant).

Table 29 Rates of therapy-associated mucosal healing

Drug	CD		UC	
	Week[1]	MH, %	Week[1]	MH, %
5-ASA			4-8	25-71
Prednisone	4-9	0-27		
AZA	16-96	40-73	26	53-69
MTX	12-60	36-38		
IFX	4-104	29-100	8-52	62-71
ADA	12	27	8	30
CER	10	5-55		

[1]Time for endoscopic assessment of mucosal healing. CD: Crohn's disease; UC: Ulcerative colitis; MH: Mucosal healing; 5-ASA: 5-Aminosalicylic acid; AZA: Azathioprine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab; CER: Certolizumab.

be maintained long-term on IFX, or if they should simply be maintained on AZA or 5-ASA.

Primary non-response has been noted in 22% of UC patients treated with IFX[126]. IFX dose optimization was required in 45%, and colectomy was required in 19%. In a single-centre, prospective out-patient cohort of UC patients, response to induction therapy with IFX was 96% and 80% for ADA[144]. For maintenance therapy response to IFX and ADA, the rates were 78% and 70%. None of these differences was statistically significant.

Recent meta-analysis has considered the efficacy and safety of biological therapies in inducing remission in moderately to severely active UC (Table 27)[61].

The summary points are as follows: (1) No data on the efficacy and safety of biological therapies *vs* placebo in preventing relapse of disease activity in quiescent UC; (2) "IFX was highly effective in inducing remission in patients with moderate to severely active UC who had failed therapy with first and second-line agents, and who had also failed to respond to a course of high-dose corticosteroids, with a NNT of only 4[61]"; (3) IFX for rescue therapy in severe UC, with a therapeutic gain of (71%-33%) 38% (NNT, 3); (4) IFX will induce and maintain clinical remission and mucosal healing in treatment–refractory, moderate/severe UC; (5)Unknown if

Table 30 Complete mucosal healing[1]

	ADA	PL [ADA induction only (3 doses)]	TG (%)
ITT		PL	
week 12	27%	13% (P = 0.056)	14
week 52	24%	0% (P < 0.001)	24
PP			
week 12	28%	13% (P = 0.046)	15

[1]Extend: Open-label adalimumab[182]. Of patients with CMH at week 12, 46% of ADA every other weeks had CMH at week 52 *vs* 0% CMH in those receiving only ADA induction. ITT: Intention-to-treat; PP: Per protocol; TG: Therapeutic gain; PL: Placebo; ADA: Adalimumab.

IFX plus AZA is superior to monotherapy with AZA or IFX; (6) Before starting IFX in UC, discuss options of AZA, cyclosporine A (Cy A), and pan proctocolectomy with ileoanal pouch procedure; (7) Unknown comparison of Cy A *vs* TNFB for rescue therapy; (8) Short term: young age, absent pANCA (in CD, presence of pANCA, ASCA, or pANCA + ASCA had no predictive valve); and (9) Long-term (predictors for no colectomy): short term clinical response to CRP < 5 mg/L, no previous treatment with IV steroids or Cy A.

Importance of mucosal healing

There are a number of potential advantages in achieving mucosal healing in CD (Table 28). In both CD and UC, the rates of mucosal healing range widely depending upon the therapeutic agent and the duration of treatment (Table 29).

In clinical trials, there has been no uniform standard for defining mucosal healing (MH), no agreed-upon optimal time for assessing MH. It is not surprising then that the ranges of MH are very wide.

The clinical trials of agents used to treat IBD have used a variety of clinical scores heavily based on symptoms (CDAI), measures of QoL, appearance of the mucosa on diagnostic imaging, endoscopic appearance, or histopathology. Some patients with CD treated with TNFB achieved MH and the question was raised whether MH by itself, independent of patient's symptoms or QoL, was an important outcome variable. MH would be an important outcome if it were to be shown that it was associated with reduced risk of recurrence (i.e., greater likelihood of maintenance of remission), reduced risk of extraintestinal complications, reduced risk of medical treatment-associated complications, long-term risk of hospitalization, surgery or development of colorectal cancer. The risk of development of stricture or fistula in CD increases over time[46,145], so maintaining mucosal healing may decrease these problems. However, there is no evidence to answer the above asked questions.

It seems self-evident that MH should become an outcome objective of therapy in IBD. However, this needs to be proven. Because the prevalence of MH may decline over time, and the number of the week of study repeating MH varied widely, it is not possible to assert which class of drug has the highest rate of MH (Table 29), nor is it plau-

Table 31 Meta-analysis efficacy of 5-aminosalicylic acid/sulfasalazine to induce remission or to prevent relapse in Crohn's disease

	Induce remission (RR of not being in remission) on active therapy	Prevent relapse (RR of relapse)
CD	SASP (only 2 RCTs) 0.83 (95%CI: 0.69-1.00; trend)	SASP/5-ASA, ITT analysis, no benefit
	5-ASA 0.91 (95%CI: 0.77-1.06; not significant)	5-ASA, PP analysis: RR = 0.79 (95%CI: 0.66-0.95; NNT = 13 to prevent relapse)
UC	0.79 (95%CI: 0.73-0.85; NNT = 6)	0.65 (95%CI: 0.55-0.76; NNT = 4 to prevent relapse)

CD: Crohn's disease; ITT: Intention-to-treat; PP: Per protocol; SASP: Sulfasalazine; 5-ASA: 5-Aminosalicylic acid; CI: Confidence interval; UC: Ulcerative colitis; NNT: Needed to treat; RR: Relative risk; RCT: Randomized controlled trial.

sible to compare IFX with ADA or CER. In an open-label study of ADA in which there was a "placebo" (3 doses of ADA for induction), the therapeutic gain in the 12 wk complete mucosal healing (CMH) was 14% in the intention-to-treat analysis (ITT) and 15% in the per-protocol analysis, with 52 wk ITT CMH of 24% (Table 30).

When and how to start TNFB therapy: Start early, vs follow the current guidelines

This question may also be considered as "step-up" *vs* "start high". When considering a comparison in outcomes from a management approach starting early with biological therapy in the patients' lifelong CD course, *vs* the standard of care of step-up therapy, we must reflect the strength of the evidence supporting each of the components of the traditional approach in CD of using 5-ASA products, antibodies, GCS, and the immune suppressants AZA and MTX. The excellent systematic reviews and meta-analyses in the "Red Journal"[61] are very helpful in this regard, and will be reviewed briefly here to demonstrate the potential limitations of the currently accepted standard of care.

5-aminosalicylates therapy: In contrast to the supportive data in UC for the use of 5-ASA to induce remission and to prevent relapse, the data for CD is poor (Table 31)[61]. Thus, for the usually accepted ITT analyses, neither SASP nor 5-ASA can any longer be recommended in persons with CD to induce or maintain remission.

Antibiotic therapy: There is diversity in the design of antibiotic studies in IBD, including which antibiotic, as well as dose and duration of therapy. The antibiotics often used in clinical practice for this indication were metronidazole, cipro', and rifaximin (not yet available in Canada). While taking into account these events, it may surprise some readers that antibiotics are superior to placebo for the induction and maintenance of remission in CD, as well as induction of remission UC (Table 32)[146].

Glucocorticosteroid therapy: Standard GCS remains

Table 32 Efficacy of various antibiotics to induce remission or to prevent relapse in Crohn's disease

		Induce remission (RR of not remission) on active therapy	Prevent relapse (RR of relapse)
CD	Inflammatory disease	0.85 (95%CI: 0.73-0.99, P = 0.03)	0.62 (95%CI: 0.46-0.84)
	Perianal fistula	0.80 (95%CI: 0.66-0.98)	
UC		0.64 (95%CI: 0.43-0.96)	

UC: Ulcerative colitis; CD: Crohn's disease; CI: Confidence interval; RR: Risk ratio.

the standard for inducing remission in moderate-to-severe UC, but must never be used for maintenance of disease because of its lack of efficacy and its high adverse effects risk profile. While the trials of GCS in active CD individually showed efficacy because of heterogeneity between studies (I^2 = 88%, P = 0.004) and the small size of the studies. When the risk difference was used as the summary statistic, the treatment effect became significant, with NNT = 3 (95%CI: 2-11)[61]. Furthermore, systematic review and meta-analysis showed that standard GCS were superior to the locally acting budesonide in achieving remission in active CD (RR = 0.82; 95%CI: 0.68-0.98); oral budesonide itself was superior to placebo for CD remission (RR = 0.73; 95%CI: 0.63-0.84). Oral budesonide used for up to one year delays the time to recurrence of active CD.

Adverse effects of GCS therapy in the setting of treating active CD could not be determined in the studies of Ghosh *et al*[34] and Summers *et al* Standard GCS-related adverse effects were more frequent than with budesonide (RR = 1.64; 95%CI: 1.34-2.00). Thus, GCS and budesonide will continue to be used for short term treatment of active CD, and the choice between standard GCS *vs* budesonide must be made on the trade-off of efficacy, adverse effects and cost.

Immunosuppressive therapy: The meta-analysis of the use of Immunosuppressants in CD was disappointing; example (Table 33), the relative risk of not being in remission for active CD was not different from placebo. However, if OR was used rather than RRs as the summary statistic, then the result was significant, using a random effects model (OR = 0.42; 95%CI: 0.2-0.89). While some clinicians would accept significant ORs of AZA/6-MP/MTX *vs* placebo as reason to use immunosuppression to induce remission of active disease, the onset of biological benefit takes 2-3 mo. While many gastroenterologists use AZA/6-MP/MTX to prevent relapse in CD, this is likely based on the evidence of individual trials or previous meta-analysis. Surprising to some, while immunosuppressant therapy in UC was not significantly superior to placebo to induce remission, azathioprine was efficacious to prevent relapse (Table 33).

In summary then of the standard step-up approach to the care of patients with CD, in the treatment of active disease to achieve remission, the step using 5-ASA/

Table 33 Meta-analysis of efficacy of immunosuppressive therapy to induce remission or to prevent relapse in Crohn's disease and ulcerative colitis

		Induce remission (RR of not being in remission on active therapy)	Prevent relapse (RR of relapse)
CD	AZA/6-MP *vs* placebo/no therapy	0.87 (95%CI: 0.71-1.06)	0.64 (95%CI: 0.34-1.23)
		If OR was used rather than RRs as the summary statistic, then "the result was significant, using a random effects model (OR = 0.42; 95%CI: 0.2-0.89)	There was no statistically significant benefit for continuing AZA to prevent CD relapse (RR = 0.39; 95%CI: 0.21-0.74)
	MTX *vs* placebo	0.82 (95%CI: 0.65-1.03), no statistically significant benefit	IM MTX: 0.57 (95%CI: 0.35-0.94); NNT = 4 (95%CI: 2-25) to prevent one relapse
		If or used, then the result was statistically significant: OR = 0.47; 95%CI: 0.23-0.99	PO MTX to facilitate steroid withdrawal: 0.82 (95%CI: 0.58-1.17)
	Cyclosporine *vs* placebo (only one trial)	0.84 (95%CI: 0.62-1.07; tacrolimus, one study, no improvement: RR = 0.64; 95%CI: 0.44-0.92)	0.96 (95%CI: 0.77-1.20)
UC	AZA *vs* placebo	0.85 (95%CI: 0.71-0.01, *P* = 0.07)	0.60 (95%CI: 0.37-0.95, *P* = 0.03); NNT = 4, 95%CI: 2-10)
	MTX *vs* placebo	0.59 (95%CI: 0.04-7.90)	

UC: Ulcerative colitis; AZA: Azathioprine; MTX: Methotrexate; RR: Risk ratio; OR: Odds ratio; NNT: Needed to treat; CI: Confidence interval; IM: Intramuscular; PO: Oral; CD: Crohn's disease; 6-MP: 6-mercaptopurine.

Table 34 Benefits of mucosal healing in Crohn's disease[183]

Positive: IBSEN, Step-up/top-down, SONIC
IBSEN (Froslie): 5-yr longitudinal study of IBD patients not on TNFB and enjoying mucosal healing
CD: ↓ Inflammation, ↓ Future use of steroids
UC: ↓ Risk of future colectomy
Negative: Music

IBSEN: The inflammatory bowel south-eastern Norway (study group of gastroenterologists); SONIC: Study of biologic and immunomodulator naive patients in Crohn's disease; TNFB: Tumor necrosis factor-α blockers; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

Table 35 SONIC (Crohn's disease diagnosed within 4 years) %

Conventional step-up (steroids→AZA→IFX)	Step-down (IFX x3, with AZA)		
2 yr	30	73	(43)
3-4 yr			
No flare, no steroids, on demand IFX	about 65	about 20	(about 45)
No flare, no steroids, no TNFB	about 60	about 15	(about 45)
New or persistent fistulas	about 2	about 22	(about 20)

IFX: Infliximab; AZA: Azathioprine; TNFB: Tumor necrosis factor-α blockers.

SASP should be removed; the antibiotic and GCS/budesonide steps remain, and the AZA/6-MP/MTX step is rather shaky and quite slow.

In the end, we may believe that early use of biologics is too aggressive a step, which needs to be shored up by more data, but we need to acknowledge that the standard step-up approach is not as good as we used to accept.

The presence of two mutant nucleotide-binding oligomerization domain-containing protein 2 (NOD2) alleles may be quite specific for future complicated disease. Although the sensitivity is poor and the area under the sROC curve is poor, the high degree of specificity may be sufficient to recommend testing for double mutations if we can identify therapies that can truly change the outcomes for this high-risk stratum of patients. Testing for double mutations would likely miss many patients with aggressive disease. However, the presence of double mutations, particularly mutations in P.Leu1007fsX1008, does identify a high risk stratum of reagent-grade patients that should be identified and analyzed as a distinct subgroup in clinical trials. If prospective trials can show a change in outcomes in these patients, this would be a strong justification for targeted top-down therapy in this group of high-risk patients to prevent stricturing or fistulising complications.

The other major finding of this meta-analysis is the lack of prognostic value of the presence of one mutant allele for predicting CD behaviour. Although we specifically studied NOD2 mutations, recent genome-wide association studies have identified numerous other genes that contribute to the susceptibility of developing CD. It is likely that the level at which genetics contributes to phenotypic disease expression is complex and multiple risk alleles contribute to complex interactions. Further studies addressing disease behaviour will likely require large-scale, multicenter trials to obtain sufficient clinical data to accurately ascertain the cumulative risk from clinical, genetic, and environmental factors. Once additional predictive factors are identified and validated, we will be better able to study targeted therapies in high-risk patients.

In a prospective extension of a patient cohort study of 133 newly-diagnosed CD patients recruited from 18 European Centres, half of the patients were initially treated with as "top-down", and the other half as step up[147]. In the 46 persons who had endoscopically proven mucosal healing at two years, ileocolonoscopies were continued at year three and four. The results can be interpreted as being either for or against mucosal healing as an important therapeutic outcome: complete mucosal healing at year two was associated with a greater proportion of steroid-free clinical remissions (71% *vs* 27%, respectively; *P* = 0.036). While MH may be associated with

Table 36 Sonic naive (no azathioprine, no tumor necrosis factor-α blockers) %

	Clinical remission, off steroids		
	AZA	IFX	AZA + IFX
26 wk	30	44	57
50 wk	24	35	46
	17	30	44[1]

[1]AZA + IFX *vs* IFX (P = 0.55) or *vs* AZA ($P \leq 0.001$). IFX: Infliximab; AZA: Azathioprine.

later steroid-free remission, it is not clear if this correlates with fewer adverse effects, or better outcomes such as QoL, hospitalizations or surgery. There are studies in which the support for the benefit of MH is positive, or negative (Table 34). The case remains "unproven" about the long-term importance of mucosal healing rather than symptom relief as the major end point of treatment of CD.

In accordance with recommendations from current guidelines, the TNFBs are usually initiated in the CD patient who has failed the "step up" approach of sequential use of GCS, ASA/MTX. Initial clinical trials of TNFB were designed to take this conventional sequence into account, and to use TNFB as "add-on" therapy. The terms "step-up, step-down" "top-down" and "fast forward" all related to the concept of whether CD patients will do better in the long-term if using TNFBs earlier *vs* relatively later after the diagnosis of CD, in an attempt to possibly alter the course of the disease and to thereby improve patient outcomes.

Is the response to TNFBs greater when used early in the history of the person's CD? Is there benefit of starting recently diagnosed CD patients on IFX or IFX+AZA immediately, i.e., "top down" *vs* the "step-up" of GCS→AZA/MTX→TNFB? In the open label trial of D'Haens *et al*[110], newly diagnosed CD patients were treated either with AZA + IFX, or conventional therapy with GCS, followed by AZA, and then followed by IFX (GCS→AZA→IFX). With the early use of AZA + IFX with GCS (top-down), at week 26 and 52 the proportion who were steroid-free and who had not undergone IBD-related surgery was 60% and 62%, respectively. In the step up group, the corresponding figures were lower at 36% (P = 0.006) and 42%, respectively. Although the top down group achieved remission faster than did the GCS-AZA-IFX group (P = 0.018), at 53 wk a similar percent of patients in each group were in remission (Table 35).

What then is the influence of confounders such as CD duration, or being TNFB- or AZA/MTX naives? In a post-hoc analysis of the use of ADA in the Charm study[57], after adjustment for potential confounders, disease duration had an effect on clinical remission (RR: 0.96, 95%CI: 0.94, P = 0.002). In the Sonic study (REMICADE), patients with CD who had not previously received treatment with anti-TNFBs or immunomodulators (anti-TNF naive as well as anti-AZA/MTX naive) were randomized to IFX, AZA, or IFX + AZA. At 26 wk, the steroid-free remission rate was higher with IFX monotherapy (P = 0.022) or AZA monotherapy (P = 0.009) groups (Table 36).

When IFX was used within one year of the diagnosis of CD, 78% of the early treatment group responded and 76% were in remission, as compared to 47% and 37% (P < 0.001 and P = 0.002, respectively)[51]. This suggests that earlier use of TNFB may be better when looked from the perspective of this relatively short term follow-up. But of course, those persons started earlier on TNFBs might possibly have more total adverse events (AEs), because of their potentially longer duration of total exposure to drug.

Clearly, current guidelines must be respected. More data is necessary, but it looks promising that the early use of TNFBs (or AZA) may provide outcome advantages. If this possibility becomes evidence based, then guidelines and clinical practice will need to change appropriately.

ADVERSE EFFECTS

Anti-IFX antibodies

In some CD patients who develop secondary nonresponse to TNFB, this may be due to the development of neutralizing antibodies, what proportion of patients on IFX or ADA develop antibodies to IFX (ATIs), or antibodies to ADA [antibodies to adalimumab (ATAs)]? In patients given scheduled IFX 5 mg/kg, 10%-16% develop ATI, whereas ATA occur in 0.04%-2.60%. Infusion reactions with IFX are more common if there are ATI (36%-50% with, and 21%-24% without ATI). Concurrent immunosuppressant (CI) therapy reduces ATI (63%-75% with concurrent AZA/MTX, *vs* 24%-43% with no concurrent AZA/MTX). This beneficial effect of concurrent immunosuppressant on the rate of development of ATI or ATA is most pronounced with episodic rather than with the scheduled use of IFN. This is because the development of ATIs is greater with episodic than with scheduled infusion of 5 mg/kg or 10 mg/kg IFX: 30%, 10%, and 7%, respectively (P < 0.001)[113]; 39% episodic *vs* 16% scheduled (P = 0.036)[128].

The presence of ATI/ATA is of clinical importance. In fact, there is a negative correlation between the concentration of ATIs and the duration of response to IFX (P < 0.001)[112]. Furthermore, the median ATI concentration was 15-fold higher in those with loss of response to IFX than in those who maintained response (P < 0.0001)[148].

The development of ATAs is uncommon: in Classic Ⅰ, the rate of ATA formation at week 4 was 0.04%[149]. In Classic Ⅱ, 2.6% showed ATA[55]. The presence of ATA is also clinically important: there is a significant relationship between the presence of ATAs and nonresponse to ADA (OR = 12.7, 95%CI: 1.7-92.6, P = 0.05)[83].

Protection against the development of ATIs may be achieved with intravenous hydrocortisone premedication given immediately before each IFX infusion, with lower

Table 37 Adverse effects for infliximab and adalimumab[1]

	IFX	ADA
All AEs	-	-
Serious AEs	-6.6	-4.7[2]
Nausea	+7.9[3]	+2.4
Pharyngitis	+16.1	+3.7[2]
Injection site infection	-	+3.8[2]
Injection site reaction	-	+10.5[2]
Arthralgias	-	+4.3[2]
Urinary tract infections	-	+3.5[2]

[1]The rates are shown as the percentage differences in the rates of AEs in tumor necrosis factor-α blocker minus placebo groups. AEs are shown, with the increased (+) or decreased (-) difference from placebo. Note that the duration of follow-up observation varied between study, and has not been standardized here (e.g., per 100 treatment years); [2]The statistically significant or trends: (1) Note that the rates of all AEs as well as serious AEs were lower than in the placebo groups; and (2) Serious AEs include lymphoma and other cancers; [3]Significant difference. IFX: Infliximab; ADA: Adalimumab; AEs: Adverse effects.

median levels of ATI[150]. However, in this study, even though ATIs were lower, the use of hydrocortisone did not affect the rate of infusion reactions or increase clinical response. As noted previously, the major role of concurrent immunosuppression with maintenance TNFB may be when TNFB is not given in the recommended scheduled manner. Therefore, due to higher rate of ATI with episodic treatment, it is not a recommended route of therapy.

Other adverse effects

When considering the safety of TNFB, it is important to consider whether the TNFB was given with immunosuppressants or steroids, and for how long therapy was undertaken. In one study, steroid use in persons on IFX were the only independent risk factor for infections [or 2.69 (95%CI: 1.18-6.12, P = 0.018)][151]. The overall rate of AEs in IFX-treated patients is about 13%[77,111,151,152], which is not statistically significantly different from the 19% AE rate quoted for non-IFX treated patients [OR = 1.33 (95%CI: 0.56-3.00, P = 0.45)][151].

In a case-controlled family practice, population-based retrospective study of 15 471 IBD patients followed for an average of 6.4 years, AZA did not increase the risk for solid organ cancer development (OR = 1.04, 95%CI: 0.89-1.21), but the risk of lymphoma was enhanced 3.22-fold[153]. If myelosuppression occurs with AZA in naive CD patients, it does so in the first 6 wk of AZA therapy[154].

AE data has been collected from 10 TNFB randomized controlled trials in IBD [(five IFX[45,52-54,69], four ADA[55,56,85,93] and one with etanercept[31]). More AEs (serious AEs, pharyngitis, urinary tract infection, injection site reaction or infection)] were reported with ADA *vs* placebo than IFX *vs* placebo (Table 37). From four ADA placebo-controlled trials[54-56,93], there was no difference between ADA and placebo in the percentage of persons with overall AEs (77% *vs* 80%, P = 0.145). AEs that were more common in the ADA than the placebo group were pharyngitis (10% *vs* 6%, P = 0.047), injection site infection (4.9% *vs* 1.1%, P = 0.005), injection site reaction (22% *vs* 12%, P = 0.003), arthralgia (10.4% *vs* 6.1%, P = 0.014) and urinary tract infection (5.0% *vs* 1.5%, P = 0.017) (Table 37). Curiously, ADA had significantly fewer serious AEs than did placebo (6% *vs* 10%, P < 0.001), and the placebo group from one study had numerically more AEs than another placebo group (11% *vs* 18%, P = 0.065).

The medium-term safety of TNFB has been reviewed: In 21 studies enrolling 5356 CD patients, TNFBs did not increase the risk of serious infection malignancy or death[3]. However, as cautioned by these authors, "definitive conclusions cannot be drawn because of methodological limitations (safety analyses satisfied by concomitant therapy were not performed in the individual studies". Fortunately, not all trials reported death as an outcome. In ACCENT I [54], with IFX 5 mg/kg, two patients unfortunately died from sepsis and one from a myocardial infection.

The most common AEs with CER were upper respiratory tract infection (20%), urinary tract infection (7%) and arthralgia (6%)[155]. From five IFX placebo-controlled trials[52-54,78,156], there was no difference between IFX and placebo in the percentage of persons with overall AEs (93% *vs* 95%, P = 0.355). The absolute serious AE was 7% higher in IFX *vs* the placebo groups (24% *vs* 17%, P = 0.019), and only sinusitis was reported more frequently in UC given IFX *vs* placebo (9% *vs* 5%, P = 0.04). Concomitant immunosuppression with maintenance IFX does not significantly extend the duration of clinical response[111]. Nausea was the only AE which was more frequent in IFX than placebo (15% *vs* 7%, P < 0.031), with a higher trend for pharyngitis (18.9% *vs* 2.8%, P = 0.056). Importantly, the increased role of infections in CD or in CD patients on IFX may be from their concomitant use of GCS[129,151].

Life-threatening side effects occur in 2%-5% of persons on IFX, with twice the rate of sepsis, myelotoxicity or risk of lymphoma as those on thiopurines[157]. However, IFX has been used by approximately one million persons worldwide. As a class, the main complications of TNFBs are infections, lymphoproliferative disorders and malignancy[158]. As well, neurological, dermatological, autoimmune and cardiac complications commonly occur with these agents (Table 37). Deaths have been reported in persons using TNFB. The risk of sepsis, malignancies and myelosuppression is higher if AZA/MTX is given with TNFB. For example[159], the risk of opportunistic infections is increased with TNFB (OR = 4.4), and is increased much more if two or more immunosuppressants and used (OR = 12.9).

It is recommended that the complete blood count (CBC) be performed at 1 wk, 2 wk, 3 wk, 4 wk, 6 wk and 8 wk after starting AZA therapy, and then to continue a monthly CBC thereafter. During the first 8 wk of AZA therapy, the risk of leucopenia (white blood cell, < 1.0 × 10^9/L), neutropenia (platelet-neutrophil complexes, < 1.0 × 10^9/L), and thrombocytopenia (platelet count, < 20 × 10^9/L) were 0.3%, 1.0% and 0.2%, respectively[154]. For

treatment intervals of greater than 26 wk, these hematological risks were 0.2%, 1.1% and 0.1%.

IBD patients, including those on GCS and AZA/MTX, have an increased risk of TB (RR = 2.36, 95%CI: 1.17-4.74). TB reactivation is an accepted risk of TNFBs, with the RR for reactivation 2-20 times that of the general population[160,161]. In high-risk areas, IBD patients who are scheduled to be placed on TNFBs must be screened for histoplasmosis and coccidiomycosis[162].

Some authors suggest that CD itself, in the absence of the use of AZA/MTX/TNFB, does not have an increased risk for the development of lymphoma[163]. Others would argue that CD itself does carry a small increased risk. However, these drugs used to treat CD certainly are associated with as much as a 4-fold increased risk of the development of lymphoma[157]. And yet, in the Treat registry of IBD patients, the incidence of malignancies was 0.53 per 100 patient years in the IFX group and 0.49 in the non-IFX group[164]. A small number of adolescents with CD on IFX and thiopurines have been reported as developing hepatosplenic T-cell lymphoma[165]. This consensus is that the concurrent use of both AZA/MTX plus TNFB should not be used in non-adults.

ECONOMIC EVALUATION

Within the context of an economic evaluation, there are several special considerations: (1) Safety (immunizations complete, no TB, no personal/ or family history or demyelation disorders; (2) Perhaps early after the diagnosis of IBD has been made; (3) Perhaps in those with predictors of likely response to TNFB; (4) About 1/3 of persons treated with IFX, ADA and CZP will have a primary failure; (5) Use same TNFB in higher dose or same dose more frequently or, switch to another TNFB; (6) With a secondary failure, the initial response to another TNFB is lower; and (7) Exclude concurrent infection/abscess as a reason for "failed" effect on luminal inflammation.

Anti-TNF therapy is expensive. While there are reductions in all areas of disease-related resource use following IFX therapy, in the United Kingdom, this was not sufficient to cover the cost of TNFB therapy[166]. Future studies of these pharmacoeconomic issues will also need to include considerations on indirect cost-savings and changes in the quality of life[167]. A cost-utility analysis was determines as a part of the CADTH report, giving cost per QALY. The cost of usual standard of care (SOC) was $17 107, that for ADA was $45 480, and for IFX $54 084. The QALY for SOC was 2.555, and the value for ADA and IFX were only slightly higher at 2.701 and 2.721, respectively. This resulted in ICUR of $193 305 and $222 955 for ADA and IFX, respectively. When comparing IFX with ADA for the ICUR efficiency using a five-year time horizon, the values were $193 305 *vs* $451 165. These cost efficiencies of IFX *vs* ADA were not so much due to variations in the values of their QALY, but rather to higher initial remission and response rates with IFX including with fistulizing CD, lower relapse rates with ADA, and higher remission rates amongst the patients who stopped responding to initial IFX and were switched to ADA[72,108]. It is important to stress that this comment is based on very preliminary data. From an economic perspective, it is preferential to switch an IFX non-responder to ADA, rather to increase the dose from 5 mg/kg to 10 mg/kg. There is insufficient evidence of the use of treating an IFX non-responder to a reduced dose interval. Also, the lower rates of hospitalization and need for IBD-related surgery in the TNFB groups was partly offset by the higher drug costs. The true economic impacts of SOC *vs* TNFBs will depend upon longer term data.

For interest, the cost of usual care for UC patients was $24 268 and the QALY was 2.015. For a strategy of IFX 5 mg/kg every 8 wk for maintenance, and for those who do not respond to IFX or lose the initial benefit, the patient is switched to ADA. This escalated the cost to $82 756 and is associated with a very small increase in QALY from 2.015 to 2.178. For an incremental QALY of 0.163, the ICUR was an astounding $308 088. In contrast, an economic evaluation in the United Kingdom gave a more favourable ICUR for IFX of about $70 000 (using the 2008 currency exchange rate). While there are other economic evaluations of TNFB[168-170], the CADTH study is the first to compare the initiation and maintenance therapy costs of IFX and ADA. The authors of the CADTH review suggested, with regards to the economics of the use of TNFBs, that "...the incremental cost-effectiveness in managing this IBD patient population may not be perceived as favourable for anti-TNFα drugs compared with usual care without these drugs. This finding was supported by deterministic sensitivity analysis and probabilistic sensitivity analysis".

Over the past four years (2006 to 2010), and based on acceptable incidence and prevalence estimates of CD and UC[2], the number of IBD patients has risen by about 5% (1% per year), yet between 2006 to 2008, the annualized increases in public spending on IFX and ADA in IBD have risen 25% and 18%, respectively. There is no evidence that over the past five years there has been a marked increase in the number of IBD sufferers who have suddenly developed severe or non-responsive disease. This 5-fold greater use in TNFBs as compared with the increase in the number of sufferers may reflect the wider dissemination and acceptance of TNFB guidelines, or may be the result of inappropriate over usage.

The economic analysis of the health services impact suggest that "…Adalimumab and IFX for the treatment of IBD may not be perceived to be a cost-effective use of health care resources"[51]. There are no head-to-head trials comparing standard-of-care *vs* anti-TNF therapy. What do TNFBs add to the annual cost of care of every IBD patient in Canada? "Based on trends in expenditures over the past three years, total public expenditures on anti-TNFs for IBD are projected to be $94 832 652 in fiscal year 2010-2011. The estimated annual drug costs for IBD patients who are on maintenance therapy with

Table 38 Some of the important anti-tumor necrosis factor studies still to be reported

Direct head-to-head comparisons of various anti-TNFs
Further evidence that treating with anti-TNFs earlier after the diagnosis of CD is better
Further clarification of the role of using scheduled anti-TNF therapy without concomitant AZA/MTX
Head-to-head trials comparing the effectiveness of IFX, ADA, and CER peg with conventional therapy in refractory CD (other than newly diagnosed and treatment naive patients)
Long-term safety
Evidence for efficacy of switch from ADA → IFX for ADA secondary loss of response
Optimal therapy for anti-TNF therapy primary non-responders
The comparison of standard-of-care *vs* anti-TNF therapy for maintenance therapy in UC
Are physicians correctly distinguishing mild from moderate or severe CD?
What is the profile of the patient who is most likely to respond? Are they genotypic or phenotypic characteristics?
When is the optimal time in the course of the patient's illness to begin anti-TNF therapy?

TNF: Tumor necrosis factor; CD: Crohn's disease; AZA: Azathioprine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab; CER: Certolizumab; UC: Ulcerative colitis.

anti-TNF drugs vary from $23 000 to $38 000[159]. Similar benefits of IFX on rate of hospitalization, surgery and procedures were described in the Accent I and Ⅱ trails[54,164].

This reduction in health serious utilization could be partially offset by the high cost of biologics, so that cost effectiveness and cost-utility analysis are necessary. A UK cost utility analysis reported that maintenance treatment with IFX every 8 wk was cost-effective for patients with luminal and fistula CD[171]. For example, for luminal CD, an incremental cost per QALY gained for IFX was £26 128 (using the exchange rate at the time of publication of this paper, this equates to approximately $70 000 Cdn). However, the use of QALY for the measurement of cost-effectiveness is of questionable value for diseases such as IBD, which have a young patient base and low mortality rate but high morbidity[167]. Also, indirect costs are high in persons with IBD: In Sweden or Scotland, about two thirds of the costs are indirect[172,173], and in the Accent I trial of IFX, about half the patients at baseline were not employed[54]. Because of this the result of studies of cost-effectiveness and cost-utility need to be challenged, since direct costs are often not taken into consideration[174].

To quote from the report of Canadian Agency of Drugs and Technologies in Health[51], "Using cost-utility analysis cost per QALY allows for the incorporation of quality-of-life impact of clinical effects of CD treatment strategies…" and "allow for comparison with evaluation of disease area that use this outcome." The incremental cost-utility ratio for ADA and IFX, compared with usual care, was $193 305 and $451 165, respectively[51]. "….IFX and ADA have proven clinical benefit when compared to placebo, the primary and secondary non-response rates are high, the therapeutic gain is small, and…"[51] the costs associated with these treatments could be perceived as high. Furthermore, based on incremental cost utility findings from our primary economic evaluations. Adalimumab and IFX for the treatment of IBD may not be perceived to be a cost effective use of health care resources"[51] (Table 38).

CONCLUSION

The results of these and the recent meta-analyses may differ because of ".... a less contemporaneous search data, the inclusion of non-FDA-approved therapies in the analysis, and different time points for data extraction, which were not the primary end points of the included trials"[61].

In persons with luminal CD who have failed treatment with first and second-line agents or who are corticosteroid dependent, for induction of remission of active luminal CD, "IFX, natalizumab, and adalimumab appear to have the most evidence for their use, although the latter two therapies performed only moderately in this setting"[61].

"The most marked effect of biological therapies was in preventing relapse of luminal CD once remission had been achieved, with a NNT of only 4. This benefit was observed for both IFX and certolizumab, but not for adalimumab"[61].

"In terms of fistulizing CD, there is less evidence for the use of biological therapies, though there was a clear beneficial effect when only IFX was studied, although in one trial, and when only RCTs with follow-up in excess of 4 wk were considered in the analysis. There was also a benefit in favour of biological therapies over placebo when only studies that reported fistula healing in the longer term were included in the analysis."

"In preventing recrudescence of fistula, there was only one RCT reporting on this end point, which demonstrated that IFX was superior to placebo. Although concerns about adverse events, arising from the long-term use of these drugs is understandable, the data from the RCTs included in this meta-analysis did not demonstrate any increase in overall adverse events, or serious adverse events, with use for up to 1 year."

"Clinical trials for the three anti-TNFα agents approved for CD or UC have used different end point criteria and response time, in addition to the different modes of administration. Interpretations of these differences and their overall clinical relevance remain contro-

versial."

"The efficacy of anti-TNFα is limited. A total of 20%-30% of patients with refractory CD and roughly 40% of patients with refractory UC do not respond to anti-TNFα treatment and are defined as primary failures."

"Long-term therapy with biologics is associated with significant loss of response (up to 40%)."

"TNFBs represent a major advancement in the management of carefully selected patients with IBD, such as those with active fistulizing CD and active CD not responding to adequate doses of steroids and/or immunosuppressants. It is important to give these agents for an adequate duration before declaring failure of treatment. The specific time period of use is unknown, but it includes side effects to any medications or failure to achieve remission of disease."

It must be appreciated that in most TNFB studies, there is a run-in interval, and the initially approximately 30% non-responsive patients may be excluded from the find reporting of response rates; the methodology for the statistical analysis of each study must be carefully considered.

There is insufficient current evidence to support "top-down" TNFB therapy (starting early in the course of the patient's disease with TNFBs rather than the SOC progression to steroids and immunosuppressants.

The use of mucosal healing as an endpoint of successful therapy, rather than clinical response, has not been proven.

The initial response rate of fistulizing CD for induction may be higher with IFX than ADA, but longer term maintenance with ADA may be superior to IFX.

There are wide ranges in the reported rates of minor or severe adverse effects in short-term users of TNFBs. The increased risk of serious infection, lymphoma and cervical dysplasia may be as much related to the concurrent use of immunosuppressants (AZA/MTX) with TNFBs, as the TNFBs themselves. The longer term risk of TNFBs remains to be established.

It remains controversial whether immunosuppressants (AZA/MTX) should be used concurrently with TNFBs when starting TNFBs for induction therapy or continuing TNFBs for maintenance of remission. For now the international guidelines recommended using both immunomodulators and TNFB for induction of remission and mucosal healing. As well, combination therapy is preferred in moderate to severe CD as data shows reduced efficacy with single agent therapy (immunomodulators or TNFB).

It is unknown if TNFBs change the natural history of IBD.

In the UC patient who is experiencing frequent clinical recurrences while on 5-ASA, AZA or MTZ maintenance therapy, or who is hospitalized for an acute episode of their disease and fail to respond to IV steroids, it remains unclear whether IV cyclosporine followed by colectomy, AZA or IFX maintenance represent optimal therapy.

In persons with chronic continually or intermittently active UC or Crohn's colitis, it is unknown if colonoscopic surveillance protocols need to be changed to offset any potential TNFB-associated alteration in the risk of dysplasia.

When standard methods are used for economic evaluation, it is difficult to justify the use of TNFBs, based on available clinical data.

Given that some persons with IBD require TNFBs because of non-response to conventional therapies SOC, future research must solve the dilemma of predicting who will need TNFBs in the future who will be a TNFB primary or secondary non-responder, and who would benefit from early use of TNFBs. Above all, the pathogenesis of IBD needs to be identified in order than modifiable risk factors may be treated, and the diseases eventually may be prevented and cured.

The estimated 2010 expenditure on TNFBs is over $10 million (CDN), and a per IBD patient TNFB cost of $19 412 is higher than the estimated annual cost for standard of care of $17 107 for CD and $24 268 for UC.

"Making recommendations requiring TNFBs about anti-TNFα therapies based on less-than-conclusive scientific evidence has ethical implications for physicians..." (CADTH, 2009, page 60).

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REVIEW

Role for mycobacterial infection in pathogenesis of primary biliary cirrhosis?

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Abstract

Primary biliary cirrhosis (PBC) is a progressive cholestatic liver disease characterized by the immune-mediated destruction of biliary epithelial cells in small intrahepatic bile ducts. The disease is characterized by circulating antimitochondrial antibodies (AMAs) as well as disease-specific antinuclear antibodies, cholestatic liver function tests, and characteristic histological features, including granulomas. A variety of organisms are involved in granuloma formation, of which mycobacteria are the most commonly associated. This has led to the hypothesis that mycobacteria may be involved in the pathogenesis of PBC, along with other infectious agents. Additionally, AMAs are found in a subgroup of patients with mycobacterial infections, such as leprosy and pulmonary tuberculosis. Antibodies against species-specific mycobacterial proteins have been reported in patients with PBC, but it is not clear whether these antibodies are specific for the disease. In addition, data in support of the involvement of the role of molecular mimicry between mycobacterial and human mitochondrial antigens as triggers of cross-reactive immune responses leading to the loss of immunological tolerance, and the induction of pathological features have been published. Thus, antibodies against mycobacterial heat shock protein appear to cross-recognize AMA-specific autoantigens, but it is not clear whether these autoantibodies are mycobacterium-species-specific, and whether they are pathogenic or incidental. The view that mycobacteria are infectious triggers of PBC is intriguing, but the data provided so far are not conclusive.

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Key words: Antimitochondrial antibodies; Autoantibody; Autoimmunity; Cholestasis; Heat shock; Infection; Liver disease; Liver failure; *Mycobacterium*; Tuberculosis

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic, autoimmune liver disease characterized by inflammatory destruction of the small intrahepatic bile ducts, fibrosis progressing to cirrhosis[1-5], and subsequent liver failure[6,7]. PBC primarily affects middle-aged women, with an increased incidence within families[2,8,9]. The prevalence of the disease is estimated to be less than 1/2000[10]. Despite the variance noted amongst countries/ethnic groups, there is a notion that the incidence of the disease is increasing[10-13].

Several autoantibody profiles have been found to be specific for the disease[14], and aid in the diagnostic workup of PBC. These include antimitochondrial antibodies (AMAs)[15-19], and/or disease-specific antinuclear antibodies (ANAs)[20-22], which are found in both symptomatic and asymptomatic patients[23]. Most common symptoms at presentation are nonspecific and include fatigue, pruritus, Sicca symptomatology and arthralgia. In more severe cases, symptoms relate to portal hypertension and hepatic decompensation (jaundice, ascites or variceal bleeding), which may indicate the need for liver transplantation[2,5,24]. The progression of PBC is generally slow and unpredictable[6,24].

The diagnosis of PBC is based on the presence of serum AMA, biochemical markers of cholestasis and histological features on liver biopsy[2]. The diagnosis of PBC is likely when at least two of these criteria are met[2,4]. Biochemical indices of cholestasis include increased levels of alkaline phosphatase and γ glutamyltransferase (GT). The diagnostic hallmark of PBC is the presence of AMA in high titers, with less than 3%-10% of patients with PBC being negative for AMA[23]. Positivity for AMA appears to be predictive of future PBC development in asymptomatic individuals[17,25]. The prevailing notion is that the presence of AMAs and their titers/concentrations do not correlate with the severity of the disease[23]. AMA seropositivity appears unable to identify patients at risk for faster progression of their liver disease compared to the seronegative cases[19,26]. Disease-specific ANAs[27-30] are also found in PBC, to a lesser degree than AMAs (20%-50% *vs* 90%-97%)[31-34], but tend to have elevated titers, and more importantly, appear to characterize patients with a more aggressive form of the disease[35-38]. Levels of IgM are also raised in most cases[2,4]. Histologically, PBC features include destruction of biliary epithelial cells and loss of small bile ducts with portal inflammatory cell infiltration, and granuloma formation (see below)[2,4,5].

The antigenic specificity of AMA[15-19] and ANA[20-22] responses have been extensively studied. PBC-specific ANAs recognize either nuclear body proteins such as the speckled protein 100 and the promyelocytic leukemia protein, or the gp210 and nucleoporin 62 nuclear membrane proteins[28,29,39-41].

AMAs in PBC are directed against the 2-oxo-acid dehydrogenase complex family of enzymes, and in particular the E2 subunits of pyruvate dehydrogenase complex (PDC-E2), branched-chain 2-oxo acid dehydrogenase complex (BCOADC) and 2-oxoglutarate dehydrogenase complex (OGDC)[2,42-44]. PDC-E2 reactivity is found in > 95% of patients with PBC, and 70% recognize BCOADC-E2 and/or OGDC-E2[2]. Reactivity to all three antigens occurs in < 50% of patients[19]. The immunodominant antigenic regions recognized by (CD4 and CD8) T lymphocytes[45-47] on PDC-E2[48-50] comprise a region within the inner lipoyl-binding domain of the subunit, spanning amino acids 212-226 (PDC-E2$_{212-226}$)[51-53]. This region is also the core target of B cell receptors, which are antibodies in their soluble form[54-56].

Medical treatment of PBC includes ursodeoxycholic acid, with the best response seen in patients who initiate treatment early in the disease[2]. These patients often show decreased or even normalized levels of alkaline phosphatase (ALP), γGT and other markers of cholestasis[2,4,6]. Studies reporting findings in large North American and European patient cohorts indicate that the percentage of patients with PBC who require liver transplantation has fallen significantly[2,57].

The cause of PBC remains undetermined[58-61], but it is believed to be the result of a genetic predisposition compounded with several lifetime exposures[62,63], similar to a "multi-hit" model[61,64-67] of disease pathogenesis[50,68,69]. Recent genome-wide association studies[70,71] have identified several HLA[72] and non-HLA[73-76] genes to be associated with PBC. Environmental factors implicated are numerous[77-81], and range from cosmetic products and xenobiotics[82], to estrogen deficiency and infectious organisms[50,58,83-85] including bacteria and viruses[50,54,58,59,63,65,66,68,69]. Mycobacteria have been included in the list of infectious organisms, partially due to the presence of granulomas in the histopathology of PBC, and the association of granulomas with mycobacteria[2,86]. In addition, AMA is found in some patients with mycobacterium-related infections[87,88]. This review will critically analyze the evidence surrounding the role of mycobacteria in the pathogenesis of PBC.

GRANULOMAS IN PBC

Granulomas consist of focal collections of inflammatory cells and cellular debris[89-92]. Their formation occurs when nondegradable products persist, as well as in hypersensitivity reactions or a combination of the two[91]. They form in a complex process involving the interaction of the infectious organism, antigen, macrophages, T cell responses, B cell hyper-reactivity, and circulating media-

Table 1 Prevalence of granulomas in liver disease *n* (%)

Ref.	Origin	Total number liver biopsies	Total number liver biopsies with granulomas	Diagnosis of PBC in granuloma group
Drebber *et al*[100]	Germany	12 161	442 (3.6)	215 (48.6)
Dourakis *et al*[101]	Greece	1768	66 (3.7)	41 (62)
Gaya *et al*[95]	United Kingdom	1662	63 (3.8)	15 (23.8)
McCluggage *et al*[96]	Northern Ireland	4075	163 (4)	90 (55)
Satti *et al*[99]	Saudi Arabia	404	59 (14.6)	Unknown

PBC: Primary biliary cirrhosis.

tors[91]. T cells involved in granuloma formation may be of the T helper (Th)1 or Th2 type[91]. Several organisms may initiate granuloma formation, including *Mycobacterium*, *Yersinia*, *Toxoplasma gondii* and *Bartonella henselae*[93,94]. Zumla *et al*[91] have noted three categories of granulomatous infections: those due to well recognized organisms; those due to organisms that are detected by molecular techniques but not by conventional microbiological techniques; and those due to organisms that have not been identified, but which are suspected. *Mycobacterium tuberculosis* (*M. tuberculosis*) is the most common organism associated with granulomas, and is histologically characterized by epithelioid cells, lymphocytes, histiocytes, Langerhans giant cells and fibroblasts surrounding a core of necrotic debris[91]. The necrotic core is usually caseating, although non-caseating granulomas may also occur[91,92].

The presence of granulomas in liver biopsies has been well documented, with a prevalence ranging from 2% to 15% in some studies, with geographical differences in prevalence rates (Table 1)[95-99]. A German study conducted by Drebber *et al*[100] examined 12 161 liver biopsies for the presence of granulomas, in addition to determining the etiology of the granuloma through histology, clinicopathological data, and polymerase chain reaction (PCR) for the detection of infectious organisms. Granulomas were found in 442 (3.6%) of the liver biopsies and interestingly, 215 were from PBC patients (1.8% of all biopsies, and 48.7% of all biopsies with granulomas)[100]. PCR demonstrated the presence of infectious organisms in 15 samples (3.4%), with *M. tuberculosis* being detected in three of the 15 (20%). It was not indicated whether any of the samples with positive PCR results came from PBC patients[100]. Although only 1.8% of liver biopsies contained granulomas, nearly half of these were obtained from patients with PBC. However, if mycobacterial infection with associated granuloma formation is a feature of PBC, a much higher percentage of granulomas would be expected. The preponderance of granulomas in liver biopsies from PBC patients has been demonstrated in other studies as well, which show geographical differences in prevalence rates. In a Northern Irish study, 55% of liver biopsies with granulomas were from PBC patients[96], compared to 23.8% in a United Kingdom study[95]. A Greek study indicates an underlying diagnosis of PBC or overlap syndrome in 62% of liver biopsies with granulomas, followed by viral hepatitis in 7.5%, and autoimmune hepatitis (AIH) in 6%[101]. Despite these findings, it should be noted that only a small percentage of granulomas can be attributed to mycobacteria, with the liver disease in these patients being due to infection and not to PBC. Figure 1 illustrates PBC-related liver granulomas contrasted with liver granulomas seen in conditions not directly related to PBC such as sarcoidosis and schistosomiasis.

The predominance of PBC as the underling liver disease in most cases of hepatic granulomas and the association of granulomas with mycobacteria, raise the question as to whether mycobacteria play a role in the pathogenesis of PBC[58,86,102]. If mycobacteria play a role in the pathogenesis of PBC (or at least in some cases of PBC), it would be expected that certain PBC-specific features, such as disease-specific AMA positivity, would also be present in patients with mycobacterial infections. Likewise, evidence of mycobacterial infection would be expected in PBC patients. This evidence may comprise more prevalent immune responses against mycobacterial antigens, or detectable mycobacterial DNA in liver tissues of PBC patients, compared to healthy and pathological controls. If mycobacterial infection plays a role in the pathogenesis of PBC, it is likely that it is limited to a triggering event, because PBC has been known to recur following transplantation[103-106] in up to 35% of patients[107]. The recurrence rate ranges from 21% to 37% at 10 years, to 43% at 15 years[108-111]. Immunosuppression with tacrolimus, as well as warm ischemic time, is associated with an increased risk of recurrence[108-112]. The more aggressive course of recurrent disease suggests that the pathological process involved in the development of PBC persists after transplantation[113]. This does not, however, suggest that the triggers of PBC also persist, and may indicate their transient involvement in the pathogenesis of PBC[113]. Neuberger highlights that hepatitis A virus can trigger autoimmune hepatitis after viral clearance, and also indicates there are several similarities between the recurrence of hepatitis C virus and PBC after transplantation, suggesting a possible infectious trigger of PBC in some individuals[113]. Although these theories are plausible, no conclusive evidence exists to demonstrate an infectious agent (including mycobacteria) as the trigger of PBC.

PBC-specific features in patients with mycobacterial infection

In an analysis of sera from 28 patients with active pul-

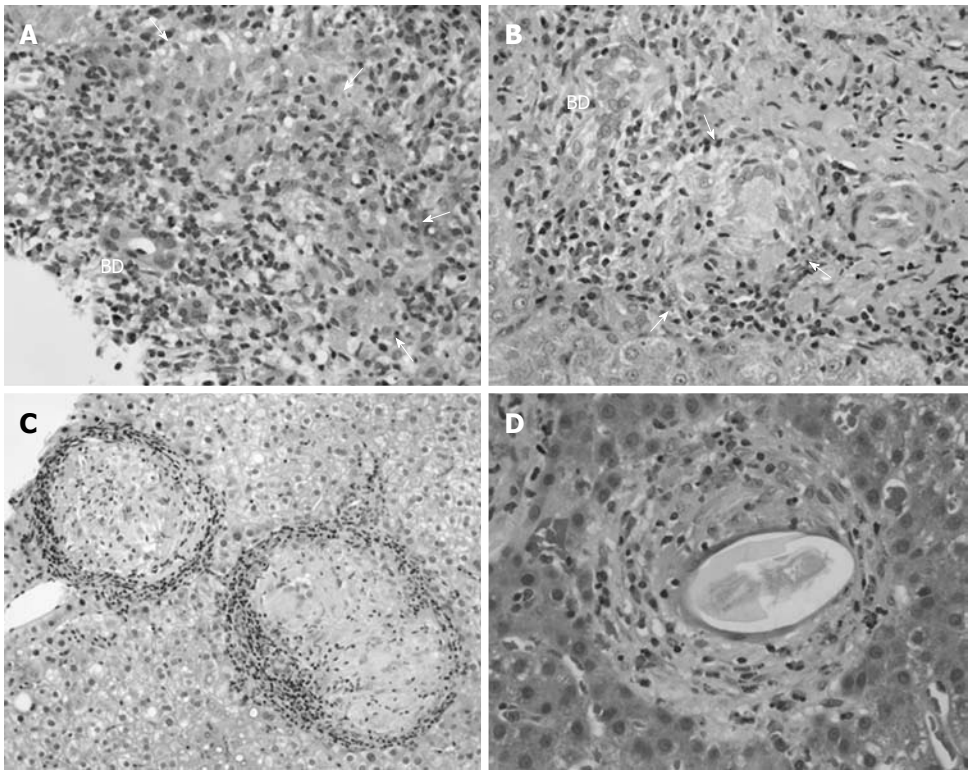

Figure 1 Epithelioid granuloma in liver biopsy. A, B: Liver specimens from patients with primary biliary cirrhosis show granuloma (arrows) close to bile ducts (BD) in portal tracts; C: Granuloma in sarcoidosis is more discrete and large in size; D: Granuloma associated with schistosomiasis contains a large number of eosinophils.

monary tuberculosis (*M. tuberculosis* infection), Klein and colleagues[88] found that 43% of these sera recognized PDC-E2 by immunoblotting based on purified mitochondrial fraction derived from beef heart mitochondria as an antigenic source. Only 2% of sera from individuals with other viral and bacterial infections [including 25 individuals with *Escherichia coli* (*E. coli*)] reacted with PDC-E2, and there was no reaction with sera from healthy controls[88]. The titers of anti-PDC-E2 antibodies were low, with no seropositive cases showing immunofixation of the 70-kDa band corresponding to PDC-E2, at a dilution of < 1/500[88]. Of relevance, none of the anti-PDC-E2 antibody-positive sera (1/10 dilution) gave an immunofluorescent pattern typical of PBC when tested by indirect immunofluorescence based on rat kidney and stomach tissue as substrates[14,16,17,88]. Among the 12 AMA-positive cases with active tuberculosis, six had abnormal levels of γGT and four had slightly elevated levels of alkaline phosphatase; only two of the cases had increased IgM levels[88]. Two of the 12 cases had alcoholic disease and of the remaining 10, none had clinical evidence of chronic liver disease[88].

A variety of autoantibodies have also been reported in the sera of patients with leprosy [caused by *Mycobacterium leprae* (*M. leprae*)], including antibodies against mitochondrial antigens[87,114,115]. In one study, the most common autoantibodies found in leprosy patients were antibodies to SS-B and mitochondria, including cardiolipin[115]. Despite the presence of AMA in the sera of leprosy patients, very few develop liver disease. Gilburd and colleagues[87] screened 69 leprosy patients with no clinical or biochemical evidence of liver disease, for the presence of anti-PDC antibodies. Positive controls consisted of three PBC patients and 18 healthy individuals served as negative controls[87]. Twenty-seven (39%) patients were found to have elevated anti-PDC antibodies by enzyme linked immunosorbent assay, but the absorbance values were relatively low; none of the normal controls had detectable AMA reactivity[87]. By immunoblotting, AMA reactivity was directed against the 54-, 41- and 35-kDa PDC subunits, and only two leprosy patients reacted with PDC-E2[87]. Inhibition studies showed that in contrast to PBC sera, none of the AMA-positive sera gave significant PDC enzyme inhibition; the rate of inhibition being similar to that noted in normal controls. Immunofluorescence testing was not performed[87].

Although AMAs are found in patients infected with mycobacteria, the reactivity patterns observed differ, with *M. tuberculosis*-infected patients showing reactivity to PDC-E2, which has not been observed in those infected with *M. leprae*[87,88]. The source of the AMAs in either case remains unknown. Gilburd *et al*[87] have suggested that the AMAs seen in leprosy patients may arise from the presence of bacterial antigens (such as *M. leprae*), which share sequence homology with the 35-, 41- and 54-kDa subunits. It is not clear whether all bands correspond to PDC subunits (such as PDC-E1 α and β) but may indeed be contaminants of other 2-oxo-acid dehydroge-

nase complexes (formerly known as M2 antigen) such as BCOADC-E2 and OGDC-E2, but reactivity to these antigens has not been tested[87].

Evidence of mycobacterial infection in PBC

In the search for evidence of antimycobacterial antibody reactivity in patients with PBC, Vilagut *et al*[86] investigated a cohort of 19 PBC patients from Spain, using membrane extracts from 10 atypical mycobacteria and found that all cases specifically reacted with *Mycobacterium gordonae* (*M. gordonae*) antigens of approximately 65 kDa and 55 kDa. It remained elusive why the other nine atypical mycobacteria were not targets of antibody responses. The unreactive mycobacterial membranes were those prepared from *Mycobacterium chelonei*, *Mycobacterium flavescens*, *Mycobacterium fortuitum* (*M. fortuitum*), *Mycobacterium intracellulare*, *Mycobacterium kansasii* (*M. kansasii*), *Mycobacterium malmoense* (*M. malmoense*), *Mycobacterium scrofulaceum*, *Mycobacterium xenopi* (*M. xenopi*), and *Mycobacterium terrae*[86]. The extraction of the 10 atypical mycobacterial membranes was performed using the same protocol, therefore, Vilagut *et al*[86] considered that their findings were not a consequence of methodological problems and suggested a biological significance for the role of *M. gordonae* in the pathogenesis of PBC.

This intriguing finding has initiated a series of subsequent studies from the same investigators as well as from independent groups[116-120]. Following the original Spanish report, a study was conducted at King's College Hospital in London investigating reactivity to *M. gordonae* and seven other atypical mycobacteria[119]. O'Donohue *et al*[119] found that 23 of 26 (88%) PBC sera reacted with a 65-kDa protein in extracts of six of the mycobacterial species tested (*M. gordonae*, *M. kansasii*, *M. fortuitum*, *Mycobacterium chelonae*, *Mycobacterium szulgai* and *M. malmoense*). Also, 15 and nine of these samples reacted with the 65-kDa band in membrane extracts of *Mycobacterium avium-intracellulare* and *M. xenopi*, respectively. However, the antibody reactivity to *M. gordonae* or other atypical mycobacteria was not restricted to PBC, but was also present in a similar prevalence in patients with other chronic liver diseases as well as in normal controls[119].

Several studies have attempted to provide evidence of mycobacterial infection in liver tissues from PBC patients. Broomé and colleagues[121] conducted an immunohistochemical study investigating liver biopsy specimens from 10 PBC cases, 13 from primary sclerosing cholangitis, five chronic hepatitis C, four alcoholic liver disease, and six healthy controls. Samples were studied using a monoclonal antibody specific for mycobacterial heat shock protein (hsp) 65. Positive staining was observed in nine of the 10 PBC cases, all primary sclerosing cholangitis, three chronic hepatitis C, and three alcoholic liver disease cases, but in none of the healthy controls[121]. Both interlobular and septal bile duct staining was observed in the PBC cases, with perinuclear staining in all positive interlobular ductal cells, and perinuclear as well as apical staining in the positive septal cells[121].

Direct evidence of microbial products in an affected tissue is best studied at the molecular level using PCR techniques. Vilagut *et al*[116] assessed the presence of *M. gordonae* DNA in liver tissue from PBC patients and controls using PCR based on amplification of a 565-bp fragment of mycobacterial gene coding for 16S rRNA on M. *gordonae*. They detected *M. gordonae* in nine of 11 (82%) PBC liver tissues but in none of the six control livers from patients with other liver diseases[116]. Contradictory results were obtained by O'Donohue *et al*[118] who investigated whether mycobacterial DNA could be detected in archival liver biopsy material from PBC and AIH. Archival material was obtained from 11 PBC and 11 AIH cases, with five lymph nodes from patients with tuberculous lymphadenopathy acting as positive controls[118]. Three of the positive controls also had liver biopsies taken for concurrent tuberculous hepatitis. No mycobacterial DNA was detected in PBC or AIH cases, while four of the five positive controls had detectable mycobacterial DNA[118]. A similar study also utilized a PCR approach for the detection of mycobacterial and other organisms in archived (paraffin embedded) liver tissues from 29 cases of PBC, as well as pathological and healthy controls. Again, no mycobacterial DNA was detected in PBC samples, and only *Helicobacter pylori* DNA was found in one case of PBC[122].

Mycobacteria and PBC: The role of molecular mimicry

The above studies do raise doubt as to whether mycobacteria play a role in PBC, although it is unclear whether a negative detection test only implies that there is no current active infection or can rule out previous infection as well. However, it has been suggested that mycobacteria and other infectious agents may not be actively present in PBC patients, but rather involved in the initiation of autoimmunity by microbial/self immunological cross-reactivity[55,56,123,124]. We and others have studied the role of molecular mimicry[68,69,123,125-128] and immunological cross-reactivity[129-133] as a mechanism responsible for the induction of autoantigen-specific immune responses in viral-hepatitis-triggered autoimmunity and several autoimmune diseases (including those affecting the liver)[134-138] in susceptible individuals[139,140]. Impairments in T-regulatory functions also appear to be a feature[141,142]. Cross-reactivity between PBC-specific mitochondrial antigens and mycobacterial proteins has been investigated by two groups[86,117]. Vilagut *et al*[86] have found that antibody responses to the 65-kDa *M. gordonae* antigen cross-reacted with anti-PDC-E2 antibodies. The same group of investigators went on to demonstrate that the 65-kDa protein was the mycobacterial hsp65, and that preincubation with PBC sera prevented binding of antibodies against hsp65[117]. The apparent cause of this cross-reactivity remained elusive until another group noted a sequence similarity between human PDC-E2 and *M. gordonae* hsp65[120]. These authors have found that the hexameric motif [GDL(IL)AE)] is shared by *M. gordonae* $hsp65_{94-99}$ and the major PBC-specific mitochondrial autoepitopes,

Table 2 Summary of studies in support or against a role of mycobacteria as triggers of primary biliary cirrhosis

Ref.	Year	Mycobacterium	M2 antigen	In support	Against
Klein *et al*[88]	1993	*M. tuberculosis*	Bovine heart	Anti-M2 AMAs (70 kDa) found in 12/28 (43%) tuberculosis patients	None of the patients had PBC AMA by IIFL were negative Anti-PDC-E2 titers were low
Gilburd *et al*[87]	1994	*M. leprae*	Bovine heart	Anti-M2 against 54-, 41- and 35-kDa bands found in 27/69 (39.1%) leprosy patients	None of the patients had PBC Only 2/27 (1%) sera reacted with the PDC-E2 band Anti-M2 inhibited anti-PDC activity in 19.1%
Vilagut *et al*[86]	1994	*M. gordonae*	Porcine heart	Anti-hsp65 *M. gordonae* antibodies found in 19/19 (100%) of PBC patients. No reactivity to other atypical mycobacteria was found Anti-porcine AMAs (anti-PDC-E2 and BCOADC-E2) cross-reacted with anti-hsp65 *M. gordonae* antibodies and *vice versa*	
O' Donohue *et al*[119]	1994	*M. gordonae*, *M. kansasii*, *M. fortuitum*, *M. chelonae*, *M. szulgai and M. malmoense*, *M. avium-intracellulare and M. xenopi*		23 of 26 (88%) PBC sera reacted with a 65-kDa protein in extracts of six of the mycobacterial species tested Sera from 15 PBC patients reacted with *M. avium-intracellulare* Sera from 9 PBC patients reacted with *M. xenopi*	Reactivity to the atypical mycobacterial mimics was found in non-PBC liver disease patients and controls
Bogdanos *et al*[54]	2004	*M. gordonae*	Primate liver	The motif GDL(IL)AE is shared by *M. gordonae* hsp65$_{94-99}$ and PDC-E2$_{216-221}$	Reactivity to *M. gordonae* hsp65$_{90-104}$ /human PDC-E2$_{212-226}$ seen in Spanish (47.5%), but rarely British patients (4%)
Broome *et al*[121]	1993	-	-	Immunohistochemistry demonstrated positive mycobacterial hsp65 staining in PBC liver tissue	Positive staining was also observed in pathological controls
Vilagut *et al*[116]	1996	*M. gordonae*	-	*M. gordonae* genetic material detected in 9 of 11 (82%) PBC liver tissues	
O'Donohue *et al*[118]	1998	*M. gordonae*	-		Mycobacterial DNA not detected in PBC liver tissues, but was detected in positive controls
Tanaka *et al*[122]	1999	Mycobacterial genus specific primers	-		Mycobacterial DNA not detected in PBC liver tissues

M. tuberculosis: *Mycobacterium tuberculosis*; *M. leprae*: *Mycobacterium leprae*; *M. gordonae*: *Mycobacterium gordonae*; *M. kansasii*: *Mycobacterium kansasii*; *M. fortuitum*: *Mycobacterium fortuitum*; *M. chelonae*: *Mycobacterium chelonae*; *M. szulgai*: *Mycobacterium szulgai*; *M. malmoense*: *Mycobacterium malmoense*; *M. avium-intracellulare*: *Mycobacterium avium-intracellulare*; *M. xenopi*: *Mycobacterium xenopi*; IIFL: Indirect immunofluorescence; PDC: Pyruvate dehydrogenase complex; PBC: Primary biliary cirrhosis; AMAs: Antimitochondrial antibodies; hsp: Heat shock protein.

namely, the inner lipoyl PDC-E2$_{216-221}$ and the outer lipoyl domain human PDC-E2$_{102-107}$[120]. No other sequences were found to be in common with *M. gordonae* hsp65 and human PDC-E2[120]. A database search analysis has found that, among bacteria, the motif SxGDL[IL]AE is virtually unique to mycobacterial hsp, and the only human sequence containing that motif was the inner lipoyl domain of PDC-E2[120]. This excellent and almost unique match between sequences of the dominant epitope of human PDC-E2 and of mycobacterial hsp65 has led the authors to investigate whether the corresponding sequences were targets of immunological cross-reactivity specifically present in patients with PBC. That study involved testing sera from 40 Spanish and 50 British PBC patients, with antibody reactivity to *M. gordonae* hsp 65$_{90-104}$/human PDC-E2$_{212-226}$ being detected in 47.5% of Spanish PBC patients and in only 4% of British PBC patients[120]. No reactivity was observed in controls. Inhibition studies confirmed that the reactivity to the mimics was due to cross-reactivity. In addition, the affinity of anti-*M. gordonae* hsp 65$_{90-104}$ antibodies was higher than that against human PDC-E2$_{212-226}$, raising the possibility that antibody reactivity to the microbe long precedes that against the human homolog[120]. Table 2 provides a summary of the above evidence for and against the role of mycobacteria in PBC.

It remains unclear whether the differing reactivity patterns noted among PBC patients from differing geographical locations (highly prevalent in Spanish PBC cases, practically absent in British PBC cases), could be explained by differing prevalence exposure rates of certain atypical mycobacteria in geographical locations. It should also be noted that multinational epidemiological studies on PBC have not indicated an association with mycobacterial infection, in contrast to what has been reported for *E. coli* or other microbes[77,78,80,81].

CONCLUSION

The presence of granulomas in liver biopsies of PBC patients raises the suspicion that bacterial infections are involved in the pathogenesis of PBC. Although infection with several bacterial species is associated with granulomatous disease, mycobacteria are the most common culprits. It is therefore not surprising that mycobacteria have been added to the list of infectious agents implicated in the pathogenesis of PBC. Furthermore, it has been observed that AMAs, which are characteristic of PBC, are also found in many patients with mycobacterial infections but their titers are low, their epitope specificity differs from that seen in PBC, and they do not appear to be detectable by conventional immunofluorescence. Additionally, mycobacteria have not been found in any significant proportion of hepatic granulomas associated with PBC. When found, the liver disease is often attributed to infection and not PBC.

It is unlikely that ongoing mycobacterial infection is a characteristic of PBC, but an early, transient infection with immunological clearance may be capable of inducing cross-reactivity. Genetic studies on PBC are now demonstrating a genetic susceptibility to the disease, and it is likely that a variety of other factors act in an additive fashion towards PBC development. These factors probably not only vary from patient to patient, but also from one geographical location to the next. A correlation between PBC and certain organisms in the context of their geographical prevalence is warranted. In addition, sequence homology between PDC-E2 and mycobacterial epitopes at the T-cell level has not been fully explored, which is of interest given that differing reactivity patterns have been observed between a variety of mycobacteria and the major mitochondrial epitope of PBC. Experimental studies in animal models of PBC involving mycobacteria may provide useful hints as to whether mycobacteria play a role in the induction of PBC. They may also distinguish between mycobacteria being a trigger of the disease, or being epiphenomena secondary to an increased susceptibility to infection in PBC patients.

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ORIGINAL ARTICLE

Indomethacin but not a selective cyclooxygenase-2 inhibitor inhibits esophageal adenocarcinogenesis in rats

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Abstract

AIM: To evaluate the effects of indomethacin [dual cyclooxygenase (COX)-1/COX-2 inhibitor] and 3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl) phenyl)-2-(5H)-furanone (MF-tricyclic) (COX-2 selective inhibitor) in a rat experimental model of Barrett's esophagus and esophageal adenocarcinoma.

METHODS: A total of 112 surviving post-surgery rats were randomly divided into three groups: the control group (n = 48), which did not receive any treatment; the indomethacin group (n = 32), which were given 2 mg/kg per day of the COX-1/COX-2 inhibitor; and the MF-tricyclic group (n = 32), which received 10 mg/kg per day of the selective COX-2 inhibitor. Randomly selected rats were killed either 8 wk or 16 wk after surgery. The timing of the deaths was in accordance with a previous study performed in our group. Only rats that were killed at the times designated by the protocol were included in the study. We then assessed the histology and prostaglandin E2 (PGE2) expression levels in the rat esophagi. An additional group of eight animals that did not undergo esophagojejunostomy were included in order to obtain normal esophageal tissue as a control.

RESULTS: Compared to a control group with no treatment (vehicle-treated rats), indomethacin treatment was associated with decreases in ulcerated esophageal mucosa (16% *vs* 35% and 14% *vs* 17%, 2 mo and 4 mo after surgery, respectively; P = 0.021), length of intestinal metaplasia in continuity with anastomosis (2 ± 1.17 mm *vs* 2.29 ± 0.75 mm and 1.25 ± 0.42 mm *vs* 3.5 ± 1.54 mm, 2 mo and 4 mo after surgery, respectively; P = 0.007), presence of intestinal metaplasia beyond anastomosis (20% *vs* 71.4% and 0% *vs* 60%, 2 mo and 4 mo after surgery, respectively; P = 0.009), severity of dysplasia (0% *vs* 71.4% and 20% *vs* 85.7% high-grade dysplasia, 2 mo and 4 mo after surgery, respectively; P = 0.002), and adenocarcinoma incidence (0% *vs* 57.1% and 0% *vs* 60%, 2 mo and 4 mo after surgery, respectively; P < 0.0001). Treatment with the selective COX-2 inhibitor, MF-tricyclic, did not prevent development of intestinal metaplasia or adenocarcinoma. In parallel, we observed a significant decrease in PGE2 levels in indomethacin-treated rats, but not in those treated with MF-tricyclic, at both 2 mo and 4 mo. Compared to control rats that did not undergo surgery

(68 ± 8 ng/g, P = 0.0022 Kruskal-Wallis test) there was a significant increase in PGE2 levels in the esophageal tissue of the rats that underwent surgery either 2 mo (1332 ± 656 ng/g) or 4 mo (1121 ± 1015 ng/g) after esophagojejunostomy. However, no differences were found when esophageal PGE2 levels were compared 2 mo *vs* 4 mo post-esophagojejunostomy. At both the 2- and 4-mo timepoints, we observed a significant decrease in PGE2 levels in indomethacin-treated rat esophagi compared to those in either the control or MF-tricyclic groups (P = 0.049 and P = 0.017, respectively). No differences in PGE2 levels were found when we compared levels in rats treated with MF-tricyclic to not-treated rats.

CONCLUSION: In this rat model of gastrointestinal reflux, indomethacin was associated with a decrease in the severity of esophagitis and reduced development of esophageal intestinal metaplasia and adenocarcinoma.

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Key words: Intestinal metaplasia; Esophageal adenocarcinoma; Indomethacin; MF-tricyclic

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INTRODUCTION

The incidence of esophageal adenocarcinoma (EA) in the United States and Western Europe has risen dramatically in the last few decades[1-3]. The reasons for this substantial increase are not entirely understood, but it has been linked to an increase in the incidence of gastroesophageal reflux (GERD) and Barrett's esophagus (BE), which is a recognized risk factor for the development of EA. BE is a consequence of chronic GERD, which represents the starting point of the sequence: GERD, inflammation, BE, low-grade dysplasia, high-grade dysplasia, carcinoma *in situ*, and invasive adenocarcinoma[4]. The molecular changes in the progression from GERD to BE and then to EA are not entirely understood[5], but in the last few years, cyclooxygenase-2 (COX-2) has emerged as a key player in carcinogenesis arising from BE; therefore, it is a potential therapeutic target in the sequence leading to EA. Evidence supporting the involvement of COX-2 in this sequence includes epidemiological studies that have demonstrated a protective effect of nonsteroidal antiinflammatory drugs (NSAIDs) against EA and *in vivo* animal studies in experimental models of chronic esophagitis and EA, in which administration of COX-2 inhibitors induced a reduction in the incidence of esophageal cancer. In addition, upregulation of COX-2 has been described at both mRNA and protein levels in humans with GERD, BE, or EA[6]. COX-1, the other cyclooxygenase isoenzyme, was traditionally considered to be a constitutively expressed isoform that generates prostaglandins for normal physiological functions such as platelet aggregation, gastric mucosal protection, and renal electrolyte metabolism; it was thought not be involved in carcinogenesis. Recent evidence, however, has demonstrated that COX-1 may be important in the development of different neoplasias, because elevated levels of COX-1 expression have been found in prostate, breast, and cervical cancers[7]. Furthermore, studies in $Min^{+/-}$ mice showed that homologous disruption of either *COX-2* or *COX-1* genes is followed by a reduction in polyp formation of approximately 80%[7]. In the esophagus, the role of COX-1 in the development of EA has not been investigated extensively. In a previous study, expression of both COX-1 and COX-2 were analyzed at the mRNA level in 123 primary human esophageal adenocarcinomas; mRNA levels of both isoenzymes were increased. Interestingly, RNA expression levels of COX-1 and COX-2 correlated significantly with one other, and expression of both isoenzymes correlated with the expression of vascular endothelial growth factors A and C (VEGF-A and VEGF-C)[8]. Another study reported an increase in COX-1 expression in a rat model of esophagitis induced by acid reflux[9]. Therefore, to analyze the effects of COX inhibitors in the development of EA due to gastrointestinal reflux, we assessed the effect of a dual COX-1/COX-2 inhibitor (indomethacin) in a rat model of esophageal adenocarcinoma induced by gastroenteroesophageal reflux, and compared it with the effect of a selective COX-2 inhibitor (3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl) phenyl)-2-(5H)-furanone) (MF-tricyclic).

MATERIALS AND METHODS

Animals

All of the animal studies were carried out in the Biomedicine and Biomaterials group of the University of Zaragoza, which is officially recorded as a research establishment that practices adequate husbandry and use of all research animals in accordance with Good Laboratory Practices guidelines. All procedures were approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes. Throughout the experiment, all rats were housed

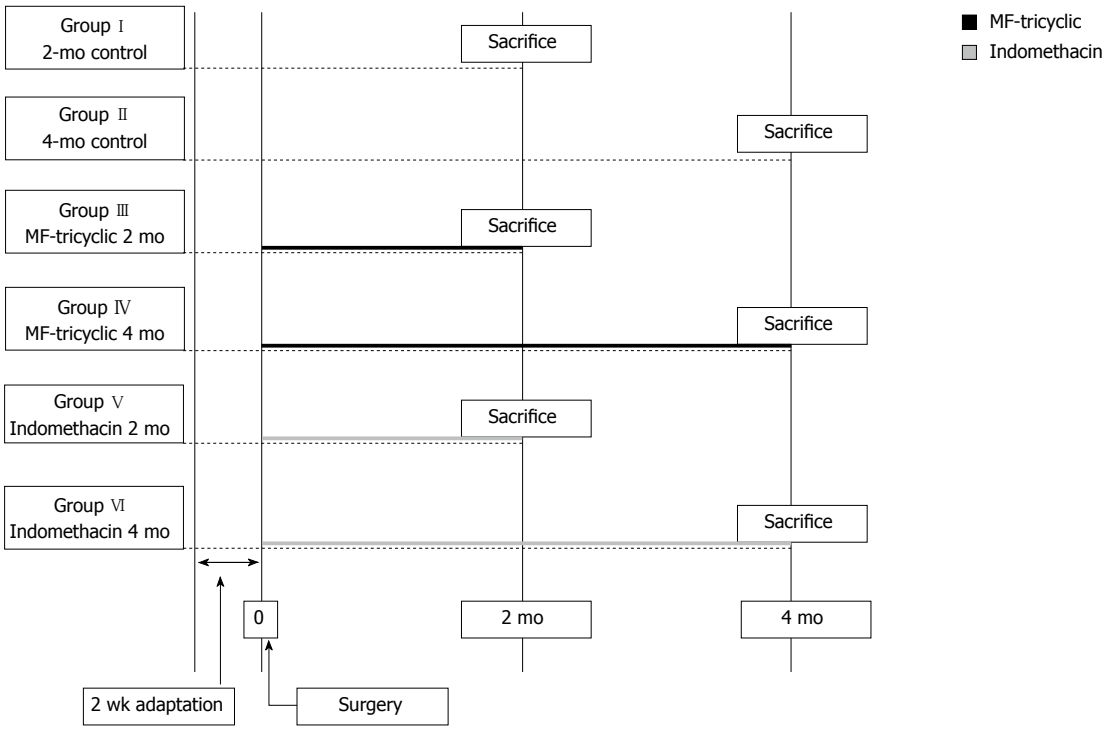

Figure 1 Scheme of the study design.

in specifically designed cages for this type of animal (EU Dim; IFFA Credo) and kept in computer-controlled conditions of light (12-h light/dark cycle), noise (45-50 dB), temperature (21 ± 2 ℃), and humidity (60%-70%). The animals were cared for by veterinary staff. A total of 128 five-week-old female Wistar rats (Harlan, Barcelona, Spain) weighing 220-300 g were used for this study.

Experimental model

Solid food was withdrawn for 24 h and water for 6-8 h before surgery. Anesthesia consisted of 75 mg/kg weight ketamine (50 mg/mL, Ketolar; Parke-Davis, Madrid, Spain) and 0.5 mg/kg weight medetomidine (1 mg/mL, Domtor; Pfizer, Madrid, Spain), which were given as a mixture in a single intramuscular injection. Before surgery, an intramuscular injection of 100 mg/kg weight cefazolin (Kefol; Lilly, Madrid, Spain) was administered as antibiotic prophylaxis. Rats underwent esophagojejunostomy with gastric preservation, allowing the gastroduodenal content to flow back into the esophagus. This surgery was performed according to a previously described protocol[10]. After surgery, the rats were housed in hanging cages to prevent bed ingestion and allowed to drink water *ad libitum*, with fasting until day 3. Water with 0.3 mg/mL buprenorphine (Buprex; Esteve, Barcelona, Spain) was provided during the first 72 postoperative hours. An additional intramuscular injection of cefazolin was administered 24 h after surgery. Rats received 50 mg/kg iron dextran (Difortin; Econatura, Madrid, Spain) intramuscularly once every 4 wk until the end of the experiment. Rats were killed by carbon dioxide inhalation either 2 mo or 4 mo after surgery. A blood sample was obtained for drug analysis from all of the animals immediately before death. Immediately after death, the esophagus was removed and opened longitudinally. Two-thirds of the esophageal specimen was prepared for pathological study. The rest of the esophagus was cut into two slices: the lower and the upper halves of the esophagus. The lower half samples were frozen immediately in liquid nitrogen and then kept at -80 ℃ until biochemical studies were performed.

General design of experiment

Two weeks after surgery, the 112 surviving post-surgery rats were randomly divided into three groups: the control group (n = 48), which did not receive any treatment; the indomethacin group (n = 32), which were given the COX-1/COX-2 inhibitor; and the MF-tricyclic group (n = 32), which received the selective COX-2 inhibitor. Randomly selected rats were killed either 8 or 16 wk after surgery. The timing of the deaths was in accordance with a previous study[10]. Only rats that were killed at the times designated by the protocol were included in the study. A scheme of the study design is shown in Figure 1. An additional group of eight animals that did not undergo esophagojejunostomy were included in order to obtain normal esophageal tissue.

Drug administration and dosage selection

MF-tricyclic was administered at a dose of 10 mg/kg body weight/d. This dose was selected according to a previous study of esophageal adenocarcinoma prevention in the same model[11]. The drug was kindly provided by Merck Frost (Quebec, Canada), which produced a pre-

mixed chow containing the drug. The concentration of MF-tricyclic in the chow was calculated using the average weight of Wistar rats and a daily consumption of 16-20 g of chow/d. This rate of chow consumption was evaluated in previous experiments[10]. Indomethacin (Sigma, Madrid, Spain) was given at a dose of 2 mg/kg body weight/d. This dose was selected according to a previous study of the inhibition of colorectal cancer[12]. The drug was added to drinking water. Indomethacin was replaced on a daily basis and administered in dark bottles in order to avoid light degradation.

Histopathological analysis

Rat esophagi were examined by two independent pathologists who were unaware of the experimental conditions. Changes in the squamous epithelium were classified into the following five categories: (1) Reactive changes: characterized by the presence of basal cell hyperplasia, increased length of papillae, and hyperkeratosis with areas of inflammation and ulceration; (2) Columnar-lined metaplasia (intestinal metaplasia): squamous epithelium was replaced with columnar-lined epithelium comprising occasional and incomplete differentiation of goblet cells; the length of the epithelial transformation was greater than 3 mm starting from the anastomosis site or was intercalated, or it could be seen beyond the surgical anastomosis, and was surrounded by squamous epithelium; (3) Dysplasia: characterized by nuclear atypia, partial loss of mucosecretory function and cell polarity, and an increase in mitotic figures. Dysplasia was classified as low-grade dysplasia (LGD) and high-grade dysplasia (HGD) according to the criteria proposed by Haggitt[13], as previously described in this experimental model[14]. In LGD, the crypt architecture is preserved or minimally distorted; the nuclei may be stratified but without reaching the apical surface of glands, nuclei are enlarged, crowded and hyperchromatic, mitotic figures may be found in the upper portion of the crypt; goblet and columnar cell mucus is usually diminished or absent, but goblet cells in which the mucous droplet does not communicate with the luminal surface may be observed. The abnormalities extend to the mucosal surface. In HGD, distortion of crypt architecture usually is present and may be marked. It is composed of branching and lateral budding of crypts, a villiform configuration of the mucosal surface or intraglandular bridging of epithelium to form a cribriform pattern of "back-to-back" glands. Nuclear abnormalities are present as in LGD, and stratification reaches the crypt luminal surface. There may be a loss of nuclear polarity, and nuclei often vary markedly in size, shape, and staining characteristics. Goblet and columnar cell mucus is usually absent. The abnormalities extend to the mucosal surface; (4) Adenocarcinoma: glandular structures of dysplastic columnar epithelial with stromal invasion and deep infiltration; and (5) Squamous carcinoma: characterized by accumulation of atypical cells with nuclear hyperchromasia, abnormally clumped chromatin, and loss of polarity. The two pathologists showed a high concordance (kappa coefficient > 0.8). However, in cases where there was disagreement, a third pathologist was required to resolve the discrepancy.

Measurement of prostaglandin E2 levels in esophageal mucosa

Prostaglandin E2 (PGE2) analysis was performed in the lower half of the esophagus. Tissue samples were added to 1 mL of cold PBS containing 10 μmol indomethacin. They were then homogenized (DIAX 900, Heidolph, Kelheim, Germany) on ice and centrifuged for 15 min at 1200 rpm (4 ℃). Supernatants were collected in 2 mL tubes. Prostaglandin extraction was performed as previously described by Powell[15]. PGE2 was measured by radioimmunoassay [prostaglandin E2 (I125); Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom].

Plasma drug levels

MF-tricyclic concentration analysis in rat plasma was performed by Merck Frost Canada; the results were disclosed to us after the study was complete. Indomethacin levels were determined as follows: 50 μg (10 μL of a 5 mg/mL methanolic solution) of the internal standard probenecid was added to 1 mL of serum before each analysis. Afterwards, samples were extracted with 2 mL of ethyl acetate and an aliquot of the organic phase was withdrawn, evaporated to dryness under a nitrogen stream at room temperature, and redissolved in 300 μL of methanol. Fifty microliters of these solutions were analyzed by high-performance liquid chromatography (Waters 600 Controller, 996 Photodiode Array Detector). A method capable of quantifying indomethacin was developed using a C-18 column (150 mm × 3.9 mm, 4 μm particle size; Waters, Barcelona, Spain) and probenecid as the internal standard. The column was equilibrated at a flow rate of 1 mL/min with a mobile phase consisting of acetonitrile in water (51:49, v/v) and 0.1% trifluoroacetic acid. The eluate was monitored at 257 nm[16].

Statistical analysis

Data management and statistical analysis were performed using SPSS software. Results from biochemical assays were expressed as mean ± SE. Data were compared between groups by nonparametric tests (Kruskall-Wallis and Mann-Whitney); $P < 0.05$ was considered to be statistically significant. Covariance analysis was used to analyze the quantitative variables of the drug, introducing the number of months as a covariant. Multiple comparisons were corrected by means of the Bonferroni method. Logistic models were adjusted for qualitative variables, evaluating in turn the effect of the drug and the number of months.

RESULTS

Macroscopically, all of the rats that underwent esophagojejunostomy showed dilatation and thickening of the middle and lower esophagus. The distal esophagus

Table 1 Macroscopic results

Group	Ⅰ (*n* = 14)	Ⅱ (*n* = 15)	Ⅲ (*n* = 11)	Ⅳ (*n* = 6)	Ⅴ (*n* = 5)	Ⅵ (*n* = 6)
Inflammation	14	15	11	6	5	6
Ulcerated mucosa,%	35.7	37.3	33.18	39.58	16	14.17
Squamous hyperplasia,%	10	14	2	5	2	2
Intestinal metaplasia in continuity with anastomosis, mm	2.29	3.5	2.32	2.83	2	1.25
Intestinal metaplasia beyond anastomosis,%	10	9	5	4	1	0
Dysplasia	14	14	10	6	5	5
High-grade dysplasia/low-to-medium grade dysplasia	10/4	12/2	4/6	4/2	0/5	1/4
Neoplasia	8	9	4	3	-	-
Mucinous adenocarcinoma	7	9	3	2	-	-
Squamous adenocarcinoma	1	-	1	1	-	-

showed ulceration. Macroscopically, we did not identify any esophageal tumors. The esophagi of all of the rats that were operated on presented inflammatory infiltrate. In most of the animals, the upper and middle parts of the esophagus showed squamous hyperplasia. The esophagi of all of the rats that were operated had columnar-lined metaplasia in continuity with the jejunal epithelium. Dysplasia had developed in all of the rats that underwent surgery. Neoplastic transformation of the epithelium was always found near the anastomosis, surrounded by columnar epithelium. Histologically, 87.5% of tumors were adenocarcinomas, and 12.5% were squamous carcinoma (Table 1). Microscopically, in some animals, a second type of metaplasia was found far above the anastomosis. This metaplasia contained isolated or grouped goblet cells, which formed authentic mucous glands in some cases. However, in any case, these metaplastic foci progressed to adenocarcinoma.

Effect of treatment on the occurrence of lesions

Inflammation, ulceration, intestinal metaplasia, dysplasia, and neoplasias were present in all of the rats that underwent esophagojejunostomy. When the percentage of ulcerated surface was analyzed, there was a significant decrease in the groups treated with indomethacin compared to the control and MF-tricyclic groups. The indomethacin treatment groups showed a significant reduction in the length of metaplasia; we also observed a significant decrease in the percentage of metaplasia beyond the anastomosis (Figures 2, 3A and B).

Most of the rats developed some degree of dysplasia. When we analyzed the occurrence of low-grade *vs* high-grade dysplasia, we found that the percentage of rats that developed high-grade dysplasia was significantly lower in rats treated with indomethacin compared to the MF-tricyclic and control groups (Figures 3C, D). The absence of neoplasias in the indomethacin-treated group (0% *vs* 36.4% in the MF-tricyclic group and 57.1% in the control group 2 mo after surgery, and 0% *vs* 50% in the MF-tricyclic group and 60% in the control group 4 mo after surgery), did not allow us to estimate the risk effect as odds ratio. Nevertheless, when we applied a log-linear model, we concluded that time was a nonsignificant factor in the presence of neoplasia. When we analyzed the effect of treatment, we observed a lower incidence of neoplasia in the indomethacin group ($P \leqslant 0.001$) (Figure 4).

Prostaglandin E2 levels

There was a significant increase in PGE2 levels in the esophageal tissue of the rats that underwent surgery either 2 mo (1332 ± 656 ng/g) or 4 mo (1121 ± 1 015 ng/g) after esophagojejunostomy compared to control rats that did not undergo surgery (68 ± 8 ng/g; $P = 0.0022$ Kruskal-Wallis test). However, no differences were found when esophageal PGE2 levels were compared 2 mo *vs* 4 mo post-esophagojejunostomy.

At both the 2- and 4-mo timepoints, we observed a significant decrease in PGE2 levels in indomethacin-treated rat esophagi compared to those in either the control or MF-tricyclic groups ($P = 0.049$ and $P = 0.017$, respectively). Rats treated with MF-tricyclic did not show differences in PGE2 levels compared to non-treated animals (Table 2).

Serum drugs levels

All of the rats treated with either MF-tricyclic or indomethacin had detectable levels of the drugs in their plasma. The serum drug concentration in rats receiving 10 mg/kg body weight MF-tricyclic was 1.62 ± 0.19 μg/mL. The rats receiving 2 mg/kg indomethacin had plasma levels of 1.97 ± 0.43 μg/mL. As expected, the rats in the untreated control group did not have detectable MF-tricyclic or indomethacin levels.

DISCUSSION

In the present study, we have used an enteroesophageal reflux model in rats to evaluate the impact of dual COX-1/COX-2 *vs* selective COX-2 inhibition in the neoplastic transformation of esophageal epithelium. Although in this experimental model the pH of the refluxate increases with increasing distance from the stomach to the anastomosis, non-acidic refluxate has been shown to enhance the occurrence of intestinal metaplasia with dysplasia and esophageal adenocarcinoma[17]. In addition, these models are widely used because they reproduce the sequence of histological changes that take place in human esophageal adenocarcinogenesis and show morphological and phenotypic characteristics similar to those observed in human Barrett's esophagus and associated

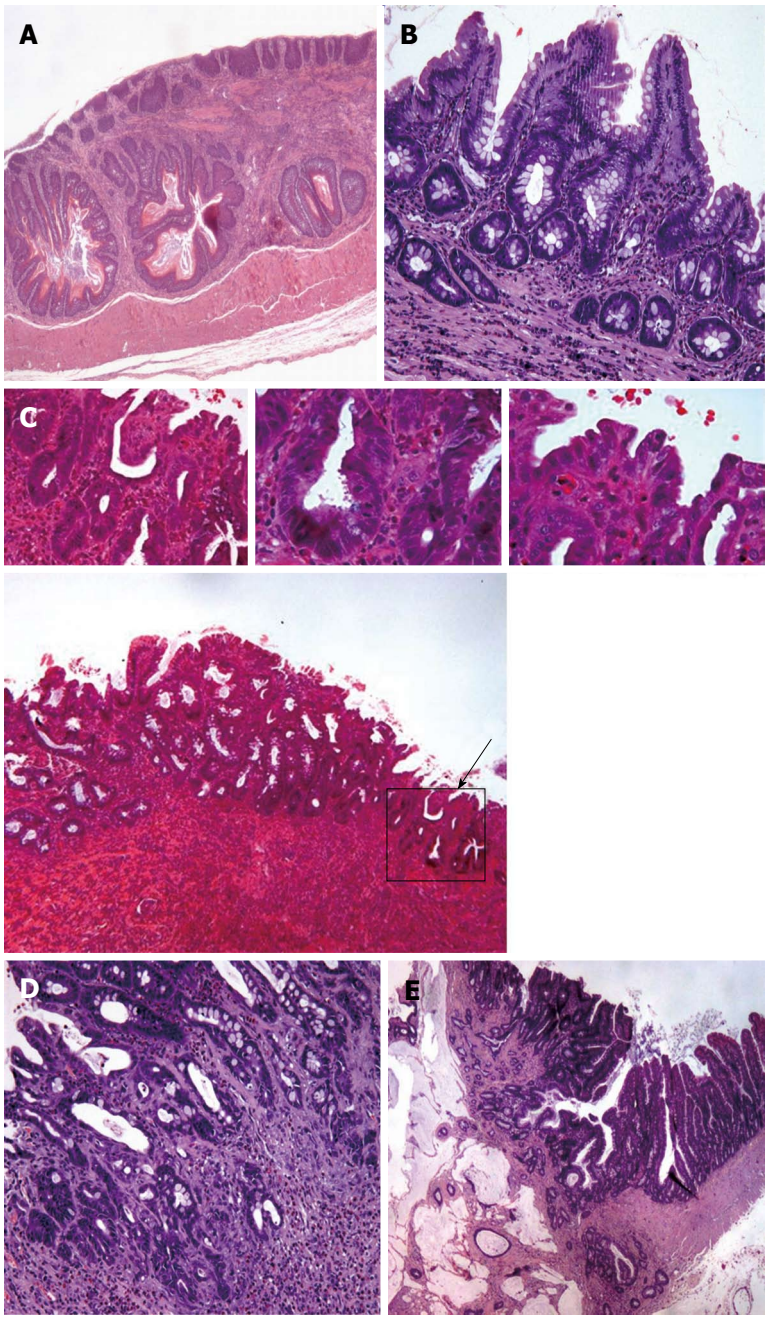

Figure 2 Pathological findings in the esophagus of rats with esophagojejunostomy. A: Hyperplasia of squamous epithelium in a control rat esophagus; B: Hematoxylin and eosin (HE) staining (× 100) showing an area of intestinal metaplasia without dysplasia. The top picture shows in more detail a gland (× 400); C: Indomethacin-treated rat. HE staining (× 40) of an area of intestinal metaplasia showing a focus with low-grade dysplasia (black arrow). The top pictures show in more detail the area with low grade dysplasia (× 200; × 400). Glands conserve their architecture. Nuclear pseudostratification can be seen, nuclear size is also increased but nuclei do not reach the lumen; D: HE staining of an area of intestinal metaplasia showing high grade dysplasia. Magnification × 100; the top picture illustrates at higher magnification (× 400), some glands with high grade dysplasia, characterized by loss of nuclear polarity and atypia; E: Photomicrograph shows an adenocarcinoma developed inside the intestinal metaplasia of the esophagus near the jejunum epithelium in a rat that had undergone an esophagojejunostomy. HE staining shows that the esophageal adenocarcinoma consists of dilated, cystic glands with abundant mucin secretion and epithelial dysplasia. (Magnification × 40; the top picture shows the same fields at × 100 magnification).

adenocarcinoma, including mucin features and expression of differentiation markers and markers of neoplastic progression[14,18].

Our results showed that treatment with the dual COX-1/COX-2 inhibitor indomethacin induced a significant reduction in the area of ulcerated mucosa, the length of metaplasia in continuity with anastomosis, the incidence of metaplasia beyond the anastomosis, the severity of dysplasia, and the incidence of adenocarcinoma in this rat model of chronic gastrointestinal reflux. In contrast, treatment with the selective COX-2 inhibitor, MF-tricyclic, did not prevent the development of these lesions in this model. Our results are consistent with those reported by Heath *et al*[19] in a human clinical trial, in which treatment with 200 mg/d of celecoxib, another selective inhibitor of COX-2, did not prevent the progression of Barrett's dysplasia to cancer. In parallel with our histopathological results, we found a significant decrease in PGE2 levels in indomethacin-treated rats, but not in the MF-tricyclic group, which suggests that PGE2 inhibition is also linked to the effect observed with these two agents. Increased levels of PGE2 during chronic inflammation were previously correlated with carcinogenesis. In an esophagitis rabbit model, Lanas *et al*[20] observed high PGE2 levels associated with increased activity of both COX-1 and COX-2. The levels decreased when rabbits were treated with the nonselective COX inhibitor, indomethacin.

In an experimental model, Buttar *et al*[11] showed that treatment with sulindac had a preventive effect against EA. In contrast with our findings, they observed a similar, although numerically lower, beneficial effect with MF-

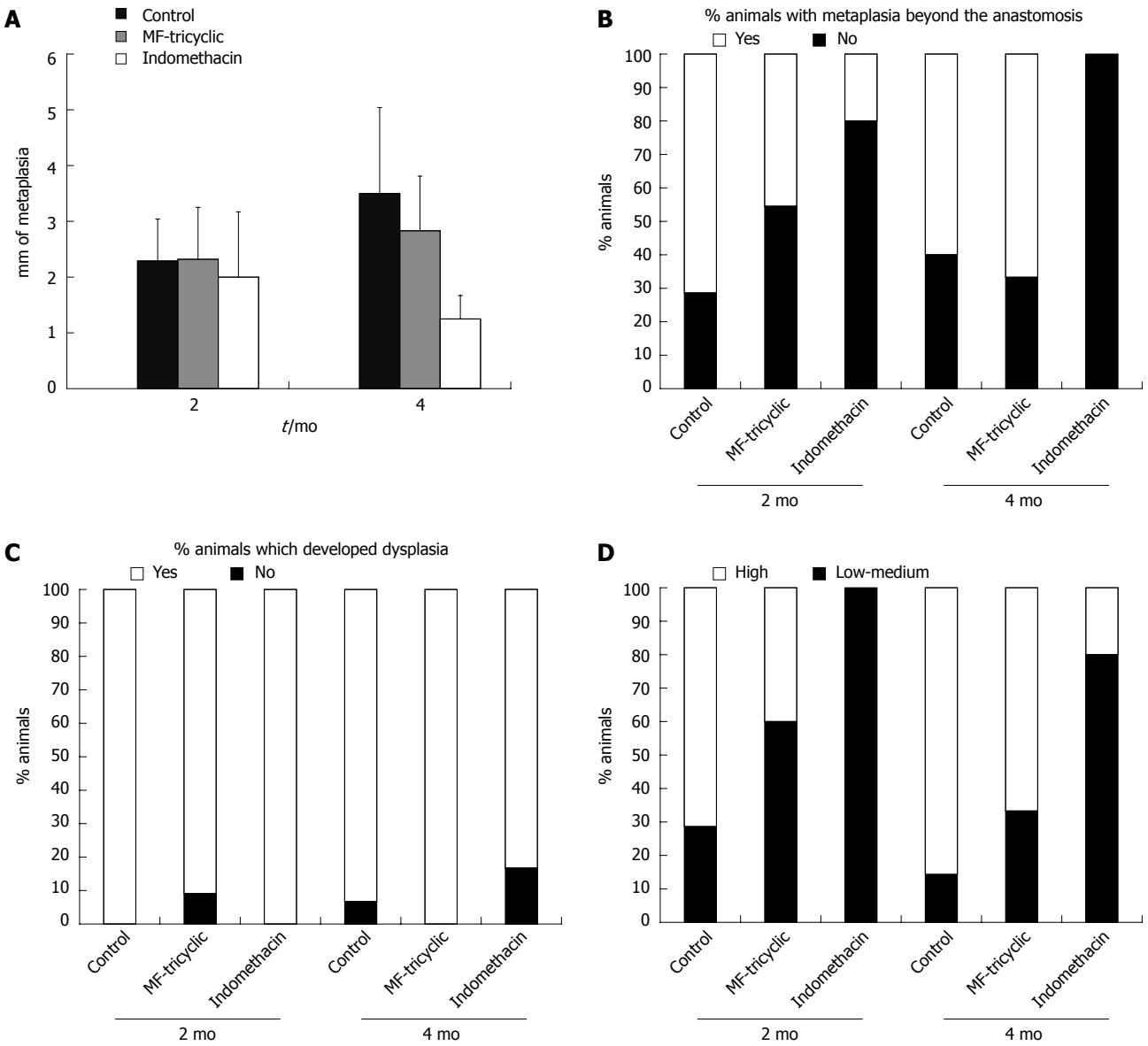

Figure 3 Macroscopic findings. A: Effect of treatment on metaplasia in continuity length. A significant decrease was observed in indomethacin-treated groups compared to control groups (P = 0.007); B: Treatment effect on the percentage of animals that developed metaplasia beyond the anastomosis. Percentage of animals that developed metaplasia beyond the anastomosis was significantly higher in both control and MF-tricyclic groups (P = 0.009 and P = 0.036, respectively); C: Percentage of animals that developed dysplasia; D: Because most animals developed dysplasia, low-to-medium grade *vs* high-grade dysplasia was analyzed. Percentage of rats that developed high-grade dysplasia was significantly lower in indomethacin groups compared to control groups (P = 0.002). When we analyzed the effect of treatment, we observed lower incidence of neoplasia in the indomethacin group (P = 0.000). MF-tricyclic: 3-(3, 4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(5H)-furanone.

tricyclic, although they also did not see significant mucosal PGE2 inhibition with this agent. In our study, we used the same dose of MF-tricyclic as that reported in Buttar's experiment, but the serum drug levels found in our rats were higher. Therefore, we suggest that the apparent differences between these results could be due to the different experimental periods of treatment (28 and 16 wk in Buttar's and our study, respectively). In a 40-wk experimental study, Oyama *et al*[21] showed that pathological changes associated with accelerated cell proliferation, such as regenerative thickening and basal cell hyperplasia, were significantly suppressed in the treated group (celecoxib 500 ppm, 100 mg/kg/d) throughout the experiment. However, the incidence of columnar lined epithelium and EA were significantly suppressed only at the end of the study (40 wk). Similarly, Chen *et al*[22] showed beneficial effects in animals treated with celecoxib over a 40-wk experimental period. We selected the endpoint of the present study according to previous research performed in our group, in which rats under esophagojejunostomy showed a high incidence of esophageal adenocarcinoma and intestinal metaplasia 4 mo after surgery. It is possible that this period of time is sufficient to see a positive effect with profound inhibition of both types of COX activity as with indomethacin, but is not long enough to see the results of weaker or partial COX inhibition with a selective COX-2 agent.

Most studies, including ours, showed a greater decr-

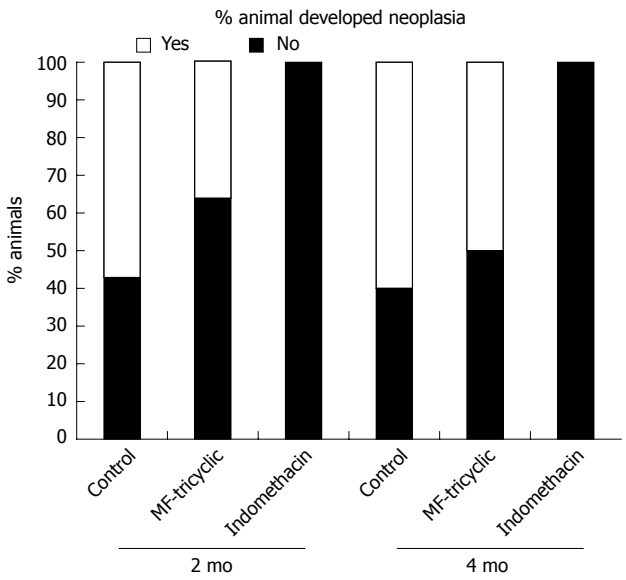

Figure 4 Percentage of animals that developed neoplasia.

ease in tissue PGE2 levels with a nonselective COX inhibitor compared to COX-2 selective inhibitors[11,20,23]. This effect suggests that PGE2-derived COX-1 inhibition may play a role in the progression from gastrointestinal reflux to EA. In addition, additional mechanisms not linked to either COX-1 or COX-2 inhibition may be involved in this process. Orido *et al*[24] observed that treatment with indomethacin, but not sulindac, decreases arachidonic acid uptake in a human colorectal cell line. They suggested that the anticancer effect of indomethacin could be independent of COX-2 inhibition. Moreover, Kim *et al*[25] evaluated the induction of NAG-1 and apoptosis by various NSAIDs, such indomethacin, sulindac, and diclofenac. Indomethacin was the most potent inducer of both NAG-1 expression ($P < 0.05$) and apoptosis ($P < 0.05$). Kopp *et al*[26] and Castro *et al*[27] reported that sodium salicylate, acetylsalicylic acid, and indomethacin inhibit activation of NF-κB[26,27]. Hanif *et al*[28] observed that NSAIDs inhibit the proliferation rate of two colon cancer cell lines independent of their ability to inhibit prostaglandin synthesis.

In addition to the above observations, it is important to note that much higher doses of NSAIDs are required for inhibition of proliferation and angiogenesis and induction of apoptosis, some of the mechanisms that have been suggested to contribute to antitumor activity, than for inhibition of prostaglandin synthesis[29].

In conclusion, treatment with indomethacin, a dual COX-1/COX-2 inhibitor, in our experimental model of EA, prevented the development of intestinal metaplasia and esophageal adenocarcinoma and reduced PGE2 production. In contrast, treatment with MF-tricyclic, a selective COX-2 inhibitor, was not associated with these effects. The profound inhibition of mucosal PGE2 with indomethacin, but not with MF-tricyclic, might partly explain the different effects, but other reasons, such as the length of therapy, activity of COX-1, and involvement of COX-independent mechanisms, could also explain the results, and deserve further investigation.

Table 2 Esophageal prostaglandin E2 levels in rats at 2 mo and 4 mo (mean ± SE, ng/g)

	2 mo	4 mo
Without treatment	1332.7 ± 656.7	1121.6 ± 1015.6
MF-tricyclic	1776.8 ± 1403.3	1914.9 ± 1905.5
Indomethacin	480.3 ± 333.4	243.9 ± 85.7

MF-tricyclic: 3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(5H)-furanone.

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COMMENTS

Background

Over the last decades, the incidence of esophageal adenocarcinoma in the United States and Western Europe has dramatically risen. Epidemiological studies have reported that consumption of cyclooxygenase (COX) inhibitors leads to a decrease in the chance of developing esophageal cancer.

Research frontiers

COX-2 has become a target for the prevention of esophageal adenocarcinoma. There are several studies in which administration of COX-2 inhibitors showed a reduction in the incidence of esophageal adenocarcinoma in rat reflux-induced models. In contrast to previous studies, the research showed no effect when a selective COX-2 inhibitor was administered, while a non-selective COX inhibitor, indomethacin, inhibited the complete carcinogenic sequence in the rat esophagus.

Innovations and breakthroughs

Recent evidence has demonstrated that COX-1 may be important in the development of different neoplasias, because elevated levels of COX-1 expression were found in prostate, breast, and cervical cancers. In the study, treatment with indomethacin, a dual COX-1/COX-2 inhibitor, prevented the development of intestinal metaplasia and esophageal adenocarcinoma and reduced prostaglandin E2 (PGE2) production. In contrast, treatment with 3-(3, 4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(5H)-furanone (MF-tricyclic), a selective COX-2 inhibitor, was not associated with these effects. These results suggest that COX-1 also plays an important role in the development of esophageal adenocarcinoma.

Applications

The inhibitory effect of the complete carcinogenic sequence observed with indomethacin in this study suggests two new aspects that should be further investigated; firstly, the potential role of COX-1 in esophageal adenocarcinogenesis and secondly, the involvement of cyclooxygenase-independent anticancer actions of indomethacin.

Peer review

The authors evaluated the effect of indomethacin, a COX non-selective inhibitor, and MF-tricyclic, a COX-2 selective inhibitor, in an experimental model of Barrett's esophagus and EA in rats. The study showed that indomethacin but not MF-tricyclic prevented the development of intestinal metaplasia and esophageal adenocarcinoma and reduced PGE2 production. The results suggest that COX-1 plays an important role in the development of esophageal adenocarcinoma.

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ORIGINAL ARTICLE

Effect of Tangweian Jianji on upper gastrointestinal remodeling in streptozotocin-induced diabetic rats

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Abstract

AIM: To investigate the effect of Tangweian Jianji (TWAJJ) on the biomechanical and morphometrical remodeling of the upper gastrointestinal tract in diabetic rats.

METHODS: Diabetes was induced in 27 rats by injecting streptozotocin (40 mg/kg body weight), the animals were then divided into three groups (n = 9 in each group), i.e., diabetic control (DM); high dose (10 g/kg, T1) and low dose (5 g/kg, T2). Another 10 rats acted as normal controls (Control). TWAJJ was administered by gavage once daily. Blood glucose and serum insulin levels were measured. Circumferential length, wall thickness and opening angle were measured from esophageal, duodenal, jejunal and ileal ring segments. The residual strain was calculated from the morphometric data. Step-wise distension was carried out on esophageal and jejunal segments. The obtained data on the length, diameter and pressure changes were then used to calculate the circumferential and longitudinal stresses and strains. Real-time reverse transcription polymerase chain reaction was used to detect the receptor of advanced glycation end-products (RAGE) mRNA level in jejunal tissues.

RESULTS: At the end of the experiment, the blood glucose level was significantly higher and the serum insulin level was significantly lower in DM, T1 and T2 groups than in the control group (Glucose: 30.23 ± 0.41 mmol/L, 27.48 ± 0.27 mmol/L and 27.84 ± 0.29 mmol/L *vs* 5.05 ± 0.04 mmol/L, $P = 1.65 \times 10^{-16}$, $P = 5.89 \times 10^{-19}$ and $P = 1.63 \times 10^{-18}$, respectively; Insulin: 1.47 ± 0.32 μg/L, 2.66 ± 0.44 μg/L, 2.03 ± 0.29 μg/L and 4.17 ± 0.54 μg/L, P = 0.0001, P = 0.029 and P = 0.025, respectively). However, these levels did not differ among the DM, T1 and T2 groups. The wet weight per unit length, wall thickness and opening angle of esophageal and intestinal segments in the DM group were significantly higher than those in the control group (from P = 0.009 to P = 0.004). These parameters in the T1 group were significantly lower than those in the DM group (wet weight, duodenum: 0.147 ± 0.003 g/cm *vs* 0.158 ± 0.001 g/cm, P = 0.047; jejunum, 0.127 ± 0.003 g/cm *vs* 0.151 ± 0.002 g/cm, P = 0.017; ileum, 0.127 ± 0.004 g/cm *vs* 0.139 ± 0.003 g/cm, P = 0.046; wall thickness, esophagus: 0.84 ± 0.03 mm *vs* 0.94 ± 0.02 mm, P = 0.014; duodenum: 1.27 ± 0.06 mm *vs* 1.39 ± 0.05 mm, P = 0.031; jejunum: 1.19 ± 0.07

mm *vs* 1.34 ± 0.04 mm, P = 0.047; ileum: 1.09 ± 0.04 mm *vs* 1.15 ± 0.03 mm, P = 0.049; opening angle, esophagus: 112.2 ± 13.2° *vs* 134.7 ± 14.7°, P = 0.027; duodenum: 105.9 ± 12.3° *vs* 123.1 ± 13.1°, P = 0.046; jejunum: 90.1 ± 15.4° *vs* 115.5 ± 13.3°, P = 0.044; ileum: 112.9 ± 13.4° *vs* 136.1 ± 17.1°, P = 0.035). In the esophageal and jejunal segments, the inner residual stain was significantly smaller and the outer residual strain was larger in the DM group than in the control group (P = 0.022 and P = 0.035). T1 treatment significantly restored this biomechanical alteration (P = 0.011 and P = 0.019), but T2 treatment did not. Furthermore, the circumferential and longitudinal stiffness of the esophageal and jejunal wall increased in the DM group compared with those in the control group. T1, but not T2 treatment, significantly decreased the circumferential wall stiffness in the jejunal segment (P = 0.012) and longitudinal wall stiffness in the esophageal segment (P = 0.023). The mRNA level of RAGE was significantly decreased in the T1 group compared to that in the DM group (P = 0.0069).

CONCLUSION: TWAJJ (high dose) treatment partly restored the morphometric and biomechanical remodeling of the upper gastrointestinal tract in diabetic rats.

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Key words: Biomechanics and morphometric remodeling; Diabetes rats; Gastrointestinal tract; Mechanism; Tangweian Jianji

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INTRODUCTION

Diabetic gastrointestinal disorder (DGID) is a common complication of diabetes. Up to 76% of diabetic patients express gastrointestinal symptoms including dysphagia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting and diarrhea[1,2]. Duration of the disease and poor glycemic control seem to be associated with the severity of gastrointestinal (GI) problems. In general, the pathogenesis of DGID includes high blood glucose level, smooth muscle degeneration, abnormal autonomic neuropathy, gastrointestinal hormone secretion disorders and oxidative stress[3,4].

The GI tract is functionally subjected to dimensional changes. Hence, biomechanical properties such as the stress-strain relationships are of particular importance[5]. These properties are remodeled in response to growth[6], fasting[7] and disease[8]. The biomechanical properties are crucial for GI motor function because peristaltic motion which propels the food through the GI tract is a result of an interaction between the passive and active tissue forces and the hydrodynamic forces in the food bolus. Remodeling of the mechanical properties reflects the changes in the tissue structure that determine a specific motor dysfunction. During the past few years, several studies have demonstrated that experimental diabetes induces GI morphological and biomechanical remodeling[9-13]. Following the development of diabetes, the GI wall becomes thicker and stiffer in a time-dependent manner. Therefore, diabetic gastrointestinal morphological and biomechanical remodeling play an important role in DGID and has become the new perspective of diabetic gastrointestinal pathogenesis[11].

Some studies on diabetic arteries have demonstrated that non-enzymatic glycation of arterial wall tissues is associated with remodeling of the wall[14,15]. We believe that the same applies to the diabetic GI wall. Advanced glycation end-products (AGEs) may contribute to diabetic GI morphological and biomechanical remodeling by two major mechanisms. The first is receptor-independent alteration of the extracellular matrix architecture by non-enzymatic glycation and the formation of protein cross-links. The second mechanism is receptor-dependent and consists of modulation of cellular functions through ligation of specific cell surface receptors[16-18].

Western medical treatment of DGID is mainly focused on symptomatic control with improvement of gastric motility, using promoting agents and supportive measures based on blood glucose control[2]. Although these therapies can partially improve the clinical symptoms, they do not fundamentally reverse the diabetes-induced changes and result in a very high relapse rate. In clinics it was shown that Tangweian Jianji (TWAJJ) significantly improved DGID with a low relapse rate, however, the mechanism involved in this improvement is not fully understood. Therefore, the aim of the present study was to investigate whether TWAJJ treatment can improve the morphometric and biomechanical remodeling of the GI tract in streptozotocin (STZ)-induced diabetic rats. Furthermore, the receptor of advanced glycation end-products (RAGE) mRNA level in the jejunal tissues was also investigated to explore the possible mechanism of TWAJJ in the treatment of DGID.

MATERIALS AND METHODS

Animal model and groups

Forty male SD rats weighing 220-250 g were included in this study. Diabetes was induced in 30 rats by a single tail vein injection of 40 mg/kg body weight of strepto-

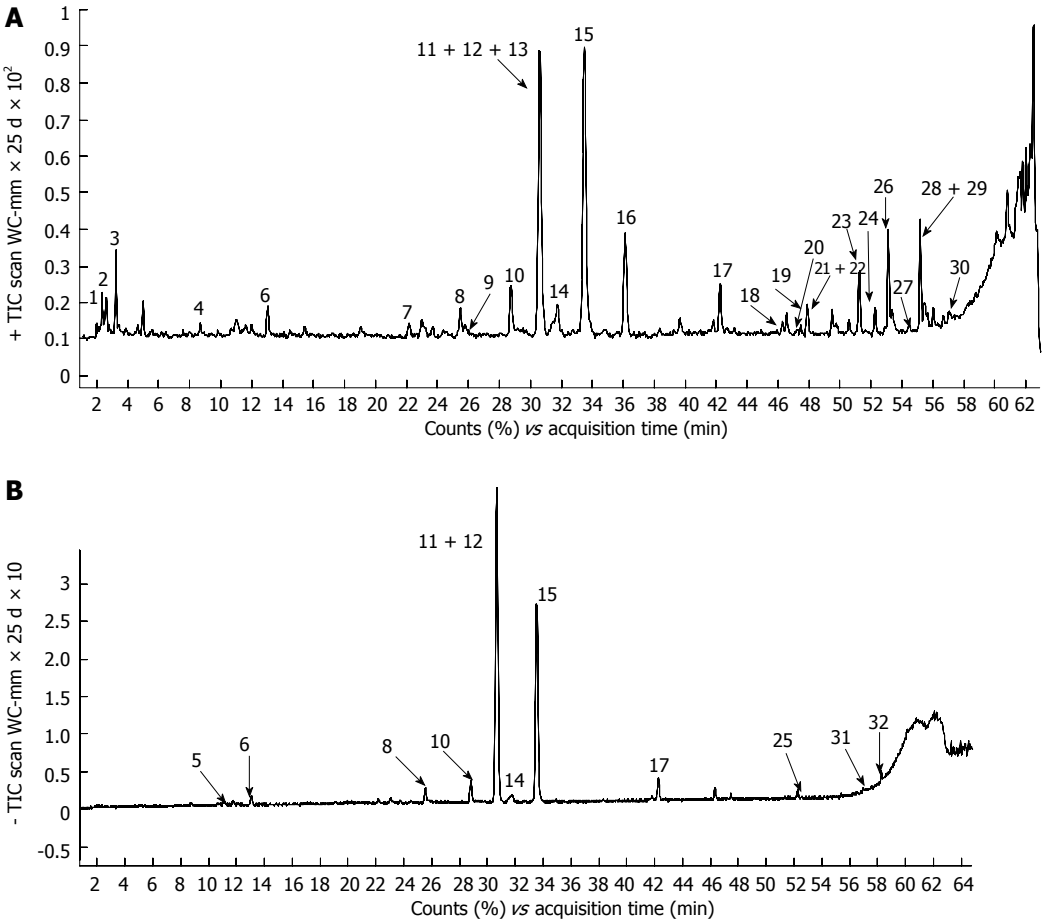

Figure 1 High pressure liquid chromatograph/mass spectrometry analysis of Tangweian Jianji extract. A: Representative total ion current (TIC) chromatograms of the Tangweian Jianji (TWAJJ) extract were obtained in positive ion electrospray; B: TIC chromatograms of the TWAJJ extract was obtained in negative ion electrospray. Visual inspection of the two TIC plots shows that the two modes of ionization generate different constituent information based on the ionizability differences of the components in TWAJJ. A total of 32 peaks were observed. The most abundant compounds identified are Naringin (peak 12), Neohesperidin (peak 15), Lobetyolin (peak 16), Atractylenolide (peak 26) and Emodin (peak 23). WC: Wave chromatogram.

zotocin (STZ, Sigma-Aldrich, China). This dose of STZ resulted in a random blood glucose level ⩾ 16.7 mmoL/L in 90% of rats 7 d after the injection. The remaining 10% of rats were excluded from this study. Twenty-seven STZ-induced diabetic rats were subdivided into three groups (n = 9 in each group), i.e., diabetic control group (DM); high dose of TWAJJ group (T1) and low dose of TWAJJ group (T2). Another 10 rats of similar age and body weight from the same vendor were used as a non-diabetic control group (Control).

Drugs and administration methods

TWAJJ is composed of Citrus aurantium, Codonopsis, fried Atractylodes and wine Rhubarb and was provided by Guang'anmen Hospital, China Academy of Chinese Medical Sciences. The medicine was administered by gavage which passed though the esophagus and reached the stomach lumen. The drug was perfused directly into the stomach once daily from the beginning of the experiment. The dosage was 10 g/kg for T1 and 5 g/kg for T2, respectively. The rats in the DM and control groups were perfused with physiological saline.

Chemical analysis of TWAJJ

The formulation of TWAJJ was prepared according to the corresponding monograph in the 2005 Edition of Chinese Pharmacopoeia. There were quantitative control limits for the raw herbs (for example, the content of Naringin in Citrus aurantium was not less than 4.0% and Neohesperidin was not less than 3.0%) and for the final drug product. The chemical composition of TWAJJ was determined by using an ultra-fast high performance liquid chromatography-electrospray-ionization-quadrupole time-of-flight mass spectrometry method. An example chromatographic fingerprint is shown in Figure 1. The major compounds in the final dosage form were identified as Naringin, Neohesperidin, Lobetyolin, Atractylenolide and Emodin. These compounds were used as the quality control markers in the final TWAJJ formulation.

Experimental procedures

Weight and blood glucose levels were measured at 2-wk intervals after initiating the experiment. For blood glucose measurement, one drop of blood was obtained from rat tail vein and blood glucose was measured by a John-

son and Johnson One Touch Ultra Blood Glucose Meter. For insulin measurement, blood was obtained from the abdominal aorta and 0.2 mL serum was separated. The serum insulin was measured by radioimmunoassay (rat insulin radioimmunoassay kit, Linco Company, United States) at the end of the experiment.

The experimental period was 60 d. At the end of the experiment, the rats were fasted overnight and anesthetized with 4% Chloral hydrate (10 mL/kg, *ip*). Following laparotomy, the whole esophagus and ten centimeters of duodenum, jejunum and ileum were harvested. The duodenum was taken from the descending section, 1 cm down from the pylorus; the jejunum from 5 cm distal to the ligament of Treitz, and the ileum from 5 cm proximal to the ileo-cecal valve. After gently cleaning the lumen of the segments with saline, the length and wet weight were measured.

The esophageal and jejunal segments were divided into three sections, the proximal 1 cm segment was immediately stored at -70 ℃ for protein and RAGE mRNA detection. The adjacent 1 cm long segment was used for the zero-stress state experiment. The remaining section was used for the distension test. The duodenal and ileal segments were divided into two and used for protein and mRNA detection and the zero-stress state experiment. In this experiment, RAGE mRNA detection was performed only on the jejunal segments.

Zero-stress state experiment

To obtain data on the zero-stress state, three 1-2 mm wide esophageal and intestinal rings were cut and placed in Krebs solution at room temperature. The composition of Krebs solution (mmol/L) was: NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; NaH_2PO_4, 1.0; $MgCl_2$, 1.2; and ascorbic acid, 0.11. A photograph was taken of the cross-section of the rings using a Canon camera (Canon, Japan) and was presented as the no-load state. Each ring-shaped segment was then cut radially from the opposite mesentery site and the photographs were taken about 60 min after the radial cutting to allow viscoelastic creep to take place. This is presented as the zero-stress state.

Distension test

The distal end of the remaining esophageal and jejunal segments was tied with a suture and the proximal end was cannulated with a tube for the distension experiment. After preconditioning of the segments, they were inflated with Krebs solution using a step-wise distension protocol from 0 cm to 20 cm H_2O (0 cm, 1 cm, 2 cm, 3 cm, 5 cm, 10 cm, 15 cm and 20 cm H_2O) for the esophageal segment and from 0 cm to 10 cm H_2O (0 cm, 0.5 cm, 1 cm, 2 cm, 4 cm, 6 cm, 8 cm and 10 cm H_2O) for the jejunal segment. The segments conformed to a cylindrical geometry during the distensions. Each pressure lasted for 2 min and then the outer diameter and length of the segments were photographed (Canon, Japan).

Mechanical data analysis

The morphometric data were obtained from digitized images of the segments in the zero-stress, no-load and pressurised states. Measurements were undertaken using image analysis software (Sigmascan ver. 4.0, Sigma Corp., San Rafael, CA, United States). The following data were measured from each specimen: the circumferential length (C), the wall thickness (h), the wall area (A), and the opening angle at zero-stress state (α). The subscripts i, o, n, z and p refer to the inner (mucosal) surface, outer (serosal) surface, no-load state, zero-stress state and pressurised condition. The opening angle α was defined as the angle subtended by two radii drawn from the midpoint of the inner wall to the inner tips of two ends of the specimen. Furthermore, the outer diameter (D) and the length (L_p) were measured from the images of the pressurised segments.

The measured data were used for the calculation of biomechanical parameters defined as:

Residual Green's strain at the mucosal surface:

$$E_i = \frac{(\frac{C_{i-n}}{C_{i-z}})^2 - 1}{2} \quad (1)$$

Residual Green's strain at the serosal surface:

$$E_o = \frac{(\frac{C_{o-n}}{C_{o-z}})^2 - 1}{2} \quad (2)$$

The stress and strain of the esophageal and intestinal segments in the pressurised state were determined under the assumptions that the wall was homogenous and the organ shape was cylindrical. Calculations were performed knowing the no-load state dimensions, the outer diameters and lengths of the specimens at varying pressures, and assuming incompressibility of the wall. The longitudinal stretch ratio, $\lambda_\varphi = \frac{L_p}{L_z}$;

the luminal radius, $_{i-p} = \sqrt{r_{o-p}^2 - \frac{A_n}{\pi\lambda_\varphi}}$;

the wall thickness, $h_p = r_{o-p} - r_{i-p}$; the mucosal circumferential length, $C_{i-p} = 2 \times \pi \times r_{i-p}$; the serosal circumferential length, $C_{o-p} = 2 \times \pi \times r_{o-p}$; the mid-wall circumferential length, $C_{m-p} = \frac{C_{i-p} + C_{o-p}}{2}$;

the circumferential stretch ratio, $\lambda_\theta = \frac{C_{m-p}}{C_{m-z}}$

(where the middle-wall circumferential length at

zero-stress state, $C_{m-z} = \frac{C_{i-z}}{C_{o-z}}$) were calculated.

Then the Kirchhoff's stress and Green's strain in a wall at a given pressure were calculated according to the following equations:

Circumferential Kirchhoff's stress: $S_\theta = \frac{\Delta P r_{i-p}}{h_p \lambda_\theta^2}$ (3)

Longitudinal Kirchhoff's stress: $S_\varphi = \frac{\Delta P r_{i-p}^2}{h_p \lambda_\varphi^2 (r_{o-p} + r_{i-p})}$ (4)

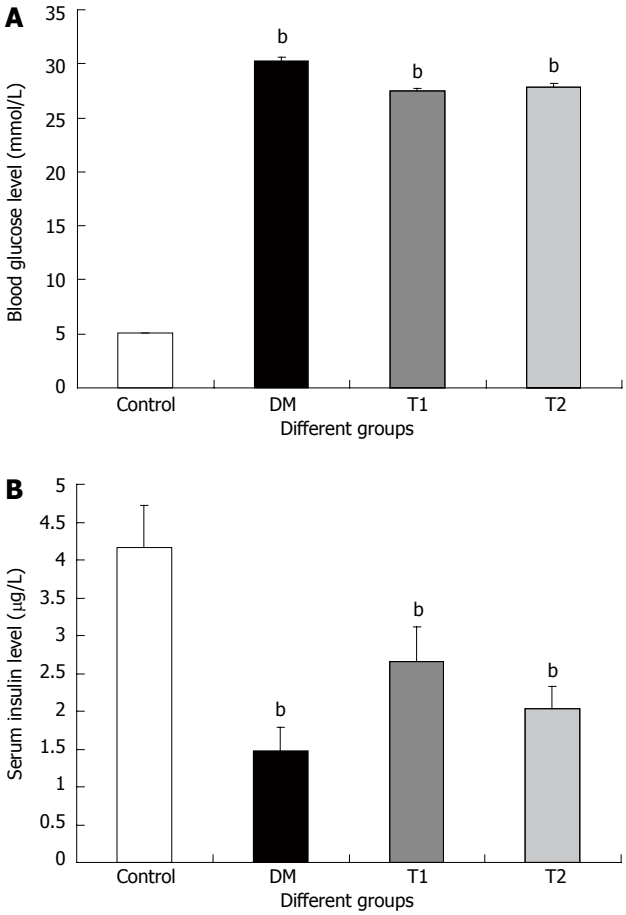

Figure 2 Blood glucose levels and serum insulin levels in the different groups. A: Blood glucose levels; B: Serum insulin level. DM, T1 and T2 groups compared with the control group. $^{b}P < 0.01$ *vs* control group. DM: Diabetic control; T1: High dose; T2: Low dose.

Circumferential mid-wall Green's strain: $E_\theta = \frac{\lambda_\theta^2 - 1}{2}$ (5)

Longitudinal Green's strain: $E_\varphi = \frac{\lambda_\varphi^2 - 1}{2}$ (6)

ΔP is the transmural pressure difference. The longitudinal mid-wall stretch ratio was referenced to the no-load state because tissue strips could not be cut to obtain the zero-stress state in the longitudinal direction. However, the longitudinal mid-wall length in rat intestine does not differ between the no-load and zero-stress states[19].

Receptor of advanced glycation end-products mRNA detection

Real-time polymerase chain reaction (RT-PCR) was used to detect RAGE mRNA level in the jejunal tissues. Total RNA was isolated from 100 mg tissue of each specimen using ultra-pure Trizol reagent according to the procedure described by the manufacturer (Invitrogen, United States). RNA concentration was determined when equal amounts of total RNA (3 μg) were used in the reverse transcription with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, United States). RT-PCR was used to quantify the mRNA expression of RAGE. Amplification was performed using iQ Supermix with the iCycler iQ Real-Time Detection System according to the manufacturer's instructions (Applied Biosystems). A standard curve was generated for each primer pair and a probe to determine PCR efficiency. Mean threshold cycle (Ct) values for each sample were normalized to β-actin and calibrated to the control group to obtain the threshold cycle difference (ΔΔCt) with 2-ΔΔCt being the fold change relative to the control group. The probes and primer sequences used were as follows: RAGE: Sense: 5'-TCT CAG AAG CCC AAG GAA GAG T-3'; Antisense: 5'-CCT AGG TCT GAA GGC CCT GAG T-3', amplified cDNA length: 173 bp; β-actin: Sense: 5'-AGA TCC ACA ACG GAT ACA TT-3'; Antisense: 5'-TCC CTC AAG ATT GTC AGC AA-3', amplified cDNA length: 309 bp.

Statistical analysis

The data were representative of a normal distribution and accordingly the results were expressed as mean ± SE. The stress-strain curve for each direction was fitted using the exponential function equation $S = (S^* + \beta)\, e^{\alpha (E-E^*)} - \beta$. S* and E* are the stress and strain at a physiological reference level[5]. The constants α and β from the above exponential function were used for the statistical evaluation of the stress-strain data. Analysis of variance was used to detect differences in the parameters and groups (Sigmastat 2.0™). The results were regarded as significant when $P < 0.05$.

RESULTS

Blood glucose and serum insulin levels

Blood glucose and serum insulin levels at the end of the experiment are shown in Figure 2. The blood glucose level was 4-fold higher in the DM group compared with the control group (Figure 2A, $P < 0.01$). The serum insulin level was significantly lower in the DM group compared with the control group (Figure 2B, $P < 0.01$). Compared with the DM group, the blood glucose and insulin levels did not significantly change in the T1 and T2 groups.

The weight/cm, wall thickness and wall area

The wet weight per unit length (Figure 3A), no-load wall thickness (Figure 3B) and cross-sectional wall area (Figure 3C) of the esophageal and intestinal segments increased in the DM group compared with the control group ($P < 0.01$, $P < 0.05$). After treatment with T1, the wall thickness decreased in all segments (Figure 3B, $P < 0.05$), whereas the wet weight and wall area decreased in the duodenal, jejunal and ileal segments (Figure 3A, D, $P < 0.01$, $P < 0.05$), but not in the esophageal segment ($P = 0.12$). All the above parameters did not change significantly in the T2 group.

Opening angle and residual strain

At the end of the experiment, the opening angle of all three intestinal segments was significantly increased in

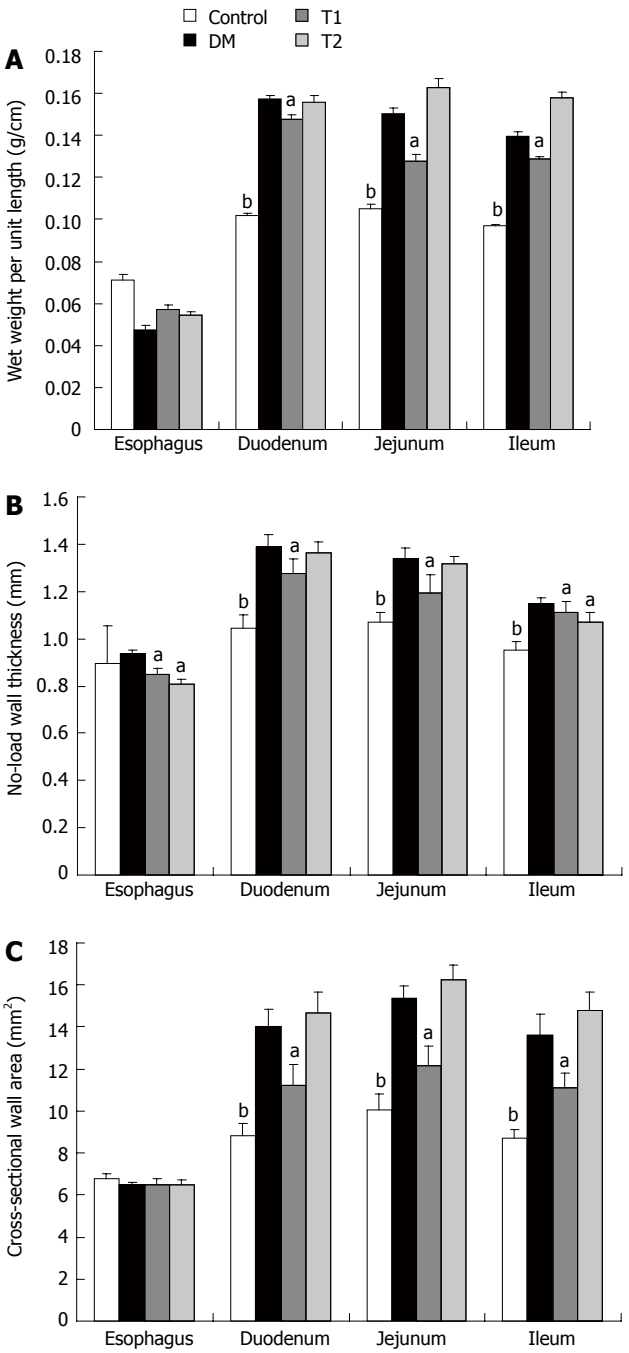

Figure 3 Wet weight per unit length, wall thickness and wall area in different segments of the different groups. A: Wet weight per unit length; B: Wall thickness; C: Wall area. $^aP < 0.05$, $^bP < 0.01$ *vs* DM group. DM: Diabetic control; T1: High dose; T2: Low dose.

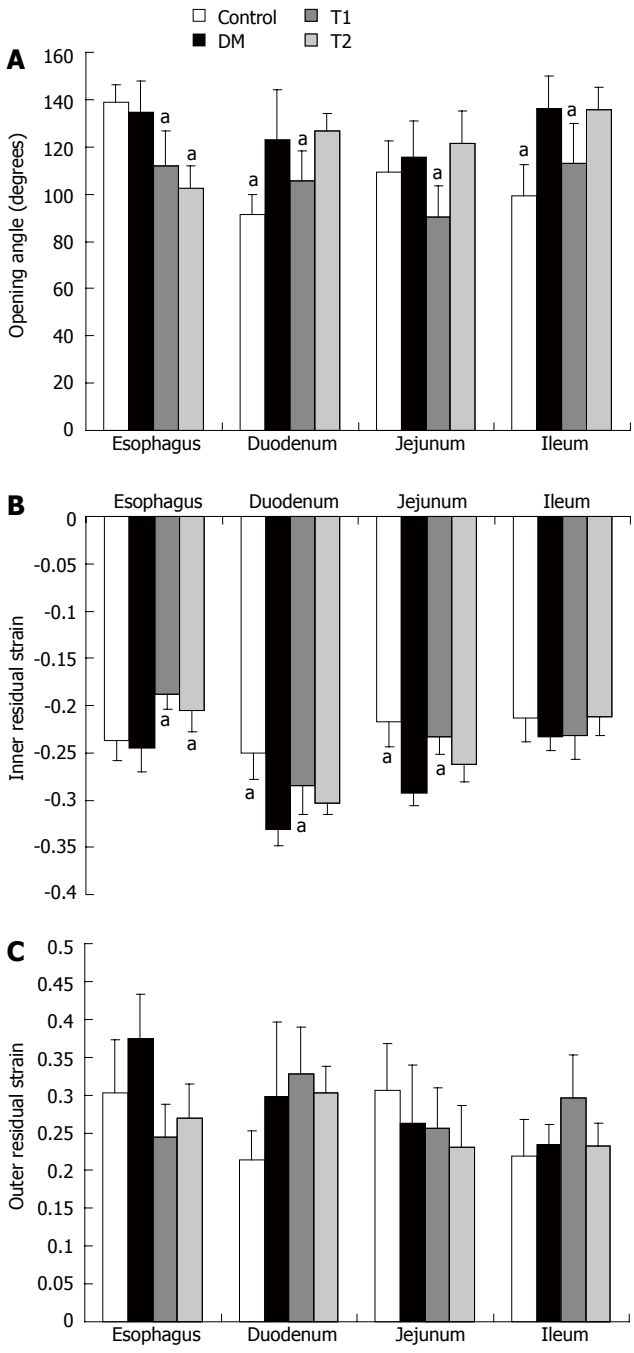

Figure 4 Opening angle, inner residual strain and outer residual strain in different segments of the groups. A: Opening angle; B: Inner residual strain; C: Outer residual strain. $^aP < 0.05$ *vs* DM group. DM: Diabetic control; T1: High dose; T2: Low dose.

the DM group compared with the control group (Figure 4A, $P < 0.05$). However, the opening angle in the esophageal segment did not change. Treatment with high dose TWAJJ decreased the opening angle in all segments studied (Figure 4A, $P < 0.05$). Interestingly, the opening angle of the esophageal segment was also decreased in the T2 group ($P < 0.05$).

A similar pattern to that found in the opening angle was found in the inner residual strain for all segments and groups, with the exception of the ileal segment (Figure 4B), where no difference was found between the different groups ($P > 0.05$). The outer residual strain did not differ between the groups and segments (Figure 4C).

Stress-strain distribution

At the end of the experiment, the stress-strain analysis showed that the circumferential stress-strain curves of esophageal and jejunal segments (Figure 5A, C), and the longitudinal stress-strain curve of the esophageal segment (Figure 5B) in the DM group shifted to the left

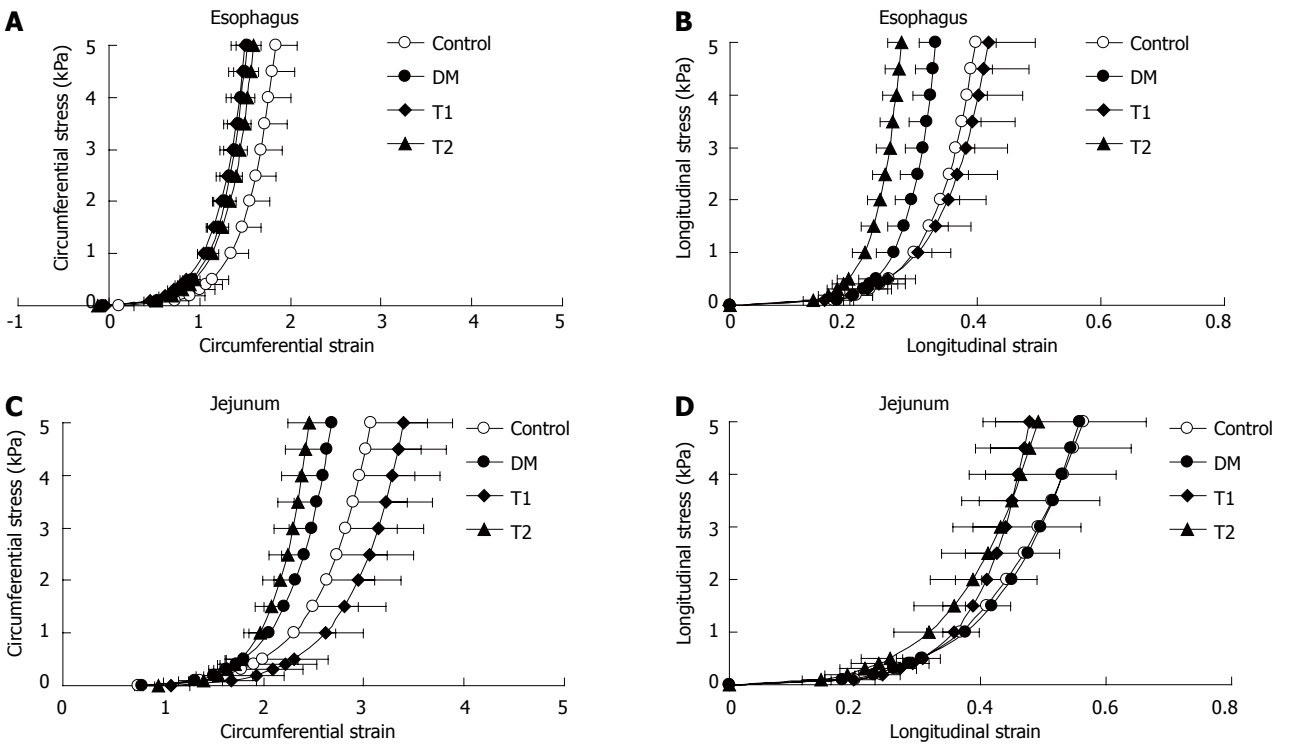

Figure 5 Circumferential and longitudinal stress-strain curves of esophageal and jejunal segments in the different groups. DM: Diabetic control; T1: High dose; T2: Low dose.

Table 1 Comparison of constant a among the different groups

	Circumferential direction		Longitudinal direction	
	Esophagus	Jejunum	Esophagus	Jejunum
Control (n = 10)	3.56 ± 0.35	2.65 ± 0.37	18.33 ± 2.36	13.51 ± 3.66
DM (n = 9)	4.59 ± 0.79[a]	3.81 ± 0.31[a]	27.62 ± 3.93[a]	14.79 ± 2.26
T1 (n = 9)	3.77 ± 0.39	2.55 ± 0.51[c]	19.43 ± 4.13[c]	14.67 ± 1.56
T2 (n = 9)	3.84 ± 0.44	4.02 ± 0.71[a]	27.82 ± 1.74[a]	15.41 ± 4.36

[a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* DM group. DM: Diabetic control; T1: High dose; T2: Low dose.

compared with those in the control group, indicating that the diabetic esophageal and intestinal wall became stiffer. Calculation of the mechanical constants demonstrated a difference between the DM group and the control group ($P < 0.05$, Table 1). However, the longitudinal stress-strain distribution of the jejunal segment did not differ between the DM and control groups ($P > 0.05$, Figure 5D). High dose TWAJJ (T1) decreased the stiffness of the esophageal wall in the longitudinal direction (Figure 5B and Table 1, $P < 0.05$) and the intestinal wall in the circumferential direction (Figure 5C and Table 1, $P < 0.05$). Low dose TWAJJ did not improve the stiffening of the esophageal and jejunal wall caused by diabetes ($P > 0.05$, Figure 5 and Table 1).

Receptor of advanced glycation end-products mRNA level

RAGE mRNA level of the jejunal segment increased in the DM group compared to the control group (Figure 6, $P < 0.01$). T1 treatment reduced the RAGE mRNA level (Figure 6, $P < 0.01$). We did not observe a significant difference between the T2 group and the DM group (Figure 6, $P > 0.05$).

DISCUSSION

Diabetes mellitus is a pandemic disease. According to WHO´s statistical report; 370 million diabetic patients are expected by 2030. The serious impact caused by diabetes and its complications does not only affect patients but is also a heavy financial burden to the health care system and on families[20]. DGID is a common complication of diabetes. A previous study showed that prominent proliferations of the GI wall, especially the small intestine and esophagus mucosae, occur in experimental diabetes[21]. Other studies demonstrated morphological and biomechanical remodeling of the upper GI tract in diabetes[9-10,22], which may be caused by over-expression of AGE and RAGE in the diabetic GI tract. Furthermore, a linear association between the glucose level and morphometric and biomechanical remodeling was observed[23].

In the view of traditional Chinese medicine, DGID is in the category of fullness, epigastric pain, vomiting, diarrhea, and constipation concurrent with diabetes. The major syndrome of DGID includes spleen deficiency and qi stagnation. Therefore, the principal treatment is to strengthen the spleen and remove stagnation. TWAJJ is a practical Chinese medicinal compound for DGID[24-26] and is composed of several herbs. Atractylodes mac-

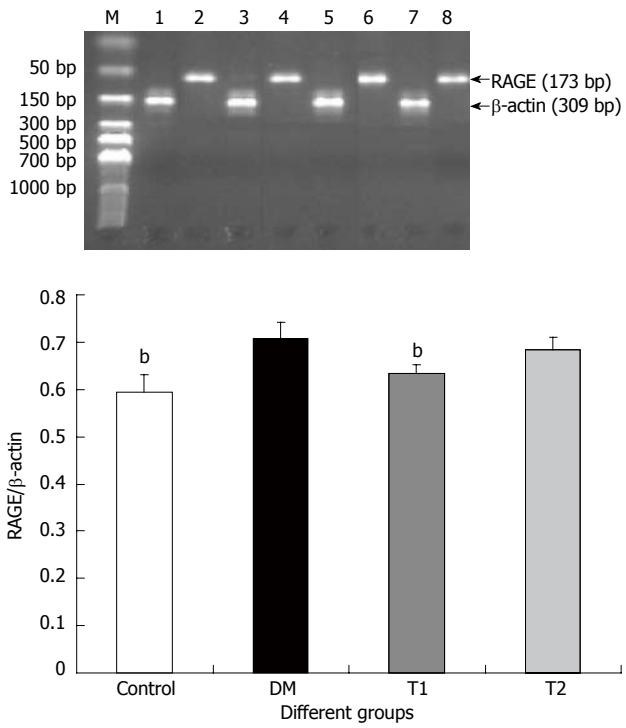

Figure 6 Receptor of advanced glycation end-products mRNA level of the jejunal segment in the different groups. $^{b}P < 0.01$ *vs* DM group. M: Ladder marker; 1, 3, 5, 7: β-actin; 2, 4, 6, 8: RAGE; 1, 2: Control; 3, 4: DM; 5, 6: T1; 7, 8: T2. DM: Diabetic control; T1: High dose; T2: Low dose; RAGE: Receptor of advanced glycation end-products.

rocephala can strengthen the spleen, harmonize the stomach, regulate GI motility and protect the gastric mucosa[27]. Citrus aurantium and wine rhubarb move qi and accumulation. Pinellia downbears counterflow and inhibits vomiting. Ginseng tones qi and has a beneficial affect on body fluid. Citrus aurantium has a non-competitive antagonist effect on acetylcholine and histamine in intestinal smooth muscle contraction, possibly by influencing Ca^{2+} channels. Its total alkaloid level can prevent vomiting and can promote GI tract movement in rats[28-30]. The active ingredients of ginseng ginsenosides can reduce blood sugar by stimulating insulin secretion and anti-apoptosis of pancreatic β-cells[31,32]. Furthermore, the results of a retrospective clinical study on TWAJJ treatment in DGID patients have been reported[24,25]. After treatment with TWAJJ, symptoms such as fullness, eructation, nausea (vomiting) and constipation were significantly improved. Although the fasting blood glucose tended to decrease after treatment, this decrease was not statistically significant. However, clarification of the clinical validity of TWAJJ in randomized controlled clinical trials is necessary. In this study, we also found that TWAJJ had a tendency to decrease blood glucose and increase serum insulin, however, statistical significance was not reached. Therefore, we consider that the functional mechanism of TWAJJ in DGID is through other pathways rather than through blood glucose regulation.

The esophagus and small intestine are tubular organs. One important function is transportation of food by wall movement; therefore the biomechanical properties of the wall are important for this function[8]. The biomechanical properties of the esophagus and small intestine depend on their structure and can be evaluated by the opening angle, residual stress and strain, and stress-strain relationship[11]. Previous studies have demonstrated that the esophageal wall[12,33,34] and intestinal wall[9-11] were remodeled during the development of diabetes. Therefore, it is important to determine whether TWAJJ can improve DGID through a pathway involved in changing the biomechanical properties of the GI tract.

The present study confirmed previous findings that morphometric and biomechanical remodeling of the esophageal and intestinal wall occur in STZ-induced diabetic rats. Although the treatment with TWAJJ did not significantly change the blood glucose and serum insulin levels, high dose TWAJJ, to a large extent, improved the morphometric and biomechanical remodeling caused by diabetes. The improvement in morphometric remodeling was expressed as reduced wall thickness and area. Improvements in biomechanical remodeling were expressed as reduced opening angle, reduced absolute value of residual strain and decreased wall stiffness. The present study indicated that the effect of TWAJJ on improvements in DGID in the clinic may be partially associated with improvements in biomechanical remodeling of the GI wall. Due to the complex geometry of the stomach[22,35], we did not focus on the biomechanical parameters of the gastric wall in the present study. Future studies may focus on the effect of TWAJJ on the stomach due to the importance of stomach function in diabetic patients.

The alterations in residual strain and wall stiffness in diabetes will change the tension and stress distribution in the location of mechanosensitive afferents in the GI tract[36,37]. Therefore, biomechanical remodeling of the diabetic GI wall indirectly affects GI motor function. High dose TWAJJ partially restored the changes in biomechanical properties caused by diabetes. This may be associated with improved motor and sensory function of the diabetic GI tract.

AGEs is a heterogeneous group of macromolecules formed by the non-enzymatic glycation of proteins, lipids and nucleic acids[38]. Endogenous AGEs are generated at higher rates in diabetes due to abnormal glucose metabolism. Chronic high blood glucose can cause excessive glycoprotein accumulation in the body including the GI tract causing GI wall stiffening[8]. There is growing evidence to show that AGEs and RAGE are implicated in various disorders. AGE levels in serum and tissues are associated with chronic complications of DM including DGID. Furthermore, RAGE is involved in signal transduction of AGEs in a variety of cells[39]. Our previous studies showed that AGE and RAGE were over-expressed in diabetic GI tissues[40]. In the present study, we found that the RAGE mRNA level in the jejunal segment increased in diabetic rats compared with normal rats. Following high dose TWAJJ administration, this alternation was reversed to the normal state. Therefore, the observed

improvement in the mechanical factors due to TWAJJ treatment in DGID may be through the inhibition of AGEs accumulation. However, in future studies we need to clarify this mechanism by immunohistochemistry of RAGE to demonstrate whether the change in mRNA expression actually translates to protein expression. Furthermore, it is also important to study whether specific knockdown of RAGE in the GI mucosa can improve biomechanical remodeling of the GI tract in diabetes and reverse DGID.

In conclusion, high dose TWAJJ treatment improves biomechanical and morphometric remodeling of the diabetic esophageal and intestinal wall. The mechanism may, at least partly, be explained by decreased RAGE mRNA levels in STZ-induced diabetic rats. Therefore, it seems feasible to develop Chinese herbs, such as TWAJJ, to improve the morphometric and biomechanical remodeling caused by diabetes. This may have an impact on GI dysfunction in diabetes and be used in clinical practice.

COMMENTS

Background

Diabetic gastrointestinal disorder (DGID) is a common complication of diabetes. However, neither the pathophysiology nor the pathogenesis of patients' gastrointestinal (GI) symptoms associated with diabetes are well understood. Furthermore, many patients with DGID do not receive appropriate treatment. Although Western medicine can partially improve the clinical symptoms, it does not fundamentally reverse the diabetes-induced changes and results in a very high relapse rate. In the clinic, it was shown that Tangweian Jianji (TWAJJ) significantly improved DGID with a low relapse rate, however, the mechanism involved in this improvement is not fully understood.

Research frontiers

The GI tract is functionally subjected to dimensional changes. Hence, biomechanical properties such as the stress-strain relationship are of particular importance. Several studies have demonstrated that diabetes induces GI morphological and biomechanical remodeling. The non-enzymatic glycation of the intestinal wall in diabetes is associated with remodeling. Therefore, the present study investigated whether TWAJJ treatment can improve morphometric and biomechanical remodeling of the GI tract in streptozotocin (STZ)-induced diabetic rats. Furthermore, the receptor of advanced glycation end-products (RAGE) mRNA level in the jejunal tissues was also investigated to explore the possible mechanism of TWAJJ in the treatment of DGID.

Innovations and breakthroughs

Compared with previous studies, this study is the first to use biomechanical test methods combined with RAGE detection to investigate the mechanism of the effect of the Chinese medicine, TWAJJ, on diabetic GI dysfunction. We showed that high dose TWAJJ improves the biomechanical and morphometric remodeling of the diabetic esophageal and intestinal walls. The mechanism may, at least partly, be related to the decreased RAGE mRNA levels in STZ-induced diabetic rats. As knowledge of GI biomechanics is new and was only recently developed and the non-enzymatic glycation of the GI tract is closely related to its biomechanical properties, determination of biomechanical properties and expression of advanced glycation end products and their receptor (AGE/RAGE) may be useful tools to investigate the mechanism of TWAJJ in DGID.

Applications

The findings of this study suggest that it seems feasible to develop Chinese herbs, such as TWAJJ, to improve the morphometric and biomechanical remodeling caused by diabetes. This may have an impact on GI dysfunction in diabetes and be used in clinical practice.

Terminology

DGID is a common complication of diabetes related to the GI tract. Up to 76% of diabetic patients express GI symptoms including dysphagia, early satiety, reflux, constipation, abdominal pain, nausea, vomiting and diarrhea; GI biomechanical properties are used to define the GI wall deformation (strain) and force (stress), which can be described by the opening angle, residual stress and strain, stress-strain relationship, stress relaxation and creep; TWAJJ is a practical Chinese medicinal compound for diabetic gastrointestinal dysfunction and is composed of several herbs including Citrus aurantium, Codonopsis, fried Atractylodes and wine Rhubarb.

Peer review

This is an interesting article. DGID is clearly a big topic especially in developed countries, thus this topic is interesting. Further, it was also fascinating to see that high dose TWAJJ was able to "treat" DGID without treating hyperglycemia. The manuscript is well written, the experiments are well planned, methods are adequately described and suitable to achieve the goals and results are appropriately described.

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BRIEF ARTICLE

Clinical usefulness of adherence to gastro-esophageal reflux disease guideline by Spanish gastroenterologists

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Abstract

AIM: To investigate usefulness of adherence to gastro-esophageal reflux disease (GERD) guideline established by the Spanish Association of Gastroenterology.

METHODS: Prospective, observational and multicentre study of 301 patients with typical symptoms of GERD who should be managed in accordance with guidelines and were attended by gastroenterologists in daily practice. Patients (aged > 18 years) were eligible for inclusion if they had typical symptoms of GERD (heartburn and/or acid regurgitation) as the major complaint in the presence or absence of accompanying atypical symptoms, such as dyspeptic symptoms and/or supraesophageal symptoms. Diagnostic and therapeutic decisions should be made based on specific recommendations of the Spanish clinical practice guideline for GERD which is a widely disseminated and well known instrument among Spanish in digestive disease specialists.

RESULTS: Endoscopy was indicated in 123 (41%) patients: 50 with alarm symptoms, 32 with age > 50 years without alarm symptom. Seventy-two patients (58.5%) had esophagitis (grade A, 23, grade B, 28, grade C, 18, grade D, 3). In the presence of alarm symptoms, endoscopy was indicated consistently with recommendations in 98% of cases. However, in the absence of alarm symptoms, endoscopy was indicated in 33% of patients > 50 years (not recommended by the guideline). Adherence for proton pump inhibitors (PPIs) therapy was 80%, but doses prescribed were lower (half) in 5% of cases and higher (double) in 15%. Adherence regarding duration of PPI therapy was 69%; duration was shorter than recommended in 1% (4 wk in esophagitis grades C-D) or longer in 30% (8 wk in esophagitis grades A-B or in patients without endoscopy). Treatment response was higher when PPI doses were consistent with guidelines, although differences were not significant (95% *vs* 85%).

CONCLUSION: GERD guideline compliance was quite good although endoscopy was over indicated in patients > 50 years without alarm symptoms; PPIs were prescribed at higher doses and longer duration.

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Key words: Gastro-esophageal reflux disease; Clinical practice guidelines; Adherence to guidelines; Treatment of gastro-esophageal reflux disease

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most prevalent gastrointestinal disorders in the general population[1]. GERD diagnosis is made mainly according to the presence of symptoms. Patients with heartburn and/or regurgitation (typical GERD symptoms) received the diagnosis. Typical symptoms of GERD are experienced by 25% of people at least once a month, 12% at least once per week, and 5% describe daily symptoms[2]. The prevalence of GERD among the Spanish population is 15%, with 33% and 22% of subjects reporting monthly episodes of heartburn and regurgitation, respectively[3]. Reported consultation rates range from 5% to 56%[4].

GERD is perceived as a benign disease but the spectrum of chronicity and severity of upper gastrointestinal symptoms frequently affects health-related quality of life (HRQoL)[5]. The wide-ranging effects of GERD on health and well-being can have consequences for the work productivity, psychological and social performance of affected individuals, particularly in patients with severe symptoms or night time acid reflux and sleep disturbance[5-8]. In some respects the HRQoL burden of these patients is similar to or greater than that observed in patients with conditions such as diabetes, hypertension or coronary heart disease[9,10].

Although treatment with proton pump inhibitors (PPIs) is satisfactory in many cases and results in cicatrisation of esophagitis when esophagitis is present, complete disappearance of symptoms is difficult to achieve[11]. Nevertheless, in many cases PPI therapy leads to rapid symptom improvement that is associated with a better HRQoL. For example, in a large Italian study, 96% of patients were satisfied with the results of 4 wk of PPI therapy and the average HRQoL score improved significantly during this period[12]. An effective management strategy is particularly important because, as mentioned, GERD imposes a significant burden of illness[13,14]. For that purpose several clinical practice guidelines have been developed by different medical societies and expert consensus meetings[15-19]. However, although many national and international guidelines are currently available, the impact of these recommendations on the behaviour of physicians has generally been limited[20-25]. Only in some disease states guidelines have been shown to influence physician behaviour, which depends on the prevalence of the disorder and access to the guidelines among other factors[26-28].

The objective of this study was to assess the level of adherence of gastroenterologists to the Spanish clinical guidelines for GERD and the results obtained in the care of patients with typical symptoms of GERD attended in daily practice.

MATERIALS AND METHODS

Study design

This multicentre and observational study was prospectively designed with two purposes: (1) to investigate whether patients with typical GERD symptoms seeking consultation from a specialist in gastroenterology were managed in accordance with the clinical practice guideline for GERD developed by the Spanish Association of Gastroenterology, the Spanish Society of Family and Community Medicine and the Iberoamerican Cochrane Center[15]; and (2) to assess the results obtained. The study was focused on the "acute phase" of GERD (4-8 wk after diagnosis) and not on the long-term; therefore, evaluation of maintenance treatment was not done. Thirty-three gastroenterologist from 33 different medical centers throughout Spain participated in the study, that was conducted under routine clinical practice conditions. Gastroenterologists participating in the study were not aware they were included in an adherence study. Consecutive patients were recruited between December 12, 2007 and November 5, 2008. All patients provided written informed consent. The protocol was approved by the Ethics Committees of the participating centers.

Inclusion and exclusion criteria

Consecutive patients (aged > 18 years) attending to gastroenterologists offices were eligible for inclusion if they had typical symptoms of GERD (heartburn and/or acid regurgitation) more than twice a week for at least 2 mo as the major complaint, in the presence or absence of accompanying atypical symptoms, such as dyspeptic symptoms and/or supraesophageal symptoms. All enrolled patients were required to sign the informed consent. Patients who had taken drug treatment for GERD within 2 mo prior to the study were excluded. Also, patients were not eligible they were currently taking non-steroidal anti-inflammatory drugs or platelet antiaggregant agents as well as if previous treatment with PPIs had been unsuccessful. Patients with history of esophageal or gastric surgery except for suture repair of a perforated gastric ulcer were excluded.

Study procedures

The study protocol included a baseline visit and a final visit, which was scheduled after 4 wk or 8 wk. At the baseline visit, eligibility criteria were assessed and the following data were recorded: demographic and anthropometric variables, and GERD-related symptoms including

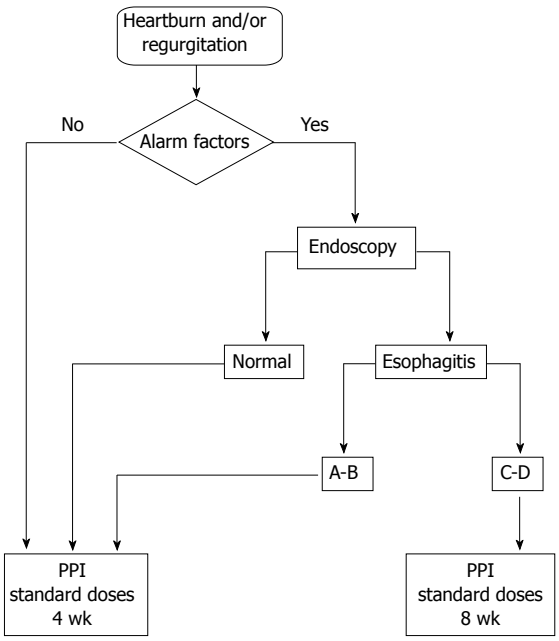

Figure 1 Management algorithm of patients with heartburn or regurgitation that should be used according to recommendations of the Spanish Clinical Practice Guideline. PPI: Proton pump inhibitor.

duration of symptoms, previous drug treatment, and frequency and intensity of typical symptoms. The frequency of each symptom was graded as 0 = absent or present less than 2 d per month, 1 = present more than 2 d per month, 2 = present weekly less than 3 d, and 3 = present 3 or more days per week. Intensity was graded as 0 = none, 1 = awareness of symptom but easily tolerated, 2 = discomfort sufficient to cause interference with normal activities, and 3 = incapacitating, with inability to perform normal activities. The product of frequency and intensity was used to define the severity of each symptom, which was expressed as the absolute number (0-1 = absent or irrelevant, 2 = mild, 3-4 = moderate, 6 = severe, and 9 = very severe).

Diagnostic and therapeutic decisions should be made based on specific recommendations of the Spanish clinical practice guideline for GERD[15], which is a widely disseminated and apparently well known instrument among specialists in digestive disease. The algorithm for the diagnosis and treatment of patients with GERD is summarized in the Figure 1. When upper gastrointestinal endoscopy was indicated, reasons and endoscopic findings should be recorded. The presence and severity of erosive esophagitis were determined using the Los Angeles classification[29]. According to the guideline, in the absence of erosive esophagitis or mild esophagitis (Los Angeles grade A or B) the duration of treatment was 4 wk but in the presence of esophagitis grades C and D, the duration of treatment was 8 wk. Treatment-related data included prescription of PPI, dose and duration of treatment (4 wk or 8 wk), and non-pharmacological measures (acid reflux diet, elevated position of the bed). The PPI selection (omeprazole, lansoprazole, pantoprazole, rabeprazole or esomeprazole) was made by individual medical preference.

Assessments at the end of treatment (4 wk or 8 wk) included frequency and intensity of symptoms to quantify the severity of disease after PPI treatment. Response to treatment was considered when patients became asymptomatic, that is, in the category of "absent or irrelevant". Patients were interviewed regarding their compliance with treatment, which was classified as excellent (> 95%), very good (> 90%), good (> 80%), fair (≥ 70%), bad (< 70%) and very bad (< 50%).

Statistical analysis

Sample size calculation was based on proportion of symptomatic treatment response after 4-8 wk. Considering a treatment response around 58%, with a ± 6% deviation, the needed sample size was 260 patients. Expecting a 15% lost during follow-up the final calculated sample size was 306 patients.

Categorical data are expressed as absolute number and percentages, and continuous data as mean and standard deviation. Response to PPI therapy according to adherence to guidelines in the doses prescribed was analyzed with the χ^2 test or fisher exact test. All analyses were performed using SAS 8.2 (SAS Institute, Cary, NC). A P value of less than 0.05 was considered statistically significant.

RESULTS

A total of 306 patients were recruited and 301 patients (58.5% men; age of 45 ± 14 years) fulfilled the inclusion criteria and were included in the study. All of them completed follow-up. The remaining 5 patients were excluded because of refusal to take part in the study (n = 2) and lack of fulfilment of the inclusion/exclusion criteria (n = 3). Mean time from the onset of GERD symptoms was 4.5 ± 6.3 years. Previous pharmacological treatment for GERD (not within 2 mo prior to the study) was recorded in 156 patients (51.9%), 87 of which had been treated with PPIs. Presenting complaints were heartburn in 99% of cases (nocturnal heartburn in 78%), regurgitation in 86%, and both heartburn and regurgitation in 85%. Only 3 patients complained of regurgitation only. A total of 273 patients (90.7%) presented dyspeptic symptoms and 174 (57.8%) supraesophageal symptoms. Distribution of heartburn, nocturnal heartburn and regurgitation according to severity of symptoms is shown in Figure 2. Symptoms were rated as severe or very severe in 56% of cases for heartburn, 34% for nocturnal heartburn and 35% for regurgitation.

Upper gastrointestinal endoscopy was performed in 123 patients (40.8%). According to participating gastroenterologists opinion indication of endoscopy was justified because of alarm symptoms in 50 patients and age over 50 years in 32. Adherence to the guidelines in relation to indication of upper gastrointestinal endoscopy

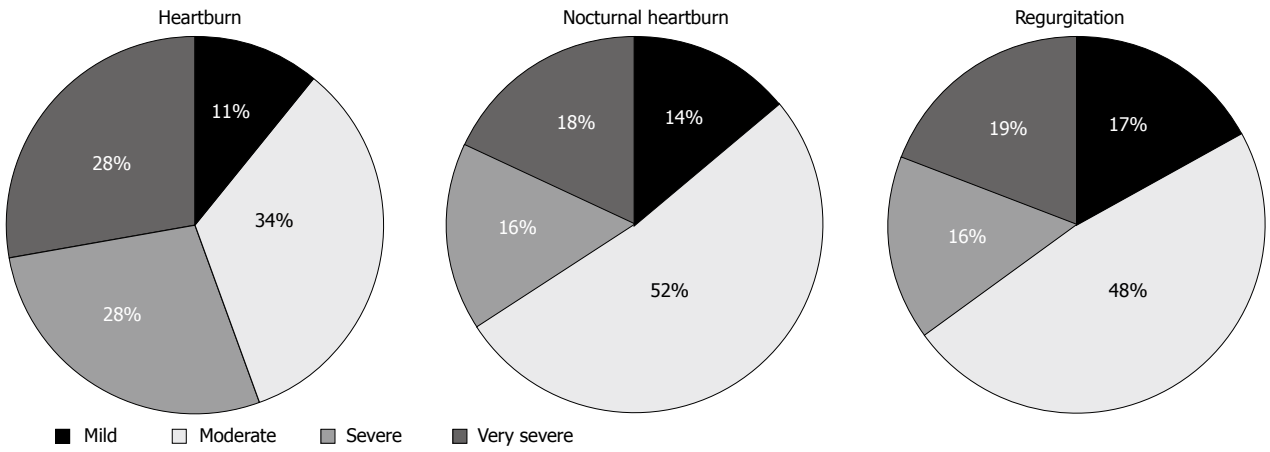

Figure 2 Distribution of typical gastro-esophageal reflux disease symptoms according to severity.

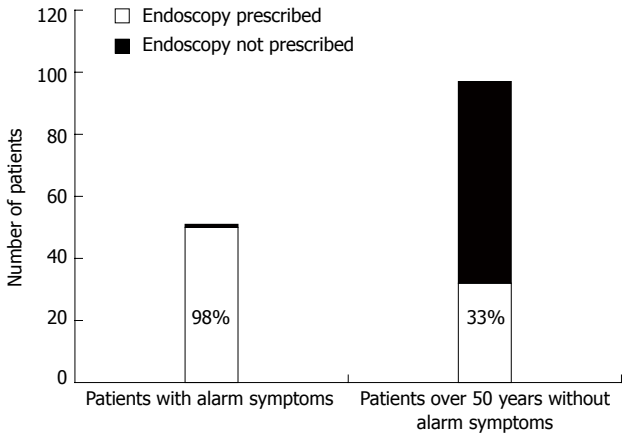

Figure 3 Upper gastrointestinal endoscopy was prescribed in 98% of patients with alarm symptoms (in accordance with the guideline) and in 33% of patients older than 50 years of age without alarm symptoms (not in accordance with the guideline).

was good due to the presence of alarm symptoms in 98% of cases. On the other hand, endoscopy was performed in 32 of 97 patients (33%) older than 50 years of age without alarm symptoms (Figure 3).

Esophagitis was diagnosed in 72 (58.3%) patients (grade A, 23, grade B, 28, grade C, 18, grade D, 3). Metaplastic changes of the esophageal mucosa suggestive of Barrett's oesophagus were documented in 9 patients and peptic stenosis in 1. Hiatal hernia was reported in 37 patients and Schatzki ring in 5.

Treatment with PPIs was prescribed to 298 patients (99%). In most cases (80%), there was adherence to the guidelines as the recommended dose of PPI was prescribed. However, doses prescribed were lower (half) in 4.8% of cases and higher (double) in 14.7%. The duration of PPI therapy was 4 wk in 63% of cases and 8 wk in 37%. In respect to duration of PPI therapy, adherence to guidelines was shown in 69% of cases (62% for the indication of 4 wk and 7% for 8 wk). Lack of adherence (31% of cases) included duration of treatment of 4 wk in patients with esophagitis grades C-D (0.7%) or 8 wk in patients esophagitis grades A-B (7.8%) or in patients without endoscopic studies (22.8%).

Compliance with pharmacological treatment was excellent in 58% of cases, very good in 30%, good in 8%, fair in 3% and bad or very bad in 1%. Acid reflux disease diet was recommended in 79% of patients and raising the bed position in 34%.

Response to PPI therapy was higher among patients who received PPI doses recommended by the guidelines as compared with patients treated with lower doses (95% *vs* 85%), although differences were not statistically significant ($P = 0.2$). Treatment response was similar in patients in which adherence to guidelines regarding duration of treatment was good or poor (95% *vs* 93%; $P = 0.5$).

Symptomatic response to treatment according to esophagitis grade was 87% in grade A, 89% in grade B and 100% in grades C and D. The indication to perform an upper gastrointestinal endoscopy had no effect on the rate of response to PPI treatment ($P = 0.9$) or whether there was good or poor adherence to the guidelines in terms of indication of the endoscopic study ($P = 0.7$).

DISCUSSION

GERD is a chronic condition in many cases and frequently requires prolonged therapy. Although complications are infrequent, the symptoms of reflux disease have a profound effect on quality of life and work performance, making GERD an expensive disease to manage. When different management strategies are compared, the cost of each strategy must be balanced against its effectiveness. For that purpose, various clinical practice guidelines have been developed based on the scientific evidence and recommendations made by experts[15-19]. In 2007, the Spanish Association of Gastroenterology, the Spanish Society of Family and Community Medicine and the Iberoamerican Cochrane Center developed a clinical practice guideline for GERD, in which two of the authors have collaborated (FM, JP), which was distributed throughout Spain both in printed version and through

the Internet[15]. Afterwards, it was investigated whether the GERD guideline had a practical impact through the Spanish gastroenterologists in the care of patients with GERD in routine daily practice.

The main findings of this study can be summarized as follows: (1) upper gastrointestinal endoscopy was requested in 41% of patients, mainly because of the presence of alarm symptoms but also because of age over 50 years without alarm symptoms; (2) severe esophagitis was infrequently diagnosed even though patients were selected for endoscopy; (3) in almost all cases (99%), treatment with PPIs was prescribed, in the majority of cases (80%) in accordance with doses recommended in the guidelines (lower doses were prescribed in only 5% of cases and higher doses in 15%); (4) adherence regarding duration of PPIs therapy was lower (69%) and most non-compliant behaviours were related to prolonged duration of treatment in patients with mild esophagitis or in those in which endoscopy was not performed; and (5) response to PPI treatment was higher in patients treated with doses of PPIs recommended in the clinical practice guidelines as compared with patients receiving other doses, although the difference was not statistically significant (95% *vs* 85%).

It is to note that, although GERD guideline compliance was quite good, endoscopy was overindicated. Thus, it was prescribed in 32 out of 97 patients (33%) older than 50 years of age without alarm symptoms. We have to emphasize here that not in the Spanish guideline neither in other very prestigious ones[30,31] is endoscopy recommended in patients with typical GERD symptoms (heartburn and/or regurgitation) older than 50 years and no alarm symptoms.

GERD is a very frequent cause of consultation in specialized gastroenterology practices as well as in the primary care setting. Consultation for GERD is associated with increased symptom severity and frequency, interference with social activities, sleep disturbance, higher levels of comorbidity, and psychological distress. Patients are less likely to consult if they feel that their doctor would trivialise their symptoms[4]. GERD has a negative impact on HRQoL being involved factors such as female gender, increased body mass index and nocturnal symptoms[5]. Nevertheless, when symptoms are properly treated, HRQoL improves in most of the cases. Thus, in a 5-year follow-up study of a large GERD population under routine care only a small minority of patients reported a clinically relevant decrease in HRQoL[32].

Although several clinical practice guidelines for GERD have been developed and published, to our knowledge, their clinical impact on gastroenterologists practice has not been evaluated. On the other hand, it is known that current GERD guidelines are infrequently used by primary care physicians. It has been shown that among 352 practitioners from 17 countries only 33% used an international and 14% used a national guideline in managing GERD patients[33]. However, in a retrospective Australian study it was shown a significant improvements in the diagnosis and management of GERD after a clinical guideline distribution and a 3-year self-audit process revealed a decrease in use of endoscopy, improved identification of risk factors, increase in recommendations for patient weight loss and reduction in use of medications that may exacerbate reflux symptoms[34]. Regarding antisecretory therapy it is well accepted that these medication are frequently overprescribed; when concordance between use of PPIs and prescribing guidelines was evaluated drug utilisation data indicated widespread use of PPIs outside current prescribing guidelines[35]. Another study tried to evaluate variability in dyspepsia management among Italian general practitioners; it was found that 44% of endoscopies prescribed for uninvestigated patients (more than half complaining of GERD symptoms) did not comply with the European Society for Primary Care Gastroenterology guideline[36].

In conclusion, compliance with the clinical practice guidelines for GERD by Spanish gastroenterologists is quite good, although a trend to indicate endoscopy in patients older than 50 years without alarm symptoms and treatments with higher PPI doses and longer duration than recommended was observed. Diagnosis of severe esophagitis is extremely infrequent, even in selected patients for endoscopy. Therapeutic response trended to be better when guideline recommendations were followed.

COMMENTS

Background

Gastro-esophageal reflux disease (GERD) is one of the most prevalent gastrointestinal disorders in the general population. GERD diagnosis is made mainly according to the presence of symptoms, especially heartburn and/or regurgitation. An effective management strategy is particularly important because GERD imposes a significant burden of illness. For that purpose several clinical practice guidelines have been developed by different medical societies and expert consensus meetings. However, the impact of these recommendations on the behaviour of physicians has generally been limited.

Research frontiers

It is a prospective, observational and multicentre study evaluating 301 patients with typical symptoms of GERD who should be managed in accordance with guidelines and were attended by gastroenterologists in daily practice. Diagnostic and therapeutic decisions should be made based on specific recommendations of the Spanish clinical practice guideline for GERD which is a widely disseminated and well known instrument among Spanish in digestive disease specialists.

Innovations and breakthroughs

The main findings of this study can be summarized as follows: Upper gastrointestinal endoscopy was requested in 41% of patients, mainly because of the presence of alarm symptoms but also because of age over 50 years without alarm symptoms. Severe esophagitis was infrequently diagnosed even though patients were selected for endoscopy.

Applications

In almost all cases (99%), treatment with Adherence for proton pump inhibitor (PPIs) was prescribed, in the majority of cases (80%) in accordance with doses recommended in the guidelines (lower doses were prescribed in only 5% of cases and higher doses in 15%). Adherence regarding duration of PPI therapy was lower (69%) and most non-compliant behaviours were related to prolonged duration of treatment in patients with mild esophagitis or in those in which endoscopy was not performed.

Terminology

Response to PPI treatment was higher in patients treated with doses of PPIs recommended in the clinical practice guidelines as compared with patients receiving other doses, although the difference was not statistically significant (95% *vs* 85%).

Peer review

Treatment practice guidelines are helpful in securing patients uniform treatment regardless of where they seek help. Such guidelines are of no use, however, if they are not followed. A study of adherence to guidelines is thus of significant importance. The presentation of the study is concise, and was approved by relevant ethics committees.

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BRIEF ARTICLE

IL28B polymorphism as a predictor of antiviral response in chronic hepatitis C

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Abstract

AIM: To evaluate the effect of single nucleotide polymorphisms of interleukin (IL)-28B, rs12979860 on progression and treatment response in chronic hepatitis C.

METHODS: Patients (n = 64; 37 men, 27 women; mean age, 44 ± 12 years) with chronic hepatitis C, genotype 1, received treatment with peg-interferon plus ribavirin. Genotyping of rs12979860 was performed on peripheral blood DNA. Histopathological assessment of necroinflammatory grade and fibrosis stage were scored using the METAVIR system on a liver biopsy sample before treatment. Serum viral load, aminotransferase activity, and insulin level were measured. Insulin resistance index, body mass index, waist/hip ratio, percentage of body fat and fibrosis progression rate were calculated. Applied dose of interferon and ribavirin, platelet and neutrophil count and hemoglobin level were measured.

RESULTS: A sustained virological response (SVR) was significantly associated with IL28B polymorphism (CC *vs* TT allele: odds ratio (OR), 25; CC *vs* CT allele: OR, 5.4), inflammation activity (G < 1 *vs* G > 1: OR, 3.9), fibrosis (F < 1 *vs* F > 1: OR, 5.9), platelet count (> 200 × 10^9/L *vs* < 200 × 10^9/L: OR, 4.7; OR in patients with genotype CT: 12.8), fatty liver (absence *vs* presence of steatosis: OR, 4.8), insulin resistance index (< 2.5 *vs* > 2.5: OR, 3.9), and baseline HCV viral load (< 10^6 IU/mL *vs* > 10^6 IU/mL: OR, 3.0). There was no association with age, sex, aminotransferases activity, body mass index, waist/hip ratio, or percentage body fat. There was borderline significance (P = 0.064) of increased fibrosis in patients with the TT allele, and no differences in the insulin resistance index between groups of patients with CC, CT and TT alleles (P = 0.12). Spearman's rank correlation coefficient between insulin resistance and stage of fibrosis and body mass index was r = 0.618 and r = 0.605, respectively (P < 0.001). Significant differences were found in the insulin resistance index (P = 0.01) between patients with and without steatosis. Patients with the CT allele and absence of a SVR had a higher incidence of requiring threshold dose reduction of interferon (P = 0.07).

CONCLUSION: IL28B variation is the strongest host factor not related to insulin resistance that determines outcome of antiviral therapy. Baseline platelet count predicts the outcome of antiviral therapy in CT allele patients.

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Key words: IL28B polymorphism; Hepatitis C; Fibrosis; Progression; Peg-interferon alpha; Sustained viral response

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INTRODUCTION

Investigations on genetic determinants of chronic hepatitis C (CHC) established that a single nucleotide polymorphism (SNP) in the interleukin (IL)-28B gene promoter region affected the spontaneous and induced clearance of hepatitis C virus (HCV)[1-5]. Among 500 000 genetic variants which were analyzed genome-wide, a few associated with the virologic response were identified, and showed variable frequency and importance across human ethnic groups[3,5-7]. The mechanism by which SNPs influence the outcome of HCV infection and its treatment is not clear. It is suggested that regulation of the promoter region of IL28B in antiviral activity may also affect two other genes belonging to interferon (IFN)-λ family encoded in this region[6,8-12]. According to some authors, IL28B SNPs are merely indicators of more complex mechanisms of CHC pathogenesis[13]. Some factors which determine the efficacy of antiviral therapy influence the course of CHC[14]. The impact of SNPs within the IL28B region on spontaneous and induced HCV clearance suggests the possibility that polymorphism affects the chronic phase of hepatitis. On the basis of previous research, there is no clear evidence that there is a correlation between IL28B SNPs and progression of untreated CHC. In a meta-analysis by Romero-Gomez *et al*[15], an effect of IL28B polymorphism on the progression of fibrosis was disputed. Research evaluating liver cirrhosis and fibrosis progression after liver transplantation in HCV-infected patients indicated a connection with IL28B SNPs[16,17].

However, about 50% of patients with a sustained virological response (SVR) do not carry the favorable IL28B alleles[15]. The factors which increase the chance of a therapeutic response in these patients are not yet known. A detailed analysis of the course of therapy of CHC with pegylated IFNα and ribavirin (RBV) in the presence of a hazardous IL28B allele might better delineate the clinical characteristics of this difficult-to-treat group of patients.

The aim of this study was to determine the impact of IL28B polymorphism on the progression of CHC, in order to identify predictors of effective therapy with IFN and RBV in the group of patients with unfavorable IL28B polymorphism.

Table 1 Characteristics of patients

IL28B polymorphism	*n* (%)	SVR (%)	Gender (M/F)	Age (yr)	BMI (kg/m^2)
CC	13 (20)	77			
CT	34 (53)	38			
TT	17 (27)	6			
All	64	39	27/37	44 ± 12	27 ± 5

The values of BMI and age given as arithmetic mean and standard deviation. CC, CT and TT: Single nucleotide polymorphisms at location *rs12979860* IL28B; SVR: Sustained virological response; BMI: Body mass index; M/F: Male/female.

MATERIALS AND METHODS

Sixty-four patients of European origin (Caucasians) with CHC due to HCV genotype 1 were included in the present study. Patients were treated with standard antiviral therapy with pegylated IFN 2a or 2b and RBV according to standard criteria of inclusion and exclusion. A liver biopsy was performed before treatment, and HCV viral load (COBAS® TaqMan® HCV 2.0) and insulin resistance index (HOMA-IR: homeostasis model of assessment) was determined.

The histopathological assessment of necroinflammatory grade and fibrosis stage were scored using the METAVIR system, and the presence of fatty liver disease was taken into consideration. The course of therapy was monitored on the basis of early virologic response (EVR), end of treatment response (ETR) and SVR.

The presence of the IL28B-related SNP *rs 12979860* variant was determined in whole blood samples. Genomic DNA was isolated from peripheral blood samples using proteinase K ion exchange column extraction kit (A&A Biotechnology, Gdynia, Poland). An amplification product of 190 bp length was obtained using standard polymerase chain reaction with the following primers: 5'- GCC TCT TCC TCC TGC GGG ACA AG and 5'- GCG CGG AGT GCA ATT CAA CCC T. Following digestion with *Bsh*1236I (*Bst*UI) restriction endonuclease, products were separated using agarose gel electrophoresis. The variant C was digested by the enzyme into fragments. All laboratory procedures were performed on blinded samples.

Patient age, sex, measurable anthropometric parameters such as waist, waist/hip ratio, body mass index (BMI) and percentage of body fat from MALTRON® measurements were analyzed in the present study.

Based on estimated duration of infection, the fibrosis progression rate was defined as the ratio between fibrosis stage and HCV duration. The serum aminotransferase levels prior to the start of treatment and after 72 wk was assessed. Patient characteristics are presented in Table 1.

The complete blood count at baseline and its changes during the course of therapy were determined. Thresholds for drug dose reduction were established: white blood cells < 750 cells/μL platelets < 70 000/μL, and

Table 2 Odds ratios of sustained virological response, depending on type of polymorphism of IL28B, inflammation activity, fibrosis, fatty liver, baseline hepatitis C virus viral load, the level of platelets and insulin resistance

Features affecting SVR	*P* value	OR	95% CI
CC *vs* TT	< 0.001	25	3.5-177.5
Platelets CT	0.002	12.8	1.8-42.6
Staging	0.008	5.9	1.5-23.8
CC *vs* CT	0.018	5.4	1.2 -23.3
Fatty liver	0.014	4.8	1.2-13.6
Platelets	0.005	4.7	1.3-17.3
Grading	0.026	3.9	1.1-13.4
Insulin resistance	0.029	3.9	1.1-13.8
HCV viremia	0.038	3	1-8.8

Difference in the risk of a SVR between patients with the CC allele and TT and CT alleles, low and high activity of the inflammatory process (G < 1 *vs* G > 1, according to METAVIR), little and advanced fibrosis (F < 1 *vs* F > 1, according to METAVIR), low and high hepatitis C viral load (< 10^6 IU/mL *vs* > 10^6 IU/mL), and absence or presence of fatty liver, high and low platelet count (> 200 × 10^9/L *vs* < 200 × 10^9/L), low or high insulin resistance index (< 2.5 *vs* > 2.5). Platelets CT: OR of SVR in patients with genotype CT with normal *vs* low platelets. SVR: Sustained virological response; CI: Confidence interval; HCV: Hepatitis C virus; OR: Odds ratio.

hemoglobin < 10 g/L. For each patient the dose of IFN and RBV used in the therapy was established and expressed as a percentage of the recommended dosage. The significance of differences in the clinical parameters as well as anthropometric, biochemical, histological and virological features between patients with and without a SVR was determined based on analysis of variance Kruskal-Wallis rank sum, Mann-Whitney *U* and χ^2 tests. A similar analysis was performed on patients only with the IL28B CT genotype. In patients with CC and TT genotypes, analysis of the diversity of the SVR was not performed because of the disproportionate distribution and small number of patients who did or did not achieve a SVR (Table 1).

Using the Kruskal-Wallis rank sum test, we assessed variations in the investigated clinical features between patients with different IL28B polymorphisms. In the logistic regression model we evaluated the odds ratio (OR) and 95% confidence interval (CI) of SVR, depending on the degree of inflammatory-necrotic activity, fibrosis scores, platelet count, baseline viral load, insulin resistance index (IR index) and IL28B polymorphisms (Table 2). The OR was determined against cases of high inflammatory-necrotic activity, above grade 1 in the METAVIR score, advanced fibrosis according to METAVIR F > 1, presence of steatosis, viral load > 1 × 10^6 IU/mL, alleles TT and CT, baseline platelet count < 200 × 10^9/L and IR index > 2.5.

The study was performed according to ethical guidelines of the 1975 Declaration of Helsinki. All patients were informed about the study, and provided written informed consent.

RESULTS

In the Mann-Whitney *U* test, differences were found in inflammatory-necrotic activity (P = 0.011), stage of fibrosis (P = 0.009), fibrosis progression rate (P = 0.015), EVR and ETR (P = 0.00001), baseline viral load (P = 0.09), reduction in viral load at 3 mo of therapy (P = 0.0004) and the presence of a biochemical response after completion of therapy (P = 0.03) between the patients who achieved a SVR and those who did not. In the group of patients who achieved a SVR, a lower incidence of liver steatosis was observed significantly (χ^2 test, P = 0.062). In the χ^2 test, there were no significant differences between patients with and without SVR as regards sex, age, comorbidities, and anthropometric factors. Differences in the parameters mentioned above were not observed among the three groups selected on the basis of the IL28B polymorphism. The χ^2 test showed differences in the rate of achievement of EVR (P = 0.02), ETR (P = 0.069) and SVR (P = 0.001) between groups of patients with CC, CT and TT alleles. The Kruskal-Wallis rank sum test showed differences in the size of the reduction of viral load after 3 mo of therapy (P = 0.019) between groups of patients with CC, CT and TT alleles. The OR revealed that patients with the CC allele had a 25-fold greater chance of achieving a SVR compared with those with the TT allele (P < 0.001) and a 5.4-fold higher chance compared with those with the CT allele (P = 0.018) (Table 2). Patients with low liver inflammation had a 3.9 times greater probability of achieving a SVR (P = 0.026), and patients with no or little fibrosis had 5.9 times greater probability (P = 0.008). HCV viral load < 1 × 10^6 IU/mL increased the chance of achieving a SVR by 3-fold (P = 0.038), and an IR index < 2.5 increased the chance by 3.9-fold (P = 0.029). The Kruskal-Wallis rank sum test showed no differences in the IR index between groups of patients with CC, CT and TT alleles (P = 0.12). Spearman's rank correlation coefficient between IR index and stage of fibrosis and BMI was r = 0.618 and r = 0.605, respectively (P < 0.001). In the Mann-Whitney *U* test, differences were found in the IR index (P = 0.005) between patients with and without steatosis. The Kruskal-Wallis rank sum test revealed borderline significance (P = 0.064) of increased fibrosis in patients with the TT allele (Figure 1).

The Mann-Whitney *U* test established that patients who did not achieve a SVR had low platelet levels at baseline (P = 0.001) compared with subjects with a SVR. A similar trend was also found for neutrophils (P > 0.05). Patients without SVR bearing the CT allele, were characterized by lower baseline platelet count (P = 0.003) and decreased (P = 0.02) drop of platelet count upon treatment. Decreased platelet levels in patients with the CT allele and absence of a SVR were accompanied by a higher incidence of requiring a dose reduction of IFN (P = 0.07). No differences in IFN and RBV dosage in patients with the CT allele or other allelic variants were found between groups with or without a SVR.

DISCUSSION

The present study observed an impact of IL28B SNPs

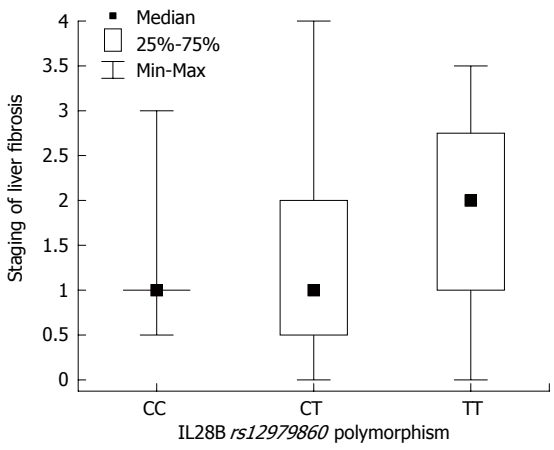

Figure 1 Liver fibrosis stage (by METAVIR) is increased in patients with the TT allele of the *rs12979860* region in the IL28B gene compared with patients with CC and CT alleles using the Kruskal-Wallis test (*P* = 0.06).

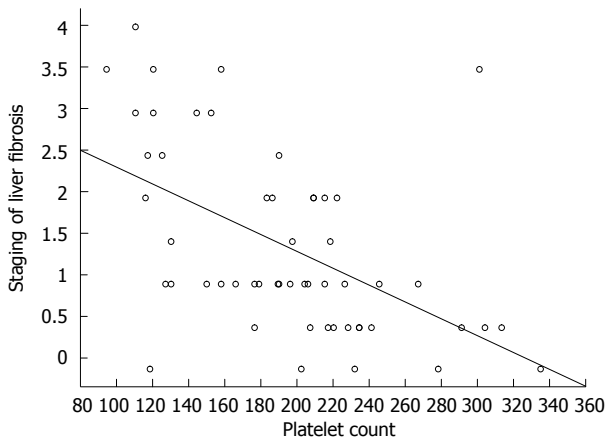

Figure 2 Correlation between liver fibrosis stage (by METAVIR) and the platelet count (10^3/L). Spearman's correlation coefficient r = -0.565, P < 0.001.

on the efficacy of IFN and RBV in CHC, which was consistent with the results from previous research. In patients with TT and CT genotypes, greater severity of liver fibrosis and necroinflammation, presence of liver steatosis, higher HCV viral load, and lower baseline platelets, had a reduced chance of therapeutic success. In the OR of the SVR, the greatest differences concerned the IL28B polymorphism between CC and TT alleles. The difference was more than 4-fold for fibrosis in the comparison of the CC allele with the CT allele. For liver steatosis, low platelet level, necroinflammatory grade, IR index and initial viral load of HCV, the difference was 5-8-fold (Table 2). The results confirm that polymorphism in the IL28B is significantly related to the outcome of antiviral therapy of CHC.

The mechanism by which genetic factors influence the progression of fibrosis in CHC remains unclear[18-21]. In the present study, patients with the TT genotype had a more active state of necroinflammation in the histological analysis, and borderline significance (P = 0.06) of greater severity of fibrosis (Figure 1). This observation is consistent with previous results describing a higher incidence of TT or CT allele in cases of cirrhosis and faster fibrosis progression in HCV-infected liver transplant recipients and liver from donors with the TT genotype[16,17]. Taking into consideration that the correlation between the degree of fibrosis and IL28B was not statistically significant and there was no association with fibrosis progression rate, it was not possible to confirm the impact of the genotype on the progress of CHC. Thus, IL28B polymorphism is probably not the factor which directly affects the outcome of untreated CHC.

The correlation between insulin resistance and stage of fibrosis, presence of the liver steatosis, efficacy of IFN/RBV therapy and higher BMI observed in the present study is consistent with the results from previous observations[22-27]. No statistically significant correlation between insulin resistance and IL28B polymorphism was observed, indicating that there are different mechanisms by which both factors can influence the course and therapy of CHC.

In the present study 60% of patients with SVR had TT (8%) or CT (52%) genotypes, which are not known factors associated with higher IFN responsiveness. These values are similar to those described in previous reports[15]. Analysis of the differences in the course of therapy in patients with the CT allele and a SVR revealed higher baseline platelet and neutrophil levels. These factors were markedly reduced in the group of patients with therapeutic failure. The present results are consistent with existing data, where the platelet level was a predictor of a rapid virological response[28]. In this study, a low baseline platelet count was significantly associated with the need for IFN dose reduction in the group without a SVR. In the group of patients with the CT allele, the platelet count was the only factor differentiating patients with and without a SVR. The chance of a SVR was 8-fold higher in patients with the CT allele and higher platelet count compared with patients with low levels of platelets.

The platelet level is one of the factors used to estimate the risk of the presence of advanced liver fibrosis[29]. In the present study, the platelet level also correlated with the stage of liver fibrosis (Figure 2). More severe fibrosis, and a lower baseline platelet level increased the risk of requiring a reduction in IFN dose and subsequent reduction in the chance of achieving a SVR[13]. Assessment of the platelet level and the IL28B polymorphism can complement the decision-making algorithm for a patient's eligibility for antiviral therapy.

Differentiation of SNPs in the IL28B promoter region is the strongest factor determining the effectiveness of therapy with IFN and RBV. Despite the greater severity of fibrosis in patients with the TT genotype, there was no additional evidence of an IL28B SNP associated with progression of CHC. In patients with the IL28B CT allele, a low baseline platelet count is associated with a requirement to reduce IFN and a decreased chance of obtaining a SVR.

COMMENTS

Background

Ineffective therapy for chronic viral hepatitis type C (CHC) determines the progression of liver disease to cirrhosis and primary hepatocellular carcinoma. Single nucleotide polymorphism (SNP) in the IL28B promoter gene region determines spontaneous and induced clearance of hepatitis C virus.

Research frontiers

Clinical aspects of the impact of IL28B SNPs on the efficacy of antiviral therapy and progression of CHC are not fully recognized. In the present study, potential factors influencing the efficacy of therapy in relation to IL28B polymorphism were investigated.

Innovations and breakthroughs

The results of the study confirm a significant role of SNPs in the IL28B gene among different predictive factors of a sustained virological response (SVR). The impact of IL28B SNPs on the SVR is not related to insulin resistance. In the case of the CT allele, a low baseline platelet count is associated with a reduced chance of a SVR. The phenomenon is connected with the stage of liver fibrosis and need for a reduction of interferon dose during therapy. The results suggest that the progression of CHC is faster in patients with the TT genotype.

Applications

Results of the study can complement the decision-making algorithm for eligibility of patients with CHC for antiviral therapy.

Terminology

Single nucleotide polymorphism is a variation of a single nucleotide usually found in non-coding microsatellite fragments of the genome. It may serve as the fingerprint for predicting susceptibility toward disease. IL28B SNPs include different variants localized in the gene promoter region, among which the most common in clinical practice is C/T SNP of *rs 12979860*.

Peer review

This study examined the association of SNPs of the IL28B, rs12979860 on progression of CHC. They found that SNPs of IL28B had any impact on CHC progression, in spite of apparently more advanced fibrosis among patients carrying TT genotype. In addition, they revealed that, in patients with the CT allele IL28B, low baseline platelets count is linked to the reduction of IFN and decreased chance of reaching the SVR. This study demonstrated a new characteristic of IL-28b in CHC.

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BRIEF ARTICLE

Analysis of colonoscopic perforations at a local clinic and a tertiary hospital

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Abstract

AIM: To define the clinical characteristics, and to assess the management of colonoscopic complications at a local clinic.

METHODS: A retrospective review of the medical records was performed for the patients with iatrogenic colon perforations after endoscopy at a local clinic between April 2006 and December 2010. Data obtained from a tertiary hospital in the same region were also analyzed. The underlying conditions, clinical presentations, perforation locations, treatment types (operative or conservative) and outcome data for patients at the local clinic and the tertiary hospital were compared.

RESULTS: A total of 10 826 colonoscopies, and 2625 therapeutic procedures were performed at a local clinic and 32 148 colonoscopies, and 7787 therapeutic procedures were performed at the tertiary hospital. The clinic had no perforations during diagnostic colonoscopy and 8 (0.3%) perforations were determined to be related to therapeutic procedures. The perforation rates in each therapeutic procedure were 0.06% (1/1609) in polypectomy, 0.2% (2/885) in endoscopic mucosal resection (EMR), and 3.8% (5/131) in endoscopic submucosal dissection (ESD). Perforation rates for ESD were significantly higher than those for polypectomy or EMR ($P < 0.01$). All of these patients were treated conservatively. On the other hand, three (0.01%) perforation cases were observed among the 24 361 diagnostic procedures performed, and these cases were treated with surgery in a tertiary hospital. Six perforations occurred with therapeutic endoscopy (perforation rate, 0.08%; 1 per 1298 procedures). Perforation rates for specific procedure types were 0.02% (1 per 5500) for polypectomy, 0.17% (1 per 561) for EMR, 2.3% (1 per 43) for ESD in the tertiary hospital. There were no differences in the perforation rates for each therapeutic procedure between the clinic and the tertiary hospital. The incidence of iatrogenic perforation requiring surgical treatment was quite low in both the clinic and the tertiary hospital. No procedure-related mortalities occurred. Performing closure with endoscopic clipping reduced the C-reactive protein (CRP) titers. The mean maximum CRP titer was 2.9 ± 1.6 mg/dL with clipping and 9.7 ± 6.2 mg/dL without clipping, respectively ($P < 0.05$). An operation is indicated in the presence of a large perforation, and in the setting of generalized peritonitis or ongoing sepsis. Although we did not experience such case in the clinic, patients with large perforations should be immediately transferred to a tertiary hospital. Good relationships between local clinics and nearby tertiary hospitals should therefore be maintained.

CONCLUSION: It was therefore found to be possible to perform endoscopic treatment at a local clinic when sufficient back up was available at a nearby tertiary hospital.

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Key words: Colonoscopy; Colon perforation; Endoscopic clipping; Endoscopic submucosal dissection; Endoscopic mucosal resection; Polypectomy

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INTRODUCTION

Colonoscopy is widely used for the diagnosis, treatment, and follow-up of colorectal diseases[1,2]. The improvement of the equipment and increased needs for screening colonoscopy has increased the number of colonoscopies rapidly not only at hospitals but also in clinics. It is convenient for patients to have lesions treated when they are identified by either a routine-check up or diagnostic endoscopy. Therefore, therapeutic endoscopy is sometimes needed at a local clinic, including on the day surgery to perform polypectomy. The occurrence of complications in such cases negatively affects the quality life of these patients. Bleeding after polypectomy is the most common complication[3]. However, the development of endoscopic clipping prevents the occurrence of bleeding after polypectomy[4]. Therefore, cases that need surgical treatment for bleeding after therapeutic procedures are quite rare. Although perforation occurs less often, it is more problematic than bleeding and should be given the most attention[5,6]. Perforations sometimes require surgical intervention and will decrease the patients' quality of life. Several large, retrospective studies have determined perforation incidences of 0.02%-0.8% and 0.15%-3% for diagnostic and therapeutic colonoscopy, respectively[6-11].

Surgery has been the mainstream treatment for iatrogenic perforation[12-14]. Surgical treatment for iatrogenic perforation should be avoided at local clinics because most such clinics do not have the appropriate equipment for such surgery. As a result, the performance of therapeutic endoscopy has so far not become common at local clinics. However, the use of endoscopic clipping to prevent the leakage of intestinal contents can circumvent the need for surgery[15,16]. Although surgical treatment should be selected when it is needed, the use of endoscopic clipping could potentially extend the therapeutic indications for such treatments at local clinics.

The aim of this study was to determine the incidences, clinical presentations, and management of iatrogenic perforations that occurred after diagnostic and therapeutic colonoscopy at a local clinic. This study compared the data between a local clinic and a tertiary hospital. Finally, the optimal strategies for performing therapeutic endoscopy and steps for dealing with complications at local clinics are also discussed.

MATERIALS AND METHODS

This study retrospectively reviewed the patient database of colonoscopies, and therapeutic procedures at Shirakawa Clinic (Maebashi, Japan) between April 2006 and December 2010. The data from Maebashi Red Cross Hospital (Maebashi, Japan), a tertiary hospital in the same region were also analyzed and compared between January 1996 and December 2010. Endoscopies at Shirakawa Clinic and Maebashi Red Cross Hospital are performed or supervised by staff gastroenterologists or fellows. The Shirakawa Clinic has 19 inpatient beds and appropriate management, such as drip infusion, can be easily carried out. However, the performance of either surgery or intensive care is restricted and patients required such case are therefore referred to the Maebashi Red Cross Hospital which is a tertiary hospital. Patients that required treatment for an iatrogenic colon perforation during the study period were analyzed. The underlying conditions, clinical presentations, perforation locations, treatment types (operative or conservative), and outcome data were analyzed. Possible complications were explained to all patients before the procedures, and all provided their written consent. Outpatients were informed to contact the clinic if they experienced any post-procedural abdominal distension or pain. This study was approved by the institutional ethical committee (No. SC2011/003; date, December 10, 2010).

Endoscopic procedures

The device for diagnostic endoscopy was a single-channel endoscope (CF260AI, Olympus Optical Co, Tokyo, Japan). A single-channel endoscope (GIF230 and/or CF260AI, Olympus) with a hood and a high-frequency generator with an automatically controlled system (Erbotom ICC200 or VIO 300D, ERBE, Tuebingen, Germany) were used for the endoscopic submucosal dissection (ESD) procedure. The patients principally received 24 mg sennoside the night before the examination for bowel preparation, and drank 200 mL polyethylene glycol (PEG) solution (Niflec®, Ajinomoto Pharma Co., Ltd., Tokyo, Japan) every 10 min on the examination day, for a total intake of 2000 mL PEG solution. Polypectomy and endoscopic mucosal resection (EMR) were performed as usual[17,18]. The ESD procedure was performed as described previously[19-21]. Abdominal X-rays were routinely performed after therapeutic procedures to check the perforation. Patients without complications were permitted to take soft food the day after the therapeutic procedures. Hemoclips (HX-600-135 and HX-600-090L, Olympus) were used for the endoscopic closure of any perforation.

Statistical analysis

The data were expressed as the mean ± SD. The data were analyzed using the Fisher's exact probability test,

Table 1 Frequency of perforation depending on the type of endoscopic procedure

		Total	Diagnostic	Therapeutic	Polypectomy	EMR	ESD
Shirakawa Clinic between April 2006 and December 2010							
Number	*n*	10 826	8201	2625	1609	885	131
Perforation	*n* (%)	8 (0.07)	0 (0)	8 (0.3)	1 (0.06)	2 (0.2)	5 (3.8)
Maebashi Red Cross Hospital between January 1996 and December 2010							
Number	*n*	32 148	24 361	7787	5500	2244	43
Perforation	*n* (%)	9 (0.03)	3 (0.01)	6 (0.08)	1 (0.02)	4 (0.17)	1 (2.3)

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

Table 2 The cases with iatrogenic perforation at the Shirakawa Clinic

Case	Age	Gender	Procedure	Site of perforation	Disease	Discovery of perforation	Abdominal pain	Peritonitis sign	Extrabowel gasses	Treatment	Max body temperature (℃)	CRP (mg/dL)	Cessation of food intake (d)	Intravenous antibiotics treatment (d)	Total hospital stay (d)
1	71	F	EMR	Ascending	Adenocarcinoma	Just after procedure	+	No	Free air	Conservative (with clipping)	36.9	4.4	1	6	7
2	57	M	ESD	Ascending	Adenocarcinoma	During	-	No	Free air	Conservative (with clipping)	36.8	0.9	1	4	9
3	69	F	ESD	Caecum	Adenoma	During	-	No	PP[1]	Conservative (with clipping)	37.5	3.8	2	8	13
4	66	M	ESD	Rectm (Rb)	Adenoma	Just after procedure	-	No	PP	Conservative (without clipping)	37.9	17.9	1	6	12
5	80	M	ESD	Caecum	Adenoma	Just after procedure	-	No	Free air	Conservative (without clipping)	37.0	4.2	2	6	10
6	68	M	Polypectomy	Transverse	Adenoma	1 d after procedure	+	Located	Free air	Conservative (without clipping)	36.9	11	4	7	10
7	64	M	EMR	Ascending	Adenocarcinoma	2 d after procedure	+	Located	Free air	Conservative (without clipping)	36.9	5.5	4	6	11
8	64	F	ESD	Ascending	Adenocarcinoma	During	+	Located	Free air	Conservative (with clipping)	37.2	2.4	3	5	11

[1]Pneumoretroperitoneum. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; CRP: C-reactive protein; M: Male; F: Female.

and Mann-Whitney's *U*-test. A *P* value less than 0.05 was considered to be significant.

RESULTS

Patients and characteristics

A total of 10 826 colonoscopies, and 2625 therapeutic procedures were performed at the Shirakawa Clinic between April 2006 and December 2010. Eight (0.07%) perforations were attributed to endoscopy (Table 1). There were no perforation cases for diagnostic procedures. On the other hand, a therapeutic procedure was performed in the 8 perforation cases (perforation rate, 0.3%; 1 per 328 procedures). The perforation rates for specific procedure types were 0.06% (1 per 1609) for polypectomy, 0.2% (1 per 443) for EMR, and 3.8% (1 per 26) for ESD. Perforation rates for ESD were significantly higher than those for polypectomy or EMR ($P < 0.01$). The cases with iatrogenic perforation are shown in Table 2. The study group included 3 females and 5 males aged from 57 years to 80 years of age (mean age 67.4 ± 6.6 years).

A total of 32 148 colonoscopies, and 7787 therapeutic procedures were performed in Maebashi Red Cross Hospital, and 9 (0.03%) perforations were attributed to endoscopy (Table 1). There were 3 (0.01%) perforation cases among the diagnostic procedures. Six perforations occurred with therapeutic endoscopy (perforation rate, 0.08%; 1 per 1298 procedures). Perforation rates for specific procedure types were 0.02% (1 per 5500) for polypectomy, 0.17% (1 per 561) for EMR, 2.3% (1 per 43) for ESD. The patients included 3 females and 6 males from 59 years to 81 years of age (mean age 69.2 ± 8.5 years). The cases with perforation during diagnostic endoscopy were complicated with diverticulitis, radiation colitis and amyloidosis, respectively. These 3 perforations during diagnostic endoscopy were treated by surgery. There were no significant differences in the perforation rates for each therapeutic procedure between the local clinic and the tertiary hospital.

Management and outcomes of perforations in the clinic

Three of 8 cases (37.5%) perforations were detected during the endoscopic procedure due to the visualization of a tear in the serosa. Six (75%) patients were diagnosed to have a perforation within 24 h of colonoscopy. Two patients were diagnosed to have a perforation more than 24 h later. These cases were considered to be delayed perforation caused by electrocautery. Four (50%) of 8 patients had been undergone clipping. Six (75%) patients had free intraperitoneal air and 2 patients showed pneumoretroperitoneum by abdominal radiography or computed tomography (CT). There were no symptoms of peritonitis in 5 patients and 3 patients showed localized peritonitis. All patients were managed conservatively because there no progression to peritonitis symptoms, Conservative treatment included the withholding of oral intake, hydration, intravenous antibiotics and serial abdominal examinations.

The mean maximum C-reactive protein (CRP) titer was 6.3 ± 5.6 mg/dL (range 0.9-17.9 mg/dL) and maximum body temperature was 37.1 ± 0.4 ℃ (range: 36.8-37.9 ℃). The mean fasting period for these patients was 2.3 ± 1.3 d (range: 1-4 d) and the mean hospital stay following perforation was 10.4 ± 1.8 d (range: 7-13 d). The points of references used to discontinue fasting were relief of abdominal pain, improvement of leukocytosis. The mean duration of total intravenous antibiotic treatment was 6.0 ± 1.2 d (range 4-8 d). Flomoxef sodium (FMOX) was administered in all cases. When fasting had been completed, oral ciprofloxacin was prescribed.

The mean maximum CRP titer was 2.9 ± 1.6 mg/dL with clipping and 9.7 ± 6.2 mg/dL without clipping, respectively. The mean maximum CRP titer was significantly lower in the patients with clipping ($P < 0.05$). The duration of fasting with and without clipping were 1.8 ± 1.0 d and 2.8 ± 1.5 d, respectively. The duration of intravenous antibiotics treatment with and without clipping was 5.8 ± 1.7 d and 6.3 ± 0.5 d, respectively. The duration of total the hospital stay with and without clipping were 10.0 ± 2.6 d and 10.8 ± 1.0 d, respectively. There were no significant differences in the total hospital stay, duration of fasting, dose of intravenous antibiotics between those that had or had not undergone clipping because of the small number of patients with perforation. However, the duration of fasting, dose of intravenous antibiotics and total hospital stay tended to be shorter when clipping was successful in comparison to when clipping was not performed.

Representative cases

Perforation discovered during the therapeutic procedure and treated with clipping: The case was 57-year-old male with no major complications. Perforation was observed during an ESD procedure for ascending colon adenocarcinoma (Figure 1A). Carbon dioxide inflation was used during ESD. Endoscopic clipping closed the small tear of the serosa. Abdominal X-ray showed a small amount of extra-bowel gasses. This case was treated conservatively. FMOX was administered for 4 d. The patient fasted for 1 d and food intake was started at day 3. The patient was discharged at day 9.

Perforation discovered more than 24 h after therapeutic procedure and treated without clipping: The case was a 64-year-old male with a past history of myocardial infarction. Abdominal pain was appeared after 2 d an EMR procedure for ascending colon adenoma. Abdominal XP and CT findings revealed free air (Figure 1B). This case was treated conservatively because the symptoms of peritonitis were localized. FMOX was administered for 6 d. The patient fasted for 4 d and food intake was started at day 6. The patient was discharged on day 11.

Perforation discovered just after a therapeutic procedure and treated with suture clippings: The case was a 71-year-old female complicated with hypertension. This case was treated by usual air inflation during the EMR procedure. Abdominal XP and CT findings revealed free air just after the EMR procedure for ascending colon adenoma (Figure 1C). Inflation was changed to CO_2 inflation and endoluminal repair with suture clipping was performed. FMOX was administered for 6 d. The patient fasted for 1 d and food intake was started on day 3. The patient was discharged on day 7.

Trouble shooting for perforation at a local clinic

A management algorithm for colonoscopic bowel perforation at a clinic is shown in Figure 2. A minor colon injury that is recognized during colonoscopy should be treated by changing to CO_2 inflation, endoluminal repair with clips and further conservative treatment could avoid immediate surgical intervention. Delayed endoscopic repair with clipping should be considered only if the condition of the patient is stable and a specific site is highly suspected. This latter recommendation should be useful when the perforation is suspected in the rectosigmoid area, because perforations in this region are easily located on scope reinsertion. An operation is indicated in the presence of a large perforation, and in the setting of generalized peritonitis or ongoing sepsis. Such patients should be immediately transferred to a tertiary hospital. A good relationship between local clinics and nearby tertiary hospitals should therefore be maintained.

DISCUSSION

The perforation risks of diagnostic and therapeutic colonoscopy are 0.02%-0.8% and 0.15%-3%, respectively[6-11]. Large studies of iatrogenic perforation related to colonoscopy are shown in Table 3[6-11,22-24]. New therapeutic approaches including ESD techniques have become more popular and the risk of perforation is increased in comparison to conventional techniques such as polypectomy or EMR. The perforation risk of a therapeutic procedure is usually higher than that of a diagnostic procedure. Fortunately, there were no perforations during diagnostic procedures at the local clinic in the present study.

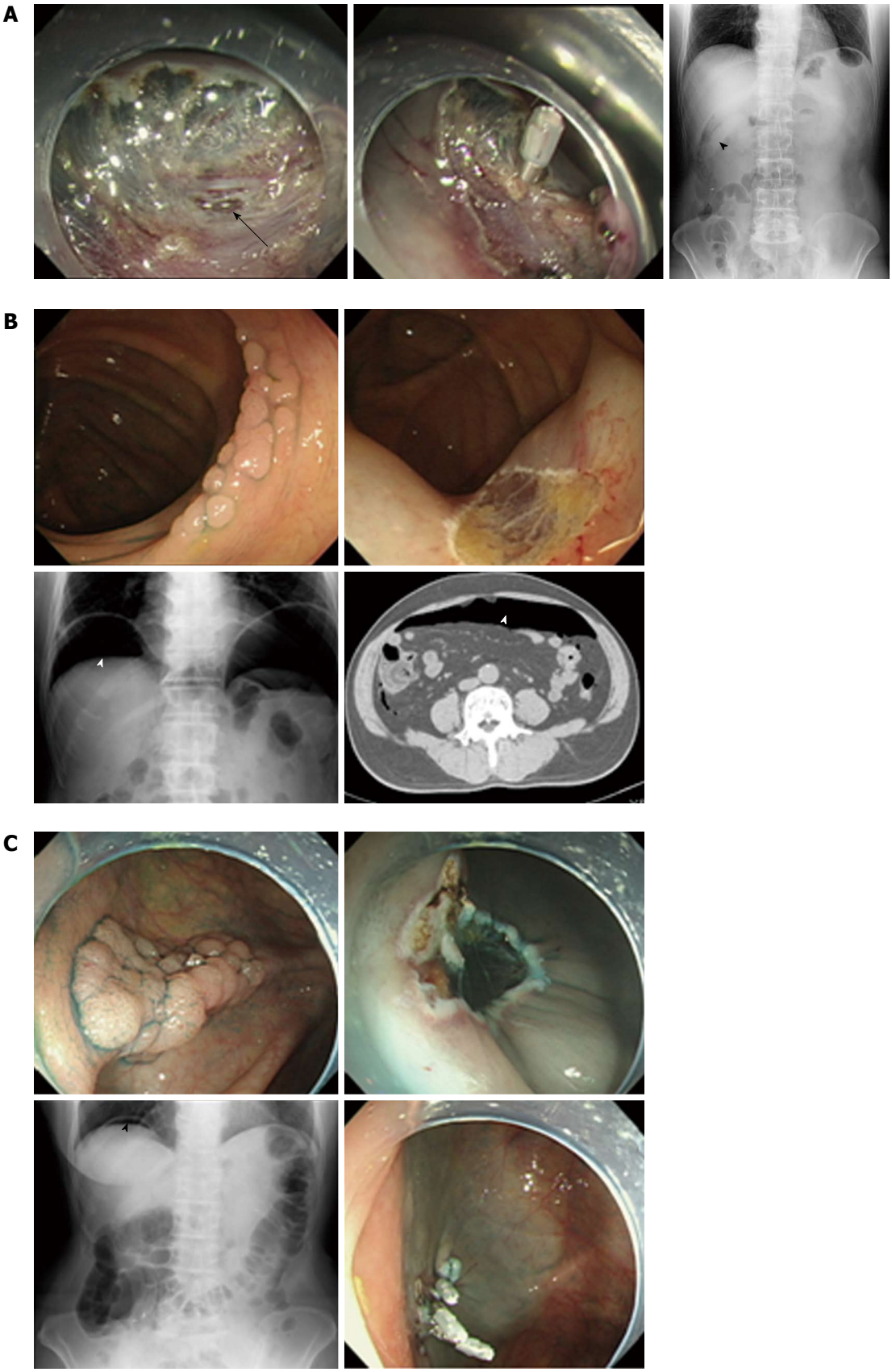

Figure 1 Representative cases of colonoscopic perforations. A: Case 1: Perforation discovered during the therapeutic procedure and treated with clipping; B: Case 2: Perforation discovered more than 24 h later after the therapeutic procedure and treated without clipping; C: Case 3: Perforation discovered just after the therapeutic procedure and treated with suture clipping.

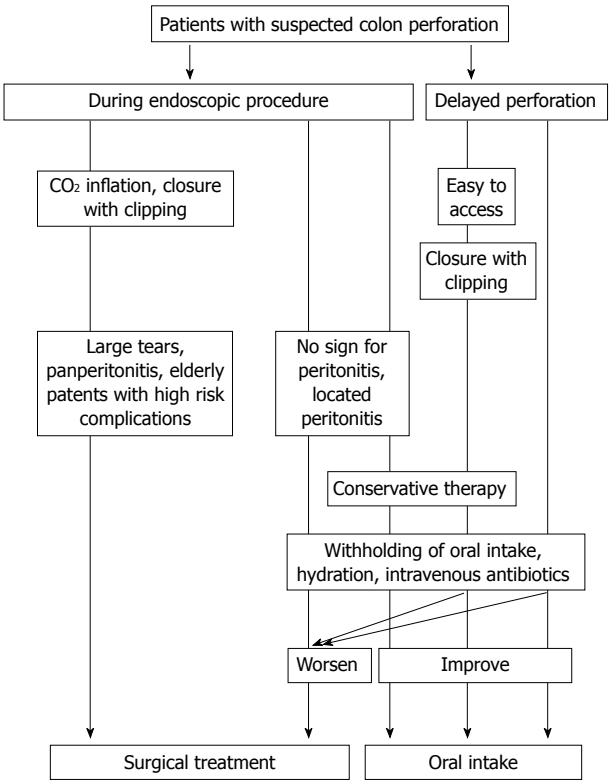

Figure 2 Trouble shooting for perforation at the local clinic.

Recent studies have demonstrated the possibility of endoscopic perforation closure using endoclips[25,26]. Closure by endoscopic clipping significantly reduced the maximum CRP titer in this study. Successful closure with endoscopic clipping in conservatively managed patients can reduce the fasting period, duration of intravenous antibiotic administration, and hospital stay[25,26]. As a result, successful clipping can improve patient quality of life and reduce medical costs. This study found no significant differences in the duration of fasting, intravenous antibiotics treatment, total hospital stay between those that had or had not undergone clipping. It may be due to the small number of patients with perforation.

Perforations associated with diagnostic procedures are usually due to applying pressure to the colonic wall, and they are noticed immediately. However, perforations that occur after therapeutic procedures are often diagnosed late. Ischemia of the colonic wall caused by electrical or thermal injury after electrocoagulation can cause a delayed perforation following therapeutic procedures[10]. Clipping can induce mucosal and submucosal healing and prevent fecal soiling of the peritoneal cavity when the perforation is small and significant colonic pathology does not exist. However, the application of clipping for perforation in delayed perforation still remains controversial. Delayed endoscopic repair with clipping should be considered only if the condition of the patient is stable and a specific site is highly suspected[6]. This recommendation is likely to be useful when the perforation is suspected to be in the rectosigmoid area, because perforations in this region are easily located on scope reinsertion[6].

Table 3 Large study of iatrogenic perforation related to colonoscopy *n* (%)

Ref.	Total	Diagnostic	Therapeutic	Polypectomy	EMR	ESD
Anderson *et al*[11]	59 987					
	22 (0.04)					
Cobb *et al*[10]	43 609					
	14 (0.03)					
Kaneko *et al*[24]	3 152 053	2 587 689	564 364	422 119	142 245	ND
	1387 (0.04)	568 (0.02)	819 (1.4)	621 (0.15)	198 (0.14)	ND
Iqbal *et al*[9]		78 702				
		66 (0.84)				
Tulchinsky *et al*[23]	120 067					
	7 (0.06)					
Lüning *et al*[7]		30 366				
		35 (0.12)				
Lüning *et al*[7]		433 816				
		393 (0.09)				
Taku *et al*[8]			15 160	8240	1906	43
			23 (0.15)	4 (0.05)	12 (0.63)	6 (14.0)
Kang *et al*[6]	44 534	37 762	6772			
	53 (0.12)	26 (0.07)	27 (0.40)			
Oka *et al*[22]			71 204	34 433	36 083	688
			62 (0.09)	6 (0.02)	33 (0.09)	23 (3.3)

Upper column: Number of cases; lower column: Perforation (%). EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; ND: No data.

The limitations of this study include the retrospective nature of the work and the inclusion of descriptions of local experience without the addition of new protocols. However, we believe that investigating a large number of patients at a local clinic will elucidate the role the local clinic plays in therapeutic endoscopies. Furthermore, selection bias between the two institutes of patients or endoscopists may have existed. The incidence of complications depends on the type of lesion or the skill of the endoscopist. Selection bias of patients or endoscopists is a problem to be evaluated in a future study.

Selective patients are likely to improve under conservative management involving hospitalization, intestinal rest, intravenous fluids, and antibiotics to limit peritonitis and allow the perforation to seal. However, conservative management requires careful observation with frequent and repeated abdominal exams. Patients successfully treated non-surgically must be clinically stable, and their abdominal symptoms should improve rapidly with no deterioration due to peritoneal signs[14]. A local clinic does not have any surgical options, so a good relationship and close contact with a tertiary hospital is needed. Patients must be immediately transferred to a tertiary hospital when either abdominal symptoms are observed or peritonitis worsens.

In conclusion, iatrogenic colonic perforation is a serious but uncommon complication of colonoscopy. However, surgery is not mandatory for perforations caused by therapeutic procedures, and endoscopic perforation closure using endoclips should be considered as a helpful adjunct to conservative treatment. It is possible to perform endoscopic treatment at a local clinic when there is appropriate back-up by a nearby tertiary hospital. Of course, close contact between local clinics and a tertiary hospital is essential.

COMMENTS

Background

The number of colonoscopies has increased rapidly, not only in hospitals, but also in clinics. The occurrence of complications negatively affects the quality life of patients. The aim of this study was to determine the incidence, clinical presentation and management of iatrogenic perforations that occurred after diagnostic and therapeutic colonoscopies at a local clinic.

Research frontiers

Data and management strategies regarding iatrogenic perforations at hospitals were reported. However, such reports from clinics were rare. Optimal strategies for performing therapeutic endoscopy and steps for managing complications at local clinics were also discussed.

Innovations and breakthroughs

This study investigated the incidence, management and outcomes of colonoscopic perforation in a local clinic. Performing endoscopic perforation closure using endoclips should be considered as a helpful adjunct to conservative treatment. It is possible to perform endoscopic treatment at a local clinic when appropriate back-up support at a nearby tertiary hospital is available.

Applications

The number of endoscopic treatments performed at local clinics will increase when appropriate back-up support at nearby tertiary hospitals is available.

Terminology

A colonoscopic perforation is a complication of diagnostic or therapeutic colonoscopy. Although perforation occurs infrequently, it is problematic and should be given proper attention. Perforations sometimes require surgical intervention, and will decrease a patient's quality of life.

Peer review

The study is well designed and the paper is well written. Although there is no novelty in the idea and only a description of the local experience is provided, the study is a nice piece of work that includes a large number of patients.

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BRIEF ARTICLE

High-risk esophageal varices in patients treated with locoregional therapy for hepatocellular carcinoma: Assessment with liver computed tomography

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Abstract

AIM: To assess the diagnostic performance of follow-up liver computed tomography (CT) for the detection of high-risk esophageal varices in patients treated with locoregional therapy for hepatocellular carcinoma (HCC).

METHODS: We prospectively enrolled 100 patients with cirrhosis who underwent transcatheter arterial chemoembolization, radiofrequency ablation or both procedures for HCCs. All patients underwent upper endoscopy and subsequently liver CT. Three radiologists independently evaluated the presence of high-risk esophageal varices with transverse images alone and with three orthogonal multiplanar reformation (MPR) images, respectively. With endoscopic grading as the reference standard, diagnostic performance was assessed by using receiver operating characteristic (ROC) curve analysis.

RESULTS: The diagnostic performances (areas under the ROC curve) of three observers with transverse images alone were 0.947 ± 0.031, 0.969 ± 0.024, and 0.916 ± 0.038, respectively. The mean sensitivity, specificity, positive predicative value (PPV), and negative predicative value (NPV) with transverse images alone were 90.1%, 86.39%, 70.9%, and 95.9%, respectively. The diagnostic performances, mean sensitivity, specificity, PPV, and NPV with three orthogonal MPR images (0.965 ± 0.025, 0.959 ± 0.027, 0.938 ± 0.033, 91.4%, 89.5%, 76.3%, and 96.6%, respectively) were not superior to corresponding values with transverse images alone ($P > 0.05$), except for the mean specificity ($P = 0.039$).

CONCLUSION: Our results showed excellent diagnostic performance, sensitivity and NPV to detect high-risk esophageal varices on follow-up liver CT after locoregional therapy for HCC.

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Key words: Liver computed tomography; High-risk esophageal varices; Locoregional therapy; Hepatocellular carcinoma; Multiplanar reformation images

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INTRODUCTION

More than one-half of patients with cirrhosis eventually develop esophageal varices[1]. Screening upper endoscopy is repeated in 1-3 years in order to evaluate esophageal varices in cirrhotic patients owing to the substantial morbidity and mortality related with variceal bleeding[2]. The presence of high-risk varices seen in up to 30% of patients with esophageal varices on endoscopy is considered as an indication for prophylaxis against variceal bleeding with either endoscopic variceal ligation (EVL) or the administration of β-blockers[1,3].

Of several recent studies that have suggested the usefulness of computed tomography (CT) scans to evaluate esophageal varices[4-8], one study[7] asserted that CT was a better initial screening modality when compared to endoscopy in terms of cost-effectiveness and patient preference. However, for only these reasons, the adoption of CT to replace ultrasound as the surveillance imaging technique in all cirrhotic patients is still subject to debate with the consideration of radiation hazards[9]. As CT is commonly used as a follow-up imaging modality in patients treated with locoregional therapy for hepatocellular carcinoma (HCC)[10], we supposed that replacement of CT for evaluating varices was more persuasive in such patients. The purpose of this study was to evaluate prospectively the diagnostic performance of radiologists for the detection of high-risk esophageal varices on regular follow-up liver multi-detector row computed tomography (MDCT) examinations in patients treated with locoregional therapy for HCC. We also assessed the usefulness of the addition of coronal and sagittal multiplanar reformation (MPR) images.

MATERIALS AND METHODS

Study design and patients

The Institutional Review Board approved the current study and all subjects provided informed consent. In this prospective study, the sample size was determined for overall diagnostic performance based on the area (A_z) under the composite receiver operating characteristic (ROC) curve using pooled data of all observers. We expected that the pooled A_z for the diagnosis of high-risk varices on CT is 0.95, with a width of the 95% confidence interval (CI) of 0.05. When the ratio of the number of patients with high-risk varices ranges from 1:1 to 1:6 and the ratio of standard deviation of pooled A_z with high-risk varices ranges from 1:1 to 3:1, the standard error of estimates of A_z in 100 subjects is $\leqslant$ 0.025. Thus, the 95%CI of A_z is $\leqslant$ 0.05[11].

From June 2009 to December 2010, we enrolled patients with cirrhosis as well as HCCs treated with transcatheter arterial chemoembolization (TACE), radiofrequency (RF) ablation or both procedures who were scheduled to undergo both a regular follow-up liver CT examination and a screening upper endoscopy. We excluded patients who had a history of undergoing EVL for esophageal varices[5]. One-hundred patients who agreed to participate in our prospective study underwent an endoscopy and a subsequent liver MDCT examination within four hours after endoscopy. All patients had undergone one or more regular (all within a 6-mo interval) follow-up liver MDCT examinations following TACE (n = 43), RF ablation (n = 35) or both procedures (n = 22) for HCCs. The patient population consisted of 79 men and 21 women (age range, 35-82 years; median age, 59 years, mean age, 58.4 years). Patients were diagnosed with cirrhosis based on liver histopathology (n = 28), or characteristic clinical features (symptoms and stigmata of cirrhosis and its complications), laboratory results (levels of viral markers, hyperbilirubinemia, hypoalbuminemia and coagulopathy), or imaging findings (liver configuration, splenomegaly, ascites and collateral vessels) (n = 72). Eighteen patients received β-blocker medication. All patients had liver cirrhosis due to hepatitis B infection (n = 76), hepatitis C infection (n = 14), alcohol use (n = 5) or an unknown etiology (n = 5). There were 89 patients with Child-Pugh class A, ten patients with Child-Pugh class B, and one patient with Child-Pugh class C disease.

Upper endoscopy

Patients underwent standard upper endoscopy performed by experienced gastroenterologists (with experience of performing more than 500 endoscopy examinations) in our endoscopy unit. Seven gastroenterologists performed endoscopy of the 100 patients in our study. All endoscopic studies were captured as digital imaging files. On endoscopy, esophageal varices were graded according to criteria proposed by the Japanese Research Society for Portal Hypertension[12]. Grade 2 (varices showing a beaded appearance) or grade 3 (varices running in an oblique course and showing tortuosity with a tumorfactive appearance) was chosen as a cutoff point to define high-risk varices, based on the possibility to develop esophageal bleeding[12]. Two specialized endoscopists (Joon Hyeok Lee and Moon Seok Choi) reviewed all of the stored endoscopic images and the procedure reports for all patients. The endoscopists determined the final grades of the esophageal varices of the study population based on consensus. Disagreement between the two endoscopists was resolved in conference along with a third endoscopist (Geum-Youn Gwak).

Liver computed tomography protocol

Liver CT examinations for follow-up were performed with a 64-slice MDCT scanner (Lightspeed VCT; GE Healthcare, Milwaukee, WI, United States). A total of 120 mL of nonionic contrast material (Ultravist 300, 300 mg/mL iopromide, Schering, Berlin, Germany) was administered intravenously with an automatic injector at a rate of 3-4 mL/s. Images were obtained at 30-40 s, 70 s and 180 s after the initiation of contrast material injection, representing the hepatic arterial, portal venous and equilibrium phases, respectively. In order to determine the appropriate scanning time for hepatic arterial phase imaging, we used an automatic bolus-tracking technique with automated scan-triggering software (SmartPrep; GE Healthcare). Hepatic arterial phase scanning was started automatically 12 s after the trigger threshold (100 HU) was reached at the level of the supraceliac abdominal aorta. Images were obtained from the dome of the diaphragm to the bottom of the liver during a single breath-hold. CT scanning parameters were as follows: 64 detector rows; beam collimation, 0.625 mm × 64 mm; pitch, 0.984; kVp/mA, 120/100-300; gantry rotation time, 0.6 s. Isotropic raw data were acquired with a slice thickness of 1.25 mm and an interval of 1.25 mm with the use of MDCT. Using the raw data, a transverse image was obtained with a slice thickness of 5 mm and an interval of 2.5 mm. During the portal venous phase, coronal and sagittal MPR images were obtained with a 3 mm interval and a slice thickness of 3 mm. No patients received oral contrast material.

Liver computed tomography image analysis

Three abdominal radiologists (Soon Jin Lee, Dongil Choi and Hangi Jo) independently interpreted the liver CT images in order to detect the presence of high-risk (grade 2 or 3) esophageal varices. The radiologists had 13, nine and five years experience, respectively, in the interpretation of abdominal CT images. The radiologists initially evaluated transverse images alone in a random order on a picture archiving and communication system (PACS) monitor. After three weeks, three orthogonal MPR images were assessed in another random order. The radiologists were blinded to the patient physical findings, laboratory values, previous imaging results and endoscopy results.

The CT images were reviewed to detect the presence of high-risk esophageal varices with a 4-point confidence scale for the development of variceal bleeding based on the endoscopic findings that were correlated with variceal bleeding: (1) definitely low-risk or no varices; (2) probably low-risk; (3) probably high-risk; and (4) definitely high-risk. The observers investigated beaded and tortuous varices (distinct enhancing nodular or linear lesions that abutted the surface of the esophageal lumen or protruded into the luminal space) on stack PACS images of CT scans. In addition, the observers measured the shortest diameter of the largest enhancing lesion (varix) during the portal venous phase by the use of an electronic caliper on one-format PACS images. A confidence score of 4 could be assigned if the diameter was definitely greater than 3 mm. A score of 3 was assigned if the diameter was between 2 mm to 3 mm. A score of 2 was assigned if the diameter was between 1 mm to 2 mm. A score of 1 was assigned if the diameter was less than 1 mm[4].

Statistical analysis

The results of liver CT imaging analyzed by three observers were correlated with the endoscopic grading of esophageal varices by the study coordinator (Hyojin Kim). Observer performance for the detection of high-risk esophageal varices was assessed by the use of ROC curve analysis with transverse images alone and with three orthogonal MPR images. A_z under each ROC curve by application of the maximum-likelihood curve-fitting algorithm was used to indicate the diagnostic performance for the detection of high-risk esophageal varices on liver CT images. A_z values were compared by using pseudovalues with the jackknife method developed by Dorfman *et al*[13]. Composite ROC curves for the performance of all observers were obtained by using the pooled data of the three observers.

The sensitivity and specificity for the CT diagnosis of high-risk esophageal varices were calculated. The sensitivity for the detection of varices was determined by use of the number of patients with varices assigned a score of 3 or 4. Sensitivities and specificities for transverse images alone and three orthogonal MPR images were compared with use of the McNemar test. The positive predicative value (PPV) and negative predicative value (NPV) were also calculated and compared with use of the χ^2 test and Fisher's exact test, respectively. A P value of less than 0.05 was considered as significant.

Interobserver agreements for the detection of esophageal varices and high-risk varices were evaluated using κ statistics. The degree of agreement was categorized as follows: κ values of 0.00-0.20 were considered to indicate poor agreement; κ values of 0.21-0.40, fair agreement; κ values of 0.41-0.60, moderate agreement; κ values of 0.61-0.80, good agreement; κ values of 0.81-1.00, excellent agreement[14]. Statistical analyses were performed using commercially available software packages (PASW Statistics, release 17.0.2; SPSS, Chicago, IL, United States; MedCalc, version 7.1; MedCalc Software, Mariakerke, Belgium).

RESULTS

In 50 patients, there were no esophageal varices (grade 0) as determined by endoscopy. Twenty-three patients were classified with grade 1 esophageal varices, 24 patients were classified with grade 2 esophageal varices (Figure 1), and three patients were classified with grade 3 esophageal varices. No patients had a significant endoscopic abnormality other than esophageal varices, such as an ulcer,

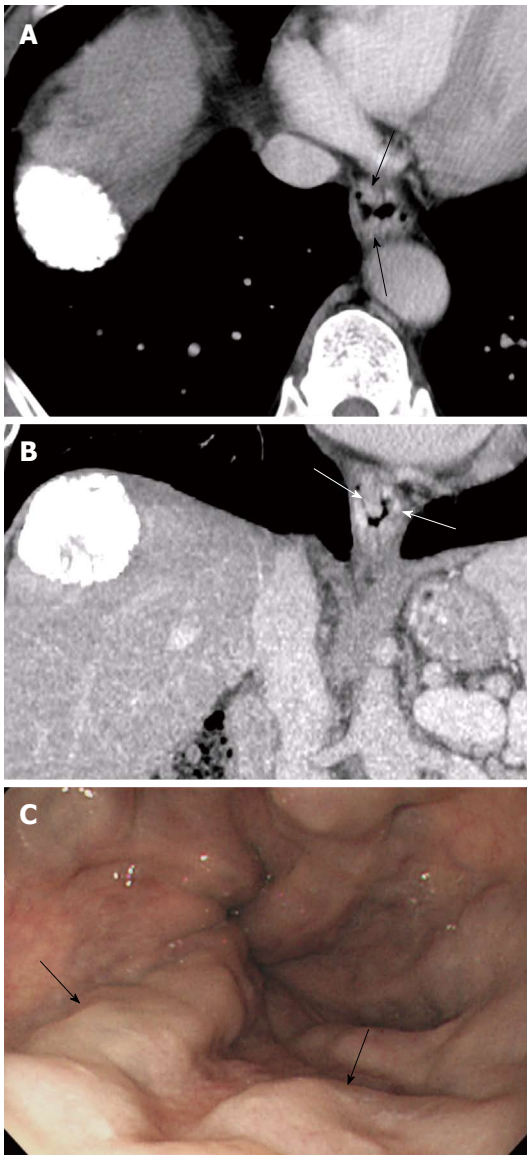

Figure 1 Imaging findings are shown for grade 2 esophageal varices in the distal esophagus in a 61-year-old man with hepatocellular carcinoma and cirrhosis. A: A transversecomputed tomography scan shows distinct enhancing nodular lesions (arrows) protruding into the luminal space. All observers recorded the lesions as high-risk esophageal varices. Periesophageal varices are not found; B: On coronal images, distinct enhancing nodular lesions (arrows) are noted, and all observers considered the lesions as high-risk esophageal varices; C: A conventional endoscopic image confirms the presence of beaded esophageal varices (arrows).

inflammation or mass. The red sign was observed in one (4.3%) of 23 patients with a grade 1 varix, 15 (62.5%) of 24 patients with a grade 2 varix and two (66.7%) of three patients with a grade 3 varix.

The A_z values of the ROC assessment of the three observers for the detection of high-risk esophageal varices with transverse images alone were 0.947 ± 0.031 (95%CI, 0.883-0.982), 0.969 ± 0.024 (95%CI, 0.913-0.993) and 0.916 ± 0.038 (95%CI, 0.843-0.962), respectively. The Az values of the ROC assessment of the three observers with the three orthogonal MPR images were 0.965 ± 0.025 (95%CI, 0.908-0.991), 0.959 ± 0.027 (95%CI, 0.899-0.988) and 0.938 ± 0.033 (95%CI, 0.871-0.976), respectively. The Az values of each observer were similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 1). In addition, there was no significant difference of mean Az values of pooled data by the three observer for transverse images alone (0.946 ± 0.018; 95%CI, 0.914-0.968) and for the three orthogonal MPR images (0.954 ± 0.017; 95%CI, 0.924-0.975, $P > 0.05$). The A_z values were also similar among the three observers both for transverse images alone and for the three orthogonal MPR images, respectively (each $P > 0.05$) (Table 1).

The mean sensitivity for the detection of high-risk esophageal varices of the three observers with transverse images alone and with the three orthogonal MPR images were 90.1% and 91.4%, respectively ($P > 0.05$). For each observer, the sensitivities of detecting high-risk esophageal varices were also similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 2). While the specificities of each observer were similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$), the mean specificity with the three orthogonal MPR images (89.5%) was better than that with transverse images alone (86.3%) ($P = 0.039$) (Table 2).

There was no significant difference for the mean PPV of three observers for transverse images alone (70.9%) and for the three orthogonal MPR images (76.3%, $P > 0.05$). For each observer, the PPVs were also similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 3). There was no significant difference of the mean NPV for transverse images alone (95.9%) and for the three orthogonal MPR images (96.6%, $P > 0.05$). For each observer, the NPVs were also similar for transverse images alone and for the three orthogonal MPR images (each $P > 0.05$) (Table 3).

All κ values for agreements among the three observers with transverse images alone showed excellent agreements (range: 0.845-0.933), and κ values for agreements among three observers with three orthogonal MPR images demonstrated good and excellent agreements (range: 0.791-0.910).

DISCUSSION

Although liver CT images always cover the distal esophagus where all varices occur, some radiologists do not comment on either the presence or the degree of esophageal varices in radiological reports. Some endoscopists are unaware that liver CT scans provide excellent images of esophageal varices. Regular follow-up CT examinations are essential to evaluate recurrence and therapeutic effectiveness in patients treated with TACE or ablation therapy for HCC[10,15]. In such patients, we can make an objective decision about the risk of variceal bleeding with the use of regular follow-up CT imaging without the use of an invasive endoscopic evaluation. The presence of periesophageal varices has been correlated with recur-

Table 1 Az in receiver operating characteristic curve analysis and *P* values of liver computed tomography for the detection of high-risk esophageal varices by three observers

	Observer 1 Transverse	Observer 1 3 MPR	Observer 2 Transverse	Observer 2 3 MPR	Observer 3 Transverse	Observer 3 3 MPR	Mean Transverse	Mean 3 MPR
ROC Az	0.947 ± 0.031	0.965 ± 0.025	0.969 ± 0.024	0.959 ± 0.027	0.916 ± 0.038	0.938 ± 0.033	0.946 ± 0.018	0.954 ± 0.017
95%CI	0.883-0.982	0.908-0.991	0.913-0.993	0.899-0.988	0.843-0.962	0.871-0.976	0.914-0.968	0.924-0.975
P value[1]	0.318		0.328		0.271		0.367	
P value[2]	Observer 1-2 0.368	Observer 1-2 0.782	Observer 2-3 0.099	Observer 2-3 0.461	Observer 3-1 0.35	Observer 3-1 0.334		

[1]Represent the differences of the A_z values for transverse images alone and for the three orthogonal MPR images by each observer and the pooled data; [2]Represent the differences (interobserver comparisons) of the Az values among three observers with both transverse images alone and with the three orthogonal MPR images.Transverse indicates transverse images alone and 3 MPR indicates three orthogonal MPR images. Az values are mean ± SD. CI: Confidence interval; MPR: Multiplanar reformation; ROC: Receiver operating characteristic.

Table 2 Sensitivity and specificity of liver computed tomography for the detection of high-risk esophageal varices by three observers

	Observer 1 Transverse	Observer 1 3 MPR	Observer 2 Transverse	Observer 2 3 MPR	Observer 3 Transverse	Observer 3 3 MPR	Mean Transverse	Mean 3 MPR
Sensitivity%	92.6 (25/27)	92.6 (25/27)	92.6 (25/27)	92.6 (25/27)	85.2 (23/27)	88.9 (24/27)	90.1 (73/81)	91.4 (74/81)
P value	1.000		1.000		1.000		1.000	
Specificity%	89.0 (65/73)	90.4 (66/73)	87.7 (64/73)	87.7 (64/73)	82.2 (60/73)	90.4 (66/73)	86.3 (189/219)	89.5 (196/219)
P value	1.000		1.000		0.070		0.039	

For sensitivity, the numbers in parentheses are the number of varices assigned a score of 3 or 4 divided by the total number of varices. *P* values represent the differences of the sensitivity and the specificity for transverse images alone and for the three orthogonal MPR images (McNemar test). MPR: Multiplanar reformation.

Table 3 Positive predictive value and negative predictive value of liver computed tomography for the detection of high-risk esophageal varices by three observers

	Observer 1 Transverse	Observer 1 3 MPR	Observer 2 Transverse	Observer 2 3 MPR	Observer 3 Transverse	Observer 3 3 MPR	All Transverse	All 3 MPR
PPV %	75.8 (25/33)	78.1 (25/32)	73.5 (25/34)	73.5 (25/34)	63.9 (23/36)	77.4 (24/31)	70.9 (73/103)	76.3 (74/97)
P value	0.947		1.000		0.352		0.482	
NPV%	97.0 (65/67)	97.1 (66/68)	97.0 (64/66)	97.0 (64/66)	93.8 (60/64)	95.7 (66/69)	95.9 (189/197)	96.6 (196/203)
P value	1.000		1.000		0.710		0.797	

P values represent the differences of the positive predicative value and the negative predicative value for transverse images alone and for the three orthogonal MPR images (χ^2 test and Fisher's exact test). MPR: Multiplanar reformation; PPV: Positive predicative value; NPV: Negative predicative value.

rence of luminal esophageal varices after treatment[16].

As with the results of previous reports[4-8], the sensitivities for the detection of high-risk esophageal varices for each observer were also high (approximately 90%) in our study (Table 2). Excellent NPVs as well as high sensitivities for the detection of high-risk varices are clinically important, as the goal of endoscopic screening for esophageal varices is to decrease morbidity and mortality resulting from variceal bleeding. As good screening tests should show a low frequency of false negative results, sensitivity and NPV are more important as compared to specificity and PPV in a screening test[17]. Even though both PPV and NPV are influenced by disease prevalence, the prevalence of high-risk esophageal varices in our study was similar to prior studies[4-8].

A recent study[7] mentioned the possibility that sagittal and coronal images with isotropic spacial resolution potentially decrease measurement variability as vessels are displayed within the imaging plane. However, based on the results of our study, additional coronal and sagittal images showed little practical help to evaluate high-risk esophageal varices when compared to assessment of transverse images alone. Moreover, the use of virtual esophagography proposed in another recent study[5] might be impractical because of time-consuming and invasive insertion of the esophagus with a catheter for air insufflation. Insufflation alters both the luminal pressure and the hemodynamics of the varices. Perri *et al*[7] pointed out that the measurement of varices in a non-distended state might provide a more proper assessment for bleeding risk as variceal bleeding occurs while the esophagus is in its normal non-distended state.

Our study has some limitations. The most important limitation is the use of endoscopy as the reference stan-

dard. Prior studies[18,19] have demonstrated a lack of good correlation between endoscopists to evaluate the grades of esophageal varices. In our study, given the semi-open access system of the endoscopy unit, many different endoscopists perform endoscopy procedures. Hence, specialized endoscopists re-evaluated the grades of esophageal varices with review of the stored endoscopic images. Second, we could not evaluate qualitative endoscopic appearances including the presence of red signs such as cherry-red spots and diffuse redness, which is one of the predictors of variceal bleeding. These red signs cannot be depicted on CT images. In a recent report[20], cases with the shortest diameter of the largest varix of more than 4 mm showed positive red signs. In our study, the endoscopic red sign was observed in only one patient with low-risk (grade 1) esophageal varices (4.3%, 1 of 23 cases)[4].

In conclusion, our results have demonstrated excellent diagnostic performance, sensitivity and NPV with low interobserver variability to detect high-risk esophageal varices on regular follow-up liver MDCT examinations in patients treated with locoregional therapy for HCCs. Careful evaluation of high-risk esophageal varices on a liver MDCT examination may be useful to avoid performing low-yield endoscopy in such patients. When compared to the assessment of transverse images alone, additional coronal and sagittal images were of little help to evaluate high-risk esophageal varices. Our results suggest that liver CT can be used as a single noninvasive surveillance tool for both esophageal varices and recurrent HCC after locoregional therapy. However, a definite change of clinical pathways may be needed in a randomized controlled study on the comparison of bleeding frequency and survival in patients treated with locoregional therapy between the use of a regular follow-up liver MDCT examination alone and with the combination of screening endoscopy.

COMMENTS

Background

The presence of high-risk varices seen in up to 30% of patients with esophageal varices on endoscopy is considered as an indication for prophylaxis against variceal bleeding with either endoscopic variceal ligation or the administration of β-blockers. Several recent studies have suggested the usefulness of computed tomography (CT) scans to evaluate esophageal varices, and one study asserted that CT was a better initial screening modality when compared to endoscopy in terms of cost-effectiveness and patient preference.

Research frontiers

As CT is commonly used as a follow-up imaging modality in patients treated with locoregional therapy for hepatocellular carcinoma (HCC), the authors suggested that replacement of CT for evaluating varices was more persuasive in such patients. Careful evaluation of high-risk esophageal varices on a liver multi-detector row computed tomography (MDCT) examination may be useful to avoid performing low-yield endoscopy in such patients.

Innovations and breakthroughs

The results of this study have demonstrated excellent diagnostic performance, sensitivity and negative predicative value with low interobserver variability to detect high-risk esophageal varices on regular follow-up liver MDCT examinations in patients treated with locoregional therapy for HCCs. When compared to the assessment of transverse images alone, additional coronal and sagittal images were of little help to evaluate high-risk esophageal varices.

Applications

The study results suggest that that liver CT can be used as a single noninvasive surveillance tool for both esophageal varices and recurrent HCC after locoregional therapy.

Peer review

This is a good prospective study in which authors evaluate the diagnostic performance of radiologists for the detection of high-risk esophageal varices on regular follow-up liver multi-detector row CT examinations in patients treated with locoregional therapy for HCC.

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BRIEF ARTICLE

Pentoxifylline in hepatopulmonary syndrome

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Abstract

AIM: To determine the effects of pentoxifylline (PTX) on clinical manifestations and evaluate arterial blood gas data in hepatopulmonary syndrome (HPS) in children.

METHODS: In a pilot study of 10 children with chronic liver disease, who had HPS, 20 mg/kg/d PTX was administered for 3 mo. Clinical data and arterial blood gas parameters were evaluated at baseline, the end of the treatment period, and 3 mo after drug discontinuation.

RESULTS: Six patients could tolerate PTX, while four patients experienced complications. Among patients who could tolerate PTX, there was a significant increase in arterial oxygen pressure (PaO_2) (P = 0.02) and oxygen saturation (SaO_2) (P = 0.04) and alveolar-arterial oxygen gradient (P = 0.02) after 3 mo of treatment. Significant decreases in PaO_2 (P = 0.02) and alveolar-arterial oxygen gradient (P = 0.02) were also seen after drug discontinuation.

CONCLUSION: PTX may improve PaO_2, SaO_2 and alveolar-arterial oxygen gradient in the early stage of HPS.

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Key words: Pentoxifylline; Hepatopulmonary syndrome; Child; Arterial oxygen pressure; Therapy; Alveolar-arterial oxygen gradient

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INTRODUCTION

The hepatopulmonary syndrome (HPS) is the triad of liver disease, arterial hypoxemia and widespread pulmonary vascular dilatation[1-3]. Prevalence of this syndrome is 10%-30% among patients with chronic liver disease[4].

The pathogenesis of HPS is unclear. The roles of eicosanoids as vasoconstrictors and the increased number of intravascular macrophage-like cells have been reported previously. Increased pulmonary production of NO in

cirrhosis has been considered as a reason for intrapulmonary venous dilatation. It is claimed that increased pulmonary NO production is due to raised pulmonary vascular endothelial NO synthase (eNOS) and inducible NO synthase expression. Recent studies have shown that increased hepatic production and release of low level of endothelin 1 (ET-1) can act as a trigger for enhancing eNO level. Tumor necrosis factor α (TNFα) and ET-1 have been implicated in the development of experimental HPS. Recent studies have suggested that progression of HPS is due to increased CO production and heme oxygenase 1 expression[5-7]. The presence of HPS increases mortality in cirrhotic patients and may influence the frequency and severity of complications of portal hypertension[8-11]. To the best of our knowledge, there is currently no effective medical therapy for HPS. Several medications such as methylene blue, garlic and aspirin have been reported to have a therapeutic effect[12-15], but curative treatment of this syndrome is still orthotopic liver transplantation[16].

Pentoxifylline (PTX), a nonspecific phosphodiesterase-4 inhibitor, blocks TNFα synthesis[17,18] and seems to prevent HPS. There are limited studies about PTX in adult patients with HPS, with varying results. Until now, no study has been carried out in children. The aim of this study was to evaluate the effect of PTX on clinical manifestations and arterial blood gas data in children with HPS.

MATERIALS AND METHODS

This pilot study was carried out in the Ghaem Medical Center of Mashhad University of Medical Sciences, Iran during a 21-mo period (March 2008-November 2010). The protocol for this study was approved by the Ethics Committee of Mashhad University. Children and their parents were given informed consent forms. A consecutive series of patients with cirrhosis attending our hospital were screened for HPS. HPS was diagnosed according to the European Respiratory Society Task Force criteria: hypoxemia plus positive contrast-enhanced echocardiography[7]. Positive contrast echocardiography was defined by the appearance of air bubbles in the left side of the heart after the first three beats during injection of 10 mL agitated isotonic saline solution in the antecubital vein[19]. Exclusion criteria were active infection, known malignancy, cardiac disease, and kidney disease.

Patient data including demographics, etiology of cirrhosis, clinical manifestations, laboratory results [complete blood count (CBC), diff, blood urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, prothrombin time, partial thromboplastin time, total bilirubin, direct bilirubin, albumin and total protein] were recorded. For each patient, Child score was determined. All patients underwent measurement of arterial oxygen pressure (PaO_2) and oxygen saturation (SaO_2) by blood sampling from the radial artery in a sitting position while breathing ambient air. For each patient, alveolar-arterial oxygen gradient was calculated. Patients were classified in four groups according to severity of hypoxemia: mild HPS with $PaO_2 \geqslant 80$ mmHg, moderate HPS with $60 \leqslant PaO_2 < 80$ mmHg, severe HPS with $50 \leqslant PaO_2 < 60$ mmHg, and very severe HPS with $PaO_2 < 50$ mmHg[7].

Table 1 Baseline patient characteristics *n* (%)

Variable	Value (*n* = 10)
Age (yr)	
Mean ± SD, (range)	9.2 ± 5.0 (2-16)
Sex	
Female/male	4/6
Cause of cirrhosis	
Wilson disease	3 (30)
Cryptogenic cirrhosis	3 (30)
Autoimmune hepatitis	2 (20)
PFIC	1 (10)
GSD	1 (10)
Staging of HPS	
Mild	2 (20)
Moderate	6 (60)
Severe	1 (10)
Very severe	1 (10)
Child score	
A	3 (30)
B	4 (40)
C	3 (30)

PFIC: Progressive familial intrahepatic cholestasis; GSD: Glycogen storage disease; HPS: Hepatopulmonary syndrome.

PTX at a dose of 20 mg/kg per day orally in three divided doses was administrated for 3 mo. In this period, patients were followed up weekly and whenever patients required, by telephone conference and monthly with outpatient visits. Clinical manifestations and laboratory data were rechecked at the end of the treatment period, 3 mo after drug discontinuation and whenever a child had any deterioration or could not tolerate PTX. Response to PTX was considered as increased $PaO_2 > 10$ mmHg from baseline[20]. Complement a three months period of treatment was considered as primary end point and development of serious side effects, death and liver transplantation was considered as the secondary end point.

After entering data into SPSS version 16.0 software, quantitative data were analyzed by the Wilcoxon signed rank test and qualitative data by the McNemar test. A P value < 0.05 was considered significant.

RESULTS

A total of 65 patients aged < 18 years with cirrhosis were screened for the presence of HPS from March to August 2009. Twelve patients had HPS. Two were excluded. One had kidney disease and one did not allow us to intervene. Ten children were enrolled. Two patients had mild HPS, six moderate HPS, one severe HPS and one very severe HPS. Four patients were girls. Mean age was 9.2 ± 5.0 years old. The most common recognized cause of cirrhosis was Wilson's disease (Table 1). Minimum alveolar-

Table 2 Clinical and laboratory data at baseline and end of treatment period (mean ± SD) *n* (%)

Variable	Baseline	End of treatment	*P* value
Clinical manifestations			
Cyanosis	9 (90)	5 (50)	0.12
Dyspnea	3 (30)	2 (20)	1.0
Clubbing	7 (70)	7 (70)	1.0
Palmar erythema	5 (50)	5 (50)	1.0
Hepatomegaly	6 (60)	6 (60)	1.0
Splenomegaly	7 (70)	7 (70)	1.0
Laboratory findings			
White blood cell count, $\times 10^3$/mm	6.4 ± 4.41	4.36 ± 2.04	0.17
Hemoglobin, g/dL	11.25 ± 2.30	11.45 ± 2.71	0.49
Platelet count, $\times 10^3$/mm^3	131.6 ± 566.74	156.2 ± 498.36	0.32
Serum creatinine, mg/dL	0.56 ± 0.16	0.6 ± 0.14	1.0
Serum urea, g/dL	12.60 ± 5.98	14.70 ± 7.20	0.34
Serum albumin, g/dL	3.88 ± 0.52	3.56 ± 0.15	0.59
Serum total protein, g/dL	7.20 ± 0.40	7.26 ± 0.81	1.0
PT, s	15.72 ± 3.07	18.51 ± 8..27	0.40
PTT, s	45.50 ± 14.54	49.42 ± 11.41	0.24
INR	1.46 ± 0.36	1.90 ± 1.14	0.67
Total bilirubin, mg/dL	5.73 ± 7.39	5.07 ± 6.04	0.17
Direct bilirubin, mg/dL	1.70 ± 2.50	1.68 ± 2.71	0.83
ALT, U/L	83.60 ± 64.28	40.60 ± 31.56	0.27
AST, U/L	92.55 ± 64.48	66.80 ± 2814	0.50
ALP, U/L	564.66 ± 198.74	536.50 ± 17.67	0.18

PT: Prothrombin time; PTT: Partial thromboplastin time; INR: International normalized ratio; ALT: Alaninaminotranferase; AST: Aspartataminotransferase; ALP: Alkaline phosphatase.

arterial oxygen gradient at baseline was 18.84 and maximum was 71.98. Clinical and laboratory characteristics of the patients at baseline and end of treatment period are summarized in Table 2.

Six patients completed the treatment period. Petechiae with platelet count 10 000/mm^3 was seen in one patient, 3 wk after the beginning of PTX. He was a 16-year-old boy with Wilson's disease and very severe HPS. His platelet count at the beginning of the study was 159 000/mm^3. Platelet count increased to 100 000/mm^3 2 wk after drug discontinuation. Other laboratory tests did not show any significant change. Although nausea was seen in all patients, only three had severe vomiting that did not respond to symptomatic treatment and caused drug withdrawal. Among them, two had moderate HPS and one had severe HPS. No significant laboratory changes were seen in these patients. Treatment periods were 10 d, 28 d and 45 d. Other adverse effects did not cause drug withdrawal and improved with symptomatic treatment (two patients with fatigue and two with headache).

Among patients who completed the treatment period, average increment in PaO_2 was 26 mmHg and SaO_2 was 4.3%. At least 10 mmHg elevations in PaO_2 level was seen in all patients. Also, PaO_2 significantly decreased 3 mo after the end of treatment. Moreover, there was significant improvement in alveolar-arterial oxygen gradient at the end of the treatment period and a significant increase after drug discontinuation (Table 3). Among these patients, two had mild HPS and four had moderate HPS.

For clinical manifestations, there were no significant

Table 3 PaO_2, SaO_2 and alveolar-arterial oxygen gradient (A-a G) before, at the end and 3 mo after treatment among patients who could tolerate pentoxifylline (mean ± SD)

Variables	PaO_2	SaO_2	A-a G
Baseline	73.3 ± 9.33	93.33 ± 6.28	40.10 ± 18.09
End of treatment	97.0 ± 3.79	97.66 ± 1.50	20.73 ± 9.46
Three months after treatment	71.83 ± 21.47	90.5 ± 8.6	43.68 ± 24.30
P value	0.02[1], 0.02[2]	0.04[1], 0.05[2]	0.02[1], 0.02[2]

[1]Baseline *vs* end of treatment; [2]End of treatment *vs* 3 mo later. PaO_2: Oxygen pressure; SaO_2: Oxygen saturation.

change between baseline, the end of treatment, and 3 mo later. There was also no significant change in other laboratory tests. In a 12-mo period of follow-up after drug discontinuation, two patients died. The first was the 16-year-old boy with Wilson's disease. He died from upper gastroesophageal bleeding from esophageal varicose veins, 8 mo after drug withdrawal. The second was a girl of 2 years with cryptogenic cirrhosis who received PTX for 10 d. She was one of the three patients who could not tolerate PTX because of severe vomiting. She died due to hepatic encephalopathy and septicemia.

DISCUSSION

In this pilot study, we found significant improvements in PaO_2, SaO_2 and alveolar-arterial oxygen gradient after 3 mo therapy among patients who could tolerate PTX. PaO_2 was also significantly decreased at 3 mo after drug withdrawal, which supports the therapeutic effects of PTX. However, we did not find any significant change in clinical parameters. In this study, 40% of patients could not complete the treatment period. In other studies of patients with liver disease, drug withdrawal rate due to adverse effects ranged from 0% to 40%[17,21,22]. It seems that gastrointestinal intolerance was the most common cause (nausea in 100% and vomiting in 30%). Previously, animal studies have shown that inhibition of TNFα with PTX in cirrhotic rats after common bile duct ligation can improve HPS[23,24].

Tanikella *et al*[25] selected nine adult patients with moderate to severe HPS. There was no significant difference in PaO_2 and alveolar-arterial oxygen gradient before and after PTX administration. High incidences of gastrointestinal adverse effects were the most important reason for intolerance. This resulted in a 44% attrition rate and only one patient (12%) could tolerate PTX for the complete treatment period of 8 wk.

Despite these results, Gupta *et al*[26] have demonstrated that 3 mo treatment with PTX can improve the symptoms and signs in about 90% of patients. There was also a significant increase in median supine, standing and postexercise PaO_2 ($P < 0.01$). Side effects were reported in a few patients (vomiting in three and dyspnea in two), but they did not require drug withdrawal.

In our study, among the four patients who could not

tolerate PTX, two had moderate, one had severe and one had very severe HPS. Also, three patients had Child score C and one had Child score B. The high incidence of intolerance in these patients was comparable with that of the Tanikella *et al*[25] study that was carried out in patients with moderate to severe HPS, and suggests that beginning PTX in the early stages of HPS may increase tolerability and effectiveness.

As far as we are aware, no study has determined the optimal dose of PTX in cirrhotic children. In animal studies, the dose of PTX used was 10-50 mg/kg per day[4,23,24]. PTX was used previously in children for treatment of Kawasaki disease as an antiplatelet drug with the same dose and no side effects were seen[27]. One potential explanation for this difference is significant first pass metabolism of PTX in the liver to an active metabolite, and consequently, serum levels are significantly high in liver dysfunction[28]. This problem does not occur in Kawasaki disease. Furthermore, finding the optimal dosage of PTX in liver dysfunction can be beneficial. In the present study, one patient experienced thrombocytopenia. Although thrombocytopenia is a manifestation of cirrhosis, decreased platelet count after administration of PTX, and increased platelet count after drug withdrawal denote the role of PTX. This implies that CBC is an important laboratory test during PTX administration. We did not observe any change in serum creatinine or liver function before and after therapy with pentoxifylline that may have contributed to changes in pentoxifylline metabolism.

To evaluate effects of PTX in HPS, we suggest double-blind studies, long-term treatment, and contrast echocardiography at the end of the treatment period for later studies. Meanwhile, it is necessary to evaluate optimal dose of PTX in cirrhotic children. In conclusion, it seems that PTX may have beneficial effects on oxygenation in early stages of HPS.

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COMMENTS

Background

Hepatopulmonary syndrome (HPS) is a triad of liver disease, arterial hypoxemia and widespread pulmonary vascular dilatation. There is currently no effective medical therapy for HPS in spite of several recommended medications. Recently, pentoxifylline (PTX), a nonspecific phosphodiesterase-4 inhibitor, which blocks tumor necrosis factor α (TNFα) synthesis, has appeared to prevent HPS. The aim of this study was to evaluate the effect of PTX on clinical manifestations and arterial blood gas data in children with HPS.

Research frontiers

This study, possibly for the first time, reported therapeutic effects of PTX in children with HPS. The authors concluded that there were no significant differences in clinical manifestations and other laboratory tests after treatment. PTX may improve arterial oxygen tension, oxygen saturation and alveolar-arterial oxygen gradient in the early stage of HPS. Thus, it seems that PTX may have beneficial effects on oxygenation in early stages of HPS.

Innovations and breakthroughs

PTX, a nonspecific phosphodiesterase-4 inhibitor, blocks TNFα synthesis and seems to prevent HPS. The authors evaluated, perhaps for the first time, the therapeutic efficacy of PTX in children with HPS.

Applications

The authors concluded that PTX may have beneficial effects on oxygenation in early stages of HPS. However, the authors suggest further double blind studies with longer treatment periods and contrast echocardiography at the end of the treatment period until the effect of PTX in HPS are defined clearly. Also, the authors recommend evaluation of the optimal dose of PTX in cirrhotic children.

Peer review

This is a prospective study with a few patients. It is accepted with minor revisions.

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BRIEF ARTICLE

Inducible nitric oxide synthetase genotype and *Helicobacter pylori* infection affect gastric cancer risk

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Abstract

AIM: To investigate the association of the inducible nitric oxide synthetase (*iNOS*) C150T polymorphism with *Helicobacter pylori* (*H. pylori*) infection and gastric cancer (GC) risk in Iran.

METHODS: In order to determine whether there was a correlation between *iNOS* genotype and GC in Iran, we conducted a case-control study using samples from 329 individuals. For each sample, the C150T *iNOS* polymorphism was genotyped by polymerase chain reaction (PCR) and restriction digestion. Patients were grouped by cancer presence, demographic and behavior characteristics, and *H. pylori* infection status. Statistical tests were conducted to determine whether any behavioral factors or a particular *iNOS* genotype was associated with GC in the study population.

RESULTS: In this population, we found that smoking, hot beverage consumption, a familial history of GC and *H. pylori* infection status were significantly associated with GC development (P = 0.015, P < 0.001, P = 0.0034, and P < 0.015, respectively). The distribution of the C150T *iNOS* genotypes among the two study groups was not statistically significant alone, but was impacted by *H. pylori* infection status. When compared to the non-*H. pylori* infected group, cancer patients who had a heterozygous CT genotype and were also infected with *H. pylori* were 2.1 times more at risk of developing GC [odds ratio (OR) = 2.1, P = 0.03] while those with a homozygous TT genotype and infected with *H. pylori* were 5.0 times more at risk of developing GC (OR = 5.0, P = 0.029). In contrast, this association was not seen in patients in the control group.

CONCLUSION: A CT or TT polymorphism at position 150 in the *iNOS* gene significantly increases the risk of GC and may be a marker for GC susceptibility.

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Key words: Inducible nitric oxide synthetase; Gastric cancer; *Helicobacter pylori*; Heterozygous CT genotype; Homozygous TT genotype

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INTRODUCTION

Gastric cancer (GC) is a major cause of morbidity and mortality throughout the world; it is estimated that GC is responsible for over 700 000 deaths per year[1,2], which makes this disease one of the leading causes of cancer-related death worldwide[3]. One of the most prominent risk factors for development of GC is infection with the bacterium *Helicobacter pylori* (*H. pylori*). Indeed, since its discovery in the early 1980s, *H. pylori* has been recognized as the causative agent of two types of cancer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma[4-8]. The tight association between infection with this pathogen and the development of GC led to the classification of *H. pylori* as a class I carcinogen[9]; it is currently the only bacterium so classified.

Although *H. pylori* infection is relatively common, only a small subset of infected individuals develop GC; some individuals colonized with *H. pylori* carry the organism their entire lives with no overt disease symptoms, while others develop severe disease. Furthermore, recent evidence has begun to suggest that *H. pylori* colonization may be beneficial in some individuals; studies indicate that *H. pylori* protects against the development of esophageal cancer, childhood asthma[10-12] and active tuberculosis[10]. These findings have led researchers to try to define risk factors associated with *H. pylori*-induced disease development. Indeed, years of study have shown that in *H. pylori*-infected individuals, disease development/carcinogenesis is a complex and multifactorial process that involves an intricate interplay between numerous factors; studies indicate that disease etiology is influenced by a combination of bacterial factors, host genetics, and environmental factors such as diet.

Numerous bacterial factors have been identified as being important for *H. pylori*-induced disease development. These include the toxins CagA[13,14] and VacA[15,16], the adhesions BabA[17] and SabA[18], and the outer membrane proteins HomA, HomB, and OipA[19-21]. These and other bacterial factors act in concert to alter host cell signaling pathways, induce epithelial cell proliferation, and disrupt the delicate balance that exists between the bacterium and the host immune system. However, although these bacterial factors play a key role in the development of gastric disease, they represent only one aspect of the story. Indeed, there is a large body of work that suggests environmental factors also affect the risk of GC development. Some of the more prominent environmental risk factors include smoking[22] and high levels of dietary salt intake[23-25].

Besides these exogenous factors, the host immune system also clearly plays a major role in the development of gastric disease. In response to infection with *H. pylori*, the localized gastric environment becomes pro-inflammatory *via* the expression of pro-inflammatory cytokines[26-28]. Because these host factors play an important role in generating and maintaining the pro-inflammatory environment, it is perhaps not surprising that specific mutations in the genes that encode these factors influence gastric disease development. For example, specific interleukin-1 (*IL-1*), *IL-8*, *IL-10*, and tumor necrosis factor-α gene polymorphisms are associated with an increased risk for GC[29,30]. While these cytokines play an important role in the development and maintenance of the pro-inflammatory environment in the gastric mucosa, there are clearly other host factors involved in the disease process.

Nitric oxide (NO) is a molecule that has multiple functions in the gastrointestinal tract. In addition to having anti-microbial properties and playing an active role in vasodilation[31], NO is also a component of the pro-inflammatory immune response that has been linked to carcinogenesis[32]. NO is synthesized from L-arginine by three distinct isoforms of the nitric oxide synthetase (NOS)[31]. Whereas 2 of the 3 isoforms (neuronal NOS and epithelial NOS) are constitutively expressed, the inducible isoform (iNOS) is primarily expressed in response to specific cytokines[31]. Furthermore, expression of iNOS is upregulated during *H. pylori* infection[33]. Several studies have shown a link between increased levels of iNOS and risk of GC development[34,35]. Since one of the end products of NO metabolism is reactive nitrogen species, it is hypothesized that higher levels of iNOS lead to more oxidative DNA damage, thus increasing the risk of cancer development[31]. Interestingly, a single amino acid substitution (Ser 608 Leu) in the iNOS reductase domain has been associated with an increased risk for GC in Asian populations[36,37]. This amino acid change is the result of a single polymorphism (C150T) in exon 16[36,37], which is believed to affect the activity of the iNOS enzyme[36].

Even though the number of GC cases has decreased in many countries in recent years, the incidence of GC in Northern Iran remains high[38]. Several provinces, including Manzadaran, Ardabil, Golestan, Semnan, and the metropolitan area of Tehran, have high rates of GC in both men and women[38]. The age-standardized incidence rate for GC in these areas has been reported as high as 49.1 in men, and 25.4 in women[38]. These incidence rates may be at least partially explained by the fact that a significant proportion of this population is colonized by *H. pylori*. Indeed, the *H. pylori* infection rate for Iranians aged 40 years and over can be as high as nearly 90%[39,40]. A similar prevalence was reported for the Babol city of Iran where the *H. pylori* infection rates are 78% for men and 82% for women[41]. In addition to high rates of *H.*

pylori infection, environmental factors such as consumption of red meat and dairy products, high salt intake, and consuming hot tea have been identified as important risk factors for GC in this region[2].

At present, virtually nothing is known concerning which host genetic factors may influence GC risk in this region. Herein, we describe an epidemiologic study in which we investigate the association of the *iNOS* C150T polymorphism with *H. pylori* infection and GC risk among men and women from the Mazandaran region of Iran. We found that in this population, individuals who are infected with *H. pylori* are at a significantly higher risk for development of GC if they also harbor a CT or TT *iNOS* genotype. To date, this report is the first study that links a host-genetic factor to GC risk in this population. Furthermore, it is the first study to investigate the importance of the *iNOS* polymorphism in a non-Asian population. *En masse*, our data indicated that the *iNOS* C150 allele may be a potential marker for susceptibility to *H. pylori*-mediated GC development in multiple populations.

MATERIALS AND METHODS

Study participants

This case-control study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Research Ethics Committee at the Mazandaran University of Medical Sciences. Written informed consent was obtained from each individual, and all study participants were given an explanation of the nature of the study prior to enrollment. Patients were enrolled in the study after seeking treatment at Touba Polyclinics or Imam Hospital between April 2008 and March 2011. Patients with GC were diagnosed by gastric endoscopy and cases were defined using the International Classification of Diseases for Oncology and Lauren criteria[42]. During the course of the study, 159 individuals with GC were enrolled. This group had a mean age of 61.32 ± 12.5 years and was composed of 75 female (approximately 47%) and 84 male (approximately 53%) patients. Patients with inflammatory diseases such as rheumatoid arthritis and/or malignancies other than gastric carcinoma were excluded from the study. Members of the control group were matched by age, sex, geographic location, and ethnic background. This group included 170 individuals with a mean age of 58.86 ± 14.2 years. Ninety-eight were male (approximately 58%) and 72 were female (approximately 42%). For this population, healthy controls who were 40 years of age or greater were recruited from the clinics after undergoing an annual medical screening that included a gastric endoscopy. Healthy controls who were less than 40 years of age were recruited after being referred to the clinic for routine laboratory testing. Demographic information, eating habits, and epidemiologic risk factors were provided by study participants during a face-to-face interview. Risk factors that were evaluated included cigarette smoking, drinking hot beverages, and consumption of salted fish and fast food. A person was considered a smoker if they reported smoking at least one cigarette per day for 12 or more months. Consumption of salted fish or fast food was defined as consuming either item one or more times a week for at least 6 mo. Criteria for drinking hot beverages were essentially as described previously[43]. Patients were considered to have a family history of GC if either of their parents currently had or had been diagnosed with GC in the past, or if any of their siblings had a history of the disease.

Inducible nitric oxide synthetase genotyping

In order to investigate *iNOS* gene polymorphisms, venous blood was collected from study participants and used to isolate genomic DNA by the salting-out method[44]. *iNOS* genotyping was performed using an amplicon-restriction fragment length polymorphism (ARFLP) essentially as previously described[37]. Briefly, for each individual a polymerase chain reaction (PCR) was performed using 2 pmol each of the iNOS-F (5'-TGTAAACCAACTTCCGTGGTG-3') and iNOS-R (5'-174 GTCTCTGCGGGTCTGAAG-3') primers, 200 mmol dNTPs, 10 mmol Tris-HCl, pH 8.3, 50 mmol KCl, 1.5 mmol $MgCl_2$, 0.25 U of Hot start Taq DNA polymerase (Fermentas, Italy) and 50 ng of genomic DNA template in a final reaction volume of 25 μL. Each reaction was denatured at 95 ℃ for 5 min followed by 35 cycles of denaturation at 94 ℃ for 60 s, annealing at 58.5 ℃ for 60 s, and extension at 72 ℃ for 40 s. A final extension at 72 ℃ for 5 min was added to ensure complete amplification. Restriction digests were performed by incubating 10 μL of PCR product with 2 U of TSP 509I enzyme (New England Biolabs, Ipswich, MA, United States) in a final volume of 15 μL for 15 h at 65 ℃. Digestion products were separated on a 1.5% SDS-polyacrylamide gel, and visualized after staining with ethidium bromide. Results of the ARFLP analysis allowed us to differentiate between the 3 possible genotypes: (1) digestion of PCR amplicons from homozygous (CC) individuals generated 2 DNA fragments of 113 bp and 175 bp; (2) digestion of amplicons from heterozygous individuals (CT) yielded 3 DNA fragments of 113 bp, 142 bp, and 175 bp; and (3) digestion of amplicons from homozygous (TT) individuals yielded 2 DNA fragments of 113 bp and 142 bp.

H. pylori detection

Infection with *H. pylori* was detected by culturing gastric tissue collected during endoscopy. Identification of clinically isolated *H. pylori* strains was confirmed by Gram stain and urease activity. Additionally, all patients were also tested for infection using an *H. pylori* specific IgG enzyme linked immunosorbent assay (ELISA) (Diagnostic Automation, CA, United States) and by urease breath test (Bahar, Afshan, Iran). Given the difficulties in culturing *H. pylori*, especially from cancerous tissue, individuals were classified as infected with *H. pylori* if they gave a positive result by ELISA.

Table 1 Patient demographics and risk behavior

	Gastric cancer n = 159 (%)	Control n = 170 (%)	P-value
Age (yr)	61.32 ± 12.5	58.86 ± 14.2	0.098
Sex (male/female)	84/75	98/72	0.367
Marital status			
Single	7 (4.4)	3 (1.8)	
Married	149 (93.7)	166 (97.6)	0.002
Divorced	3 (1.9)	1 (0.6)	
Occupation			
Unemployed	4 (2.5)	1 (0.6)	
Employee	24 (15.1)	35 (20.5)	0.001
Housewife	46 (28.9)	74 (43.5)	
Others	85 (53.5)	60 (5.4)	
Smoker	33 (20.8)	22 (12.9)	0.015
Hot beverage	76 (47.8)	38 (22.4)	< 0.0001
Salty fish	15 (9.4)	14 (8.2)	0.09
Fast food	29 (18.2)	39 (22.9)	0.037
Pickles	88 (55.3)	97 (57.1)	0.62
H. pylori positivity	94 (59.1)	77 (45.3)	0.015
Positive family history	34 (21.4)	16 (9.4)	0.0034

H. pylori: *Helicobacter pylori*.

Statistical analysis

Fisher's exact test was used to compare the different *iNOS* allele types, and the frequency of each *iNOS* genotype in the patient population(s) was assessed using a χ^2 test. The difference in age between the 2 study groups was evaluated using a two-tailed Student's *t*-test. Deviation from the Hardy-Weinberg equilibrium was evaluated using a χ^2 test. To evaluate the risk factor(s) in the study populations, unconditional logistic regression models were applied to odds ratios (ORs) and 95% confidence intervals (CIs). For all statistical tests, a *P* value of less than 0.05 was considered significant.

RESULTS

Demographics and epidemiology of the patient population

Complete epidemiologic and demographic information for the study population is given in Table 1. Across the 329 study participants, ages ranged from 28 years to 86 years. Within the control group the mean patient age was 58.9 ± 14.2 years, whereas within the cancer group the mean patient age was 62.1 ± 12.6 years. There was no significant difference between the ages of patients in the two groups (P = 0.098). The control group was comprised of 98 male and 72 female patients while the cancer group was comprised of 84 male and 75 female patients. There was no significant difference between the distribution of male and female study participants in either group (P = 0.367). Patients were categorized by marital status as single, married, or divorced. The cancer group consisted of 7 single, 149 married, and 3 divorced individuals. In contrast, the control group consisted of 3 single, 166 married, and a single divorced patient. The breakdown of marital status between the case and control groups was significant (P = 0.002). The study participants were also broken down into different groups based on occupation. Patients were considered either unemployed, employed, or worked as a housewife; those participants whose employment status did not fall into one of these 3 categories were grouped into a single category, which was named "other". In the GC group, 4 of the study members were unemployed, 24 were employed, 46 were housewives, and the remaining 85 were in the other classification. Within the control group, 1 participant was unemployed, 35 were employed, 74 were housewives, and 60 were included in the other category. The distribution of patient occupation among the case group and the control group was significantly different (P = 0.001).

Risk factors associated with GC

In addition to gathering demographic information about the study participants, we also analyzed behaviors that are known to be associated with increased GC risk. Of the 159 patients who were diagnosed with GC, 33 were smokers whereas only 22 of the healthy controls reported smoking. Since smoking is an established risk factor for development of GC, perhaps it is not surprising that the increased number of smokers in the cancer group was statistically significant (P = 0.015). Another established risk factor for GC is consumption of hot beverages; drinking hot tea is specifically associated with increased risk of esophageal cancer in Iran[43]. Seventy-six participants in the cancer group reported drinking hot beverages on a regular basis. This number was significantly different than the 38 control patients who regularly drank hot beverages (P < 0.0001). Although consuming high amounts of dietary salt has been linked to GC[23-25], we found no significant difference in the number of patients who regularly ate salted fish among the two groups. Similarly, we found no significant difference in the number of patients who consumed pickles among the groups. However, more of the healthy controls reported regularly eating fast food than did the participants who developed GC. In contrast to the other 2 food criteria, this difference was significant (P = 0.037).

Among the most widely recognized factors that are associated with GC risk are *H. pylori* infection and a family history of cancer. Among the control group, only 18 patients had a family history of GC. In contrast, 34 participants in the cancer group reported having a family history of GC (P = 0.003). As it is the most commonly associated risk factor for GC, we next compared the number of *H. pylori*-positive individuals in both of our study groups. In the cancer group, nearly 60% of the study participants tested positive for *H. pylori*. Compared to this relatively high infection rate, the percentage of healthy controls who tested *H. pylori*-positive (45%) was significantly less (P = 0.015).

Frequency and distribution of C150T inducible nitric oxide synthetase polymorphisms

Having established the relative risk factors of our population, we next sought to determine the distribution of the

Table 2 Allele and genotype frequency distribution of C150T polymorphism of the inducible nitric oxide synthetase gene in patients with gastric cancer and controls *n* (%)

iNOS C150T	Gastric cancer *n* = 159	Controls *n* = 170	Odds ratio	95%CI	*P*-value
CC	89 (56)	92 (54.1)	1.00[1]		
CT	59 (37.1)	72 (42.4)	0.86	0.54-1.35	0.5
TT	11 (6.9)	6 (3.5)	1.917	0.68-5.41	0.21
C allele	237 (74.5)	256 (75.3)	1.00[1]		
T	81 (25.5)	84 (24.7)	1.05	0.74-1.49	0.78
TT + CT	70 (44)	78 (45.9)	0.94	0.61-1.45	0.77
HWE	0.796	0.07			

[1]The first allele or genotype is the reference for statistical analysis. iNOS: Inducible nitric oxide synthetase; CC: Homozygous CC genotype; CT: Heterozygous CT genotype; TT: Homozygous TT genotype; HWE: Hardy Weinberg Equilibrium; CI: Confidence interval.

possible C150T *iNOS* genotypes among the two study groups (Table 2). Patients were genotyped essentially as described[37]; as reported in Japanese populations[36], the distribution of these specific *iNOS* polymorphisms in the control and cancer patient groups fell within the Hardy-Weinberg Equilibrium (P = 0.07 and P = 0.796, respectively). Among the 159 members of the GC group, 89 were homozygous CC, 59 were heterozygous CT and 11 were homozygous TT. The distribution of allele types within this group was not significantly different when compared to the study control group, which contained 92 homozygous CC, 72 heterozygous CT, and 6 homozygous TT individuals. There was also no difference in the frequency of each individual allele (C or T) found within the two study populations or the frequency of mutant (either CT or TT) genotypes between the GC and control groups.

CT and TT inducible nitric oxide synthetase polymorphisms contribute to GC risk in H. pylori-infected individuals

Since there was no significant difference in the frequency or distribution of *iNOS* C150T alleles in our two patient populations, we next examined any potential epidemiological interaction between *H. pylori* infection and the particular *iNOS* C150T genotype within the 159 GC patients. As shown in Table 3, the number of GC patients who were infected with *H. pylori* (n = 94) was slightly higher than the number of patients who were *H. pylori*-negative (n = 65). Among the 94 individuals who were infected with *H. pylori*, 46.8% were homozygous CC, 42.6% were heterozygous CT, and the remaining 10.6% were homozygous TT. In contrast, the *H. pylori*-negative group consisted mostly of the homozygous CC genotype (67.7%), followed by the heterozygous CT genotype (29.2%) and the TT genotype (3.1%). When compared to the non-*H. pylori* infected group, GC patients who had a heterozygous CT genotype and were also infected with *H. pylori* were 2.1 times more at risk of developing GC (OR = 2.1, P = 0.03, χ^2 test). Similarly, having a homozygous TT genotype and being infected with *H. pylori* also led to an increased risk of developing GC; the risk associated with having the homozygous TT genotype was more than twice the risk associated with having only a single T allele (OR = 5.0). This association was also significant (P = 0.029, χ^2 test). Similarly, the presence of either mutant allele (CT or TT) increased the risk of GC nearly 2.5-fold (OR = 2.4, P = 0.01, χ^2 test). Taken together, these results indicate that for individuals who are infected with *H. pylori*, carrying either a CT or TT *iNOS* polymorphism at position 150 significantly increases the risk of developing GC. Importantly, unlike those patients within the GC group, there was no significant association between *iNOS* genotype, *H. pylori* infection and risk of developing GC in the control group (Table 3).

Table 3 Allele and genotype frequency distribution of C150T polymorphism of the inducible nitric oxide synthetase gene in *Helicobacter pylori*[3] *n* (%)

	H. pylori+	*H. pylori*-	OR[2]	95%CI	*P*-value
iNOS C150T	*n* = 94	*n* = 65			
GC patients					
CC	44 (46.8)	44 (67.7)	1.00[1]		
CT	40 (42.6)	19 (29.2)	2.1	1.06-4.19	0.03
TT	10 (10.6)	2 (3.1)	5	1.03-24.14	0.029
C	128 (68.1)	107 (82.3)	1.00[1]		
T	60 (31.9)	23 (17.7)	2.2	1.26-3.76	0.004
TT + CT	50 (53.2)	21 (32.3)	2.4	1.23-4.6	0.01
Controls	*n* = 77	*n* = 93			
CC	40 (51.9)	52 (55.9)	1.00[1]		
CT	36 (46.8)	36 (38.7)	1.3	0.7-2.41	0.41
TT	1 (1.3)	5 (5.4)	0.26	0.03-2.31	0.197
C	116 (75.3)	140 (75.3)	1.00[1]		
T	38 (24.7)	46 (24.7)	0.99	0.61-1.64	0.99
TT + CT	37 (48.05)	41 (44.1)	1.2	0.64-2.15	0.6

[1]The first allele or genotype is the reference for statistical analysis; [2]Adjusted for age and gender; [3]Infected or non-infected patients with and without GC. GC: Gastric cancer; iNOS: Inducible nitric oxide synthetase; CC: Homozygous CC genotype; CT: Heterozygous CT genotype; TT: Homozygous TT genotype; C: Number of individual C alleles in the group; T: Number of individual T alleles in the group; HWE: Hardy Weinberg Equilibrium; OR: Odds ratio; CI: Confidence interval; *H. pylori*: *Helicobacter pylori*.

DISCUSSION

The study of *H. pylori*-induced disease is complicated by the fact that the etiology of disease appears to result from the intricate interplay between bacterial virulence factors, host genetics and environmental components. Moreover, an increasing body of literature indicates that colonization by *H. pylori* may even be beneficial to some hosts[10-12]. Because of these facts, there is a pressing need to develop new diagnostics that will clearly identify those individuals most at risk for development of the most severe form of *H. pylori*-induced disease-GC. To this end, numerous studies have looked at the role of virulence factors, environmental risk factors and host genetic polymorphisms in disease development. While these studies have shed an enormous amount of light on development of GC, it is becoming increasingly more evident that vari-

ous factors play different roles based on the population being studied[13-21,23-25,27-30]. Thus, there is a need for larger population-based studies to define the role of particular factors in the disease process. Despite the high prevalence of GC and *H. pylori* infection in Northern Iran, there have been relatively few studies that have examined specific risk factors associated with disease development in this population. Of those that have been conducted, one of the studies that investigated the high rate of cancer development in this region concluded that the classic *H. pylori* virulence factors were not a good predictor of disease outcome[45]. Furthermore, the authors went on to suggest that "...the association between strain virulence markers and disease in Iraq but not Iran suggests that other host and environmental factors may be more important in the disease-prone Iranian population"[45]. Given this suggestion and the deficit in our knowledge, we set out to examine the contribution of particular behavioral factors and genetic polymorphism in the *iNOS* gene to GC development in the Iranian population. Using a case-controlled study design we identified a significant association of several factors with an increased risk for GC. Among the behavioral factors that were important, smoking and consumption of hot beverages were both associated with development of GC. Both of these factors have been previously identified as risk factors for cancer in other populations[36,37]. However, our study is the first to link consumption of hot beverages with GC development in this particular region.

In addition to behavioral factors and similar to other previously reported risk factors[46], we found that a familial history of GC was significantly associated with increased risk of GC development in our study population (Table 1). Also similar to at least one other study in this region[2], we found that infection with the gastric pathogen *H. pylori* significantly increased the risk of developing GC. Finally, we found that carrying either a heterozygous (CT) allele or a homozygous (TT) allele at position 150 of exon 16 of the *iNOS* gene combined with *H. pylori* infection resulted in a significant increase in GC development.

In humans, NO is used as an important signaling molecule. However, in the presence of increased and/or sustained levels of this molecule, the beneficial effects of NO can be overcome by deleterious processes such as DNA damage and chronic inflammation. These pathologic events may be catalyzed by increased iNOS activity, which leads to increased NO production and concomitant increases in the amount of downstream free radicals. As the major source of NO in humans, perhaps it is not surprising that even minor amino acid changes in the iNOS enzyme can affect the levels of NO. The (C150T) polymorphism found in exon 16 of the *iNOS* gene was previously suggested to affect activity of this enzyme, leading to increased production of NO[36]. Interestingly, in our or other populations[36,37], the *iNOS* polymorphism alone was not a risk factor for cancer development. This fact suggests that while the C150T polymorphism may affect NO production, it does not do so to such an extent as to induce cancer development in isolation. Instead, a co-carcinogenic factor is required to synergistically interact in such a way as to promote disease progression. Within the population of Northern Iran, this co-carcinogenic factor appears to be *H. pylori* infection (Table 3). A similar result was also found within the Japanese population[36]. Alternatively, the *iNOS* polymorphism was suggested to act as a co-carcinogenic factor with past cigarette smoking and alcohol consumption within the Chinese population[37]. Thus, in multiple populations, iNOS polymorphism synergistically interacts with various factors to affect GC development.

While the molecular mechanism of synergistic interaction between the *iNOS* polymorphism and *H. pylori* infection is not clear, one plausible hypothesis is that since *H. pylori* infection alone is sufficient to increase NO levels in gastric tissue[47,48], the addition of a polymorphism that also increases iNOS enzyme activity could increase the amount of NO in the local tissue to a further degree. In turn, this combined increase in NO could skew the gastric mucosa toward a pro-inflammatory response and increase the likelihood of carcinogenic cellular damage. It is interesting to note that *H. pylori* infection alone creates a chronic pro-inflammatory environment within the gastric mucosa. Furthermore, it has been well documented that the ability of *H. pylori* to establish a persistent infection depends on maintenance of the proper balance between the host immune system and the bacterium (reviewed in[49]). In patients with either the CT or TT *iNOS* polymorphisms, the increased levels of NO may upset this delicate balance, thus perpetuating pathological and carcinogenic events.

H. pylori-mediated cancer development is a complex and multi-faceted process that involves a combination of bacterial, host, and environmental factors. Moreover, infection with *H. pylori* is inversely correlated with development of esophageal cancer[12], and is thought to provide protection against development of autoimmune disorders such as asthma[11]. Thus, the apparent benefit(s) of asymptomatically harboring this organism brings to bear the question of whether it is best to treat all patients for *H. pylori* infection; if not, the question becomes-how do we identify individuals most at risk for developing serious disease? One possible way to differentiate patients who have a high risk for disease development from those who are low-risk is to specifically examine bacterial, host-genetic and environmental factors that have been shown to be associated with cancer development in *H. pylori*-infected individuals. By screening individual patients for these three types of markers, it may be possible to specifically identify and treat those individuals who are at most risk for developing severe disease while reducing the risk of losing the apparent protective effects of *H. pylori* infection in low-risk individuals.

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COMMENTS

Background

As gastric cancer (GC) remains a serious source of morbidity and mortality worldwide, the identification of individuals most at risk for disease development is a major concern.

Research frontiers

Recent studies have highlighted the importance of polymorphisms in host genes as a risk factor for GC development. However, as many of these studies have been performed on limited patient populations, it remains unclear whether these polymorphisms are important across diverse ethnic groups and geographic areas. In this study, the authors identified an increase in GC risk in *Helicobacter pylori* (*H. pylori*)-infected Iranian patients who harbored a CT or TT polymorphism at position 150 in the inducible nitric oxide synthetase (*iNOS*) gene.

Innovations and breakthroughs

To the best of our knowledge, this study describes the first known link between a host-genetic factor and GC risk in an Iranian population. In addition, this study is the first to define the importance of the *iNOS* C150T polymorphism in a non-Asian population.

Applications

The results of this study suggest that the C150T *iNOS* polymorphism is a potential marker for susceptibility to *H. pylori*-mediated gastric disease.

Peer review

This is a study aimed at investigating the association of the *iNOS* C150T polymorphism with *H. pylori* infection and GC risk among patients from a region of Iran. The authors present an interesting study from a region that has not been addressed very often in regard to *H. pylori* and its related pathologies.

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BRIEF ARTICLE

Endothelial precursor cells promote angiogenesis in hepatocellular carcinoma

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Abstract

AIM: To investigate the role of bone marrow-derived endothelial progenitor cells (EPCs) in the angiogenesis of hepatocellular carcinoma (HCC).

METHODS: The bone marrow of HCC mice was reconstructed by transplanting green fluorescent protein (GFP) + bone marrow cells. The concentration of circulating EPCs was determined by colony-forming assays and fluorescence-activated cell sorting. Serum and tissue levels of vascular endothelial growth factor (VEGF) and colony-stimulating factor (CSF) were quantified by enzyme-linked immunosorbent assay. The distribution of EPCs in tumor and tumor-free tissues was detected by immunohistochemistry and real-time polymerase chain reaction. The incorporation of EPCs into hepatic vessels was examined by immunofluorescence and immunohistochemistry. The proportion of EPCs in vessels was then calculated.

RESULTS: The HCC model was successfully established. The flow cytometry analysis showed the mean percentage of CD133CD34 and CD133VEGFR2 double positive cells in HCC mice was 0.45% ± 0.16% and 0.20% ± 0.09% respectively. These values are much higher than in the sham-operation group (0.11% ± 0.13%, 0.05% ± 0.11%, n = 9) at 14 d after modeling. At 21 d, the mean percentage of circulating CD133CD34 and CD133VEGFR2 cells are 0.23% ± 0.19%, 0.25% ± 0.15% in HCC model *vs* 0.05% ± 0.04%, 0.12% ± 0.11% in control. Compared to the transient increase observed in controls, the higher level of circulating EPCs was induced by HCC. In addition, the level of serum VEGF and CSF increased gradually in HCC, reaching its peak 14 d after modeling, then slowly decreased. Consecutive sections stained for the CD133 and CD34 antigens showed that the CD133+ and CD34+ VEGFR2 cells were mostly recruited to HCC tissue and concentrated in tumor microvessels. Under fluorescence microscopy, the bone-marrow (BM)-derived cells labeled with GFP were concentrated in the same area. The relative levels of *CD133* and *CD34* gene expression were elevated in tumors, around 5.0 and 3.8 times that of the tumor free area. In frozen liver sections from HCC mice, cells co-expressing CD133 and VEGFR2 were identified by immunohistochemical staining using anti-CD133 and VEGFR2 antibodies. In tumor tissue, the double-positive cells were incorporated into vessel walls. These CD31 and GFP double positive cells were direct evidence that tumor vascular endothelial cells (VECs) come partly from BM-derived EPCs. The proportion of GFP CD31 double positive VECs (out of all VECs) on day 21 was around 35.3% ± 21.2%. This was much higher than the value recorded on day 7 group (17.1% ± 8.9%). The expression of

intercellular adhesion molecule 1, vascular adhesion molecule 1, and VEGF was higher in tumor areas than in tumor-free tissues.

CONCLUSION: Mobilized EPCs were found to participate in tumor vasculogenesis of HCC. Inhibiting EPC mobilization or recruitment to tumor tissue may be an efficient strategy for treating HCC.

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Key words: Hepatocellular carcinoma; Angiogenesis; Endothelial progenitor cells; Bone-marrow cells; Orthotropic hepatic cancer model

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most solid tumors, and neovascularization plays an important role in HCC development. In HCC, neovascularization status is correlated with disease progression and patient prognosis. Endothelial progenitor cells (EPCs) have been proved to be the main source of adult neovascularization. Until now, there has not been any data regarding the role of EPCs in HCC neovascularization. This study is the first to prove that EPCs incorporate into vessels directly and play an important role in HCC neovascularization.

It has been confirmed that neovascularization is vital to further multiplication, metastasis, and recurrence in malignant tumors. Without exogenous blood vessels, tumors stop growing and enter a state of suspended animation at about 2-3 cubic millimeters. The acquisition of angiogenic capacity is considered a sign of tumor development and of metastasis[1].

Recently, increasing amounts of data have revealed that bone-marrow (BM)-derived EPCs can foster the initiation and maintenance of angiogenic processes by integrating into developing vasculature under hypoxic conditions and into tumors. In 1997, EPCs were first isolated using anti-CD34 monoclonal antibodies. CD34-antigen, however, could not distinguish immature EPCs from mature circulating endothelial cells (CECs) because CD34 is expressed on both. With the discovery of CD133, an antigen restricted to immature EPCs, distinguishing between EPC and CECs has become easier[2]. VEFGR2 is another marker of EPCs. VEFGR2 signaling causes EPCs to differentiate. EPCs resemble embryonic angioblasts, which characteristically migrate, proliferate, and differentiate into mature endothelial cells[3]. In general, EPCs can be identified as cells that simultaneously express the cell surface markers CD133, CD34, and VEGFR2[4,5]. Santarelli *et al*[6] used mouse tumor models to demonstrate that BM-derived EPCs are greatly mobilized into the bloodstream, and home specifically to tumor tissues. In the tumor microenvironment, the recruited EPCs merged with the walls of a growing blood vessel, where they differentiated into endothelial cells and so promoted tumor growth. It has been reported that, during cancer proliferation, the number of circulating BM-derived progenitor cells increases dramatically. Circulating BM-derived EPCs have been used to determine the optimal dose of anti-angiogenic drugs, most successfully in breast cancer[7]. In addition, Taylor proved that high levels of circulating BM-derived progenitor cells are correlated with tumor metastatic status. Recently, the contribution of EPCs to tumor angiogenesis has also been reported in clinical tumor samples from 6 patients who received bone marrow transplants from donors of the opposite sex. The endothelial cells within the tumor vessels were of donor origin, as shown by the mismatched sex chromosomes[8].

At present, the significance of circulating EPCs and the contribution of EPCs to tumor vessels have been confirmed in only a few solid malignancies, such as malignant glioma[6], lung cancer[9], and mammal tumors[10]. However, the extent to which EPCs contribute to the generation of the tumor vessels varies significantly across different studies, from substantial to zero[11,12]. The role of EPCs in neovascularization has been the subject of great debate.

Tumor microvessel density is closely related to tumor growth, invasion, and metastasis and to patient prognosis[13,14]. It has been confirmed that the formation of new capillaries is essential to tumor development, and the status of angiogenesis is an important prognostic indicator in hepatocellular carcinoma[15,16]. There are only three papers in PUBMED, including our previous study[17], that compare the circulating EPC levels of HCC patients to those of healthy controls or those of liver cirrhosis patients[18,19]. We established an orthotropic hepatic cancer model, observed the dynamic changes of circulating EPCs, and determined whether BM-derived EPCs contributed to tumor vessels directly. We also calculated the incorporation rate of the EPCs in vessels. All the information is extremely important to evaluating the role of EPCs in neovascularization in HCC.

MATERIALS AND METHODS

Orthotropic hepatic cancer mouse model

For BM reconstruction, C57BL/6 male mice aged 8-10 wk were lethally irradiated (10 Gy) and transplanted with BM cells collected from green fluorescent protein (GFP)-C57BL/6 transgenic mice[6]. Four weeks later, the HCC

model showed a GFP positive rate of circulating nucleated cells of over 90%, suggesting full BM recovery. The mice were then used to construct an orthotropic hepatic cancer mice model by intra-hepatic injection of 1×10^5-2×10^5 H22 hepatoma cells (given to us by Dr. Ru-Tian Li of the Nanjing University Institute of Oncology, China). Sterile extract H22 hepatoma cells, normal saline to adjust the number of cancer cells to 106/mL for use. Intraperitoneal injection of sodium pentobarbital anesthesia C57BL/6 mice, disinfection shop towel, in the middle upper abdomen about the xiphoid mainly as a 1 cm incision into the abdominal cavity. Gently press out part of the lobe with bilateral costal arch, wet gauze pad in the bottom lobe to protect, and then with 1 mL medical syringe needles inserted along the long axis of lobe about 0.5 cm, the slow injection of H22 hepatoma cells 0.1 mL (cell number 1×10^5-2×10^5). Pull out the needle at the same time, rapidly at the pressure in the eye of a needle, and gently press on it with a cotton for 1 min to stop bleeding. Carefully place the liver back into the abdominal cavity. The control group received same volume of phosphate buffered solution (PBS). All mice were from the national genetically engineered mouse resource bank of China.

Degree of hepatocellular carcinoma mobilization

Flow cytometry (FCM) was used to evaluate the degree of EPC mobilization. Three hundred microliters of peripheral blood was obtained from the retroorbital plexus at 3 d, 7 d, 14 d, and 21 d after the establishment of the orthotropic HCC model. Erythrocytes were split by red blood cell lysis buffer. Mononuclear cells were incubated for 30 min at 4 ℃ with Alexa Fluor488 conjugated anti-CD133, Alexa Fluor647 conjugated anti-CD34, PE-conjugated anti-VEGFR2 (eBioscience, United States). Appropriate fluorochrome-conjugated isotypes were used as controls. In addition, serum concentration of vascular endothelial growth factor (VEGF), platelet-derived growth factor [colony-stimulating factor (CSF)] was tested by enzyme-linked immunosorbent assay kit (ADL, United States) following the collection of serum at the same times.

Identification of BM-derived CD133+CD34+VEGR2+ cells in tissue

Liver tissue was obtained from the GFP labeled BM-HCC model on day 14. After paraformaldehyde perfusion and gradient dehydration, consecutive frozen liver tissue sections 2 μm in thickness were cut in a cryostat, fixed, blocked, and incubated at 4 ℃ overnight with primary antibody rabbit anti-mouse CD133 (1:200), VEGFR2 (1:100, Abcam, United States), rat anti-mouse CD34 (1:250, ebioscience, United States) respectively. Secondary antibody was horseradish peroxidase. Positive reactions were visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Negative controls were obtained by substituting the primary antibodies with PBS.

Contribution of BM derived EPCs to vessels

Consecutive 2 μm frozen liver tissue sections were cut as stated above. Sections were blocked and then incubated overnight with rat anti-mouse CD31(1:800, eBiosicence, United States). After washing in PBS, slices were incubated with secondary antibodies Alexa Fluor 488-conjugated rabbit anti-rat IgG antibody (1:1000, molecular probes). 4',6'-diamidino-2-phenylindole hydrochloride was used to dry the nucleus. Normal mice were given PBS-sbustituted for antibodies-as a negative control. Double-labeled immunofluorescence staining sections were evaluated using fluorescence microscopy.

Proportion of EPCs in vessels

Mouse livers were obtained at 7 d or 21 d after the establishment of the GFP-BM HCC model. Two-microliter frozen anti-CD31 immunofluorescence staining sections were created as stated above. Specimens were analyzed microscopically by two independent observers who counted the number of BM-derived vascular ECs (VECs) co-expressing GFP, CD31, and total VECs in 15 random high-power fields. The quantitative contribution of EPCs to total VECs is expressed as percentages[20].

Expression of intercellular adhesion molecule 1, vascular adhesion molecule 1, and VEGF protein in tissue

Liver tissue was obtained from the GFP-labeled BM-HCC model on day 14. Conventionally processed and embedded sections cut at a thickness of 3 μm were deparaffinized, blocked, and incubated at 4 ℃ overnight with primary antibody: rabbit anti-mouse intercellular adhesion molecule 1 (ICAM1) (1:50, Protein Tech Group, United States), vascular adhesion molecule 1 (VCAM1) (1:200 Santa Cruz Biotech, Santa Cruz, CA, United States), VEGF (1:1000 Abcam, City, State, United States). The secondary antibody was horseradish peroxidase. Positive reactions were visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Negative controls were obtained by substituting the primary antibodies with PBS.

Levels of CD133 and CD34 genes in tissue

The levels of actin and CD133 mRNAs in the tumor and tumor-free areas were measured by real-time quantitative polymerase chain reaction (PCR). RNA was extracted from the livers with Trizol (Invitrogen, United States). cDNA was generated using Takara Primer ScriptTM 1st stand cDNA synthesis kits (JP). The sequences of primers were designed and synthesized by Takara. Primers for CD133 (5'-AACGTGGTCCAGCCGAATG-3', 5'-TCCCAGGATGGCGCAGATA-3'), CD34 (5'-ACCCACCGAGCCATATGCTTAC-3', 5'-GATACCCTGGGCCAACCTCA-3'), β-actin (5'-CAT CCG TAA AGA CCT CTA TGC CAA C-3', 5'-ATG GAG CCA CCG ATC CAC A-3'). The PCR reactions were performed in the light of instruction of SYBR Premix Ex TaqTM Kit (Takara, Japan) using the Stratagene

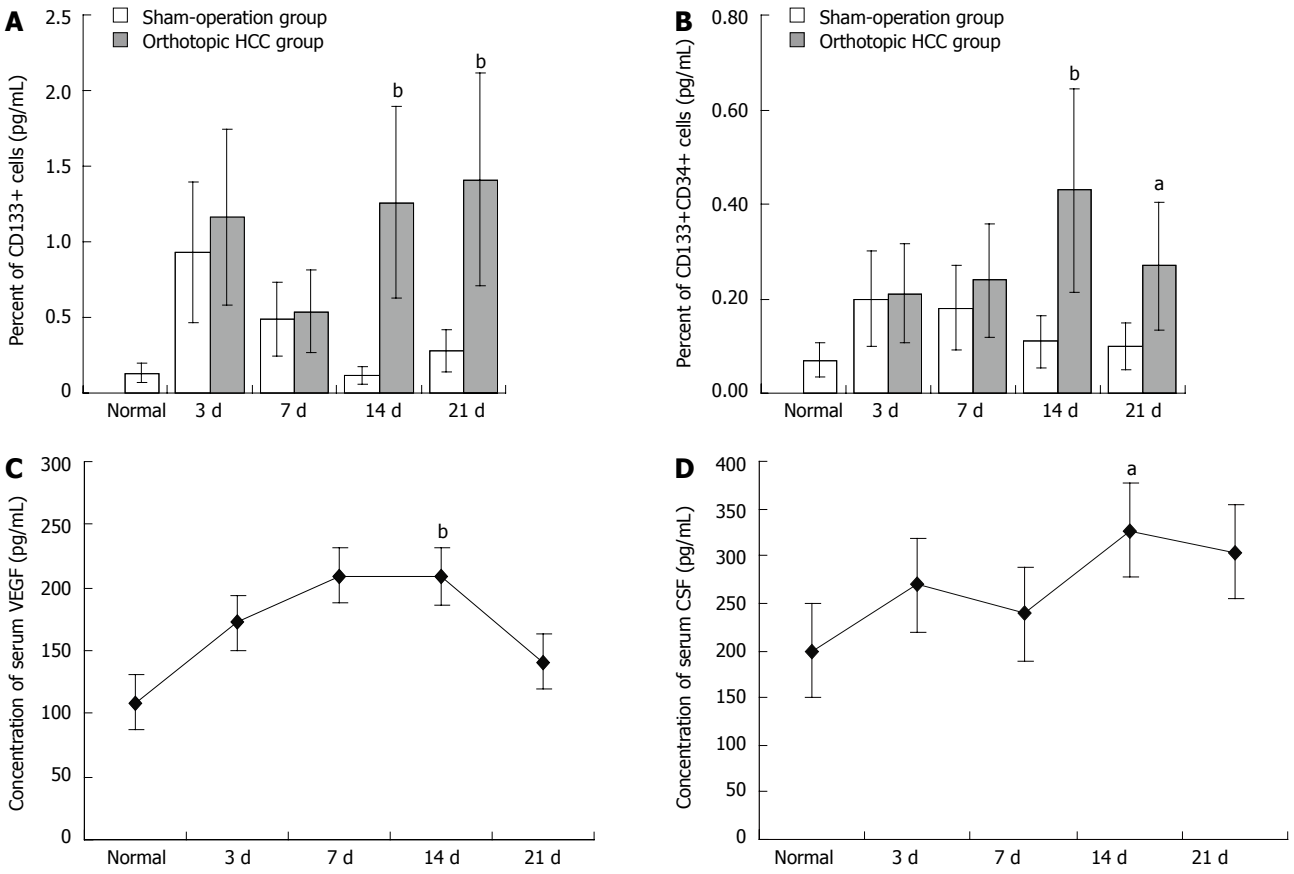

Figure 1 Mobilization of endothelial progenitor cells into circulation. A, B: Flow cytometry analysis of circulating endothelial progenitor cells in hepatocellular carcinoma (HCC) mice (day 14, n = 7; day 21, n = 9) *vs* sham-operation group (day 14, n = 9; day 21, n = 11). Normal = normal mice; C, D: Dynamic changes in serum levels of vascular endothelial growth factor and colony-stimulating factor in HCC mice (n = 8). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal control (n = 8). CSF: Colony-stimulating factor; VEGF: Vascular endothelial growth factor.

Mx3000P QPCR System (Bioscience Corporation). Each sample was measured in duplicate. The relative level of expression was determined by DDCt normalized to endogenous control (β-actin).

Statistical analysis

Statistical comparisons were performed using the t-test and analysis of variance when data were normally distributed or with separate variance estimation t-test or rank sum test. All statistical procedures were performed using SPSS 15.0. All data were presented as mean ± SD. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Establishment of BM orthotopic hepatocellular carcinoma model

The successful establishment of the HCC model was confirmed by a pathological exam confirming classic clinicopathological characteristics of HCC, intratumor hemorrhage, intrahepatic metastasis, and tumor neovascularization.

Mobilization of EPCs into circulation

The FCM analysis showed that the mean percentage of circulating CD133CD34 and CD133VEGFR2 double positive cells in HCC mice were 0.45% ± 0.16% and 0.20% ± 0.09% (n = 7), respectively. These values are much higher than in the sham-operation group (0.11% ± 0.13%, 0.05% ± 0.11%, n = 9) at 14 d after modeling. At 21 d, the mean percentage of circulating CD133CD34 and CD133VEGFR2 cells is 0.23% ± 0.19%, 0.25% ± 0.15% in HCC model *vs* 0.05% ± 0.04%, 0.12% ± 0.11% in control. The mobilization of BM cells may have been a response to the operation. Compared to the transient increase observed in controls, the higher level of circulating EPCs were induced by HCC (Figure 1A, B). In addition, the level of serum VEGF and CSF increased gradually in HCC, reaching its peak 14 d after modeling, then slowly decreased (Figure 1C, D).

Recruitment of EPCs to hepatocellular carcinoma tissue

Consecutive sections stained for the CD133 and CD34 antigens showed that the CD133+ and CD34+ VEGFR2 cells were mostly recruited to HCC tissue and concentrated in tumor microvessels (Figure 2A). Under fluorescence microscopy, the BM-derived cells labeled with GFP were concentrated in the same area (Figure 2B). The relative levels of CD133 and CD34 gene expression were elevated in tumors, around 5.0 and 3.8 times that of the tumor free area (Figure 2C, D). As determined by protein and gene expression levels, EPCs in HCC mice were recruited to tumors and tumor microvessels. The results of each three trials were largely consistent with each other.

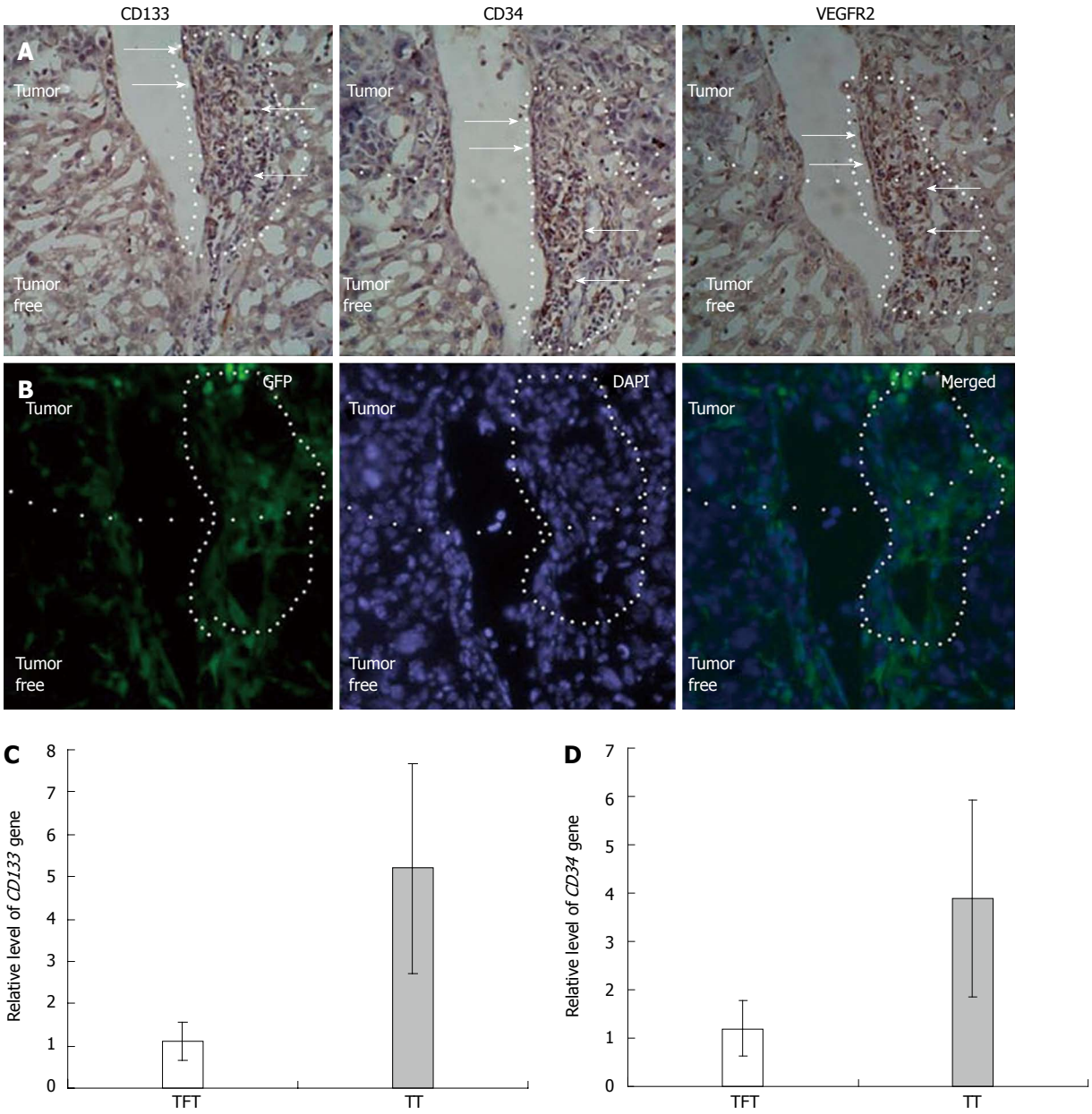

Figure 2 Recruitment of endothelial progenitor cells to hepatocellular carcinoma tissue. A: Distribution endothelial progenitor cells (EPCs) in tumor and tumor-free tissue in four consecutive 2 μm sections (arrows: Cells expressing the indicated antigens and recruited to tumors and tumor vessels); B: Bone-marrow cells labeled by green fluorescent protein recruited to tumors and tumor vessels, occupying the same position as antigen positive cells [blue regions: Nuclei stained by DAPI (frozen section, 2 μm, magnification 200)]; C: Relative levels of CD133 mRNA in tumor tissue (n = 7) and tumor-free tissue (n = 6); D: Relative level of CD34 mRNA in tumor tissue (n = 7) and tumor-free tissue (n = 7). TFT: Tumor-free tissues; TT: Tumor tissues; DAPI: 4',6'-diamidino-2-phenylindole hydrochloride; GFP: Green fluorescent protein.

Contribution of BM-derived EPCs to tumor blood vessels

In frozen liver sections from HCC mice, cells co-expressing CD133 and VEGFR2 were identified by immunohistochemical staining using anti-CD133 and VEGFR2 antibodies. In tumor tissue, the double-positive cells were incorporated into vessel walls (Figure 3C). In immunofluorescent staining, an antigen specific to mature endothelial cells CD31[21] was used to identify VECs. The image data show that cells co-expressing CD31 and GFP existed not only in sinusoid endothelial cells (Figure 3A) but also in hepatic veins (Figure 3B). These CD31 and GFP double positive cells are direct evidence that tumor VECs come partly from BM-derived EPCs. The proportion of GFP CD31 double positive VECs (out of all VECs) on day 21 was around 35.3% ± 21.2%. This is much higher than the value recorded on day 7 group (17.1% ± 8.9%) (Figure 3D).

Expression of ICAM1, VCAM1, and VEGF in tumor tissue

For further understanding the mechanism by which BM-derived EPCs home to tumors, we detected the expres-

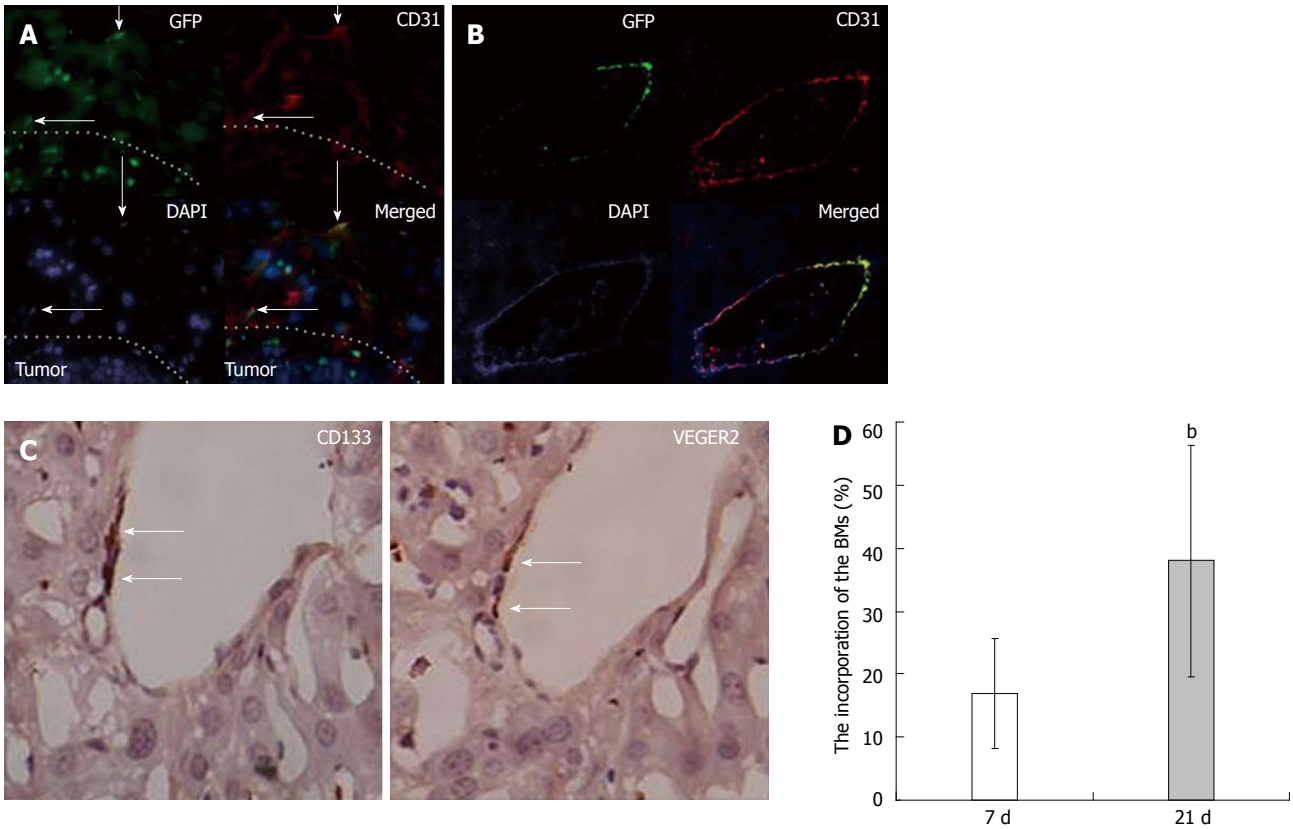

Figure 3 Bone-marrow-derived endothelial progenitor cells were found to contribute to vessel formation in hepatocellular carcinoma tissues. A: Bone marrow derived endothelial progenitor cells (EPCs) incorporated into peritumoral vessels; B: Distribution of CD133 and VEGFR2 antigens in hepatocellular carcinoma (HCC) liver vessels (Arrows: Double-positive cells, CD133+ VEGFR2+ cells incorporating into microvessels in the liver. Magnification, 400); C: Bone-marrow (BM)-derived EPCs incorporated into hepatic veins [Red: Mature vascular endothelial cells (VECs) are marked by CD31; Green: BM derived cells expressing green fluorescent protein (GFP); Blue: Nuclei stained by DAPI; Arrows: VECs co-expressing GFP and CD31, frozen, HCC, 2 μm, magnification 400]; D: The incorporation rate of the EPCs in HCC blood vessels at 7 d *vs* that observed at 21 d. $^{b}P < 0.01$ *vs* that observed at 21 d, $n = 15$. DAPI: 4',6'-diamidino-2-phenylindole hydrochloride.

sion of ICAM1, VCAM1, and VEGF in tumor tissues and tumor-free areas using immunohistochemistry. The results showed that the expression of ICAM1, VCAM1, and VEGF was higher in tumor areas than in tumor-free tissues (Figure 4).

DISCUSSION

Enhanced neovascularization is the most obvious clinicopathological characteristic of HCC, and tumor microvascular density is closely related to HCC progression and patient prognosis[2,22]. Although studies in some tumor models have shown that EPCs are mobilized into peripheral blood and contribute to tumor vascularization, to date, only Hong Kong University, our hepatic cancer institute, and the Medical University of Vienna have successively demonstrated that levels of circulating EPCs increase significantly and are correlated to clinical outcome in patients with HCC. The role of EPCs in HCC neovascularization is not currently clear. Based on our previous clinical findings[17,23], we established an orthotopic HCC model and used GFP as tracer to evaluate the role of EPCs in HCC-related angiogenesis[24,25]. According to PUBMED search, this is the first article to show direct visual evidence proving that EPCs promote angiogenesis by differentiating into vascular endothelia cells in HCC.

Thus far, the clinical significance of circulating EPCs has been confirmed in only a few solid malignancies, such as neurospongioma[25], hepatic cancer[17-19], breast cancer[26,27] and ovarian cancer[28]. Levels of circulating BM-derived stem cells have been used to determine the optimal dose of anti-angiogenic drugs, most successfully in breast cancer patients[7]. In the current investigation, which, unlike our previous clinical study, does not involve any interference from cirrhosis, it is much clearer that the mobilization of EPCs and the up-regulation of the BM-mobilization-related factors VEGF and CSF are the results of the development of HCC (Figure 1). Though wounds and injuries also can mobilize EPCs into the blood[29], the controls in this study showed only transient increases in, VEGF and CSF, and levels of circulating EPCs remained relatively low[30]. In terminal stage of the tumor, the decline in levels of serum cytokines and circulating EPCs may be the result of a disordered environment and systemic failure.

Characterization of EPCs, proof-of-principle strategies design to study the importance of vasculogenesis in tumors are current impediments. CD34-antigen could not distinguish immature EPCs from mature CECs. Recently, scientists discovered an antigen restricted to immature

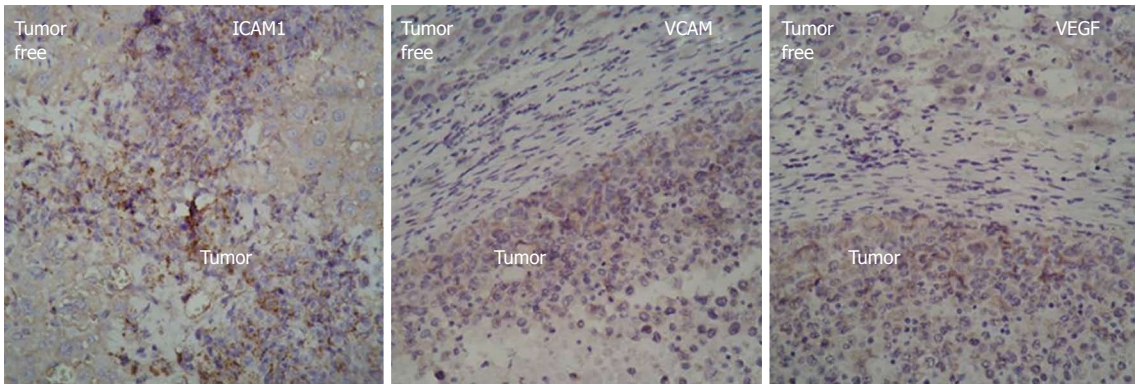

Figure 4 Expression of intercellular adhesion molecule 1, vascular adhesion molecule 1, and vascular endothelial growth factor in tumor tissues and tumor-free tissues as detected by immunohistochemistry (paraffin, liver, 2 μm, magnification, 200).

EPCs named CD133 which can distinguish between EPC and CECs. VEFGR2 is another marker of EPCs. VEFGR2 signaling causes EPCs to differentiate. EPCs resemble embryonic angioblasts, which characteristically migrate, proliferate, and differentiate into mature endothelial cells. Generally, EPCs can be identified as cells that simultaneously express the cell surface markers CD133, CD34, and VEGFR2.

In this study, we used GFP as a tracer because that tumor growth is accompanied by the formation of tumor blood vessels, we hypothesized that EPCs could home to liver tumors specifically. To test this hypothesis. The immunohistochemical image data of HCC tissue serial frozen sections showed many cells expressing EPC-specific antigens CD133, CD34, and VEGFR2, and these were concentrated in tumor tissues and tumor vessels (Figure 2A). Relative to tumor-free tissues, many BM-derived cells labeled by GFP specifically homed to tumor tissues and tumor vessels occupying the same positions as the CD133-, CD34-, and VEGFR2-positive cells (Figure 2B). This indicates that these antigen-positive cells come from bone marrow and co-express the specific antigens CD133, CD34, and VEGFR2. Real-time PCR analysis showed the levels of mRNA for stem cell markers CD133 and CD34 were around 5 and 3 times higher, respectively, than in tumor free tissues (Figure 2C). These results may be due to the fact that CD34 is expressed in both mature and immature cells, while CD133 is limited to immature cells (Figure 3B). EPCs' tropism for malignant tissues can also be observed in other cancer models, in which ICAM-1 is considered the main endothelial surface receptor responsible for recruitment of EPCs[31]. VEGF is believed to be the most important and essential mobilization factor in the induction of matrix metalloproteinase-9 (MMP-9) expression in bone marrow cells (BMCs). MMP-9, in turn, cleaves and activates the VEGF receptor of BMCs, permitting the transfer of BMCs and mobilizing EPCs into circulation[32]. In our HCC model, besides up-regulated VEGF, the levels of VEGF and ICAM1 in tumor tissue also increased significantly. This is consistent with our previously published clinical results[19]. From this, we can reasonably infer that, during tumor growth, HCC tissue releases mobilization factors, such as VEGF, into circulation. As the matrix degrades, BM-derived EPCs migrate into the liver through the bloodstream. Under the chemoattractive influence of locally high VEGF content VEGF and cellular adhesive molecules, the EPCs become come to rest in the hepatic tumor area.

Until now, the contribution of EPCs to blood vessel formation has been proved in only a few types of cancer, including malignant glioma[26] and Ewing's sarcoma[33,34]. We have identified and confirmed the clinical significance of mobilized EPCs in HCC[17]. Here, we directly prove that EPCs contribute to the formation of new HCC vessels not only in hepatic sinusoids (Figure 3A) but also in the veins of the liver (Figure 3C). Moreover, immunohistochemical image data has directly shown that cells co-expressing CD133 and VEGFR2 incorporate into liver vessels (Figure 3D). This indicates that BM-derived EPCs stimulate tumor growth by promoting neovascularization. In addition, the incorporation rate of EPCs on day 21 was found to be much higher than on day 7. In this sense, the role of EPCs in neovascularization seems to become more important as HCC growth progresses (Figure 4C). Mature ECs have relatively limited proliferative capacity. In the current body of literature, the proportion of EPCs to VECs has been found to vary widely, from zero to more than 90%[11,12]. Other studies have concluded that EPCs do not contribute to the vascular endothelium and are not needed for tumor growth[35]. The fact that these studies used different tumor models may be the source of these different results.

Ever since 1971, anti-angiogenesis and anti-vascular treatments have emerged as a clinically promising anti-cancer strategy in HCC therapy. However, though anti-angiogenic therapies to HCC have been used clinically for a long time, the actual effect is not as satisfying as would be expected. Damaging tumor vasculature or blood embolization does lead to local metabolic disturbance, acidosis, hypoxia, and eventually to necrosis. This causes the release of soluble factor into the bloodstream by tumors directly and by the responding system indirectly. These soluble factors mobilize more EPCs into the blood and

promote the formation of new blood vessels to compensate for the lost blood supply. In fact, it has been found that tumor necrosis, hypoxia, and ischemia can all cause dramatic increases in serum levels of VEGF and in the number of EPCs in circulation EPCs[36-38]. These cells home to the vasculature of treated tumors and promote tumor neovascularization. In such cases, even if tumor vasculature is damaged and local angiogenesis is inhibited, BM-derived EPCs independently form the blood vessels necessary for tumor regrowth. The existence of compensatory cellular and molecular mechanisms inhibits the efficacy current targeted anti-angiogenic therapies. We suggest inhibiting the recruitment of circulating EPCs in concert with anti-vascular treatment. We have demonstrated previously that the number of circulating EPCs rises dramatically in patients with HCC. Now we further display that, during the course of HCC, large numbers of EPCs are mobilized and recruited to tumor areas, where they promote HCC angiogenesis by integrating directly into the tumor vessels directly. As HCC develops, the proportion of EPCs in vessels seems to rise gradually.

In view of current data, we have reason to believe that the BM is the important source of cells for neovascularization in HCC. We infer that blockage of EPC mobilization or recruitment to tumors may slow down hepatic cancer growth and improve the efficacy of therapies.

COMMENTS

Background

Neovascularization is the key process for tumor development, invasiveness and metastasis. It has been accepted that tumor-neovascularization raised in two ways, one is endothelial sprouting, another is bone marrow derived cells differentiating into mature vascular endothelial cells. Hepatocellular carcinoma (HCC) is one of the most vascular solid tumors, in which neovascularization plays an important role. The status of neovascularization in HCC correlates with the disease progression and patient's prognosis.

Research frontiers

At present, the significance of circulating endothelial progenitor cells (EPCs) and contribution of EPCs to tumor vessels have been confirmed only in a few solid malignancies, such as malignant glioma, lung caner and mammal tumor, however, the extent to which EPCs contribute to the generation of the tumor vessels is highly variable in different studies, from substantial to zero. So the role of EPCs on neovascularization has been argued a lot. Till now, there is no any data to evaluate the role of bone marrow cells (BMCs) in HCC neovascularization yet.

Innovations and breakthroughs

Ever since Professor Folkman put forward the dependence of tumor growth on angiogenesis in 1971, anti-angiogenesis or anti-vascular treatment has emerged as a clinically promising anti-cancer strategy in HCC therapies. However, though anti-angiogenic therapies to HCC such as chemotherapy and embolization have been in clinical application for a long time, the actual effect is not as satisfying as expected yet. It is well known HCC is a highly vascularized solid tumor. The authors have demonstrated that circulation BMCs/bone marrow-derived stem cells rise dramatically in patients with HCC previously in clinical cancer research, now the authors further display during the course of HCC, large number of BMCs was mobilized and recruited to tumor area, then promote HCC angiogenesis by integrating into tumor vessels directly.

Applications

The study results suggest that the BMCs play a prominent role in HCC neovascularization, and inhibiting EPC mobilization or recruitment to tumor tissue may be an efficient strategy for treating HCC.

Terminology

EPCs: Endothelial progenitor cells can differentiate into mature vascular endothelial cells for formation of new vessels (vasculogenesis).

Peer review

This is a good descriptive study to investigate the role of bone marrow-derived EPC in the angiogenesis of HCC. The results are interesting and suggest that mobilized EPCs were found to participate in tumor vasculogenesis of HCC and inhibiting EPC mobilization or recruitment to tumor tissue may be an efficient strategy for treating HCC.

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BRIEF ARTICLE

Coexistence of hyperlipidemia and acute cerebral ischemia/reperfusion induces severe liver damage in a rat model

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Abstract

AIM: To investigate the correlation of hyperlipemia (HL) and acute cerebral ischemia/reperfusion (I/R) injury on liver damage and its mechanism.

METHODS: Rats were divided into 4 groups: control, HL, I/R and HL+I/R. After the induction of HL *via* a high-fat diet for 18 wk, middle cerebral artery occlusion was followed by 24 h of reperfusion to capture I/R. Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were analyzed as part of liver function tests and liver damage was further assessed by histological examination. Hepatocyte apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The expression of genes related to apoptosis (caspase-3, bcl-2) was assayed by immunohistochemistry and Western blotting. Serum tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and liver mitochondrial superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and Ca^{2+} levels were measured to determine inflammatory and oxidative/antioxidative status respectively. Microsomal hydroxylase activity of the cytochrome P450 2E1 (CYP2E1)-containing enzyme was measured with aniline as the substrate, and CYP2E1 expression in the liver tissue and microsome was determined by immunohistochemistry and Western blotting respectively.

RESULTS: HL alone induced by high-fat diet for 18 wk resulted in liver damage, indicated by histopathological analysis, and a considerable increase in serum ALT (25.13 ± 16.90 *vs* 9.56 ± 1.99, $P < 0.01$) and AST levels (18.01 ± 10.00 *vs* 11.33 ± 4.17, $P < 0.05$) compared with control. Moreover, HL alone induced hepatocyte apoptosis, which was determined by increased TUNEL-positive cells (4.47 ± 0.45 *vs* 1.5 ± 0.22, $P < 0.01$), higher caspase-3 and lower bcl-2 expression. Interestingly, compared with those in control, HL or I/R groups, massive increases of serum ALT (93.62 ± 24.00 *vs* 9.56 ± 1.99, 25.13 ± 16.90 or 12.93 ± 6.14, $P < 0.01$) and AST (82.32 ± 26.92 *vs* 11.33 ± 4.17, 18.01 ± 10.00 or 14.00 ± 6.19, $P < 0.01$) levels in HL+I/R group were observed suggesting severe liver damage, which was confirmed by liver histology. In addition, HL combined with I/R also caused significantly increased hepatocyte apoptosis, as evidenced by increased TUNEL-positive cells (6.20 ± 0.29 *vs* 1.5 ± 0.22, 4.47 ± 0.45 or 1.97 ± 0.47, $P < 0.01$), elevated expression of caspase-3 and lower expression of bcl-2. Furthermore, when compared to HL or I/R alone, HL plus I/R enhanced serum TNF-α, IL-1, liver mitochondrial MDA and Ca^{2+} levels, suppressed SOD and GSH-Px in liver mitochondria, and markedly up-regulated the activity (11.76 ± 2.36 *vs* 4.77 ± 2.31 or 3.11 ± 1.35, $P < 0.01$) and expression (3.24 ± 0.38 *vs* 1.98 ± 0.88 or 1.72 ± 0.58, $P < 0.01$) of CYP2E1 in liver.

CONCLUSION: The coexistence of HL and acute cerebral I/R induces severe liver damage, suggesting that cerebral ischemic stroke would exaggerate the damage of liver caused by HL. This effect is possibly due to en-

hanced CYP2E1 induction which further promotes oxidative damage, inflammation and hepatocyte apoptosis.

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Key words: Hyperlipidemia; High-fat diet; Cerebral ischemia/reperfusion; Liver damage; Hepatocyte apoptosis; Cytochrome P450 2E1

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INTRODUCTION

The history of hyperlipidemia (HL), an increasing public health problem, is positively associated with acute cerebrovascular diseases (ACD) such as ischemic stroke[1]. Although HL *per se* is not a direct predictor of ACD, the condition with high plasma levels of inflammatory-sensitive proteins associated with HL links to the increased incidence of stroke[2]. Positive correlations between excess lipid levels and the promotion of inflammation have been documented[2]. Moreover, it has been widely accepted that oxidative stress, an early event in the evolution of HL[3], also plays an essential role in the pathophysiology of ischemic stroke[4]. So it can be speculated that HL, as a highly prevalent risk factor for ACD, frequently accompanies ACD.

In addition, HL is also a major risk factor for liver disease[5]. The spectrum of HL-induced liver disease, also known as non-alcoholic fatty liver disease (NAFLD), ranges from "simple" steatosis to non-alcoholic steatohepatitis, which is characterized by oxidative stress, inflammation, and liver damage as well as fat deposition[6]. Of interest is that one of the main complications of the patient with ACD is the risk of secondary liver dysfunction. Ischemic stroke will further trigger the activation of inflammatory cascades leading to systemic inflammatory response[7]. In the systemic inflammatory environment developed by circulating reactive oxygen species (ROS) and pro-inflammatory cytokines after ACD, nonneurological organs, including liver, would be impaired leading to multiple organ dysfunction syndromes (MODS). The incidence of MODS in patients with ACD was reported as 11.5%, the mortality rate was 40.3%[8]. It is noteworthy that one of the major systemic complications of ACD is liver dysfunction, the presence of which is associated with mortality and overall poor outcome[9]. Clinical studies in China have showed, in patients of ACD with MODS, 26.83%[8] or 15.5%[10] had abnormal liver function. Therefore, HL or ACD is respectively known to be the independent risk factor for liver injury. It is thus reasonable to hypothesize that the coexistence of HL and ACD may contribute to more severe liver dysfunction.

As mentioned above, the pathological changes in liver caused by either HL or cerebral ischemia are associated with inflammation and oxidative stress. Inflammatory response and even apoptosis could be triggered by oxidative stress[11], which may be a key mechanism of liver injury induced by HL or cerebral ischemia. The source of oxidative stress is controversial but may result in part from over-expression of the prooxidant enzyme cytochrome P450 2E1 (CYP2E1), that has been reported to occur in human and experimental NAFLD[12]. As a major microsomal source of ROS, CYP2E1 mainly located in the endoplasmic reticulum and played a significant role in the biotransformation of fatty acid oxidation. ROS overproduction induces oxidative damage to liver DNA and contributes to hepatocellular injury[13]. The expression of CYP2E1 in liver can be enhanced in rats treated with high-fat diet[14]. In addition, brain injury or inflammation has influence on some liver cytochrome P450 enzymes[15]. But the effect of HL together with ACD on CYP2E1 is still unclear.

In this study, we hypothesized that HL or cerebral ischemia/reperfusion (I/R) injury may directly induce a series of changes leading to a pro-oxidant and pro-inflammatory state, which may finally result in the onset of liver injury, and the coexistence of HL and cerebral I/R injury may cause much more severe changes and the exacerbation of liver injury. To verify this assumption, a repeatable experimental hyperlipemic rat model with cerebral I/R injury was established to investigate the correlation of HL and/or acute cerebral I/R injury on liver damage, and further examine the effects of HL and/or I/R on hepatocyte apoptosis, CYP2E1, oxidative/antioxidant system and inflammatory process to understand the mechanism of liver damage.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats, weighing 200-250 g, were obtained from Hubei Laboratory Animals Center for Medical Science and Research (No. 00006231). The animals were allowed to acclimatize for 1 wk while being maintained at a room temperature of 22 ± 2 ℃ on a 12 h light/dark cycle with free access to standard rodent chow food and water (Standard sustain feed, from Hubei Laboratory Animals Center for Medical Science and Research, Wuhan, China). Housing facilities and all experimental protocols were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animals Care and Use Committee of Wuhan University of Science and Technology Medical College which adopts the guideline

Table 1 The examined animal groups

Group	Administration
Control	Rats given normal diet (control) for 18 wk and without I/R
HL	HL rats given high-fat diet for 18 wk and without I/R
I/R	Rats given normal diet (control) for 18 wk and with I/R
HL+I/R	HL rats given high-fat diet for 18 wk and with I/R

HL: Hyperlipidemia; I/R: Ischemia/reperfusion.

for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experiment design

The rats were fed either a standard low-fat rat chow diet (control diet) or a high-fat diet (HFD, 1% cholesterol, 10% lard, 20% sugar, 5% egg yolk, 0.2% bile salts and 63.8% standard chow, finally analyzed by the Laboratory Animal Centre of Hubei). After 18 wk of HFD, blood was withdrawn from the tail vein of overnight fasted rats for confirming HL. Serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were detected by Beckman Coulter synchron LX20 autoanalyzer (Beckman Coulter Inc., United States). The animals with serum TC level > 3.87 mmol/L were considered HL and included in the study[16]. Subsequently, the control diet group and HL group were respectively divided into two subgroups treated with or without cerebral I/R (Table 1). After HL was confirmed, the rats in I/R or HL+I/R group were subjected to middle cerebral artery occlusion (MCAO)[17] for 2 h and followed by 24 h reperfusion to induce focal cerebral I/R injury. As the sham control, rats in control or HL groups underwent the same surgical procedure as in I/R or HL+I/R group without inserting the nylon thread.

Behavioral score and brain infarct volume were evaluated at 24 h after reperfusion by researchers and another assistant who was not involved in this experiment. The neurological deficits were scored on a 5-point scale based on the report of Longa *et al*[18]. All animals were sacrificed after the evaluation of behavioral scores. The area of cerebral infarction was quantified with TTC (2, 3, 5-triphenyltetrazolium chloride) staining as previous described[13].

Measurement of liver function and serum pro-inflammatory cytokines

Blood samples were obtained from the common carotid artery to detect serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) when rats were sacrificed at 24 h after reperfusion. The levels of serum ALT and AST were measured using corresponding kits (Jiancheng, Nanjing, China) to assess the liver function. Serum TNF-α and IL-1 levels were measured by radioimmunoassay kits (Albert Poole Biotechnology, Beijing, China).

Histological and immunohistochemical examination of liver

Harvested livers were placed into 10% formalin immediately after excision, and immersed for 24 h. Liver specimens were then embedded in paraffin, and sections were cut at 5 μm. Sections were stained for: (1) Hematoxylin and eosin (HE); (2) Apoptosis was detected by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method using an *in situ* apoptosis detection kit (Boster Biological Engineering, Wuhan, China) as previously described[19]. The number of cells with TUNEL-positive nuclei was counted from 20 randomly selected fields at × 200 magnification per liver sample. Results were expressed as the mean number of TUNEL-positive apoptotic hepatocytes per microscopic field; and (3) Immunohistological analysis of CYP2E1, caspase-3, bcl-2 expression in paraffin wax-embedded liver sections were performed using a standard peroxidase-antiperoxidase technique as described previously[20], using a CYP2E1 rabbit anti-mouse polyclonal antibody (Abcam Inc, United Kingdom) or mouse monoclonal antibodies against caspase-3 and bcl-2 (Santa Cruz, CA, United States) at a 1:150 dilution, with a biotinylated goat anti-rabbit or goat anti-mouse antibody (Santa Cruz, CA, United States) as the secondary antibody. Brown colour in the cytoplasm of the hepatocytes was evaluated as positive staining.

Determination of antioxidant enzymes activities, MDA and Ca^{2+} content in liver mitochondria and microsomal CYP2E1-dependent aniline hydroxylase activity

Liver subcellular fractions were prepared by differential centrifugation according to Khemawoot *et al*[21] with slight modifications. Livers were homogenized (20% w/v) with phosphate buffer (50 mmol/L K_2HPO_4 containing 0.1 mmol/L EDTA, pH 7.4). The homogenate was centrifuged at 200 *g* for 10 min at 4 ℃, and then the supernatant was recentrifuged at 9000 *g* for 20 min. The mitochondrial pellet was collected, resuspended in 0.25 mol/L sucrose solution, and stored at -80 ℃ until detection of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and Ca^{2+} levels using the corresponding kits (Jiancheng, Nanjing, China) respectively.

Then the supernatant was recentrifuged at 105 000 × *g* for 60 min. The microsomal pellet was washed, resuspended in 0.25 mol/L sucrose solution, and stored at -80 ℃ until determination of CYP2E1 activity and expression. Microsomal protein concentration was measured according to the method of Lowry *et al*[22]. Microsomal hydroxylase activity of the CYP2E1-containing enzyme was measured with aniline as the substrate according to the procedure described previously[23].

Western blotting of liver caspase-3, bcl-2 and microsomal CYP2E1

Proteins of liver homogenate or microsome were separated by SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane by electroblotting.

Table 2 Blood lipids in different groups after high-fat diet treatment for 18 wk and before middle cerebral artery occlusion (mean ± SD, mmol/L)

Group	*n*	TC	TG	HDL-C	LDL-C
Control	10	1.69 ± 0.14	0.21 ± 0.08	1.14 ± 0.05	0.40 ± 0.10
HL	6	5.11 ± 0.55[b,e]	0.84 ± 0.20[a,f]	0.68 ± 0.15[a,f]	1.72 ± 0.52[a,e]
I/R	10	1.57 ± 0.19	0.19 ± 0.02	1.21 ± 0.16	0.36 ± 0.10
HL+I/R	10	5.33 ± 0.44[b,e]	0.79 ± 0.17[a,f]	0.70 ± 0.19[a,f]	1.64 ± 0.48[a,e]

HL: Hyperlipidemia; I/R: Ischemia/reperfusion; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [e]$P < 0.05$, [f]$P < 0.01$ *vs* I/R group.

Membrane was blocked overnight and then incubated for 2 h with a 1:1000 dilution of a rabbit polyclonal anti-rat CYP2E1 antibody (Abcam Inc, United Kingdom) or a rabbit polyclonal anti-mouse, caspase-3, bcl-2 antibody (Santa Cruz, CA, United States). After incubation with the secondary antibody, proteins were detected with an electrochemiluminescence chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom), and scanned with a Typhoon 9200 scanner (Amersham Biosciences Europe GmbH, Freiburg, Germany). The amount of protein expression was corrected by the amount of β-actin in the same sample.

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed using Student's *t* test. Probability values less than 0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

Evaluation of animal model

After 18 wk of HFD and prior to ischemia, serum levels of TC, TG and LDL-C in HL or HL+I/R group were significantly higher than those in control or I/R group, whereas HDL-C was lower ($P < 0.05$ or $P < 0.01$) (Table 2). There was no significant difference between the HL group and the HL+I/R group.

At 24 h after reperfusion, rats in the I/R or HL+I/R groups that were successfully operated with MCAO and reperfusion appeared to have a significantly higher neurological deficit score and brain infarct volume than those in the control or HL group ($P < 0.01$) (Table 3). There was no significant difference between the I/R group and the HL+I/R group.

These results strongly suggested that the animal model of HL combined with I/R was established successfully.

Liver damage caused by HL plus I/R

Plasma ALT and AST levels are common biomarkers for liver damage[24]. As compared to the control group, plasma activities of ALT and AST were elevated in the HL group ($P < 0.05$; $P < 0.01$), but not affected significantly by I/R alone; marked changes in serum ALT and AST activities suggesting hepatic damage were shown in the HL+I/R group ($P < 0.01$). The most severe liver damage was found in the HL+I/R group compared to HL or I/R alone ($P < 0.01$) (Table 3).

Table 3 Brain infarct volume and serum alanine aminotransferase and aspartate aminotransferase in different groups after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion (mean ± SD)

Group	*n*	Neurological deficit score	Infarct volume (mm^3)	ALT (U)	AST (U)
Control	10	0	0	9.56 ± 1.99	11.33 ± 4.17
HL	6	0	0	25.13 ± 16.90[b]	18.01 ± 10.00[a]
I/R	7	3.25 ± 0.36[b,d]	33.4 ± 4.16[b,d]	12.93 ± 6.14	14.00 ± 6.19
HL+I/R	6	3.53 ± 0.45[b,d]	34.5 ± 6.23[b,d]	93.62 ± 24.00[b,d,f]	82.32 ± 26.92[b,d,f]

HL: Hyperlipidemia; I/R: Ischemia/reperfusion; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* HL group; [f]$P < 0.01$ *vs* I/R group.

To analyze the extent of liver injury, liver sections were stained with HE (Figure 1A). No apparent damage was found in liver sections from the control or I/R group. Hepatocytes of the HL group showed fatty infiltration changes, light vacuolar change, scattered inflammatory cells and mild hepatocyte necrosis. However, extensive damage was detected in liver sections from HL+I/R rats. The affected livers displayed remarkable fatty accumulation, infiltration of a mixed population of inflammatory cells, hepatocyte ballooning degeneration and focal necrosis. These changes were more prominent when compared to HL or I/R alone.

Hepatocyte apoptosis caused by HL plus I/R

We assessed hepatocyte apoptosis using the TUNEL assay. TUNEL-positive cells appeared occasionally in liver sections of control or I/R groups, diffused widely in the HL group, and were most frequently observed in the HL+I/R group (Figure 1B). The number of TUNEL-positive apoptotic hepatocytes (6.2 ± 0.29 apoptotic cells/× 200 field) in the HL+I/R group was significantly greater than those in the control group (1.5 ± 0.22 apoptotic cells/× 200 field), HL group (4.47 ± 0.45 apoptotic cells/× 200 field), and I/R group (1.97 ± 0.47 apoptotic cells/× 200 field) ($P < 0.01$).

Encouraged by the severe liver damage and hepatocyte apoptosis caused by HL plus I/R, we further investigated whether the CYP2E1, oxidative/antioxidant system and pro-inflammatory cytokines are involved in this pathological condition.

Induction of hepatic CYP2E1 by HL plus I/R

The activities of CYP2E1-dependent aniline hydroxylase activity in the microsome, where CYP2E1 is mainly located in liver, were higher in the HL or the I/R group ($P < 0.01$; $P < 0.05$ *vs* control), and increased consider-

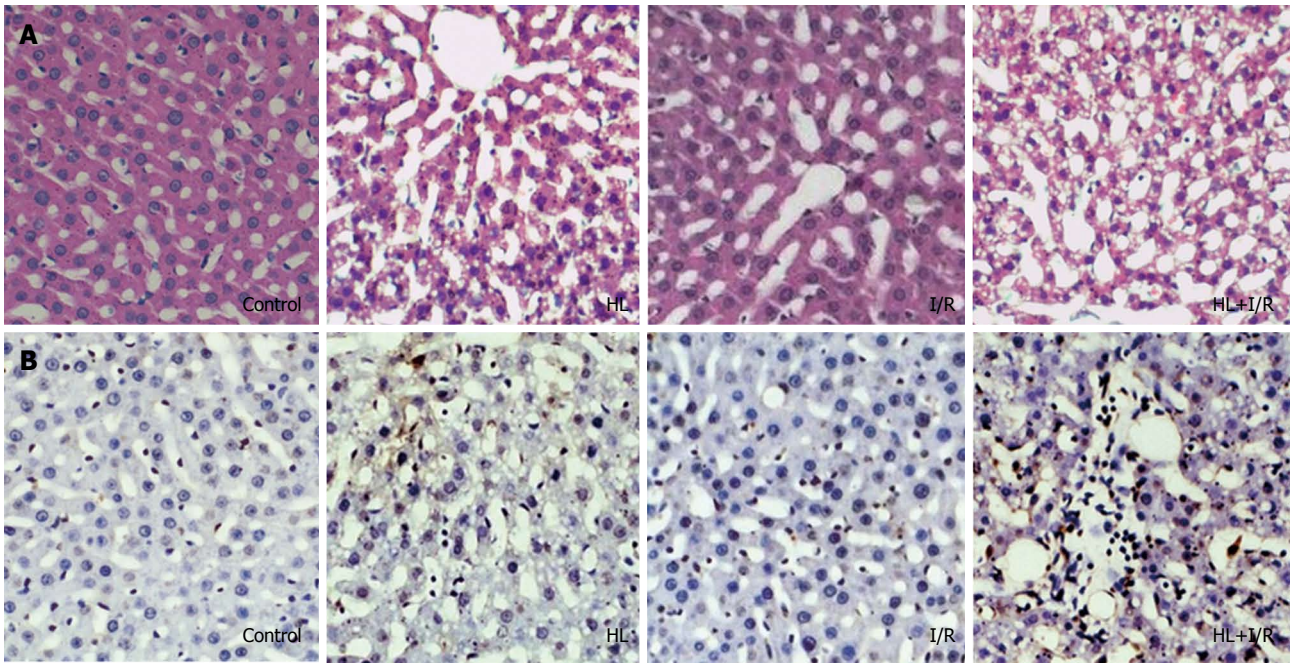

Figure 1 Histology and in situ apoptosis of liver sections from rats after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. A: HE staining showed liver damage in the hyperlipemia (HL) group and especially in the HL+ ischemia/reperfusion (I/R) group (× 200); B: Transferase dUTP nick-end labeling (TUNEL) staining showed TUNEL-positive apoptotic hepatocytes in the HL+I/R group are significantly greater than those in control, HL or I/R groups (× 200). Slides are representative of 6-10 animals per group.

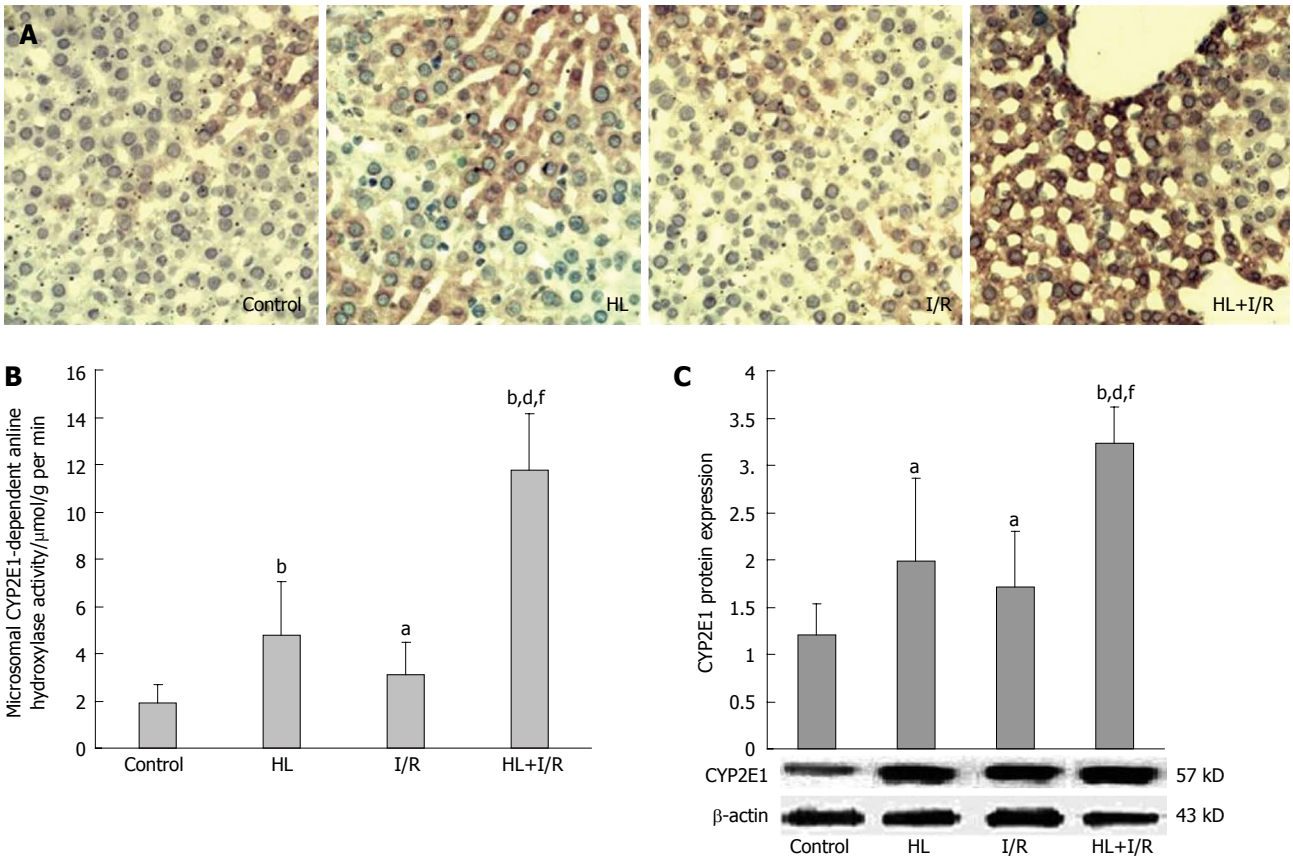

Figure 2 Effect of hyperlipemia plus ischemia/reperfusion on the activity and expression of CYP2E1 in the liver of rats after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. A: Hepatocytes in the hyperlipemia (HL) + ischemia/reperfusion (I/R) group showed increased cytochrome P450 2E1 (CYP2E1) expression examined by immunohistochemical staining (× 200) than those in other groups. Slides are representative of 6-10 animals per group; B: Microsomal hydroxylase activity of the CYP2E1-containing enzyme measured with aniline as the substrate was much higher in the HL+I/R group; C: HL+I/R enhanced the expression level of CYP2E1 protein in liver microsomes, which was confirmed by Western blotting. Values are the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* HL group; $^{f}P < 0.01$ *vs* I/R group.

Table 4 Levels of antioxidant enzymes, malondialdehyde and Ca^{2+} in liver mitochondria of different groups after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion (mean ± SD)

Group	*n*	SOD (U/μg protein)	GSH-Px (nmol/g protein per min)	MDA (mmol/g protein)	Ca^{2+} (μmol/g protein)
Control	10	244.93 ± 33.70	2172.26 ± 241.34	7.02 ± 2.58	1.329 ± 0.279
HL	6	100.59 ± 40.72[b]	937.57 ± 228.68[b]	21.84 ± 6.01[b]	2.552 ± 0.317[b]
I/R	7	172.15 ± 57.31[a]	1762.65 ± 409.18	12.58 ± 5.34	2.180 ± 0.431[b]
HL+I/R	6	59.01 ± 21.48[b,c,f]	486.41 ± 230.52[b,d,f]	47.27 ± 7.11[b,c,f]	3.136 ± 0.517[b,c,f]

HL: Hyperlipidemia; I/R: Ischemia/reperfusion; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; MDA: Malondialdehyde. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* HL group; [f]$P < 0.01$ *vs* I/R group.

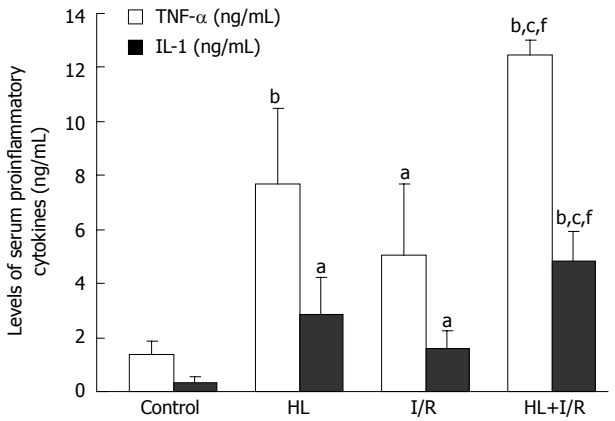

Figure 3 Effect of hyperlipemia + ischemia/reperfusion on the level of serum pro-inflammatory cytokines after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. Serum pro-inflammatory cytokines in the hyperlipemia (HL) + ischemia/reperfusion (I/R) group were significantly higher compared to those in other groups. A: Tumor necrosis factor (TNF)-α; B: Interleukin (IL)-1. Values are the mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$ *vs* HL group; [f]$P < 0.01$ *vs* I/R group.

ably especially in the HL+I/R group ($P < 0.01$ *vs* control, HL or I/R groups) (Figure 2B). To understand the basis of aniline hydroxylase activity in rat liver microsome, we carried out immunohistochemistry and Western blotting analysis of CYP2E1. Immunohistochemistrical staining for hepatic CYP2E1 was occasionally detectable in the control group, but increased in the HL or I/R groups. In the HL+I/R group, strong and dense CYP2E1 immunoreactivity was observed, which paralleled the improvement in histological severity of liver damage (Figure 2A). Similar effects were observed in Western blotting. The protein expression of microsomal CYP2E1 was induced by HL ($P < 0.05$ *vs* control) and I/R ($P < 0.05$ *vs* control), and highly enhanced by HL+I/R ($P < 0.01$ *vs* control, HL or I/R group) (Figure 2C).

Effect of HL plus I/R on the levels of antioxidant enzymes, MDA and Ca^{2+} in liver mitochondria

The activities of antioxidant enzymes, including SOD and GSH-Px, fell dramatically in the HL+I/R group ($P < 0.01$ *vs* control, $P < 0.05$ or $P < 0.01$ *vs* HL alone, $P < 0.01$ *vs* I/R alone). SOD activities of the HL, I/R and HL+I/R groups were, respectively, 41.1%, 70.3% and 24.1% of control level; GSH-Px activities were 43.2%, 81.1% and 22.4% of control level, respectively (Table 4).

MDA and Ca^{2+} levels in liver mitochondria were higher in the HL group than the control group ($P < 0.01$). Liver mitochondrial Ca^{2+} levels also increased in the I/R group ($P < 0.01$ *vs* control). In the HL+I/R group, MDA and Ca^{2+} levels were most significantly increased compared with all other groups ($P < 0.01$ *vs* control or I/R group, $P < 0.05$ *vs* HL group) (Table 4).

Effect of HL plus I/R on the level of serum pro-inflammatory cytokines (TNF-α and IL-1)

The levels of serum TNF-α and IL-1 in HL, I/R or HL+I/R groups were significantly higher compared to those in the control group ($P < 0.01$; $P < 0.05$). When compared with HL or I/R alone, the increase of these two pro-inflammatory cytokines in the HL+I/R group also had statistical significance ($P < 0.05$ *vs* HL, $P < 0.01$ *vs* I/R group) (Figure 3).

Effect of HL plus I/R on the expressions of caspase-3 and bcl-2

We further investigated the expression levels of two key apoptosis-related genes- caspase-3 and bcl-2-in liver tissue, which were determined by immunohistochemistry and Western blotting. Immunohistochemistry assay showed protein expression of caspase-3 (Figure 4A) in livers of HL and HL+I/R rats was markedly increased, and the anti-apoptosis gene bcl-2 (Figure 4B) was dramatically decreased. These findings were confirmed by Western blotting, which showed increased expression of caspase-3 (Figure 4C) and decreased expression of bcl-2 (Figure 4D), especially in the HL+I/R group.

DISCUSSION

Dyslipidemia is usually responsible for liver disease including cirrhosis, hepatocellular carcinoma and liver failure[5]. It has also been reported that "high-fat diet" can induce lipid accumulation in rat liver by nutritional intervention[25]. In agreement with previous work[26-28], our study also demonstrated that HL alone induced by HFD for 18 wk resulted in considerable increases in serum ALT and AST levels, marked pathological changes in the liver, increased TUNEL-positive cells, higher caspase-3 and lower bcl-2 expression, suggesting liver damage and hepatocyte apoptosis. Oxidative stress and subsequent

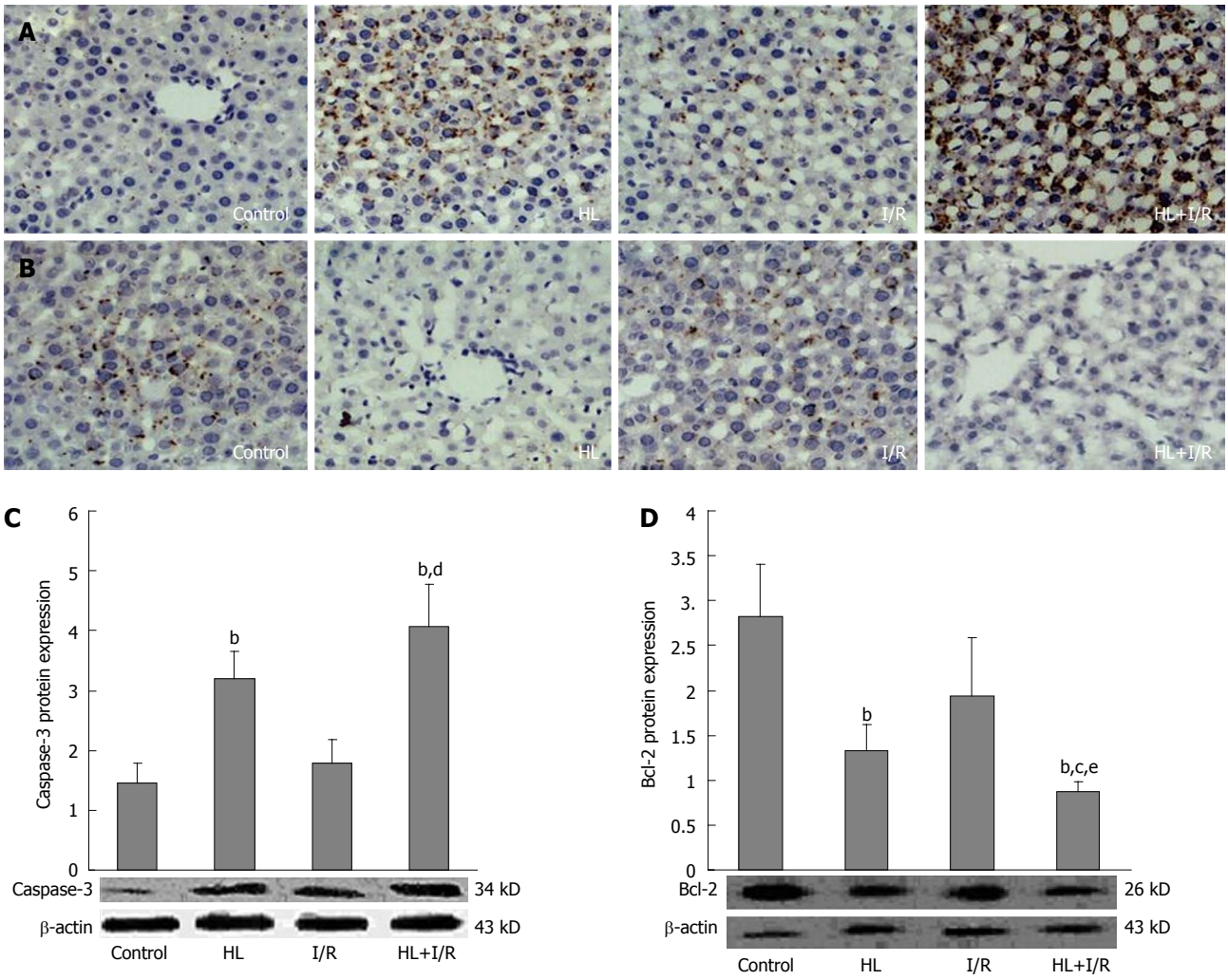

Figure 4 Expressions of bcl-2 and caspase-3 protein in liver after high-fat diet treatment for 18 wk and/or 2 h transient focal cerebral ischemia followed by 24 h reperfusion. Liver sections from different groups were immunohistochemically stained to assay the expression of caspase-3 and bcl-2 (× 200). A: Protein expression of caspase-3 was markedly elevated in the hyperlipemia (HL) + ischemia/reperfusion (I/R) group; B: Protein expression of bcl-2 was dramatically decreased in the HL+I/R group. C, D: Slides are representative of 6-10 animals per group. Liver homogenates were prepared, and then the expression levels of caspase-3 (C) and bcl-2 (D) proteins in liver were confirmed by Western blotting. Values are the mean ± SD. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* HL group; $^{e}P < 0.05$ *vs* I/R group.

lipid peroxidation, together with the production of pro-inflammatory cytokines have been shown to play important roles in the pathogenesis of nonalcoholic fatty liver disease induced by HFD[29,30], which were also confirmed in our current experiment.

Besides liver damage caused by long-term HFD, our findings are consistent with the literature stating ischemic stroke *per se* is possibly associated with liver dysfunction[31]. Although no significant change in serum ALT and AST levels, histologic examination of liver, the number of TUNEL-positive cells, caspase-3 and bcl-2 expression in liver was found, systemic inflammation and the imbalance in oxidant production and antioxidant defense systems, which are strongly associated with the development of MODS due to I/R, were indeed discovered in the I/R group. Actually, circulating pro-inflammatory cytokines, for example, TNF-α and IL-1, and systemic oxidative stress would influence the nonneurological organs including liver[32].

Taken together, HL *per se* induced systemic inflammation and oxidative stress, and eventually caused hepatocyte apoptosis and liver damage, while I/R *per se* also induced systemic inflammation and oxidative stress but did not cause obvious hepatocyte apoptosis and liver damage.

Most interestingly, we demonstrated that, as expected, the combined action of HL and I/R brought about an abrupt reduction in hepatic function with enhanced hepatocyte apoptosis, which is much worse than the damage caused by I/R or HL alone. The incidence of ischemic stroke increases dramatically with advancing age. Due to aging, many patients with cerebral ischemia also suffer from HL, which is another common geriatric disease. It has also been reported that HL, as a cardiovascular risk factor, would lead to intracranial artery atherosclerosis or blockage, aggravate cerebral infarction, and exacerbate the deleterious effects of I/R on the brain[33]. In our

study, we found the coexistence of HL and cerebral I/R injury would be more dangerous because it induces more severe visceral dysfunction i.e., liver damage than HL alone. This kind of systemic complication may be fatal subsequently.

This liver damage secondary to the coexistence of HL and cerebral I/R injury can be explained readily by the high levels of circulating pro-inflammatory cytokines and oxidative stress, which possibly result from the synergy of systemic inflammatory and oxidative milieu induced by HL *per se* and those induced by I/R *per se*. In support of these concepts, we showed that, compared with I/R or HL alone, enhanced CYP2E1 induction, an increase in serum TNF-α, IL-1, liver mitochondrial MDA, and a decrease in SOD and GSH-Px in liver mitochondria were also measured in the HL+I/R group, which indicated that the coexistence of HL with cerebral I/R injury is associated with increased systemic inflammation and oxidative stress.

A significant change in CYP2E1 should be noted in the liver of HL rats with cerebral I/R injury. CYP2E1, sometimes mediating liver damage through oxidative stress[34,35], is a major adaptive system in the CYP superfamily. CYP2E1 has adjustability, and is inducible under a variety of physiological or pathophysiological conditions. It has been demonstrated that CYP2E1 levels increased in obese rats by feeding with an energy-dense diet[14] and responded quickly to acute stress, such as hypoxia, cold or hunger[36]. In fact, acute cerebral I/R injury also induces an acute stress condition in animals. In the present study, we have corroborated the induction of CYP2E1 by HL *per se* or I/R *per se*, especially I/R combined with HL, as an increase in hepatic CYP2E1 activity and protein expression was found in the HL and I/R groups, and especially in the HL+I/R group. CYP2E1 is known to be a key indicator of lipid peroxidation, and a significant source of ROS[12] contributing to hepatocellular injury. Enhanced CYP2E1 induction oxidizes mitochondrial DNA, proteins and lipids, and triggers hepatic TNF-α formation by activating nuclear factor-κB, which further increases mitochondrial ROS formation. ROS overproduction leads to inflammatory recruitment and apoptosis from oxidative stress[11], and finally exacerbates the disease.

Consistent with the change in MDA, we also revealed that mitochondrial Ca^{2+} content increased in the HL+I/R group. An imbalance of Ca^{2+} homeostasis is thought to be the "common pathway" of hepatocyte damage. Mitochondrial and cellular Ca^{2+} overload facilitates lipid peroxidation, which attenuates the oxidative phosphorylation of the mitochondrion resulting in damage to the mitochondrial structure and function and then the reduction of ATP synthesis. On the other hand, lipid peroxidation will in turn exacerbate the Ca^{2+} overload by acting on the membrane structure[37]. Accordingly, mitochondrial Ca^{2+} overload of hepatocytes is closely relevant to lipid peroxidation, and both are likely to be involved in hepatic injury after HL+I/R.

Moreover, we have evaluated, by immunohistochemistry and Western blotting analysis, two key mediators of apoptosis (caspase-3 and bcl-2), which appear to be adjusted by ROS and cytokines, including TNF-α and IL-1[32,38]. Increased protein expression of key pro-apoptotic factor caspase-3 and markedly down-regulated anti-apoptotic bcl-2 detected in livers of HL rats with cerebral I/R injury in our study which resulted in hepatocyte apoptosis and liver damage.

In summary, based on an experimental HL rat model with cerebral I/R, the present study revealed that the coexistence of HL and acute cerebral I/R induced severe liver damage. These findings suggested cerebral ischemic stroke would exaggerate the damage of liver caused by HL. So it can be reckoned that patients of HL combined with cerebral I/R injury have a higher risk of liver damage compared with patients with HL or cerebral I/R injury alone, the mechanism of which is possibly due to enhanced CYP2E1 induction, which can further increase mitochondrial ROS formation, oxidative damage, inflammation and apoptosis with increased protein expression of key pro-apoptotic factor caspase-3, and markedly down-regulated anti-apoptotic bcl-2. Therefore, special precautions against subsequent liver injury are required after the burst of cerebral ischemic stroke in patients with HL. It seems that the therapeutic approaches to inhibit CYP2E1 activation, rebalance the oxidative/antioxidant system, and suppress inflammation or apoptosis might be efficacious in preventing the liver damage induced by HL plus cerebral I/R injury.

COMMENTS

Background

The history of hyperlipidemia (HL), an increasingly public health problem, is positively associated with acute cerebrovascular diseases (ACD) such as ischemic stroke. Moreover, HL is also a major risk factor for liver disease. One of the main complications of the patient with ACD is the risk of secondary liver dysfunction. It is thus reasonable to hypothesize that the coexistence of HL and ACD may contribute to more severe liver dysfunction.

Research frontiers

The pathological changes in liver caused by either HL or cerebral ischemia are associated with inflammation and oxidative stress. An inflammatory response and even apoptosis could be triggered by oxidative stress, which may be a key mechanism of liver injury induced by HL or cerebral ischemia. The source of oxidative stress is controversial but may result in part from over-expression of the prooxidant enzyme cytochrome P450 2E1 (CYP2E1). CYP2E1 has adjustability, and is inducible under a variety of physiological or pathophysiological conditions. It has been demonstrated that CYP2E1 levels are enhanced in obese rats by feeding with an energy-dense diet and respond quickly to acute stress, such as hypoxia, cold or hunger.

Innovations and breakthroughs

HL or ACD have been known to be independent risk factors for liver injury. In this study, the authors developed a repeatable experimental hyperlipemic rat model with acute cerebral ischemia/reperfusion injury, and investigated the correlation of HL and/or acute cerebral ischemia/reperfusion injury on liver damage, and further examine the effects of HL and/or ischemia/reperfusion on hepatocyte apoptosis, CYP2E1, the oxidative/antioxidant system and inflammatory process to understand the mechanism of liver damage.

Applications

The study results suggest that cerebral ischemic stroke would exaggerate the

damage of liver caused by HL. So it can be reckoned that patients with HL combined with acute cerebral ischemia/reperfusion injury have a higher risk of liver damage compared with patients with HL or cerebral ischemia/reperfusion injury alone, the mechanism of which is possibly due to enhanced CYP2E1 induction, which can further increase mitochondrial reactive oxygen species formation, oxidative damage, inflammation and apoptosis with increased protein expression of key pro-apoptotic factor caspase-3, and markedly down-regulated anti-apoptotic bcl-2. Therefore, special precautions against subsequent liver injury are required after the burst of cerebral ischemic stroke in patients with HL. It seems that therapeutic approaches to inhibit CYP2E1 activation, rebalance the oxidative/antioxidant system, suppress inflammation or apoptosis might be efficacious in prevention and treatment of liver damage induced by HL plus cerebral ischemia/reperfusion injury.

Peer review

The topic is of great clinical importance as the prevalence and incidence of hyperlipidemia and related cerebrovascular diseases as well as non-alcoholic fatty liver disease are increasing in industrialized countries; hence the associated financial problems is a major point to cope with. The rat model based study is well designed and the results are significant.

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BRIEF ARTICLE

Does gemcitabine-based combination therapy improve the prognosis of unresectable pancreatic cancer?

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Abstract

AIM: To assess whether gemcitabine-based combination therapy improves the prognosis of unresectable pancreatic cancer compared with gemcitabine treatment alone.

METHODS: A quantitative up-to-date meta-analysis was undertaken to investigate the efficacy of gemcitabine-based combination treatment compared with gemcitabine monotherapy in locally advanced or metastatic pancreatic cancer. Inclusion was limited to high-quality randomized clinical trials.

RESULTS: Twenty-six studies were included in the present analysis, with a total of 8808 patients recruited. The studies were divided into four subgroups based on the different kinds of cytotoxic agents, including platinum, fluoropyrimidine, camptothecin and targeted agents. Patients treated with gemcitabine monotherapy had significantly lower objective response rate [risk ratio (RR), 0.72; 95% confidence interval (CI): 0.63-0.83; $P < 0.001$], and lower 1-year overall survival (RR, 0.90; 95%CI: 0.82-0.99; $P = 0.04$). Gemcitabine monotherapy caused fewer complications, including fewer grade 3-4 toxicities: including vomiting (RR, 0.75; 95%CI: 0.62-0.89; $P = 0.001$), diarrhea (RR, 0.66; 95%CI: 0.49-0.89; $P = 0.006$), neutropenia (RR, 0.88; 95%CI: 0.72-1.06; $P = 0.18$), anemia (RR, 0.96; 95%CI: 0.82-1.12; $P = 0.60$), and thrombocytopenia (RR, 0.76; 95%CI: 0.60-0.97; $P = 0.03$) compared with gemcitabine combination therapies.

CONCLUSION: Gemcitabine combination therapy provides a modest improvement of survival, but is associated with more toxicity compared with gemcitabine monotherapy.

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Key words: Pancreatic cancer; Gemcitabine; Combination therapy; Outcome; Meta-analysis

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INTRODUCTION

Pancreatic cancer is a highly lethal disease, and represents the fourth cause of cancer-related death in the Western

world. In the United States, the incidence of pancreatic cancer almost equals its mortality, with 43 140 estimated new cases and 36 800 deaths in 2010. Globally, the incidence of new cases is approximately 230 000 each year[1,2]. Despite advances in diagnosis, staging, and surgical management of the disease during the past decade, the 5-year survival rate in the United States, Europe, and Australia is less than 5%[3-5]. Pancreatic cancer is characterized by a rapid disease progression and highly invasive tumor phenotype, with most patients having unresectable disease at diagnosis, and chemotherapy being the only possible treatment option for these patients[6].

Gemcitabine is a pyrimidine antimetabolite and analog of deoxycytidine. It was approved by the Food and Drug Administration as a first-line treatment for patients with locally advanced (stage Ⅱ or stage Ⅲ disease when surgery is not an option) or metastatic (stage Ⅳ) cancer of the pancreas, and has been widely used during the last decade. However, because of high levels of intrinsic and acquired chemoresistance, a large number of patients do not respond to gemcitabine[7]. To improve clinical efficacy, systemically administered gemcitabine is often combined with a second cytotoxic agent, such as platinum analogs, fluoropyrimidine, or a targeted cytotoxic agent. Numerous clinical trials have aimed at proving the superiority of gemcitabine-based combination therapy over single-agent gemcitabine treatment. However, most of the results of clinical trials have important limitations, including lack of statistical power because of small study populations. Thus, there remain several areas of controversy and uncertainty concerning optimal treatment regimens.

In the present study, we undertook a quantitative up-to-date meta-analysis to investigate the efficacy of gemcitabine-based combination treatment in locally advanced or metastatic pancreatic cancer. The aim of this study was to assess whether gemcitabine-based combination therapy improves the prognosis compared with gemcitabine treatment alone and to discuss possible mechanisms.

MATERIALS AND METHODS

Literature search strategy

We carried out a comprehensive search of the literature, including PUBMED (http://www.ncbi.nlm.nih.gov/pubmed), EMBASE (http://www.embase.com/home), American Society of Clinical Oncology abstracts (http://www.asco.org), and the Cochrane Database (http://www.thecochranelibrary.com). The following keywords were used in the search: ("Gemcitabine" or "Gemzar"), and ("pancreatic cancer" or "pancreatic tumor" or "pancreatic carcinoma"), and ("clinical trial"). The deadline for a publication to be eligible for this study was November 5, 2011. In addition to the online search, references from reviews and original articles were also scanned manually to identify further trials that met the eligibility criteria. No language restrictions were applied.

Inclusion and exclusion criteria

Studies included in the analysis had to meet all of the following criteria: (1) prospective, randomized, controlled open or blinded trial; (2) patients with histologically confirmed locally advanced or metastatic pancreatic ductal adenocarcinoma; and (3) assessment of the efficacy of gemcitabine combination therapy *vs* gemcitabine alone. Non-randomized trials and quasi-randomized trials, studies of curatively aimed resection, and studies where patients had multiple cancers, were excluded to avoid clinical heterogeneities between different studies.

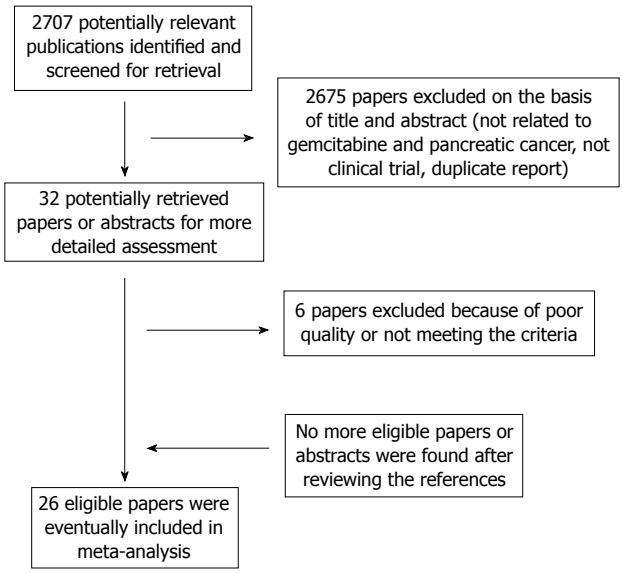

Figure 1 Flow chart of study selection.

Data extraction and quality assessment

Data extraction and quality assessment were performed independently by two reviewers (CS and DA). Any disagreements between the reviewers were discussed with a third reviewer (RA) to achieve a consensus. The data extracted from the eligible studies included: first author, year of publication, patient characteristics, intervention, and clinical outcome (toxicity, response rate, overall survival and progression-free survival). If the same trial appeared on sequential or multiple publications, the data from the most recent or comprehensive one was included.

The methodological quality of included studies was assessed using the "Jadad scale" or "Oxford quality scoring system"[8]. This tool is an evidence-based quality assessment tool. There are three items (randomization, double blinding, withdrawals and dropouts) directly related to bias reduction for assessment. Each item is given a score of 1 point for each "yes" or 0 points for each "no", and 1 additional point for appropriate randomization and double blinding. Every eligible study were assessed and given a score from 0-5.

Data analysis

The outcome measures were objective response rate [objective response rate (ORR) = complete response (CR) + partial response (PR)], as previously defined[9], 1-year overall survival (OS), median progression-free survival (PFS), median OS and toxicity. The analysis of ORR

Table 1 Characteristics of selected trials

Ref.	Intervention	*n*	Male (%)	Age (range)	Metastatic (%)	Response rate (%)	Median PFS (mo)	1-yr OS	Median OS (mo)	Quality
Louvet *et al*[15]	Gem	163	53	60.1 (22-75)	70	17.3	3.7	27.8	7.1	4
	Gem + Oxaliplatin	163	60	61.3 (35-77)	68	26.8	5.8	34.7	9.0	
Poplin *et al*[16]	Gem	275	56.4	64 (31-88)	90.2	6	2.6	16	4.9	4
	Gem + Oxaliplatin	272	45.6	63 (29-96)	89.3	9	2.7	21	5.7	
Heinemann *et al*[17]	Gem	95	61.9	66 (43-85)	78.9	9.0	3.1	24.7	6.0	4
	Gem + Cisplatin	95	65.3	64 (37-82)	80	11.5	5.3	25.3	7.5	
Colucci *et al*[18]	Gem	54	50	63 (43-75)	65	9.2	2	11	5	4
	Gem + Cisplatin	53	35	60 (33-71)	68	26.4	5	11.3	7.5	
Colucci *et al*[19]	Gem	199	56.8	63 (37-75)	82.9	10.1	3.9	34.0	8.3	4
	Gem + Cisplatin	201	62.2	63 (35-75)	84.6	11.4	3.8	30.7	7.2	
Kulke *et al*[20]	Gem	64	66	58.9 (31-81)		14	3.3	NR	6.4	4
	Gem + Cisplatin	66	56	58.9 (36-84)	100	13	4.5	NR	6.7	
	Gem + Irinotecan	64	68	60.8 (32-77)		14	4.0	NR	7.1	
Berlin *et al*[21]	Gem	162	53.7	64.3 (33-85)	90.1	5.6	2.2	NR	5.4	4
	Gem + 5-fluorouracil	160	51.8	65.8 (28-84)	89.4	6.9	3.4	NR	6.7	
Herrmann *et al*[22,24]	Gem	159	53	62 (36-84)	79	8	3.9	30	7.2	4
	Gem + Capecitabine	160	54	62 (27-83)	80	10	4.3	32	8.4	
Cunningham *et al*[23]	Gem	266	58	62 (26-83)	71	12.4	3.8	22.0	6.2	4
	Gem + Capeitabine	267	60	62 (37-82)	70	19.1	5.3	24.3	7.1	
Scheithauer *et al*[25]	Gem	42	55	66 (39-75)	100	14	4.0	37.2	8.2	4
	Gem + Capeitabine	41	66	64 (40-75)	100	17	5.1	31.8	9.5	
Costanzo *et al*[26]	Gem	49	48	64 (34-75)	73	8	3.5	18	7.75	4
	Gem + 5-fluorouracil	45	63	62 (44-75)	67	11	4.5	20	7.5	
Abou-Alfa *et al*[27]	Gem	174	57	62.3 (30-84)	78	5.7	3.8	21	6.2	4
	Gem + Exatecan	175	53	63.0 (36-85)	79	7.1	3.7	23	6.7	
Stathopoulos *et al*[28]	Gem	74	42	64 (44-83)	66	10	2.9	21.8	6.5	4
	Gem + Irinotecan	71	39	64 (31-84)	60	15	2.8	24.3	6.4	
Lima *et al*[29]	Gem	180	53.3	60.2 (32-82)	80.6	4.4	3.0	22	6.6	4
	Gem + Irinotecan	180	57.2	63.2 (38-81)	82.2	16.1	3.5	21	6.3	
Moore *et al*[38]	Gem	284	57	64 (36.1-92)	75.0	8.0	3.55	17	5.91	4
	Gem + Erlotinib	285	47.7	63.7 (37-84)	76.5	8.6	3.75	23	6.24	
Cutsem *et al*[39]	Gem	347	58	62 (30-88)	77	8	3.6	24	6.06	4
	Gem + Tipifarnib	341	57	61 (29-89)	76	6	3.7	27	6.43	
Eckhardt *et al*[40]	Gem	120	59	60 (35-86)	73	NR	3.03	NR	7.36	5
	Gem + Tipifarnib	124	64	63 (35-81)	71	NR	2.3	NR	6.73	
Philip *et al*[37]	Gem	371	54	64.3	78	7	3.0	NR	5.9	4
	Gem + cetuximab	372	51	63.7	79	8	3.4	NR	6.3	
Kindler *et al*[36]	Gem	300	51	65.0 (35-86)	85	10	2.9	NR	5.9	4
	Gem + Bevacizumab	302	58	63.7 (26-88)	84	13	3.8	NR	5.8	
Kindler *et al*[35]	Gem	316	59	62 (35-89)	72	1.6	4.4	NR	8.3	5
	Gem + Axitinib	316	61	61 (34-84)	72	4.9	4.4	NR	8.5	
Spano *et al*[41]	Gem	34	47	61.0 (36-78)	19	3	3.7	23.5	5.6	4
	Gem + Axitinib	69	51	65.0 (44-81)	40	7	4.2	36.8	6.9	
Friess *et al*[34]	Gem	43	42	66 (56-80)	91	14	3.83	0.24	7.7	4
	Gem + Cilengitide	46	57	68 (40-80)	94	17	3.66	0.15	6.7	
Richards *et al*[33]	Gem	44	72.7	64.1 (41-83)	86.4	5.3	3.0	17	5.1	4
	Gem + Enzastarin	86	53.5	68.3 (39-86)	90.7	8.6	3.4	19	5.6	
Bramhall *et al*[31]	Gem	119	71	62 (37-85)	62	16	3.2	17	5.46	5
	Gem + Marimastat	120	69	62 (32-83)	59	11	3.08	18	5.51	
Richards *et al*[32]	Gem	85	60.2	65 (36-83)	83.0	13.9	3.43	NR	7.13	5
	Gem + CA-994	85	59.3	62 (32-82)	82.6	11.8	3.06	NR	6.47	
Oettle *et al*[30]	Gem	282	53.5	63 (28-82)	91.1	7.1	3.3	20.1	6.3	4
	Gem + PPemetrexed	283	60.4	63 (27-82)	90.1	14.8	3.9	21.4	6.2	

NR: No record; PFS: Progression-free survival; OS: Overall survival.

(number of partial and complete responses, as defined by Response Evaluation Criteria in Solid Tumors[10]), PFS (time from randomization to progression or death) and OS (time from the date of random assignment until date of death or date last known to be alive) were based on the intent-to-treat population, consisting of all patients randomly assigned onto every study. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, and based on the safety population. The median PFS and median OS were assessed using the paired *t*-test. $P < 0.05$ was considered statistically significant.

Meta-analysis was performed using the Review Manager (version 5.1, provided by The Cochrane Collaboration). The strength of the associations between treatment and outcomes were estimated by risk ratio (RR, a ratio

Table 2 Grade 3 or 4 toxicity of selected clinical trials

Ref.	Intervention	Vomiting	Diarrhea	Neutropenia	Anemia	Thrombocytopenia
Louvet *et al*[15]	Gem	3.2	1.3	27.6	10.3	3.2
	Gem + Oxaliplatin	8.9	5.7	20.4	6.4	14.0
Poplin *et al*[16]	Gem	7	4	33	10	13
	Gem + Oxaliplatin	16	6	22	6	11
Heinemann *et al*[17]	Gem	5.9	4.7	NR	10.6	10.6
	Gem + Cisplatin	22.2	3.3	NR	13.3	4.4
Colucci *et al*[18]	Gem	2	0	9	4	2
	Gem + Cisplatin	2	4	18	6	2
Colucci *et al*[19]	Gem	< 1	1.5	13.7	1	18.9
	Gem + Cisplatin	2.6	< 1	24.7	4.8	15.3
Kulke *et al*[20]	Gem	24.1	NR	82.8	20	43.1
	Gem + Cisplatin	29	NR	74.2	25.8	79
	Gem + Irinotecan	16.7	NR	41.7	8.3	23.3
Berlin *et al*[21]	Gem	8	NR	5	10	11
	Gem + 5-fluorouracil	7	NR	7	10	19
Herrmann *et al*[22,24]	Gem	4	2	19	6	8
	Gem + Capeitabine	4	5	23	8	4
Cunningham *et al*[23]	Gem	6	4	22	6	6
	Gem + Capeitabine	6	5	35	4	11
Scheithauer *et al*[25]	Gem	0	0	7.6	0	2.5
	Gem + Capeitabine	0	5	10	5	0
Costanzo *et al*[26]	Gem	0	0	2	6	0
	Gem + 5-fluorouracil	2	0	2	7	2
Abou-Alfa *et al*[27]	Gem	3.1	< 1	14.6	7.6	4.4
	Gem + Exatecan	5.3	1.1	30.3	5.9	15.4
Stathopoulos *et al*[28]	Gem	1.4	2.8	15.7	4.2	0
	Gem + Irinotecan	1.6	3.2	26.6	5	5
Lima *et al*[29]	Gem	8.2	1.8	31.9	13	14.2
	Gem + Irinotecan	13.9	18.5	37.6	16.2	19.6
Moore *et al*[38]	Gem	NR	< 1	27%	NR	11%
	Gem + Erlotinib	NR	2	24%	NR	10%
Cutsem *et al*[39]	Gem	9	3	30	16	12
	Gem + Tipifarnib	7	4	40	20	15
Eckhardt *et al*[40]	Gem	9.3	0	33.9	11	15.3
	Gem + Tipifarnib	4	0	35.5	12.1	16.1
Philip *et al*[37]	Gem	2.2	2.5	23.9	6.2	8.5
	Gem + cetuximab	6.6	2.8	23.3	9.7	6.6
Kindler *et al*[36]	Gem	NR	NR	29	8	12
	Gem + Bevacizumab	NR	NR	33	5	12
Kindler *et al*[35]	Gem	3.2	1.6	< 1	< 1	< 1
	Gem + Axitinib	3.9	1.3	0	0	0
Spano *et al*[41]	Gem	10	0	33	17	13
	Gem + Axitinib	6	6	28	8	20
Richards *et al*[33]	Gem	0	2.6	28.2	5.1	25.6
	Gem + Enzastarin	2.4	3.7	18.3	3.7	14.6
Bramhall *et al*[31]	Gem	14%	NR	NR	7%	NR
	Gem + Marimastat	6%	NR	NR	3%	NR
Richards *et al*[32]	Gem	9	3	NR	5	11
	Gem + CA-994	9	5	NR	13	25
Oettle *et al*[30]	Gem	3.7	0.7	12.8	2.9	6.2
	Gem + Pemetrexed	3.3	2.9	45.1	13.9	17.9

NR: No record.

of the probability of the event occurring in the exposed group versus a non-exposed group) and 95% confidence interval (CI)[11]. The heterogeneities between different studies or different subgroups were estimated using Cochran's Q test[12,13]. In addition, the I^2 value was used for evaluating the extent of heterogeneity between studies or subgroups[14]. The I^2 index measures the extent of true heterogeneity dividing the difference between the result of the Q test and its degrees of freedom by the Q value itself, and multiplied by 100. I^2 indicated the possibility of the variability among effect sizes caused by true heterogeneity between studies, not by sampling error[14]. If a significant Q test ($P < 0.1$) or $I^2 > 50\%$ indicated that heterogeneity existed between studies, the random effects model was used for meta-analysis, otherwise the fixed effects model was used. Publication bias was assessed by visual inspection of funnel plots.

RESULTS

Literature search and trial flow

The results of the literature search are depicted in Figure

Study or subgroup	Monotherapy Events	Monotherapy Total	Combination Events	Combination Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
1.1.1 Gem *vs* gem + platinum						
Colucci *et al*[18]	5	54	14	53	3.0	0.35 (0.14, 0.90)
Colucci *et al*[19]	20	199	26	201	5.5	0.78 (0.45, 1.35)
Heinemann *et al*[17]	8	89	10	87	2.2	0.78 (0.32, 1.89)
Kulke *et al*[20]	6	43	7	56	1.3	1.12 (0.40, 3.08)
Louvet *et al*[15]	27	156	42	157	8.9	0.65 (0.42, 0.99)
Poplin *et al*[16]	17	275	26	272	5.6	0.65 (0.36, 1.16)
Subtotal (95%CI)		816		826	26.4	0.67 (0.52, 0.87)
Total events	83		125			
Heterogeneity: χ^2 = 3.20, df = 5 (P = 0.67); I^2 = 0%						
Test for overall effect: Z = 3.01 (P = 0.003)						
1.1.2 Gem *vs* gem + fluoropyrimidine						
Berlin *et al*[21]	9	162	11	160	2.4	0.81 (0.34, 1.90)
Costanzo *et al*[26]	4	48	5	43	1.1	0.72 (0.21, 2.50)
Cunningham *et al*[23]	33	266	51	267	10.8	0.65 (0.43, 0.97)
Herrmann *et al*[22,24]	12	153	15	150	3.2	0.78 (0.38, 1.62)
Scheithauer *et al*[25]	6	42	7	41	1.5	0.84 (0.31, 2.28)
Subtotal (95%CI)		671		661	19.0	0.71 (0.53, 0.96)
Total events	64		89			
Heterogeneity: χ^2 = 0.45, df = 4 (P = 0.98); I^2 = 0%						
Test for overall effect: Z = 2.23 (P = 0.03)						
1.1.3 Gem *vs* gem + camptothecin						
Abou-Alfa *et al*[27]	9	157	12	168	2.5	0.80 (0.35, 1.85)
Kulke *et al*[20]	6	43	7	51	1.4	1.02 (0.37, 2.80)
Lima *et al*[29]	8	180	29	180	6.2	0.28 (0.13, 0.59)
Stathopoulos *et al*[28]	7	70	9	60	2.1	0.67 (0.26, 1.68)
Subtotal (95%CI)		450		459	12.1	0.53 (0.35, 0.81)
Total events	30		57			
Heterogeneity: χ^2 = 5.63, df = 3 (P = 0.13); I^2 = 47%						
Test for overall effect: Z = 2.92 (P = 0.003)						
1.1.4 Gem *vs* gem + targeted agent						
Bramhall *et al*[31]	14	88	11	97	2.2	1.40 (0.67, 2.93)
Cutsem *et al*[39]	28	347	20	341	4.3	1.38 (0.79, 2.39)
Friess *et al*[34]	6	43	8	46	1.6	0.80 (0.30, 2.12)
Kindler *et al*[36]	30	300	39	302	8.3	0.77 (0.49, 1.21)
Kindler *et al*[35]	4	255	12	247	2.6	0.32 (0.11, 0.99)
Moore *et al*[38]	23	284	24	285	5.1	0.96 (0.56, 1.66)
Oettle *et al*[30]	20	282	42	283	8.9	0.48 (0.29, 0.79)
Philip *et al*[37]	23	331	28	329	6.0	0.82 (0.48, 1.39)
Richards *et al*[32]	10	85	8	85	1.7	1.25 (0.52, 3.01)
Richards *et al*[33]	2	44	7	86	1.0	0.56 (0.12, 2.58)
Spano *et al*[41]	1	34	5	69	0.7	0.41 (0.05, 3.34)
Subtotal (95%CI)		2093		2170	42.5	0.82 (0.67, 0.99)
Total events	161		204			
Heterogeneity: χ^2 = 14.40, df = 10 (P = 0.16); I^2 = 31%						
Test for overall effect: Z = 2.02 (P = 0.04)						
Total (95%CI)		4030		4116	100.0	0.72 (0.63, 0.83)
Total events	338		475			
Heterogeneity: χ^2 = 26.81, df = 25 (P = 0.37); I^2 = 7%						
Test for overall effect: Z = 4.80 (P < 0.00001)						
Test for subgroup differences: χ^2 = 3.71, df = 3 (P = 0.29); I^2 = 19.2%						

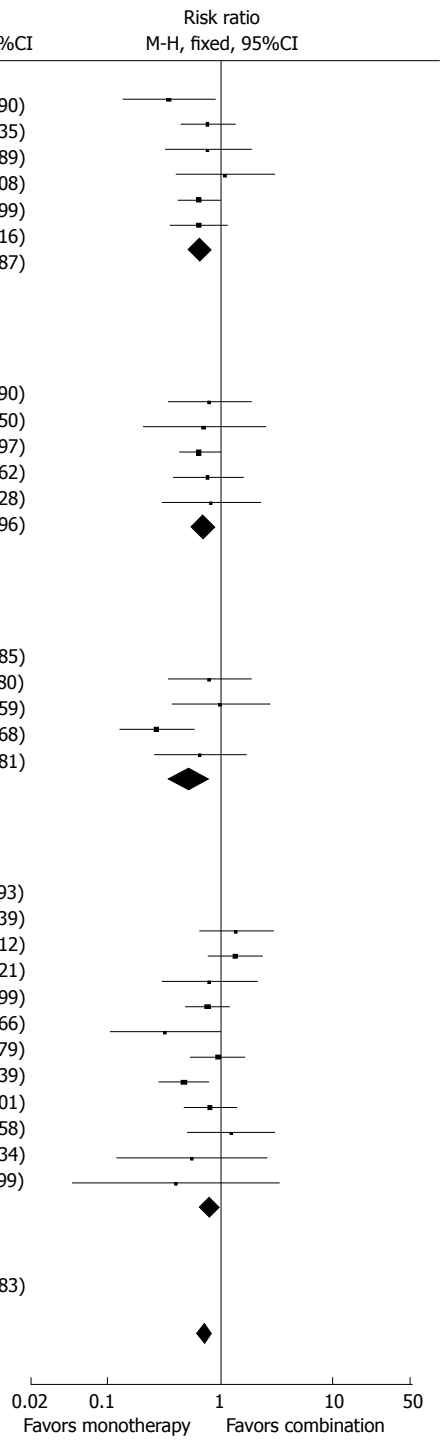

Figure 2 Fixed effect model of risk ratio of objective response rate. CI: Confidence interval.

1. Some 2707 studies were initially retrieved. After reading the title and abstract, 2675 studies were excluded, and 32 studies were left for further review. Six of these were excluded because of poor quality or failure to meet the inclusion criteria.

Characteristics of selected trials

The characteristics of the included studies are shown in Table 1. Twenty-six studies were included in the present analysis, with 8808 recruited patients. The studies were divided into four subgroups based on types of cytotoxic agents used, including platinum[15-20], fluoropyrimidine[21-26], camptothecin[21,27-29] and targeted agents[30-41]. All included studies enrolled patients that were ⩾ 18 years; had a histologically confirmed locally advanced or metastatic pancreatic cancer not amenable to surgical resection; no previous chemotherapy; a life expectancy of at least 12 wk; a Karnofsky performance status ⩾ 50, or World Health Organization performance status of 0-2; and adequate liver, renal, and hematopoietic functions.

Study or subgroup	Monotherapy Events	Monotherapy Total	Combination Events	Combination Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
1.2.1 Gem *vs* gem + platinum						
Colucci *et al*[18]	6	54	6	53	0.9	0.98 (0.34, 2.85)
Colucci *et al*[19]	64	199	57	201	8.1	1.13 (0.84, 1.53)
Heinemann *et al*[17]	22	89	22	87	3.2	0.98 (0.59, 1.63)
Louvet *et al*[15]	43	156	54	157	7.7	0.80 (0.57, 1.12)
Poplin *et al*[16]	44	275	57	272	8.2	0.76 (0.53, 1.09)
Subtotal (95%CI)		773		770	28.2	0.91 (0.77, 1.09)
Total events	179		196			
Heterogeneity: χ^2 = 3.67, df = 4 (P = 0.45); I^2 = 0%						
Test for overall effect: Z = 1.03 (P = 0.30)						
1.2.2 Gem *vs* gem + fluoropyrimidine						
Costanzo *et al*[26]	9	49	9	45	1.3	0.92 (0.40, 2.11)
Cunningham *et al*[23]	58	264	65	267	9.3	0.90 (0.66, 1.23)
Herrmann *et al*[22,24]	48	159	51	160	7.3	0.95 (0.68, 1.31)
Scheithauer *et al*[25]	16	42	13	41	1.9	1.20 (0.66, 2.17)
Subtotal (95%CI)		514		513	19.8	0.95 (0.77, 1.16)
Total events	131		138			
Heterogeneity: χ^2 = 0.72, df = 3 (P = 0.87); I^2 = 0%						
Test for overall effect: Z = 0.51 (P = 0.61)						
1.2.3 Gem *vs* gem + camptothecin						
Abou-Alfa *et al*[27]	37	174	40	175	5.7	0.93 (0.63, 1.38)
Lima *et al*[29]	40	180	38	180	5.5	1.05 (0.71, 1.56)
Stathopoulos *et al*[28]	16	74	17	71	2.5	0.90 (0.50, 1.65)
Subtotal (95%CI)		428		426	13.7	0.97 (0.76, 1.25)
Total events	93		95			
Heterogeneity: χ^2 = 0.26, df = 2 (P = 0.88); I^2 = 0%						
Test for overall effect: Z = 0.20 (P = 0.84)						
1.2.4 Gem *vs* gem + targeted agent						
Bramhall *et al*[31]	20	119	22	120	3.1	0.92 (0.53, 1.59)
Cutsem *et al*[39]	83	347	92	341	13.3	0.89 (0.69, 1.15)
Moore *et al*[38]	48	284	65	285	9.3	0.74 (0.53, 1.04)
Oettle *et al*[30]	57	282	60	283	8.6	0.95 (0.69, 1.32)
Richards *et al*[33]	7	44	16	86	1.6	0.86 (0.38, 1.92)
Spano *et al*[41]	8	34	25	68	2.4	0.64 (0.32, 1.27)
Subtotal (95%CI)		1110		1183	38.3	0.85 (0.73, 1.00)
Total events	223		280			
Heterogeneity: χ^2 = 1.97, df = 5 (P = 0.85); I^2 = 0%						
Test for overall effect: Z = 2.00 (P = 0.05)						
Total (95%CI)		2825		2892	100.0	0.90 (0.82, 0.99)
Total events	626		709			
Heterogeneity: χ^2 = 7.74, df = 17 (P = 0.97); I^2 = 0%						
Test for overall effect: Z = 2.09 (P = 0.04)						
Test for subgroup differences: χ^2 = 1.11, df = 3 (P = 0.78); I^2 = 0%						

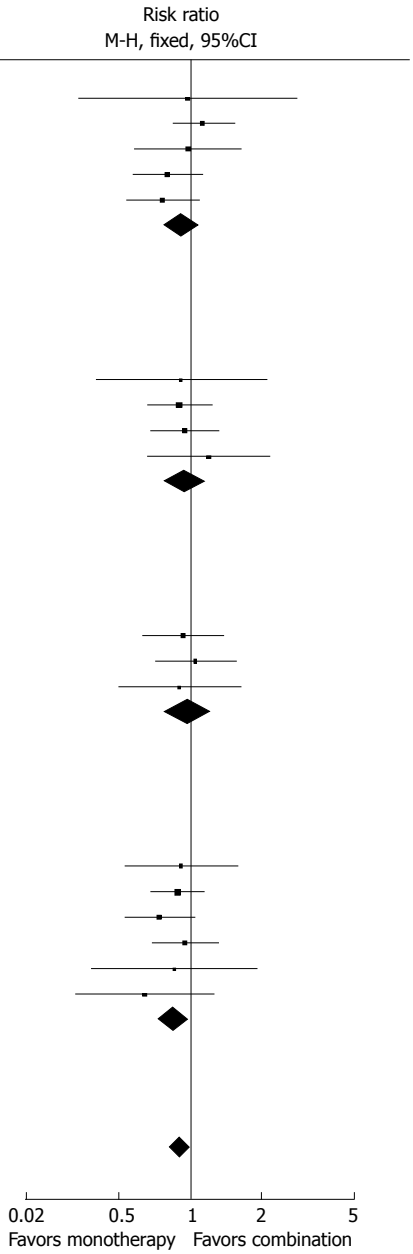

Figure 3 Fixed effect model of risk ratio of 1-year overall survival rate. CI: Confidence interval.

The details of toxicity assessment are shown in Table 2 (only grade 3 and 4 toxic effects are presented), including vomiting, diarrhea, neutropenia, anemia and thrombocytopenia.

The methodological quality of selected studies was high. Most trials had a Jadad score of 3, while three studies had a score of 4. Methods of double blinding were infrequently reported.

Gemcitabine combination therapy improves objective response rate compared with gemcitabine treatment alone

This analysis evaluated 26 trials (8146 patients) comparing gemcitabine monotherapy with the combination of gemcitabine and some other cytotoxic agent. Based on all studies, patients treated with gemcitabine monotherapy had a significantly lower ORR than gemcitabine combination therapy (RR, 0.72; 95%CI: 0.63-0.83; P < 0.001). The RRs in the different subgroups were 0.67, 0.71, 0.53 and 0.82, for platinum, fluoropyrimidine, camptothecin and targeted agents, respectively. All RRs in the subgroups were also significant. Data are shown in Figure 2.

Gemcitabine combination therapy improves 1-year overall survival rate compared with gemcitabine alone

5717 patients from 18 trials were included in this meta-analysis comparing gemcitabine monotherapy with combination therapies for 1-year overall survival. The RRs of 1-year overall survival were analyzed both totally and in the different subgroups. Subgroup analysis showed that the RRs of the monotherapy-based 1-year overall survival were lower than for the combination groups (RR:

Study or subgroup	Monotherapy Events	Monotherapy Total	Combination Events	Combination Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
1.3.1 Gem *vs* gem + platinum						
Colucci *et al*[18]	1	54	1	53	0.4	0.98 (0.06, 15.29)
Colucci *et al*[19]	1	189	5	186	1.9	0.20 (0.02, 1.67)
Heinemann *et al*[17]	5	85	20	90	7.4	0.26 (0.10, 0.67)
Kulke *et al*[20]	14	58	18	62	6.6	0.83 (0.46, 1.51)
Louvet *et al*[15]	5	156	14	157	5.3	0.36 (0.13, 0.97)
Poplin *et al*[16]	19	264	42	263	16.1	0.45 (0.27, 0.75)
Subtotal (95%CI)		806		811	37.8	0.46 (0.33, 0.64)
Total events	45		100			
Heterogeneity: χ^2 = 6.22, df = 5 (P = 0.29); I^2 = 20%						
Test for overall effect: Z = 4.61 (P < 0.00001)						
1.3.2 Gem *vs* gem + fluoropyrimidine						
Berlin *et al*[21]	13	158	11	158	4.2	1.18 (0.55, 2.56)
Costanzo *et al*[26]	0	49	1	41	0.6	0.28 (0.01, 6.69)
Cunningham *et al*[23]	14	247	15	251	5.7	0.95 (0.47, 1.92)
Herrmann *et al*[22,24]	6	156	7	159	2.6	0.87 (0.30, 2.54)
Subtotal (95%CI)		610		609	13.2	0.98 (0.62, 1.55)
Total events	33		34			
Heterogeneity: χ^2 = 0.88, df = 3 (P = 0.83); I^2 = 0%						
Test for overall effect: Z = 0.10 (P = 0.92)						
1.3.3 Gem *vs* gem + camptothecin						
Abou-Alfa *et al*[27]	5	157	9	168	3.3	0.59 (0.20, 1.74)
Kulke *et al*[20]	14	58	10	60	3.8	1.45 (0.70, 3.00)
Lima *et al*[29]	14	169	24	173	9.1	0.60 (0.32, 1.11)
Stathopoulos *et al*[28]	1	70	1	60	0.4	0.86 (0.05, 13.41)
Subtotal (95%CI)		454		461	16.5	0.80 (0.52, 1.21)
Total events	34		44			
Heterogeneity: χ^2 = 3.71, df = 3 (P = 0.29); I^2 = 19%						
Test for overall effect: Z = 1.06 (P = 0.29)						
1.3.4 Gem *vs* gem + targeted agent						
Cutsem *et al*[39]	30	342	23	331	8.9	1.26 (0.75, 2.13)
Eckhardt *et al*[40]	11	118	5	124	1.9	2.31 (0.83, 6.45)
Kindler *et al*[35]	10	308	12	305	4.6	0.83 (0.36, 1.88)
Oettle *et al*[30]	10	273	9	273	3.4	1.11 (0.46, 2.69)
Philip *et al*[37]	8	355	24	361	9.1	0.34 (0.15, 0.74)
Richards *et al*[32]	8	85	8	85	3.1	1.00 (0.39, 2.54)
Richards *et al*[33]	0	39	2	82	0.6	0.41 (0.02, 8.44)
Spano *et al*[41]	3	31	4	68	1.0	1.65 (0.39, 6.91)
Subtotal (95%CI)		1551		1629	32.5	0.96 (0.71, 1.29)
Total events	80		87			
Heterogeneity: χ^2 = 11.69, df = 7 (P = 0.11); I^2 = 40%						
Test for overall effect: Z = 0.29 (P = 0.77)						
Total (95%CI)		3421		3510	100.0	0.75 (0.62, 0.89)
Total events	192		265			
Heterogeneity: χ^2 = 33.22, df = 21 (P = 0.04); I^2 = 37%						
Test for overall effect: Z = 3.25 (P = 0.001)						
Test for subgroup differences: χ^2 = 12.39, df = 3 (P = 0.006); I^2 = 75.8%						

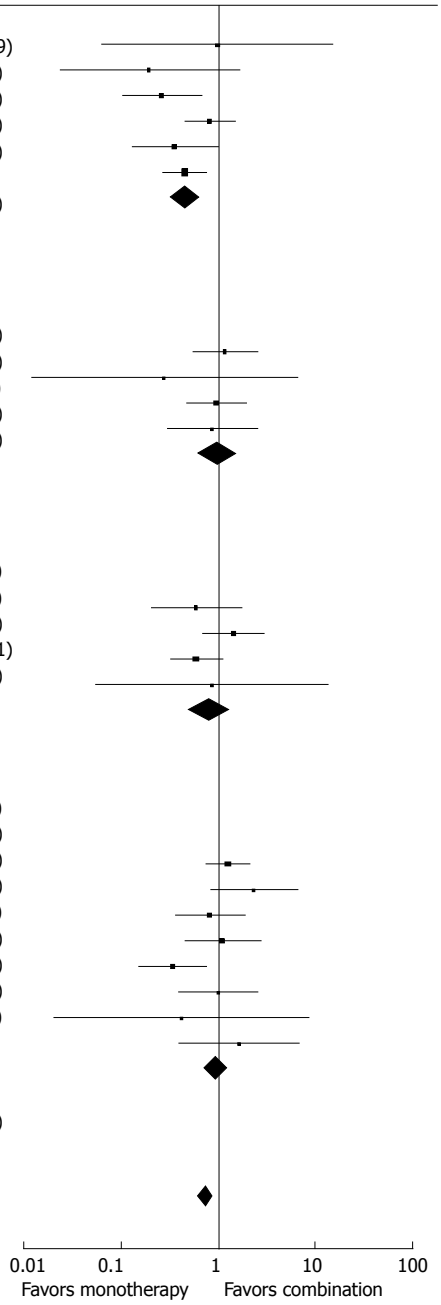

Figure 4 Fixed effect model of risk ratio of grade 3 or 4 vomiting. CI: Confidence interval.

0.91, 0.95, 0.97 and 0.85, for platinum, fluoropyrimidine, camptothecin and targeted agents, respectively), but no significant differences were found. When analyzed in terms of total events, the RR shown in Figure 3 was 0.90 [(95%CI: 0.82-0.99); P = 0.04]. The result showed that the 1-year overall survival rate of gemcitabine monotherapy was almost 90% of that of the combination therapy.

Gemcitabine combination therapy increases the toxicity effect compared with gemcitabine alone

Outcomes of the meta-analysis of the main toxicities are presented in Figures 4-8. All RRs with grade 3-4 toxicities analyzed in this study were lower in the gemcitabine monotherapy group than in the combination group. The incidence of vomiting (RR, 0.75; 95%CI: 0.62-0.89; P = 0.001), diarrhea (RR, 0.66; 95%CI: 0.49-0.89; P = 0.006) and thrombocytopenia (RR, 0.76; 95%CI: 0.60-0.97; P = 0.03) were all significantly different between the treatment groups, while no significant difference was noted regarding neutropenia (RR, 0.88; 95%CI: 0.72-1.06; P = 0.18) and anemia (RR, 0.96; 95%CI: 0.82-1.12; P = 0.60).

In subgroup analysis, the RRs were 0.46-0.98 for vomiting, 0.65-0.70 for diarrhea, 0.70-1.03 for neutropenia, 0.87-1.09 for anemia and 0.65-0.85 for thrombocy-

Study or subgroup	Monotherapy Events	Monotherapy Total	Combination Events	Combination Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
1.4.1 Gem *vs* gem + platinum						
Colucci *et al*[18]	0	54	2	53	2.3	0.20 (0.01, 4.00)
Colucci *et al*[19]	3	189	1	186	0.9	2.95 (0.31, 28.13)
Heinemann *et al*[17]	4	85	3	90	2.7	1.41 (0.33, 6.12)
Louvet *et al*[15]	2	156	9	157	8.3	0.22 (0.05, 1.02)
Poplin *et al*[16]	11	264	16	263	14.9	0.68 (0.32, 1.45)
Subtotal (95%CI)		748		749	29.2	0.65 (0.38, 1.13)
Total events	20		31			
Heterogeneity: χ^2 = 5.33, df = 4 (P = 0.26); I^2 = 25%						
Test for overall effect: Z = 1.53 (P = 0.13)						
1.4.2 Gem *vs* gem + fluoropyrimidine						
Cunningham *et al*[23]	11	247	12	251	11.0	0.93 (0.42, 2.07)
Herrmann *et al*[22,24]	3	156	8	159	7.4	0.38 (0.10, 1.41)
Scheithauer *et al*[25]	0	39	2	39	2.3	0.20 (0.01, 4.04)
Subtotal (95%CI)		442		449	20.7	0.65 (0.34, 1.25)
Total events	14		22			
Heterogeneity: χ^2 = 2.00, df = 2 (P = 0.37); I^2 = 0%						
Test for overall effect: Z = 1.28 (P = 0.20)						
1.4.3 Gem *vs* gem + camptothecin						
Abou-Alfa *et al*[27]	1	157	2	168	1.8	0.54 (0.05, 5.84)
Stathopoulos *et al*[28]	2	70	2	60	2.0	0.86 (0.12, 5.90)
Subtotal (95%CI)		227		228	3.8	0.70 (0.16, 3.14)
Total events	3		4			
Heterogeneity: χ^2 = 0.09, df = 1 (P = 0.76); I^2 = 0%						
Test for overall effect: Z = 0.46 (P = 0.65)						
1.4.4 Gem *vs* gem + targeted agent						
Cutsem *et al*[39]	10	342	13	331	12.3	0.74 (0.33, 1.67)
Kindler *et al*[35]	5	308	4	305	3.7	1.24 (0.34, 4.57)
Moore *et al*[38]	2	280	6	282	5.5	0.34 (0.07, 1.65)
Oettle *et al*[30]	2	273	8	273	7.4	0.25 (0.05, 1.17)
Philip *et al*[37]	9	355	10	361	9.2	0.92 (0.38, 2.23)
Richards *et al*[32]	3	85	4	85	3.7	0.75 (0.17, 3.25)
Richards *et al*[33]	1	39	3	82	1.8	0.70 (0.08, 6.52)
Spano *et al*[41]	0	31	4	68	2.6	0.24 (0.01, 4.32)
Subtotal (95%CI)		1713		1787	46.3	0.66 (0.43, 1.02)
Total events	32		52			
Heterogeneity: χ^2 = 4.22, df = 7 (P = 0.75); I^2 = 0%						
Test for overall effect: Z = 1.86 (P = 0.06)						
Total (95%CI)		3130		3213	100.0	0.66 (0.49, 0.89)
Total events	69		109			
Heterogeneity: χ^2 = 11.63, df = 17 (P = 0.82); I^2 = 0%						
Test for overall effect: Z = 2.77 (P = 0.006)						
Test for subgroup differences: χ^2 = 0.01, df = 3 (P = 1.00); I^2 = 0%						

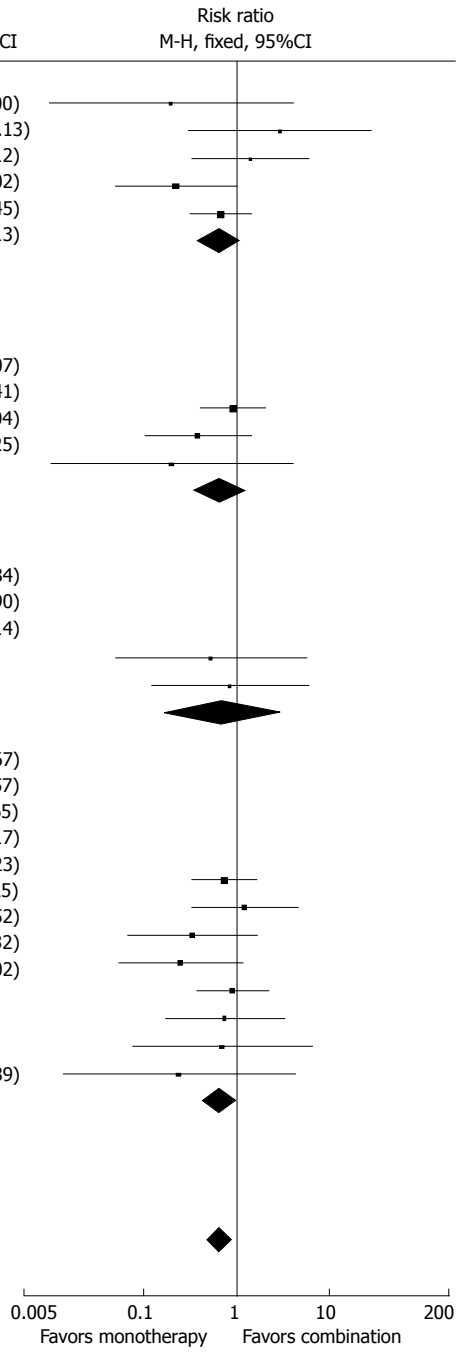

Figure 5 Fixed effect model of risk ratio of grade 3 or 4 diarrhea. CI: Confidence interval.

topenia, respectively. No subgroup differences in overall toxic outcomes, expect for vomiting, were found between the gemcitabine monotherapy group and the combination groups. Gemcitabine monotherapy decreased the incidence rate of vomiting by more than 50% compared with the gemcitabine plus platinum subgroup (RR, 0.46; 95%CI: 0.33-0.64; P < 0.001), while no significant differences for other subgroups was seen. In addition, the results showed that gemcitabine plus fluoropyrimidine therapy significantly increased the incidence of neutropenia (RR, 0.70; 95%CI: 0.55-0.88; P = 0.002).

The median PFS and median OS between gemcitabine monotherapy and combination therapies

The data of median PFS and OS in every study were extracted and assessed by a paired t-test. The results showed that only gemcitabine plus fluoropyrimidine significantly increased the median PFS (3.480 *vs* 4.520; P = 0.045) and median OS (6.950 *vs* 7.840; P = 0.038). Data are shown in Figure 9.

Publication bias

The funnel plots are shown in Figure 10. There was no

Study or subgroup	Monotherapy Events	Monotherapy Total	Combination Events	Combination Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
1.5.1 Gem *vs* gem + platinum						
Colucci *et al*[18]	5	54	9	53	2.3	0.55 (0.20, 1.52)
Colucci *et al*[19]	26	189	46	186	4.8	0.56 (0.36, 0.86)
Kulke *et al*[20]	48	58	46	62	6.1	1.12 (0.92, 1.35)
Louvet *et al*[15]	43	156	32	157	5.0	1.35 (0.91, 2.02)
Poplin *et al*[16]	87	264	58	263	5.6	1.49 (1.12, 1.99)
Subtotal (95%CI)		721		721	23.9	1.03 (0.74, 1.43)
Total events	209		191			
Heterogeneity: Tau^2 = 0.10; χ^2 = 16.61, df = 4 (P = 0.002); I^2 = 76%						
Test for overall effect: Z = 0.16 (P = 0.97)						
1.5.2 Gem *vs* gem + fluoropyrimidine						
Berlin *et al*[21]	8	158	11	158	2.8	0.73 (0.30, 1.76)
Costanzo *et al*[26]	1	49	1	41	0.5	0.84 (0.05, 12.97)
Cunningham *et al*[23]	54	247	87	251	5.6	0.63 (0.47, 0.84)
Herrmann *et al*[22,24]	30	156	36	159	4.9	0.85 (0.55, 1.31)
Scheithauer *et al*[25]	3	39	4	40	1.4	0.77 (0.18, 3.22)
Subtotal (95%CI)		649		649	15.1	0.70 (0.55, 0.88)
Total events	96		139			
Heterogeneity: Tau^2 = 0.00; χ^2 = 1.31, df = 4 (P = 0.86); I^2 = 0%						
Test for overall effect: Z = 3.09 (P = 0.002)						
1.5.3 Gem *vs* gem + camptothecin						
Abou-Alfa *et al*[27]	23	157	51	168	4.8	0.48 (0.31, 0.75)
Kulke *et al*[20]	48	58	25	60	5.5	1.99 (1.44, 2.74)
Lima *et al*[29]	54	169	65	173	5.6	0.85 (0.64, 1.14)
Stathopoulos *et al*[28]	11	70	16	60	3.6	0.59 (0.30, 1.17)
Subtotal (95%CI)		454		461	19.4	0.85 (0.44, 1.65)
Total events	136		157			
Heterogeneity: Tau^2 = 0.40; χ^2 = 32.95, df = 3 (P < 0.00001); I^2 = 91%						
Test for overall effect: Z = 0.47 (P = 0.64)						
1.5.4 Gem *vs* gem + targeted agent						
Cutsem *et al*[39]	102	342	132	331	6.0	0.75 (0.61, 0.92)
Eckhardt *et al*[40]	40	118	44	124	5.3	0.96 (0.68, 1.35)
Kindler *et al*[36]	76	263	91	277	5.8	0.88 (0.68, 1.13)
Kindler *et al*[35]	1	308	0	305	0.3	2.97 (0.12, 72.64)
Moore *et al*[38]	75	280	68	282	5.6	1.11 (0.84, 1.47)
Oettle *et al*[30]	35	273	123	273	5.4	0.28 (0.20, 0.40)
Philip *et al*[37]	85	355	84	361	5.7	1.03 (0.79, 1.34)
Richards *et al*[33]	11	39	15	82	3.6	1.54 (0.78, 3.04)
Spano *et al*[41]	10	30	18	64	3.8	1.19 (0.62, 2.25)
Subtotal (95%CI)		2008		2099	41.6	0.86 (0.63, 1.18)
Total events	435		575			
Heterogeneity: Tau^2 = 0.16; χ^2 = 52.85, df = 8 (P < 0.00001); I^2 = 85%						
Test for overall effect: Z = 0.93 (P = 0.35)						
Total (95%CI)		3832		3930	100.0	0.88 (0.72, 1.06)
Total events	876		1062			
Heterogeneity: Tau^2 = 0.15; χ^2 = 123.50, df = 22 (P < 0.00001); I^2 = 82%						
Test for overall effect: Z = 1.34 (P = 0.18)						
Test for subgroup differences: χ^2 = 3.81, df = 3 (P = 0.28); I^2 = 21.3%						

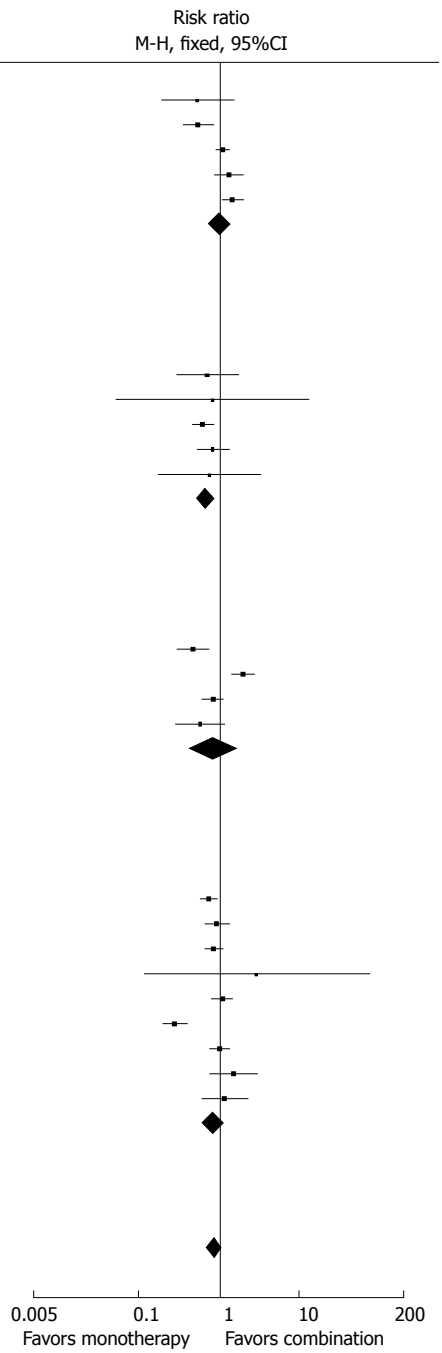

Figure 6 Random effect model of risk ratio of grade 3 or 4 neutropenia. CI: Confidence interval.

obvious publication bias found in the analysis.

DISCUSSION

Pancreatic cancer is a devastating disease with a dismal prognosis. Most of patients present with locally advanced or metastatic disease and are thus not candidates for surgical resection, thereby having to rely on palliative chemotherapy as the only treatment option. In this situation, the goals of chemotherapy should be to control tumor progression, decrease toxicity, and improve the survival rate. At present, gemcitabine monotherapy remains the standard treatment for patients with locally advanced or metastatic pancreatic cancer, but its efficacy is unsatisfactory. During the last decade, several randomized controlled clinical trials have evaluated gemcitabine in combination with various agents in an attempt to improve the prognosis of pancreatic cancer. The aim of this meta-analysis was to compare the therapeutic efficacy of gemcitabine-based combination treatments with gemcitabine alone in patients with locally advanced or metastatic pancreatic cancer. The results of this study showed that various combination therapies overall did not provide any major benefit compared with gemcitabine monotherapy.

Moreover, gemcitabine monotherapy had an almost 30% lower ORR than combination therapy regimens, but

Study or subgroup	Monotherapy Events	Monotherapy Total	Combination Events	Combination Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
1.6.1 Gem *vs* gem + platinum						
Colucci *et al*[18]	2	54	3	53	1.0	0.65 (0.11, 3.76)
Colucci *et al*[19]	2	189	9	186	3.0	0.22 (0.05, 1.00)
Heinemann *et al*[17]	9	85	12	90	3.9	0.79 (0.35, 1.79)
Kulke *et al*[20]	12	58	16	62	5.2	0.80 (0.42, 1.55)
Louvet *et al*[15]	16	156	10	157	3.3	1.61 (0.75, 3.44)
Poplin *et al*[16]	26	264	16	263	5.3	1.62 (0.89, 2.95)
Subtotal (95%CI)		806		811	21.8	1.04 (0.75, 1.43)
Total events	67		66			
Heterogeneity: χ^2 = 8.72, df = 5 (P = 0.12); I^2 = 43%						
Test for overall effect: Z = 0.22 (P = 0.83)						
1.6.2 Gem *vs* gem + fluoropyrimidine						
Berlin *et al*[21]	16	158	16	158	5.3	1.00 (0.52, 1.93)
Costanzo *et al*[26]	3	49	3	41	1.1	0.84 (0.18, 3.92)
Cunningham *et al*[23]	14	247	9	251	3.0	1.58 (0.70, 3.58)
Herrmann *et al*[22,24]	10	156	12	159	4.0	0.85 (0.38, 1.91)
Scheithauer *et al*[25]	0	39	2	40	0.8	0.20 (0.01, 4.14)
Subtotal (95%CI)		649		649	14.2	1.02 (0.68, 1.53)
Total events	43		42			
Heterogeneity: χ^2 = 2.46, df = 4 (P = 0.65); I^2 = 0%						
Test for overall effect: Z = 0.10 (P = 0.92)						
1.6.3 Gem *vs* gem + camptothecin						
Abou-Alfa *et al*[27]	12	157	10	168	3.2	1.28 (0.57, 2.89)
Kulke *et al*[20]	12	58	5	60	1.6	2.48 (0.93, 6.61)
Lima *et al*[29]	22	169	28	173	9.2	0.80 (0.48, 1.35)
Stathopoulos *et al*[28]	3	70	3	60	1.1	0.86 (0.18, 4.09)
Subtotal (95%CI)		454		461	15.2	1.09 (0.75, 1.59)
Total events	49		46			
Heterogeneity: χ^2 = 4.30, df = 3 (P = 0.23); I^2 = 30%						
Test for overall effect: Z = 0.45 (P = 0.65)						
1.6.4 Gem *vs* gem + targeted agent						
Cutsem *et al*[39]	55	342	66	331	22.4	0.81 (0.58, 1.12)
Eckhardt *et al*[40]	13	118	15	124	4.9	0.91 (0.45, 1.83)
Kindler *et al*[36]	21	263	14	277	4.5	1.58 (0.82, 3.04)
Kindler *et al*[35]	2	308	0	305	0.2	4.95 (0.24, 102.71)
Philip *et al*[37]	22	355	35	361	11.6	0.64 (0.38, 1.07)
Richards *et al*[32]	4	85	11	85	3.7	0.36 (0.12, 1.10)
Richards *et al*[33]	2	39	3	82	0.6	1.40 (0.24, 8.05)
Spano *et al*[41]	5	30	5	66	1.0	2.20 (0.69, 7.03)
Subtotal (95%CI)		1540		1631	48.9	0.87 (0.70, 1.08)
Total events	124		149			
Heterogeneity: χ^2 = 11.20, df = 7 (P = 0.13); I^2 = 37%						
Test for overall effect: Z = 1.25 (P = 0.21)						
Total (95%CI)		3449		3552	100.0	0.96 (0.82, 1.12)
Total events	283		303			
Heterogeneity: χ^2 = 28.20, df = 22 (P = 0.17); I^2 = 22%						
Test for overall effect: Z = 0.52 (P = 0.60)						
Test for subgroup differences: χ^2 = 1.55, df = 3 (P = 0.67); I^2 = 0%						

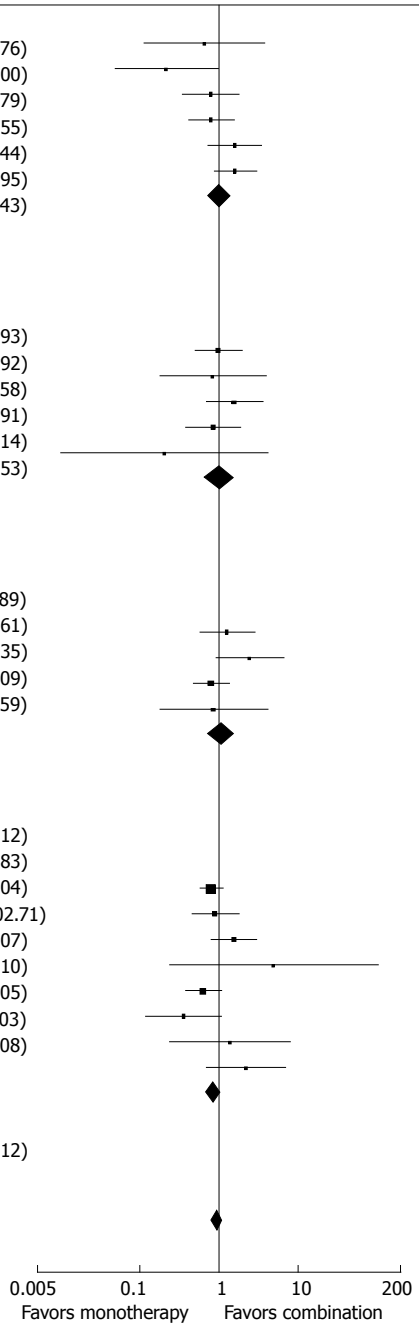

Figure 7 Fixed effect model of risk ratio of grade 3 or 4 anemia. CI: Confidence interval.

only had a 10% lower 1-year OS than combination therapy. According to the present data, 1-year OS in advanced or metastatic pancreatic cancer was low (less than 20%) after gemcitabine monotherapy; therefore, the combination therapy options studied did not improve outcome in a substantial way, the prognosis still being poor[21,31,42]. In addition, there were no significant improvements found in this analysis in median PFS and median OS after combination therapy compared with monotherapy, except for gemcitabine plus fluoropyrimidine treatment. Our results are similar to previously published analyses[23,43,44]. We also assessed the five most common toxicities related to chemotherapeutic treatment of locally advanced or metastatic pancreatic cancer, including hematological and gastrointestinal side effects. Grade 3-4 toxicities tended to be higher following combination therapy compared with monotherapy, and three of the five investigated toxicities reached significant differences. The RRs of vomiting, diarrhea, and thrombocytopenia in the monotherapy group were about 70% of that noted in the combination therapy group. Furthermore, subgroup analysis showed that the RR of vomiting after treatment with gemcitabine plus platinum was twice that of monotherapy. The results presented here showed that combination therapy induced

Study or subgroup	Monotherapy Events	Monotherapy Total	Combination Events	Combination Total	Weight (%)	Risk ratio M-H, fixed, 95%CI
1.7.1 Gem *vs* gem + platinum						
Colucci *et al*[18]	1	54	1	53	0.7	0.98 (0.06, 15.29)
Colucci *et al*[19]	10	189	29	186	4.6	0.34 (0.17, 0.68)
Helnemann *et al*[17]	9	85	4	90	2.8	2.38 (0.76, 7.45)
Kulke *et al*[20]	25	58	49	62	6.5	0.55 (0.40, 0.75)
Louvet *et al*[15]	5	156	22	157	3.4	0.23 (0.09, 0.59)
Poplin *et al*[16]	35	264	29	263	5.8	1.20 (0.76, 1.91)
Subtotal (95%CI)		806		811	23.7	0.65 (0.36, 1.18)
Total events	85		134			
Heterogeneity: Tau^2 = 0.35; χ^2 = 21.13, df = 5 (P = 0.0008); I^2 = 76%						
Test for overall effect: Z = 1.43 (P = 0.15)						
1.7.2 Gem *vs* gem + fluoropyrimidine						
Berlin *et al*[21]	17	158	30	158	5.3	0.57 (0.33, 0.98)
Costanzo *et al*[26]	0	49	1	41	0.5	0.28 (0.01, 6.69)
Cunningham *et al*[23]	14	247	28	251	4.9	0.51 (0.27, 0.94)
Herrmann *et al*[22,24]	12	156	7	159	3.6	1.75 (0.71, 4.32)
Scheithauer *et al*[25]	1	39	0	40	0.5	3.08 (0.13, 73.27)
Subtotal (95%CI)		649		649	14.8	0.73 (0.42, 1.28)
Total events	44		66			
Heterogeneity: Tau^2 = 0.14; χ^2 = 6.61, df = 4 (P = 0.16); I^2 = 39%						
Test for overall effect: Z = 1.10 (P = 0.27)						
1.7.3 Gem *vs* gem + camptothecin						
Abou-Alfa *et al*[27]	7	157	26	168	4.0	0.29 (0.13, 0.64)
Kulke *et al*[20]	25	58	14	60	5.3	1.85 (1.07, 3.19)
Lima *et al*[29]	24	169	34	173	5.7	0.72 (0.45, 1.16)
Stathopoulos *et al*[28]	0	70	3	60	0.6	0.12 (0.01, 2.33)
Subtotal (95%CI)		454		461	15.6	0.65 (0.26, 1.66)
Total events	56		77			
Heterogeneity: Tau^2 = 0.63; χ^2 = 17.34, df = 3 (P = 0.0006); I^2 = 83%						
Test for overall effect: Z = 0.89 (P = 0.37)						
1.7.4 Gem *vs* gem + targeted agent						
Cutsem *et al*[39]	41	342	50	331	6.2	0.79 (0.54, 1.17)
Eckhardt *et al*[40]	18	118	20	124	5.1	0.95 (0.53, 1.70)
Kindler *et al*[36]	32	263	33	277	5.8	1.02 (0.65, 1.61)
Kindler *et al*[35]	1	308	0	305	0.5	2.97 (0.12, 72.64)
Moore *et al*[38]	30	280	28	282	5.6	1.08 (0.66, 1.76)
Oettle *et al*[30]	17	273	49	273	5.4	0.35 (0.21, 0.59)
Philip *et al*[37]	30	355	24	361	5.5	1.27 (0.76, 2.13)
Richards *et al*[32]	9	85	21	85	4.4	0.43 (0.21, 0.88)
Richards *et al*[33]	10	39	12	82	4.3	1.75 (0.83, 3.70)
Spano *et al*[41]	4	30	13	66	3.1	0.68 (0.24, 1.90)
Subtotal (95%CI)		2093		2186	45.9	0.85 (0.62, 1.16)
Total events	192		250			
Heterogeneity: Tau^2 = 0.14; χ^2 = 23.21, df = 9 (P = 0.006); I^2 = 61%						
Test for overall effect: Z = 1.05 (P = 0.3)						
Total (95%CI)		4002		4107	100.0	0.76 (0.6, 0.97)
Total events	377		527			
Heterogeneity: Tau^2 = 0.20; χ^2 = 72.14, df = 24 (P < 0.00001); I^2 = 67%						
Test for overall effect: Z = 2.23 (P = 0.03)						
Test for subgroup differences: χ^2 = 0.83, df = 3 (P = 0.84); I^2 = 0%						

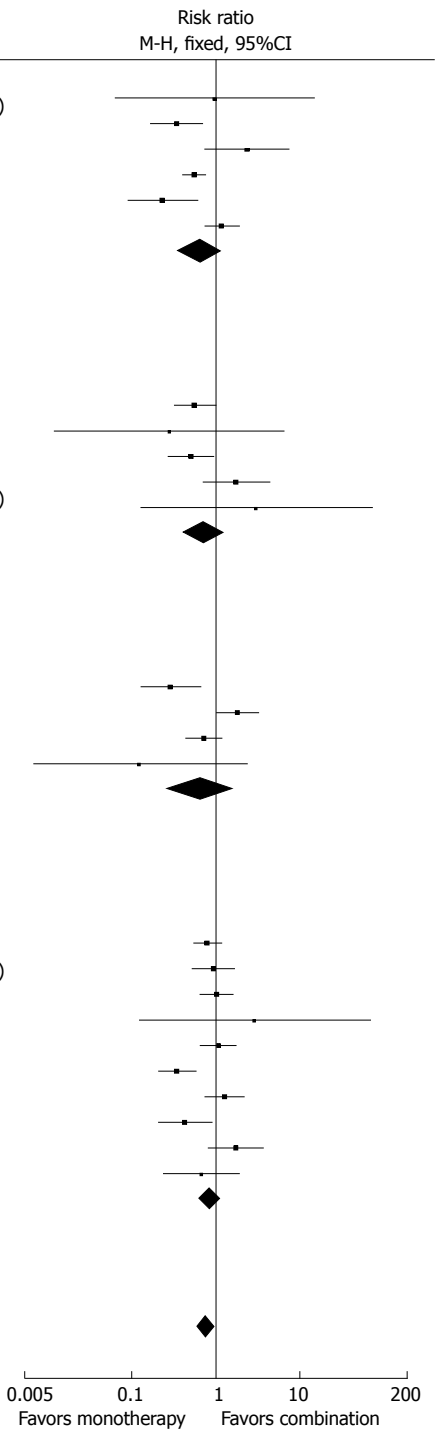

Figure 8 Random effect model of risk ratio of grade 3 or 4 thrombocytopenia. CI: Confidence interval.

more toxicity than gemcitabine alone. Similar results were found in a previous study[45]. This finding might explain why combination therapy received higher ORR, but without affecting the overall prognosis, as toxicities counterbalanced the positive effects.

In the present analysis, we included several kinds of combination therapies with gemcitabine, which could provide different anti-tumor effects and different uptake and toxicity profiles, including platinum based agents, topoisomerase inhibitors, taxanes, bevacizumab, cetuximab and other biologically targeted agents. However, no significant differences were found between the subgroups, except for side-effects (vomiting). In addition, the analysis of heterogeneity was not significant in most subgroups. This is interesting, and indicates that effectiveness of the different combinations regarding outcomes could not clearly be shown. There are two possible reasons that could explain these results. One is related to gemcitabine-uptake receptors. The other one could be the inherent drug-resistant character of pancreatic carcinoma cells and

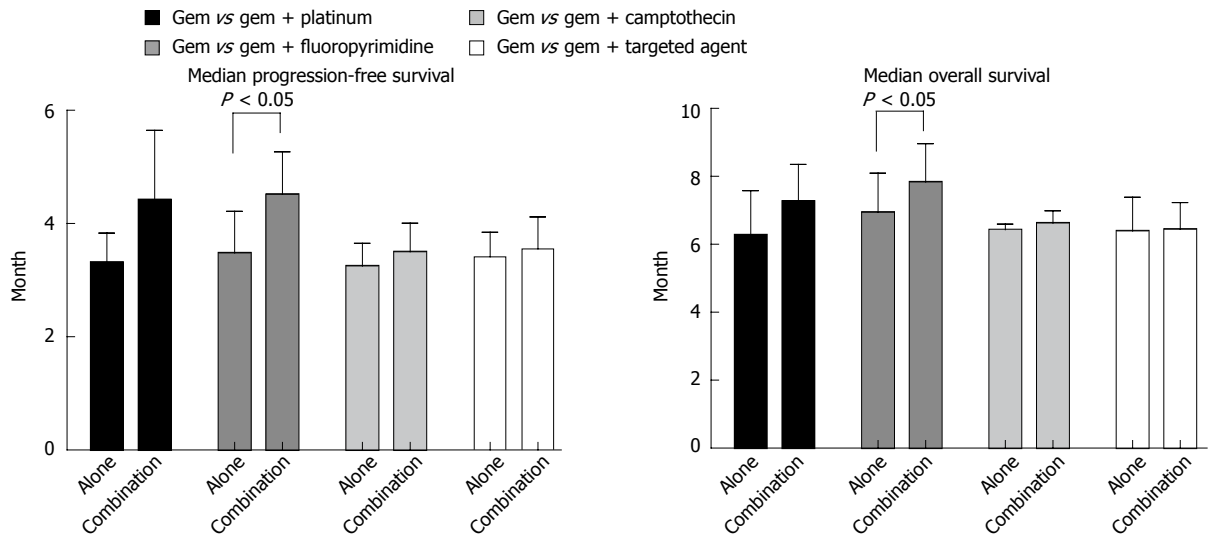

Figure 9 Median progression-free survival and overall survival between gem-monotherapy and combination therapy.

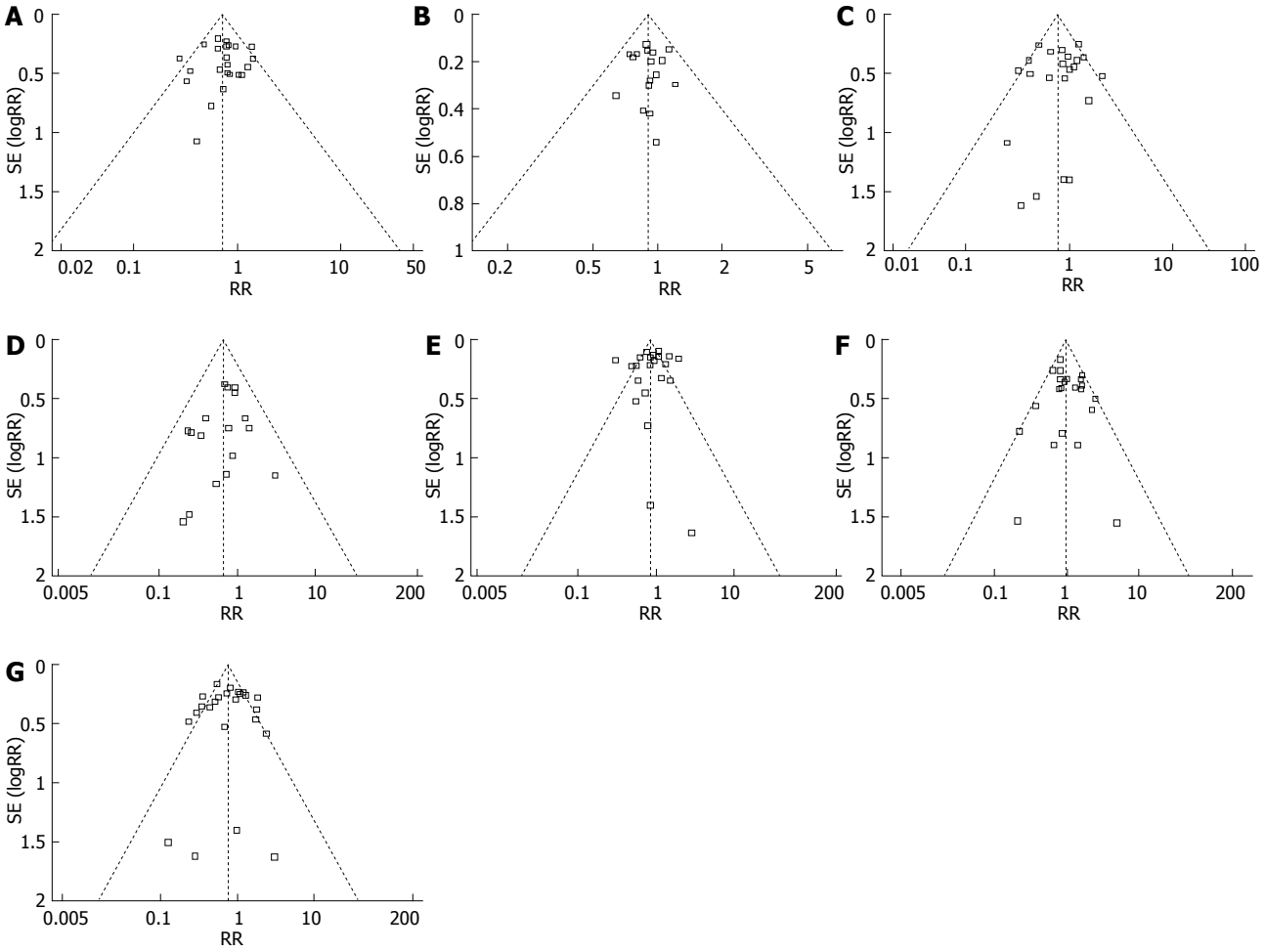

Figure 10 Funnel plots. A: Objective response rate; B: 1-year overall survival rate; C: Vomiting; D: Diarrhea; E: Neutropenia; F: Anemia; G: Thrombocytopenia. RR: Risk ratio.

the effect of the surrounding stroma.

Human equilibrative nucleoside transporter-1 (hENT-1) is the main membrane channel responsible for intracellular bioavailability of gemcitabine. Preclinical and clinical data have revealed that only a minority of patients have high hENT1 expression and reduced levels of hENT-1 expression correlate with increased resistance to gemcitabine[7]. To increase gemcitabine efficiency and

avoid overtreatment, determination of the hENT1 status at the time of diagnosis and modifying gemcitabine to allow it to bypass the receptor may represent a future mode of overcoming gemcitabine resistance[7]. Multidrug resistance proteins, including ABC transporters, have also been implied in drug resistance in pancreatic cancer and limit the efficacy of gemcitabine[46]. The pancreatic stellate cell has a key role in stroma formation and secretes factors that promote pancreatic tumor growth and resistance to radiation and gemcitabine chemotherapy[47]. The hypoxic stroma could be a physical barrier preventing chemotherapeutic drugs from reaching the pancreatic cancer cells per se, and depletion of the stroma could enhance cancer drug delivery[48]. Recently, cancer stem cells and epithelial to mesenchymal transition (EMT)-type cells have been implicated in metastasis formation and drug resistance[49]. Selective targeting of cancer stem cells and EMT-type cells might represent a future strategy to enhance chemosensitivity. The plasma circulating time of gemcitabine is short and its rapid metabolism thus limits tumor uptake. Using novel formulas such as PEGylated gemcitabine may result in a prolonged circulation time, and possibly inducing cytotoxicity at lower concentrations than those required with treatment with the native drug[50]. Pancreatic cancer, including its concurrent stroma, is complex; therefore, treatment with a single anti-tumor agent may not be effective enough. Determining the underlying mechanisms of drug resistance in pancreatic cancer will provide ways of overcoming chemoresistance and provide a foundation for novel therapies.

During the last decade, several meta-analyses have focused on the efficacy of combination therapy in locally advanced or metastatic pancreatic cancer[43,44,51-54]. Most of these studies have reported positive trends or have concluded that combination treatments may improve outcome, but only a limited number of the studies have reported toxicity data, or have only focused on one anti-tumor agent. In the present study, we collected and analyzed the outcomes in different chemotherapeutic subgroups, as well as toxicity, to make the result more comprehensive and convincing. However, several limitations in this analysis should still be considered. Firstly, some endpoints were not evaluated in all of the included randomised controlled trials. Secondly, heterogeneity of certain endpoints in some subgroups might limit the comparability between studies and affect the validity, despite the use of randomized models.

In conclusion, we compared the outcomes of different combination therapies with gemcitabine monotherapy, including ORR, OS, PFS and major toxicity. The results showed that gemcitabine combination therapy leads to a modest improvement in survival, though with more toxicity reported compared with gemcitabine monotherapy.

COMMENTS

Background

Pancreatic cancer is a devastating disease with a dismal prognosis. The majority of patients (80%-90%) have unresectable disease at diagnosis and have to rely on palliative chemotherapy alone. In this situation, the goals of chemotherapy should be to control tumor progression and improve the survival rate with minimal toxicity.

Research frontiers

Gemcitabine has long been the first-line chemotherapeutic agent in pancreatic cancer. However, because of high levels of intrinsic and acquired chemoresistance, a large number of patients do not respond to the drug. In an attempt to improve clinical efficacy, systemically administered gemcitabine has been combined with a second cytotoxic agent, such as platinum analogs, fluoropyrimidine, or a targeted cytotoxic agent. However, the added value of these additive agents is unknown.

Innovations and breakthroughs

The present meta-analysis included 26 high-quality randomized trials comparing gemcitabine monotherapy with gemcitabine combination therapy. The primary outcome variables evaluated were objective response rate, overall survival, progression-free survival and major toxicity. The results showed that gemcitabine combination therapy was associated with only a modest improvement in survival, though with more toxicity reported, compared with gemcitabine monotherapy.

Applications

The results of this study imply that current treatments for unresectable pancreatic cancer are not sufficiently effective. Further research concerning the mechanisms involved in drug resistance in pancreatic cancer could discover ways of overcoming chemoresistance and lead to novel types of therapy.

Peer review

This is a meta-analysis concerning combination chemotherapy with Gemcitabine unresectable pancreatic cancer. The manuscript is well written and represents elaborative work.

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CASE REPORT

Cameron ulcers: An atypical source for a massive upper gastrointestinal bleed

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Abstract

Cameron lesions represent linear gastric erosions and ulcers on the crests of mucosal folds in the distal neck of a hiatal hernia (HH). Such lesions may be found in upto 50% of endoscopies performed for another indication. Though typically asymptomatic, these may rarely present as acute, severe upper gastrointestinal bleed (GIB). The aim is to report a case of a non-anemic 87-year-old female with history of HH and atrial fibrillation who presented with hematemesis and melena resulting in hypovolemic shock. Repeat esophagogastroduodenoscopy was required to identify multiple Cameron ulcers as the source. Endoscopy in a patient with HH should involve meticulous visualization of hernia neck and surrounding mucosa. Cameron ulcers should be considered in all patients with severe, acute GIB and especially in those with known HH with or without chronic anemia.

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Key words: Cameron lesion; Upper gastrointestinal bleeding; Shock; Hiatal hernia

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INTRODUCTION

The incidence of hiatal hernia (HH) rises with age[1]. Given the rising demographics and the growing number of endoscopies, this condition now constitutes an increasingly common endoscopic finding. One study reported the incidence to be upwards of 50% during upper endoscopies performed for another indication[2]. Though typically asymptomatic, several complications can occur including gastroesophageal reflux disease[3], iron deficiency anemia[4], acute or chronic bleeding[5], and ulcer or erosion formation[2]. Usually an incidental endoscopic finding, Cameron lesions represent linear gastric erosions and ulcers on the crests of mucosal folds in the distal neck of a HH. Both erosions and ulcers are thought to be distinct forms of the same disease process. Lesions are found in 5.2% of patients with HH identified on upper endoscopy[6] and in over 60%, multiple lesions may be found[1].

CASE REPORT

An 87-year-old female presented to the Emergency Department (ED) with several episodes of bright red hematemesis and black, tarry stools over six hours. She complained of severe lightheadedness and crampy, lower abdominal pain. On examination, she was found to be hypotensive with systolic blood pressures in the 60s, and despite aggressive resuscitation with intravenous fluids

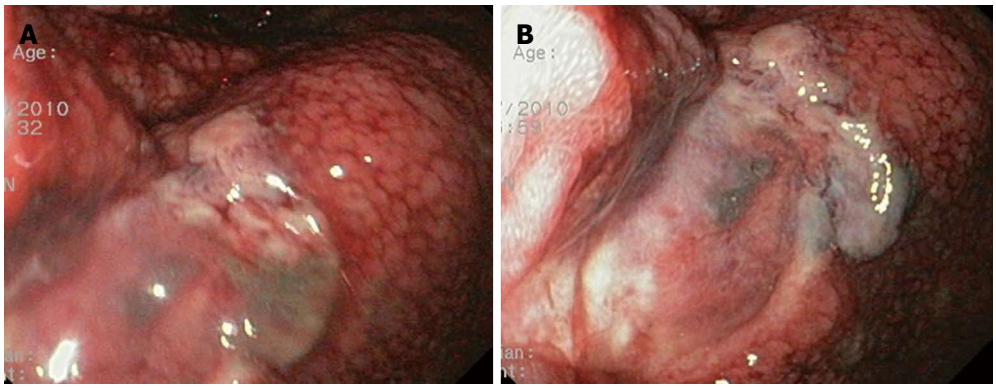

Figure 1 Ulcer with vessel in hernia sac. A: Retroflexed view of cameron ulcer; B: Second ulcer.

and blood products, she required vasopressors.

Two days earlier, she had been discharged following a brief hospital stay for atrial fibrillation and a large HH. She had been conservatively treated with proton pump inhibitor, Sucralfate, and Ondansetron as needed with fair results.

At this presentation, hemoglobin (Hgb) was 12.7 g/dL (baseline of 14.8 g/dL). Nasogastric lavage returned 500 cc of bright red blood. Intravenous proton pump inhibitor was started, patient was intubated and an emergent EGD revealed a large clot in the gastric fundus along with diffuse, friable mucosa in the mid-distal esophagus. No other bleeding site was identified. Despite multiple units of packed red blood cells and fresh frozen plasma, the patient's hemoglobin continued to decline. A repeat EGD demonstrated multiple circumferential non-bleeding Cameron ulcers at 37 cm with large placental clots in the fundus (Figure 1). Small clots were also seen at the gastroesophageal junction. In total, the patient received 14 units of packed red blood cells, multiple units of fresh frozen plasma, protamine sulfate and Vitamin K. Her Hgb stabilized on hospital day 10. During the course, she developed aspiration pneumonia, which was successfully treated with antibiotics. No further bleeding occurred and the patient recovered.

DISCUSSION

The pathogenesis of Cameron lesions is poorly understood. Some attribute lesion formation to mechanical trauma to the esophagus caused by respiration-related diaphragmatic contractions[6]. Other etiological factors may include acid reflux, ischemia, *Helicobacter pylori* infection, gastric stasis or vascular stasis[7]. It is likely that the etiology is multifactorial, and includes genotype, phenotype and patient risk factors including underlying co-morbidities and medication use. The prevalence is also likely dependent on the size of the HH with a 10%-20% risk for Cameron ulcers in hernias 5 cm in size or greater[8]. However, the absence of HH should not rule out Cameron lesions either. One study used push enteroscopy to evaluate ninety-five patients with obscure GIB previously investigated with standard endoscopy. Of the thirty-nine patients with an identifiable source, Cameron lesions were the second most commonly missed lesion (21%)[9]. Presumably, the lack of a large HH may have reduced the suspicion for Cameron lesions. Among the most concerning clinical manifestations of Cameron lesions are acute and chronic GIB. Chronic blood loss resulting in anemia has been well described. A large prospective, national, population-based study found that patients with HH had a significantly higher association of iron-deficiency anemia as compared to those with esophagitis[4]. Additionally, in a case-controlled study, Cameron showed that of 259 patients with radiographic evidence of HH, 18 were anemic compared to 1 in the control group ($P < 0.001$)[10]. Patients with Cameron lesions typically respond well to medical treatment consisting of iron supplementation with or without acid-suppression therapy. It is worth noting that our patient's MCV was 97 on admission without any supplementation.

More alarming are lesions that present as severe, acute GIB. Studies have reported rates of 29%-58%, raising the possibility of life-threatening hemorrhage[2,10]. To our knowledge, there are very few reports of life-threatening upper GIB secondary to a Cameron ulcer. One case report describes the successful treatment of a visible vessel within a Cameron ulcer with band ligation[11]. However, in these cases, surgical intervention is recommended, as endoscopic hemostasis can be technically difficult. Potential risks of endoscopy in acute, upper GIB include deep ulcers and perforation as the gastrointestinal wall around the gastroesophageal junction lacks fibrous tissue. Thus, surgery should be considered in those with either severe sliding hernias with Cameron lesions and in patients with lesion-related complications refractory to medical treatment[12]. Long-term recurrence rates are extremely low following surgery[13].

Complicating our case was the need to anticoagulate the patient for atrial fibrillation prior to endoscopy. More studies are needed to evaluate whether radiographic evidence of large HH should prompt an endoscopy prior to anticoagulation. In conclusion, endoscopy in a patient with radiographic evidence of large HH should involve meticulous antegrade and retrograde visualization with views of hernia neck and surrounding mucosa. Cameron

ulcers are a potential source of severe, acute GIB in patients with known HH with or without chronic anemia.

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CASE REPORT

Metastases of esophageal carcinoma to skeletal muscle: Single center experience

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Abstract

Metastases of esophageal carcinoma to the skeletal muscle are rare, but the incidence may be increasing because of better diagnosis resulting from widespread use of positron emission tomography/computed tomography (PET/CT). A cohort of 205 patients with esophageal carcinoma treated at our center who had PET/CT between 2006 and 2010 was retrospectively evaluated for the presence of skeletal muscle metastases. Four patients had skeletal muscle metastases of esophageal carcinoma, including two patients with squamous cell carcinoma. In another patient with squamous cell carcinoma of the esophagus and synchronous skeletal muscle metastases, muscle metastases were subsequently shown to be related to second primary pancreatic adenocarcinoma. In all cases, skeletal muscle metastases were the first manifestation of systemic disease. In three patients palliation was obtained with the combination of external beam radiation therapy, systemic chemotherapy or surgical resection. Skeletal muscle metastases are a rare complication of esophageal carcinoma.

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Key words: Drug therapy; Esophageal neoplasms; Muscle; Skeletal; Positron-emission tomography; Radiotherapy

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INTRODUCTION

The prognosis of esophageal carcinoma is, in general, poor, with the mortality rate reaching 90% of the incidence rate[1]. Both uncontrolled growth of the primary tumor or locoregional recurrence and distant metastases may be the ultimate cause of death in individual patient.

Esophageal carcinoma commonly spreads to the liver, lung, adrenals, bone, pleura, peritoneum or pericardium. Less frequent sites of metastases include thyroid, pancreas, spleen, ovary, kidney, heart, skin, gallbladder or small and large bowel[2,3]. Metastatic involvement of skeletal muscle is rare.

Until recently, asymptomatic skeletal muscle metastases (SMM) were detected only fortuitously. The widespread use of positron emission tomography/computed tomography (PET/CT) resulted in the detection of asymptomatic metastases in different organs, including skeletal muscle. A number of case reports or case series on SMM in patients with different primary tumors, including esophageal carcinoma, have been published[4-8], but the natural history of SMM in patients with esophageal carcinoma remains largely unknown. We report here five cases of patients with esophageal carcinoma and SMM observed in our center.

CASE REPORT

Records of 205 patients with esophageal carcinoma treated at our center who had PET/CT between 2006 and 2010 were searched to identify patients who had SMM diagnosed.

SMM were diagnosed in 5 patients (2%), all males. In one of these patients, SMM were subsequently shown to be related to a second primary tumor (Table 1). The details of these cases are outlined below.

Case 1

A 64-year-old male was diagnosed with adenocarcinoma of the distal esophagus in February 2004. The staging examination that included laparoscopy did not reveal distant metastases. The patient was treated with neoadjuvant chemoradiation. Transhiatal esophagectomy was performed on June 1, 2004. Histological examination of the resection specimen revealed pathologic complete response. The course of the follow-up was uneventful until October 2006 when the patient observed a resistance in the left thigh. Whole body [^{18}F] fluorodeoxyglucose (FDG) PET/CT performed in December 2006 revealed high focal uptake in the left quadriceps femoris muscle and in the left inguinal and iliac lymph nodes (Figure 1), with no evidence of a second primary tumor. An excisional biopsy established the diagnosis of SMM of adenocarcinoma.

The metastatic involvement of skeletal muscle and lymph nodes was treated with external beam radiation (50 Gy) that was administered in February and March 2007 along with 2 cycles of systemic chemotherapy (cisplatin and 5-fluorouracil). Four cycles of systemic chemotherapy using the same schedule have been administered subsequently until September 2007. A control FDG PET/CT in October 2007 demonstrated persistent uptake of radioactivity in the thigh metastasis that decreased in size. Additional metastatic lesions were discovered in medial condylus of the left femur and the retroperitoneal lymph nodes. The patient was feeling well and was subsequently only followed. In May 2008 an edema of the left lower extremity manifested. Control FDG PET/CT in June 2008 demonstrated uptake in retroperitoneal, mediastinal and left supraclavicular lymph nodes, lungs and left tibia as well as persistent accumulation in the left thigh. Between July and October 2008, the patient was treated with 5 cycles of combination of cisplatin and 5-fluorouracil. After the completion of chemotherapy, only symptomatic therapy was administered. The patient died on May 18, 2009, 31 mo after the diagnosis of skeletal muscle metastasis. An autopsy was not performed.

Case 2

76-year-old male was diagnosed in June 2006 with squamous cell carcinoma of the middle esophagus. Staging FDG PET/CT did not reveal distant metastases or evidence of a second primary tumor. The patient was treated with neoadjuvant chemoradiation that consisted of external beam radiation (50 Gy in 25 fractions of 2 Gy with systemic cisplatin and 5-fluorouracil). The control FDG PET/CT in November 2006 revealed complete disappearance of FDG uptake in the primary, but a pathologic accumulation in the right gluteus minimus muscle (21 mm × 14 mm in size) was noted. Because of complete response, patient age and uncertainty about the systemic disease, esophagectomy was not performed, and the patient was further only followed. On control FDG PET/CT, no uptake in the primary tumor was observed, but the size of the lesion in gluteus minimus muscle increased to 66 mm × 44 mm × 34 mm and involved bone. In addition, similar lesion was noted in the left psoas muscle. A biopsy performed in April 2007 confirmed metastasis of squamous cell carcinoma. Between May and July 2007 external beam radiation was administered to both lesions (70 Gy in 35 fractions of 2 Gy) concomitantly with 2 cycles of systemic cisplatin and 5-fluorouracil. Control FDG PET/CT in September 2007 demonstrated metastatic spread to the lungs, pleura, adrenals and retroperitoneal lymph nodes. Only symptomatic therapy was administered after the diagnosis of progressive metastatic disease, and the patient died on March 4, 2008, 16 mo after detection of SMM. An autopsy was not performed.

Case 3

A 57-year-old male was diagnosed in September 2006 with squamous cell carcinoma of the middle third of the esophagus. Staging FDG PET/CT performed in October 2006 revealed an accumulation of radioactivity not only in the primary tumor, but also in the skeleton of the pelvis and in the right subscapularis muscle. No evidence of a second primary tumor was found. The biopsy of the lesions was considered technically difficult, and the diagnosis of metastatic esophageal carcinoma was made based on multiplicity and appearance of lesions. Between October and December 2006 the patient was treated with 3 cycles of systemic chemotherapy (cisplatin and 5-fluorouracil). Chemotherapy had to be interrupted in January 2007 because of pneumonia. A control FDG PET/CT

Table 1 Patients with esophageal carcinoma and skeletal muscle metastases in the present series

Patient	Age at diagnosis (yr)	Histology	Stage at diagnosis	Timing	Primary therapy	Metastatic interval (mo)	Symptoms	Diagnosis	Location	Other metastases	Therapy for muscle metastases	Survival from the diagnosis of muscle metastasis (mo)
1	64	Adeno	Ⅱ	Metachronous	CXRT	32	Mass	PET/CT; biopsy	Quadriceps femoris	B; LN (subs)	CXRT	31
2	76	SCC	Ⅲ	Metachronous	CXRT	5	None	PET/CT; biopsy	Gluteus minimus; psoas	L; P; A; LN (subs)	CXRT	16
3	57	SCC	Ⅳ	Synchronous	None	0	None	PET/CT	Subscapularis	B	CRT; subs XRT	5
4	42	Adeno	Ⅳ	Synchronous	CXRT	0	None	PET/CT; biopsy	Iliacus	PR	S	16
5	60	SCC; adeno (pancreas)	Ⅳ	Synchronous	None	0	None	PET/CT; autopsy	Multiple	Multiple	None	2

A: Adrenal; Adeno: Adenocarcinoma; B: Bone; CRT: Chemotherapy; CXRT: Chemoradiation; L: Lungs; P: Pleura; PR: Peritoneum; LN: Lymph nodes; S: Surgery; SCC: Squamous cell carcinoma; subs: Subsequently; XRT: Radiotherapy; PET/CT: Positron emission tomography/computed tomography.

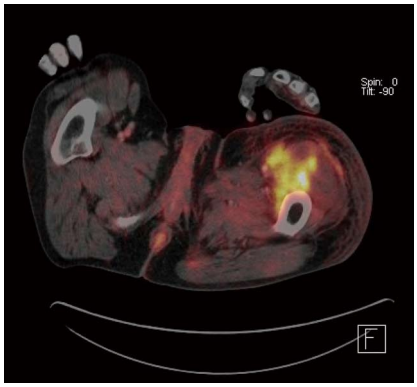

Figure 1 [^{18}F] fluorodeoxyglucose positron emission tomography/computed tomography showing a metastasis in the left quadriceps femoris muscle.

in February 2007 revealed mild progression. Palliative external beam radiation therapy (45 Gy in 18 fractions of 2.5 Gy) of all three sites of disease was then performed, but the condition of the patient deteriorated, with the progression of dysphagia. The patient died on March 24, 2007, 5 mo after the diagnosis of SMM. An autopsy was not performed.

Case 4

42-year-old male presented with adenocarcinoma of distal esophagus and cardia in April 2010. Staging FDG PET/CT demonstrated high FDG uptake in the primary tumor and a 2 cm mass in the iliacus muscle, with no evidence of a second primary tumor. As staging laparoscopy performed in May 2010 did not reveal spread in the abdominal cavity, the patient was referred for concomitant chemoradiation (50 Gy in 25 fractions of 2 Gy) and 2 cycles of systemic chemotherapy (cisplatin and 5-fluorouracil). Control PET/CT in October 2010 demonstrated only moderate progression in the primary, but marked progression of SMM (4 cm). The patient underwent subsequently (in October 2010) esophagectomy and resection of the muscle mass. Histologic examination of the muscle metastasis demonstrated poorly differentiated adenocarcinoma similar to the esophageal primary. The patient was then followed. In April 2011, the patient presented again with dysphagic complaints. Stenosis of the anastomosis was detected, and biopsy confirmed recurrence of adenocarcinoma. During the surgery to perform a jejunostomy, peritoneal carcinomatosis was detected and a large abdominal metastasis was resected. After a brief course of investigational therapy in a clinical trial the disease progressed and the patient was treated symptomatically. The patient died on August 27, 2011, 16 mo after diagnosis of SMM. An autopsy was not performed.

Case 5

60-year-old male was diagnosed in January 2010 with squamous cell carcinoma of the distal esophagus. Staging FDG PET/CT performed in February 2010 demonstrated high uptake in the tumor of distal esophagus, in lymph nodes adjacent to lesser curvature of the stomach, in the liver, left adrenal, bone and multiple metastases in skeletal muscle, including gluteus maximus muscle. Because of the extent of metastatic disease, only symptomatic therapy was indicated and histological verification of metastases was not sought. An esophageal stent was introduced to palliate the stenosis. The procedure resulted in the relief of the symptoms of short duration, but in March 2010 the condition of the patient deteriorated, he was admitted for back pain and died on March 21, 2010. Autopsy demonstrated, besides squamous cell carcinoma of the distal esophagus, a second primary pancreatic adenocarcinoma. Autopsy also revealed metastases in mediastinal and abdominal lymph nodes, peritoneum, pleura, lung, liver, kidneys, adrenal, thyroid, left submandibular gland, skeletal muscle, subcutis and bone. The histology of all metastases was adenocarcinoma pointing to pancreatic primary.

Table 2 Overview of the reports of esophageal carcinoma skeletal muscle metastases

Ref.	Sex	Age (yr)	Histology	Timing	Symptoms	Diagnosis	Location of muscle metastases	Other metastases	Therapy	Outcome
Heyer *et al*[18]	M	54	Adeno	Synchronous	Asymptomatic; fortuitously detected during CT	CT; MRI; biopsy	Multiple	None	CXRT (to the primary)	ND
Rehman *et al*[19]	M	71	Adeno	ND	Pain; unable to walk	MRI; biopsy	Thigh	ND	XRT (metastases)	Symptomatic relief 2 wk later
Kozyreva *et al*[20]	M	72	Adeno (T3N1Mx)	Synchronous	Asymptomatic; fortuitously detected during PET/CT	PET/CT; biopsy	Deltoid	None	C (oxaliplatin + 5FU/FA)	Progression of muscle metastases
Schultz *et al*[5]	ND	ND	SCC	Synchronous	ND	CT; biopsy	Gluteus minimus	ND	ND	ND
Heffernan *et al*[21]	F	67	Adeno	5 mo after surgery	Asymptomatic; fortuitously detected during PET/CT	PET/CT	Multiple	Mediastinum	ND	ND
Wu *et al*[22]	M	67	Adeno	Synchronous	Asymptomatic; fortuitously detected during PET/CT	PET/CT; biopsy	Gluteus minimus	None	ND	ND
Lekse *et al*[23]	F	78	Adeno	4 mo after diagnosis	Diplopia	CT; biopsy	Inferior rectus ocular	Lung	Symptomatic	ND
Lekse *et al*[23]	M	78	Adeno	2 mo after diagnosis	Diplopia; loss of vision	MRI	Lateral rectus ocular	Lung; bone; liver	XRT; C (5FU + FA)	ND

5FU: 5-fluorouracil; Adeno: Adenocarcinoma; C: Chemotherapy; CT: Computed tomography; CXRT: Chemoradiation; FA: Folinic acid; M: Male; F: Female; MRI: Magnetic resonance imaging; ND: Not described; PET: Positron emission tomography; XRT: Radiotherapy; SCC: Squamous cell carcinoma.

DISCUSSION

The 2% incidence of SMM in the present series may be affected by selection bias, as the primary diagnosis in one of the patients was performed before 2006. The information on esophageal carcinoma SMM is limited to individual case reports (Table 2), or to series of patients with SMM[5]. However, in most series of patients with SMM, no cases of patients with esophageal primary have been described[4,6-8]. SMM were also not reported in most autopsy series of esophageal carcinoma patients[2,3,9,10]. Cases of SMM have also been described in patients with gastric cancer[6]. To the best of our knowledge, only one case of squamous cell carcinoma of the esophagus metastatic to the skeletal muscle has been reported so far, with few details described[5]. Two of the patients reported here therefore represent the second description SMM of squamous cell carcinoma of the esophagus. The case of the patient with clinically undetected pancreatic adenocarcinoma demonstrates that histological verification may be needed not only to verify the neoplastic nature of the muscle lesion, but also to determine the primary as the frequency of second primary tumors is increasing. This case also indicates that a possible occult second primary tumor represents limitation in retrospective series of rare distant metastases. Histological verification confirming esophageal primary origin was possible in 3 out of 4 remaining cases. In all four cases SMMs were the first manifestation of metastatic disease. In patients with metachronous metastases, SMM involvement preceded the manifestation of metastatic spread to other organs by several months and survival times were relatively favorable in the context of metastatic esophageal carcinoma.

SMM detected with imaging studies were reported in 1%-2% of all patients with metastatic disease, with metastatic involvement limited to skeletal muscle reported in less than 10% of patients[11]. Similarly to the present series, most patients with SMM have metastasis only in one muscle[11]. Given the mass of skeletal muscle in the body, one would expect the metastases to this tissue to be more frequent. Experimental data suggest that muscle contractions result in mechanical destruction of tumor cells arrested in the microvasculature[12]. It has also been hypothesized that high lactate concentrations in the skeletal muscle microenvironment may decrease the responsiveness of muscle vessels to hypoxia, creating an environment unfavorable to angiogenesis[13].

PET/CT is currently being extensively utilized for pre-therapeutic staging of esophageal carcinoma because it was demonstrated that distant metastases missed by other imaging studies may be detected[14,15]. Individual cases of esophageal carcinoma SMM have been noted in cohorts of patients staged by PET/CT[16]. The increased incidence of esophageal carcinoma SMM may not only be the result of better diagnosis with PET/CT, but also of improved prognosis resulting from better control of the primary with the use of combined modality treatment. In fact, in one of the patients in the present series, the patient who had pathologic complete response after primary treatment two years earlier, the diagnosis could be made based on clinical presentation. It is therefore possible that with advances in the therapy of esophageal

carcinoma we will witness a further continuous increase of the incidence of SMM.

Little is known about the outcome of metastases affecting specific sites and optimal management of these patients, with the only source of information being anecdotal reports like the present series. In contrast to metastases affecting other organs, e.g., bone, no organ-specific drugs like bisphosphonates are available for SMM. Moreover, the potential utilization of new radiotherapy techniques in SMM is, for obvious reasons, limited. Prolonged survival has been observed in individual patients managed by combination of external beam radiation, surgery or chemotherapy[4,17]. Based on the experience in the present series and other cases reported in the literature, only general recommendations for the management of patients with SMM, including SMM of esophageal carcinoma, can be outlined. Important consideration is symptom control, and the therapeutic strategy has to be tailored according the muscle affected. Similarly to metastases of other location, the best control may be obtained with surgery which is, however, meaningful only in patients with isolated SMM. In addition, surgery may be necessary to establish the diagnosis of SMM. External beam radiation should be reserved for patients with inoperable or recurrent SMM, including patients with metastases in other sites, and best results may be obtained when combining radiation with platinum-based chemotherapy. No standard fractionation regimen or technique has been established in this rare indication. Outside of setting of chemoradiation regimens, the role of systemic chemotherapy in the management of SMM seems to be limited, but systemic chemotherapy may be an only option in patients with widespread metastases and good general condition. For patients in poor general condition, only symptomatic therapy (mostly corticosteroids and pain medication) is indicated.

In conclusion, while SMM represent a rare event in patients with esophageal carcinoma, the incidence may be increasing because of better diagnosis or improved control of the primary. SMM may represent the first manifestation of metastatic disease. An active therapeutic approach using surgery, external beam radiation and chemotherapy may result in survival that is substantially longer compared to other cases of metastatic esophageal carcinoma.

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CASE REPORT

Hepatic schwannoma: Imaging findings on CT, MRI and contrast-enhanced ultrasonography

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Abstract

A primary benign schwannoma of the liver is extremely rare and is difficult to preoperatively discriminate from a malignant tumor. We compared the imaging and pathological findings, and examined the possibility of preoperatively diagnosing a benign liver schwannoma. A 72-year-old woman was admitted to our hospital because of a 4.6-cm mass in the liver. A malignant tumor was suspected, and a right hepatectomy was performed. After this, the diagnosis of a primary benign schwannoma of the liver was made through pathological examination. Contrast-enhanced ultrasonography (CEUS) with Sonazoid showed minute blood flows into the septum and solid areas of the tumor in the vascular phase; most likely due to increased arterial flow associated with infiltration of chronic inflammatory cells. In the postvascular phase, CEUS showed contrast defect of cystic areas and delayed enhancement of solid areas; most likely due to aggregation of siderophores. Because discriminating between a benign and malignant schwannoma of the liver is difficult, surgery is generally recommended. However, the two key findings from CEUS may be useful in discriminating ancient schwannoma by recognizing the hemorrhage involved in the secondary degeneration and aggregation of siderophores.

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Key words: Liver schwannoma; Contrast-enhanced ultrasonography; Liver resection

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INTRODUCTION

A schwannoma is a benign nerve sheath tumor that

originates from the Schwann cells of the nerve sheath. Schwannomas can occur in patients at any age, but are most commonly detected in patients between 20 years and 50 years of age. Although a schwannoma can develop in any part of the body, the most common sites include the head, neck, and flexor surfaces of the extremities[1]. Schwannomas develop infrequently in the gastrointestinal tract, the retroperitoneal cavity, and any other part of the body[2]. Thus, a primary benign schwannoma of the liver is extremely rare. Preoperative discrimination between a benign and malignant schwannoma is difficult; therefore, operation is typically recommended for a diagnosis. In the current study, we used a combination of computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET), but discrimination was still difficult. The use of preoperative imaging findings to detect liver schwannoma or the use of contrast-enhanced ultrasonography (CEUS) to detect and characterize focal liver lesions has not been previously undertaken. In this paper, we report a case of benign schwannoma, which we diagnosed using a novel method that combined CEUS with CT, MRI and PET imaging. In addition, we compared the imaging and pathological findings.

CASE REPORT

A 72-year-old woman was admitted to our hospital because a 4.6-cm mass was detected in her liver by ultrasonography (US) at a private clinic. Upon physical examination, the abdomen was soft and flat without tenderness. The liver, spleen, and superficial lymph nodes were not palpable. Blood analysis showed almost normal liver function. Carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) were both negative. Hepatitis B surface antigen and hepatitis B e antibody were positive, but hepatitis C virus marker was negative. The patient's serum antibodies were negative for specific echinococcal antigens.

CT, MRI, PET and CEUS were used for the qualitative diagnosis of the liver mass. Multiphasic contrast-enhanced CT in the arterial dominant, portal venous and late venous phases was performed using a 64-slice CT scanner. A CT scan of the abdomen showed an approximately 5-cm, well-defined round tumor in segment 8 of the right hepatic lobe. The mass was hypodense and contained a minute partial calcification (Figure 1A). The mass showed heterogeneous enhancement in the arterial dominant phase (Figure 1B) and delayed enhancement until the portal venous (Figure 1C) and late venous (Figure 1D) phases. There was no evidence of fatty infiltration or cirrhosis in the background liver.

MRI revealed a hypointense mass with T1-weighted imaging (Figure 2A) and a mixed hypo- and hyperintense mass with T2-weighted imaging (Figure 2B) and diffusion-weighted imaging (DWI) (Figure 2C). The diffusion images were obtained with a b value of 1000 s/mm^2. Because the mass was hyperintense on the apparent diffusion coefficient maps, it was also hyperintense on DWI under the influence of the T2 shine-through. Gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid (Gd-EOB-DTPA) was injected at 0.025 mmol/kg body weight. Gd-EOB-DTPA-enhanced MRI showed heterogeneous enhancement in the arterial phase and delayed enhancement until the late venous phase (similar to dynamic CT imaging), but a defect in the hepatobiliary phase (Figure 2D).

An F-18 fluorodeoxyglucose PET (FDG-PET) system was used (Discovery VCT; GE Healthcare, Tokyo, Japan) with the following conditions: PET, 2-3 min/1 bed, 3.75-mm slice; CT, 120 kV, 100 mA with auto mA; tube rotation time, 0.6 s/rotation; detector collimation, 64 mm × 1.25 mm; beam pitch, 0.984; section thickness, 5.0 mm; and reconstruction pitch, 3.27 mm. FDG-PET revealed abnormal uptake in the lesion, with a maximum standardized uptake value of 3.9. There was no additional lesion in the liver, no evidence of extrahepatic disease, and no lymphadenopathy.

US showed a 4.6-cm mass that contained multiple hypoechoic cysts with mixed internal septations and solid areas (Figure 3A). CEUS with Sonazoid showed minute blood flows into the septum and solid areas of the tumor in the vascular phase (Figure 3B), and contrast defects of cystic areas and delayed enhancement of solid areas in the postvascular phase (Figure 3C).

A malignant tumor, such as a biliary cystadenocarcinoma or cholangiocellular carcinoma (CCC), was suspected. Because there was danger of seeding, we did not perform a biopsy. An operation was performed to remove the mass. At surgery, it was discovered that the tumor originated from the anterior segment of Glisson's sheath, but was tightly attached to the posterior segment of the sheath. Intraoperative cholangiography revealed a stricture in the posterior segment of the bile duct, and there was no extension of the distal bile duct. The bile duct did not have a tumor projection until strangulation. Because the tumor involved the bile duct, dissection was not possible; therefore, the patient underwent right hepatectomy.

The gross findings were a well-circumscribed, whitish/brownish, localized mass, 4.5 cm in size, which was surrounded by a fibrous capsule. The division surface of the tumor showed large and small multilocular cysts. Microscopic examination revealed that the tumor was adjacent to the peripheral nerve of the portal area, and the Antoni A hypercellular area and Antoni B hypocellular area were present in a complex (Figure 4A). In the Antoni A region, an array of irregular groups of spindle cells was seen, but nuclear palisading with Verocay bodies was not observed. In the Antoni B region, there was edematous stroma, vasodilatation, aggregation of siderophores, and slight permeation of chronic inflammatory cells of the histiocyte and the lymphocyte types (Figure 4B). Immunohistochemical staining showed a strong positive S-100 protein reaction. Vimentin, nonspecific enolase, bcl-2, and MIC2 (also known as CD99) staining

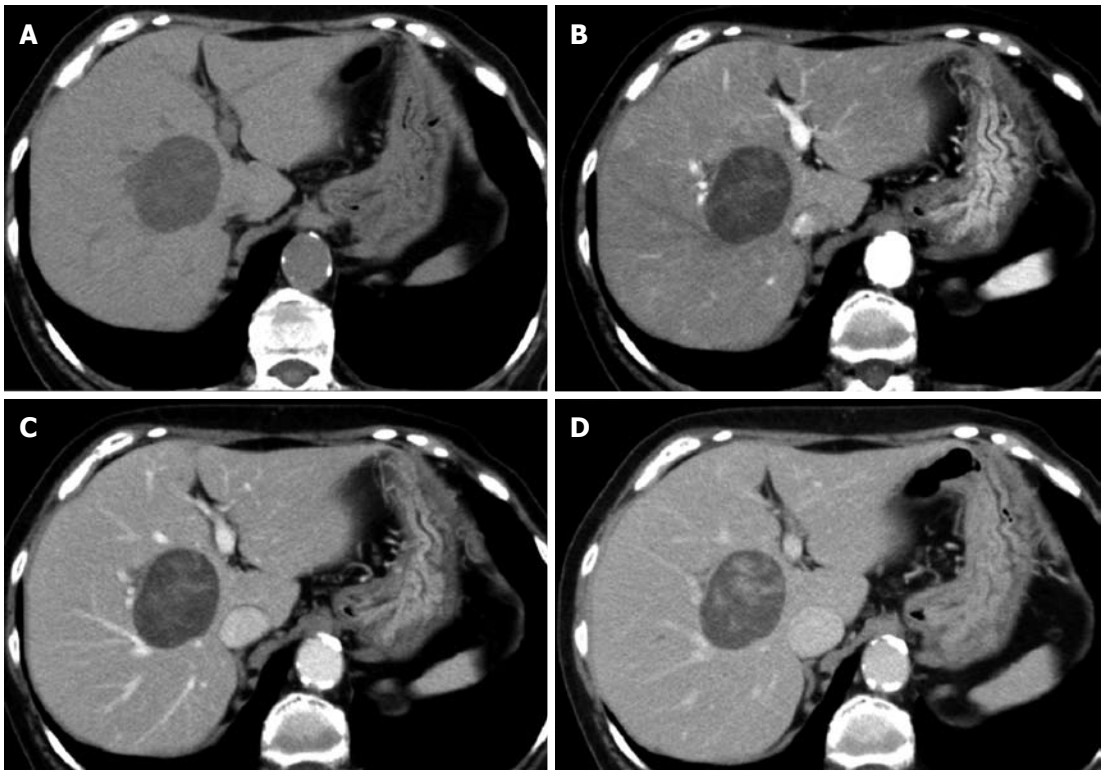

Figure 1 Abdominal computed tomography. A: Plain; B: Arterial dominant phase; C: Portal venous phase; D: Late venous phase. Abdominal computed tomography (CT) showed an approximately 5-cm well-defined round tumor in segment 8 of the right hepatic lobe. CT showed heterogeneous enhancement in the arterial dominant phase; and delayed the enhancement until the portal venous and late venous phases.

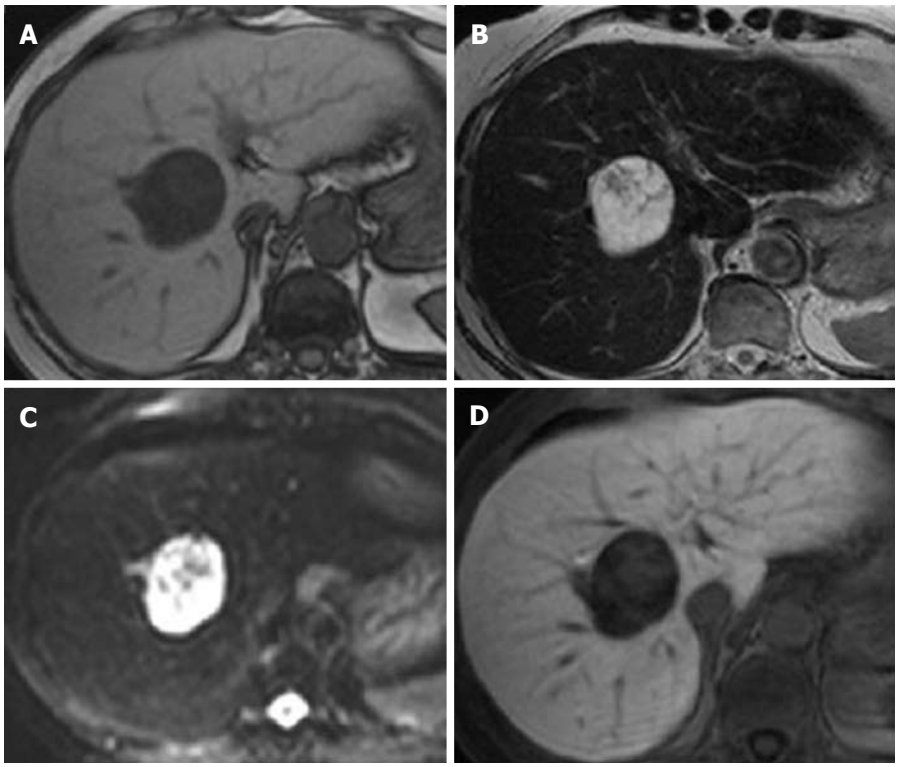

Figure 2 Magnetic resonance imaging. A: T1-weighted imaging; B: T2-weighted imaging; C: Diffusion-weighted imaging (DWI); D: Hepatobiliary phase. Magnetic resonance imaging (MRI) revealed a hypointense mass on T1-weighted imaging, and a mixed hypo- and hyperintense mass on T2-weighted imaging and on DWI. Gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid-enhanced MRI showed a defect in the hepatobiliary phase.

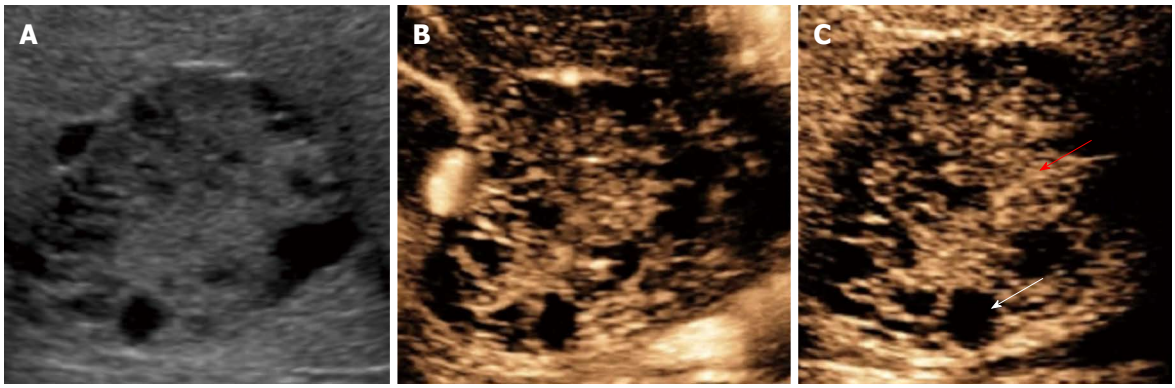

Figure 3 Contrast-enhanced ultrasonography with Sonazoid. A: B-mode ultrasound; B: Vascular phase; C: Postvascular phase. Ultrasonography showed a 4.6-cm mass that contained multiple hypoechoic cysts with mixed internal septations and solid areas. Contrast-enhanced ultrasonography with Sonazoid showed minute blood flow into the septum and solid areas of the tumor in the vascular phase, and contrast defect of cystic areas (white arrow) and delayed enhancement of solid areas (red arrow) in the postvascular phase.

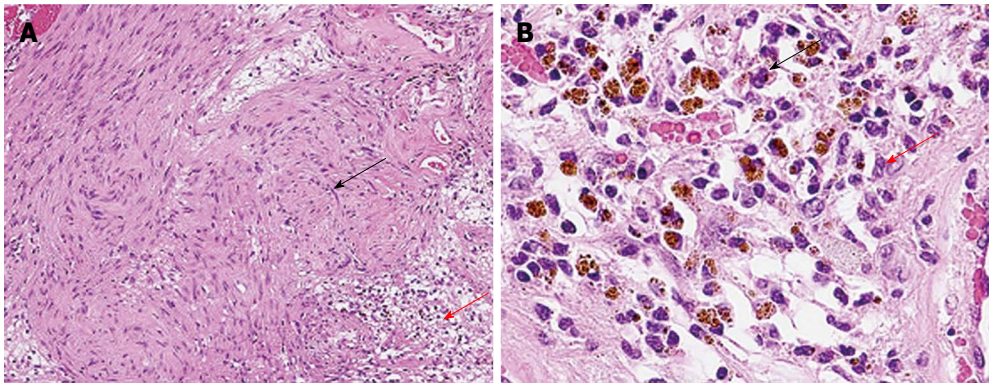

Figure 4 Microscopic examination. A: B-mode ultrasound. Microscopic examination revealed that Antoni A hypercellular area (black arrow) and Antoni B hypocellular area (red arrow) existed together in a complex; B: Vascular phase. In the Antoni B region, there were edematous stroma, vasodilatation, aggregation of siderophores (black arrow), and slight permeation of chronic inflammatory cells of the histiocyte and lymphocyte types (red arrow).

was also positive. The MIB-1 index was 2%. However, desmin, α-smooth muscle actin, CD34, c-kit, HMB-45, neurofilament, glial fibrillary acidic protein, and cytokeratin (AE1/AE3) staining was all negative.

On the basis of these findings, we diagnosed the patient as having a primary benign schwannoma of the liver. The patient was healthy and free from recurrence at a follow-up CT scan, which was performed 12 mo after surgery.

DISCUSSION

Histologically, schwannomas are encapsulated tumors that arise within nerve sheaths and consist of a highly ordered cellular component (Antoni A area) and a loose myxoid component (Antoni B area). In the subcellular component, spindle cells are present. Larger schwannomas have a tendency to undergo secondary degeneration, such as pseudocystic regression, hemorrhage, and calcification[3]. A primary benign schwannoma of the liver is an isolated benign tumor surrounded by a fibrous capsule. Immunohistochemical staining is diffusely and strongly positive for S-100 protein in a schwannoma, which is consistent with the markers for a nerve sheath tumor[3]. There are no specific characteristics with regard to the radiological appearance[1].

We reviewed the country of origin, size, age, sex, associated diseases, diagnosis, and prognosis of primary benign or malignant schwannomas; the findings are shown in Table 1[3-18]. Primary benign or malignant schwannomas were found in 14 women and 4 men. The age of onset was from 35 years to 74 years, and the mean age of onset was 57.5 years. While 13 cases were Asian in origin, there were no race differences. Although many hepatic schwannomas were Asian in origin, there has been no report of a causal relationship with hepatitis-associated viruses, and thus the reason for this correlation is unknown.

Generally, a CT scan depicts a schwannoma as a well-defined hypodense area, and enhanced CT shows peripheral enhancement, with an irregular pattern on the inside[10]. A delayed peripheral subject enhancement until the late venous phase in the CT scan reflects a fibrous capsule and an internal fibrillary element. With the aid of MRI, schwannomas have been described as masses with hypointensity on T1-weighted images and as masses with inhomogeneous hyperintensity or, in some cases, masses with internal heterogeneous mixed hypo- and hyperin-

Table 1 Review of primary benign or malignant schwannoma

Ref.	Age/sex	Country of origin	Size (cm)	Associated diseases	Diagnosis	Prognosis
Tuder *et al*[18]	74/M	North America	21 × 12 × 8	None	Surgery/autopsy	Death
Abe *et al*[17]	68/F	Asia	8.0 × 6.0 × 4.5	None	Surgery	Survival
Hytiroglou *et al*[16]	67/M	North America	13 × 11 × 10	None	-	-
Heffron *et al*[15]	38/F	North America	5	None	Surgery	Survival
Yoshida *et al*[14]	56/F	Asia	16	None	-	-
Morikawa *et al*[13]	63/M	Asia	20 × 13	None	Autopsy	Death
Sheikh *et al*[12]	35/F	Asia	-	None	-	-
Fiel *et al*[11]	49/M	North America	-	None	-	-
Wada *et al*[10]	69/F	Asia	15 × 13	None	Surgery	Survival
Wada *et al*[10]	64/F	Asia	4	Early gastric cancer	Surgery	Survival
Flemming *et al*[9]	57/F	Europa	Huge	None	Surgery	Survival
Momtahen *et al*[8]	52/F	Asia	4.4 × 3.6 × 2.9	None	Surgery	Survival
Lee *et al*[7]	36/F	Asia	5 × 4 × 2	None	Surgery	Survival
Akin *et al*[6]	66/F	Asia	3 × 5 × 4	Breast cancer	Biopsy/surgery	Survival
Shih *et al*[5]	58/F	Asia	9.1 × 7.1 × 8.9	None	Surgery	Survival
Sukegawa *et al*[4]	55/F	Asia	4.0 × 2.5 × 2.0	None	Surgery	Survival
Ozkan *et al*[3]	56/F	Asia	15 × 10 × 10	None	Surgery	Survival
Ota *et al* (present case)	72/F	Asia	4.6	None	Surgery	Survival

M: Male; F: Female.

Table 2 Comparison between preoperative imaging and pathological findings

Preoperative imaging technique		Characteristics	Pathology
Dynamic CT		Delayed peripheral subject enhancement	A fibrous capsule An internal fibrillary element
T2-weighted MRI		Internal heterogeneous mixed hypo- and hyperintensity	Secondary degeneration such as pseudocystic regression and hemorrhage
FDG-PET		Abnormal uptake	High cellular density (Antoni A area)
Ultrasonography	B mode	Mixed internal septations and solid areas	Division surface showed big and small multilocular cysts
	Vascular phase	Minute blood flows into septum and solid areas	Increased arterial vessel flows associated with infiltration of chronic inflammatory cells
	Postvascular phase	Delayed enhancement of solid areas	Aggregation of siderophores

CT: Computed tomography; MRI: Magnetic resonance imaging; FDG-PET: F-18 fluorodeoxyglucose-positron emission tomography.

tensity on T2-weighted images[8]. These findings reflect secondary degeneration, such as pseudocystic regression and hemorrhage. Abnormal uptake in FDG-PET reflects the high cellular density of schwannomas[19] (Table 2).

In this study, CT and MRI showed a tumor containing multiple cysts. Therefore, echinococcosis, hepatobiliary cystadenoma/adenocarcinoma or cystic liver metastasis of epithelial tumor, angiomyolipoma, leiomyoma, solitary fibrous tumor, schwannoma, or neurofibroma of the mesenchyma were possible differential diagnoses. However, it is very difficult to diagnose a schwannoma preoperatively based on its radiological appearance. Furthermore, abnormal uptake in FDG-PET did not aid the diagnosis.

Therefore, we used CEUS with Sonazoid for the preoperative diagnosis. Mixed internal septations and solid areas in B mode were reflected faithfully in the gross findings of a division surface with large and small multilocular cysts. CEUS showed minute blood flows into the septum and solid areas of the tumor in the vascular phase. The start of an inflow into a node can be confirmed within 18 s after the administration of Sonazoid. We accordingly determined that the minute blood flows were derived from the hepatic artery. Increased arterial flows were observed with the infiltration of chronic inflammatory cells of the histiocyte and lymphocyte types.

CEUS showed a contrast defect in the cystic areas and a delayed enhancement of the solid areas in the postvascular phase, which may reflect aggregation of siderophores. For example, in the case of echinococcosis, hepatobiliary cystadenoma/adenocarcinoma, cystic metastases, or CCC, CEUS shows contrast defects of the entire tumor in the postvascular phase (Table 2). Therefore, the two findings in CEUS (minute arterial flows into the tumor along the nodal septum in the vascular phase and contrast defects of the cystic areas and delayed enhancement of solid areas in the postvascular phase) are potentially useful for diagnosing an ancient schwannoma by recognizing the hemorrhage involved in the secondary degeneration and aggregation of siderophores.

The discrimination of a benign or a malignant schwannoma is difficult with preoperative imaging. However, some findings may indicate the presence of rare neoplasms such as a malignant schwannoma. The radiological features of hepatic malignant schwannoma include a well-demarcated, hypodense mass on CT, non-enhancement

on enhanced CT, and hypovascularity on angiography; in particular, hypovascularity may be an important finding for diagnosis, because most malignant hepatic tumors are hypervascular. Another important finding is the high level of alkaline phosphatase in serum with normal serum levels of hepatic transaminase and tumor markers, including CEA and AFP[13]. Nonetheless, because discrimination between a benign and malignant schwannoma is difficult, surgery is typically recommended.

In cases of malignant schwannoma, there are no reported chemotherapies and the prognosis is poor. Because the schwannoma may be tightly attached to Glisson's sheath (as in this case), surgical removal of the tumor could be very difficult, and hepatectomy that includes Glisson's sheath could be required.

In conclusion, here, we report a case of benign schwannoma, which we diagnosed using a novel method that combined CEUS with CT, MRI and PET. Because it is difficult to discriminate between a benign or malignant schwannoma of the liver, surgery is generally recommended for such tumors. Our findings with CEUS revealed minute arterial flow into the tumor along the nodal septum in the vascular phase, and delayed enhancement of solid areas in the postvascular phase. These two key observations may be useful in diagnosing an ancient schwannoma by recognizing the hemorrhage involved in the secondary degeneration and aggregation of siderophores.

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

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

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

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

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H. pylori*, *E coli*, *etc.*

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