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World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science
Citation Index Expanded (also known as SciSearch®),
Journal Citation Reports/Science Edition, *Index
Medicus*, MEDLINE, PubMed, Chemical Abstracts,
EMBASE/Excerpta Medica, Abstracts Journals,
PubMed Central, Digital Object Identifier,
CAB Abstracts, and Global Health.
ISI, Thomson Reuters, 2008 Impact Factor: 2.081
(32/55 Gastroenterology and Hepatology).

Volume 15 Number 33 September 7, 2009

World J Gastroenterol
2009 September 7; 15(33): 4097-4224

Online Submissions

wjg.wjgnet.com
www.wjgnet.com

Printed on Acid-free Paper

世界胃肠病学杂志

World Journal of Gastroenterology®

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2007-2009



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^[1]Passed away on October 20, 2007

^[2]Passed away on June 14, 2008



World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 33
September 7, 2009



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NAME OF JOURNAL

World Journal of Gastroenterology

RESPONSIBLE INSTITUTION

Department of Science and Technology of Shanxi Province

SPONSOR

Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHING

The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No.62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

PRINTING

Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR

Beijing Bureau for Distribution of Newspapers and Journals (Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE

September 7, 2009

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SUBSCRIPTION

RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN

ISSN 1007-9327
CN 14-1219/R

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Iatrogenic bile duct injuries: Etiology, diagnosis and management

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Received: June 25, 2009 Revised: July 21, 2009

Accepted: July 28, 2009

Published online: September 7, 2009

Key words: Iatrogenic disease; Biliary drainage; Bile ducts; Cholecystectomy; Roux-en-Y anastomosis; Surgical injuries; Surgical anastomosis

Peer reviewer: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Jabłońska B, Lampe P. Iatrogenic bile duct injuries: Etiology, diagnosis and management. *World J Gastroenterol* 2009; 15(33): 4097-4104 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4097.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4097>

Abstract

Iatrogenic bile duct injuries (IBDI) remain an important problem in gastrointestinal surgery. They are most frequently caused by laparoscopic cholecystectomy which is one of the commonest surgical procedures in the world. The early and proper diagnosis of IBDI is very important for surgeons and gastroenterologists, because unrecognized IBDI lead to serious complications such as biliary cirrhosis, hepatic failure and death. Laboratory and radiological investigations play an important role in the diagnosis of biliary injuries. There are many classifications of IBDI. The most popular and simple classification of IBDI is the Bismuth scale. Endoscopic techniques are recommended for initial treatment of IBDI. When endoscopic treatment is not effective, surgical management is considered. Different surgical reconstructions are performed in patients with IBDI. According to the literature, Roux-en-Y hepaticojejunostomy is the most frequent surgical reconstruction and recommended by most authors. In the opinion of some authors, a more physiological and equally effective type of reconstruction is end-to-end ductal anastomosis. Long term results are the most important in the assessment of the effectiveness of IBDI treatment. There are a few classifications for the long term results in patients treated for IBDI; the Terblanche scale, based on clinical biliary symptoms, is regarded as the most useful classification. Proper diagnosis and treatment of IBDI may avoid many serious complications and improve quality of life.

INTRODUCTION

Iatrogenic bile duct injuries (IBDI) remain an important problem in gastrointestinal surgery. They are most frequently caused by laparoscopic cholecystectomy, which is one of the commonest surgical procedures in the world^[1]. The early and accurate diagnosis of IBDI is very important for surgeons and gastroenterologists, because unrecognized IBDI lead to serious complications such as biliary cirrhosis, hepatic failure and death^[2,3]. The choice of the appropriate treatment for IBDI is very important, because it may avoid these serious complications and improve quality of life in patients. Therefore, the question regarding the type of treatment for patients with IBDI is still a matter of debate. Initially, endoscopic treatment is recommended in patients with IBDI. When endoscopic techniques are not effective, different surgical reconstructions are performed^[4,5]. The goal of surgical treatment is reconstruction to allow good bile flow to the alimentary tract. In order to achieve this goal, many techniques are used. There are some contradictory opinions on different surgical reconstructions in the literature.

HISTORICAL PERSPECTIVES OF RECONSTRUCTIVE BILIARY SURGERY

Anatomic knowledge of the liver and bile ducts can be traced to Babylon in 2000 BC^[6]. Gallstone disease has been found in one mummy from Amen of the 21st Dynasty. Historic notes from Mesopotamia, Greece, Egypt and Roma also show an occurrence of bile duct

disease in ancient history^[6]. The first surgical procedures performed on bile ducts were not complicated. In 1618, Fabricius removed gallstones. In 1867, Bobbs performed cholecystostomy. Cholecystostomies were also performed by Sims (1878), Kocher (1878) and Tait (1879)^[6-8]. The first planned cholecystectomy in the world was performed by Langenbuch in 1882^[6-9]. The first choledochotomy was performed by Couvoissier in 1890. Widespread use of surgical procedures on bile ducts was associated with occurrence of IBDI. The first iatrogenic bile duct injury was described by Sprengel in 1891. He also reported the first choledochoduodenostomy (ChD) for calculi (1891)^[7,10]. In 1892, Doyenn reported the first choledochocholedochostomy for the same condition^[7]. Cholecystoenterostomy to the colon was the first biliary-alimentary anastomosis and it was performed by Winiwater in 1881^[7]. The first surgical reconstruction ("end-to-side" ChD) of IBDI was performed by Mayo in 1905^[7]. The first Roux-en-Y hepaticojejunostomy (HJ) was described by Monprofit in 1908. Dahl noted Roux-en-Y HJ for surgical treatment of IBDI in 1909^[7]. In 1969, Smith created a mucosal graft anastomosis in the repair of the damaged proximal bile duct^[7]. In 1954, Hepp and Couinaud described the hilar plate and long extrahepatic course of the left hepatic duct. The left hepatic duct, after dissection of the hilar plate, was used in the repair of high strictures^[7]. In 1948, Longmire and Sanford described a technique of finding of a branch of the left hepatic duct for anastomosis in the high biliary strictures. This technique was based on partial resection of the left hepatic lobe. In 1957, this technique was modified by Soulpaut and Couinaud. They described finding much larger ductal structures in the left lobe by following the round ligament to the origin of the 3rd segment duct^[7]. In 1994, Hepp and Blumgart described a technique of hilar and intrahepatic biliary-enteric anastomosis^[11].

ETIOLOGY AND PATHOGENESIS OF IBDI

Etiology of IBDI

IBDI present about 95% of all benign biliary strictures^[12,13]. Benign biliary strictures encompass a wide spectrum involving not only IBDI, but also biliary disorders caused by other pathogenetic factors. The main causes of benign biliary strictures are presented in Table 1.

There are two main groups of surgical procedures leading to IBDI. The first group involves surgical procedures performed on the biliary tract such as open and laparoscopic cholecystectomy, choledochotomy and previous operations on bile ducts. The second group involves operations performed on other organs of the epigastrium such as gastric resection (most frequently the Billroth II partial gastric resection), hepatic resection and liver transplantation, pancreatic resections, biliary-enteric anastomoses, portacaval shunts, lymphadenectomy and other procedures within the hepato-duodenal ligament^[12,13]. IBDI occur most frequently during cholecystectomy. Recently, the number of patients with

Table 1 Main causes of benign biliary strictures

Congenital strictures	Biliary atresia and congenital cysts
Bile duct injuries	Iatrogenic: postoperative, following endoscopic and percutaneous procedures
	Following blunt or penetrating trauma of the abdomen
Inflammatory strictures	Cholelithiasis and choledocholithiasis
	Mirizzi's syndrome
	Chronic pancreatitis
	Chronic ulcer or diverticulum of the duodenum
	Abscess or inflammation of the liver or subhepatic region
	Parasitic, viral infection
	Toxic drugs
	Recurrent pyogenic cholangitis
	Primary sclerosing cholangitis
	Radiation-induced strictures
	Papillary stenosis

Table 2 Incidence of IBDI following cholecystectomy (%)

Author	IBDI incidence following OC	IBDI incidence following LC
McMahon <i>et al</i> ^[14] , 1995	0.2	0.81
Strasberg <i>et al</i> ^[15] , 1995	0.7	0.5
Shea <i>et al</i> ^[16] , 1996	0.19-0.29	0.36-0.47
Targarona <i>et al</i> ^[17] , 1998	0.6	0.95
Lillemoe <i>et al</i> ^[18] , 2000	0.3	0.4-0.6
Gazzaniga <i>et al</i> ^[19] , 2001	0.0-0.5	0.07-0.95
Savar <i>et al</i> ^[20] , 2004	0.18	0.21
Moore <i>et al</i> ^[21] , 2004	0.2	0.4
Misra <i>et al</i> ^[22] , 2004	0.1-0.3	0.4-0.6
Gentileschi <i>et al</i> ^[23] , 2004	0.0-0.7	0.1-1.1
Kaman <i>et al</i> ^[24] , 2006	0.3	0.6

IBDI: Iatrogenic bile duct injuries; OC: Open cholecystectomy; LC: Laparoscopic cholecystectomy.

IBDI has increased two-fold, which has been associated with widespread use of laparoscopic cholecystectomy^[11]. The incidence of IBDI following open and laparoscopic cholecystectomy according to different authors in the literature is presented in Table 2.

Pathogenesis of IBDI

There are several factors associated with an increased risk of IBDI. Coexisting acute or chronic inflammation around the gallbladder and hepato-duodenal ligament can increase the difficulty of the surgical procedure and increase the risk of IBDI. Other factors such as patient obesity, fat within the hepato-duodenal ligament, poor exposure and bleeding in the surgical area also increase the risk of IBDI. Poor prognostic factors are also male gender and long duration of symptoms before cholecystectomy.

Anatomical anomalies of the bile ducts and hepatic arteries significantly increase the risk of IBDI. The most frequent cause of IBDI is misidentification of the bile duct as the cystic duct in cases of anomalies of cystic duct insertion into the common hepatic duct. About 70%-80% of all IBDI are a consequence of

misidentification of biliary anatomy before clipping, ligating and dividing structures^[12,13,25,26]. Excessive dissection along the common bile duct margins during open cholecystectomy can lead to biliary stricture because of damage to the three o'clock and nine o'clock axial arteries and their branches to the pericholedochal plexus. According to the literature, distal IBDI are accompanied by damage of axial arteries (10%-15%) and proximal IBDI are usually associated with damage to the hepatic artery and its branches (40%-60%)^[26-30].

Clinical presentation of IBDI

The common clinical symptoms are jaundice, fever, chills, and epigastric pain. The clinical presentation depends on the type of injury and is divided into two groups. In patients with bile leaks, bile is present in the closed-suction drain located in the subhepatic region. If the subhepatic region is not drained, subhepatic bile collection (biloma) or abscess develops. In these patients fever, abdominal pain and other signs of sepsis occur. Generally, jaundice is not observed in these patients because cholestasis does not appear. In the second group of patients with biliary strictures, jaundice caused by cholestasis is the commonest clinical symptom^[12,13].

Diagnostics of IBDI

Laboratory and radiological investigations are used in diagnosis of IBDI. Among laboratory examinations, indicators of cholestasis and liver function play an important role: serum bilirubin, alkaline phosphatase, γ -glutamyl transpeptidase, alanine and aspartate aminotransferases. In patients with IBDI without complications, the liver is not damaged. Therefore, cholestasis indicators are increased but aminotransferases are not increased in these patients. Pathological levels of aminotransferases are present in cases of secondary biliary cirrhosis as a serious complication of unrecognized or improperly treated biliary injuries. In patients with secondary biliary cirrhosis, hypoalbuminemia and coagulation defects (prolonged prothrombin time) are observed. They are the most frequently used parameters of synthetic capacity of the liver. Imaging diagnostics in IBDI involve ultrasonography of the abdominal cavity, cholangiography, endoscopic retrograde cholangiopancreatography (ERCP), computed tomography, and magnetic resonance-cholangiography. Ultrasonography of the abdominal cavity allows imaging of intrahepatic and extrahepatic bile ducts with measurement of the diameter of the common bile duct or common hepatic duct. It also shows biloma or intraabdominal abscesses in patients with bile leaks. Computed tomography is useful for more specific investigation in doubtful cases in patients with bile leaks. Percutaneous transhepatic cholangiography is useful in assessment of the bile tract proximal to the location of the damage. ERCP is a very useful method of investigation in imaging of damaged bile ducts and it allows the repair of small bile duct injuries by insertion of a biliary prosthesis. Magnetic resonance cholangiography is a sensitive (85%-100%) and non-invasive imaging modality

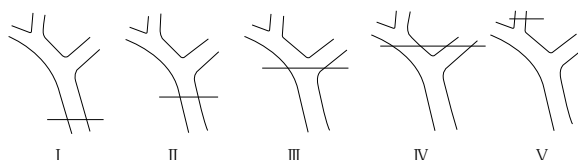


Figure 1 Bismuth classification of IBDI. I : Common bile duct and low common hepatic duct (CHD) > 2 cm from hepatic duct confluence; II : Proximal CHD < 2 cm from the confluence; III : Hilar injury with no residual CHD-confluence intact; IV : Destruction of confluence: right and left hepatic ducts separated; V : Involvement of aberrant right sectoral hepatic duct alone or with concomitant injury of CHD.

for the biliary tract. Currently, it is the “gold standard” in preoperative diagnosis of IBDI in patients qualifying for surgical reconstruction^[12,13,31,32].

Almost 85% of IBDI are not recognized during the primary iatrogenic surgical procedure^[33]. According to the literature, only 15%-30% of IBDI are recognized during the initial operation^[34]. According to other data, 70% of IBDI are diagnosed within 6 mo and 80% within 12 mo after the initial operation^[31].

Classification of IBDI

A number of classifications have been proposed by different authors. In our opinion, the Bismuth scale is the most useful and simple classification. It is based on the location of the injury in the biliary tract^[35]. This classification is very helpful in prognosis after repair, but does not involve the wide spectrum of possible biliary injuries. The Bismuth classification is described in Figure 1. Another classification is the Strasberg scale which, in contrast to the Bismuth scale, allows differentiation between small (bile leakage from the cystic duct) and serious injuries performed during laparoscopic cholecystectomy, but it does not play an important role in the choice of surgical treatment^[15,34,36]. The Mattox classification of IBDI takes into consideration the type of injuring factor (contusion, laceration, perforation, transection, diversion or interruption of the bile duct or the gallbladder)^[37]. There are several classifications in the literature for IBDI induced during laparoscopic cholecystectomy (Stewart *et al*^[38], Schmidt *et al*^[27], Bektas *et al*^[39]).

MANAGEMENT OF IBDI

Endoscopic and radiological treatment of IBDI

Non-invasive, percutaneous radiological and endoscopic techniques are recommended as initial treatment of IBDI. When these techniques are not effective, surgical management is considered. According to the literature, the effectiveness of a radiological approach with transhepatic stenting of the damaged biliary tract is 40%-85%. The common complications of the radiological procedures are as follows: hemorrhage (hemobilia, bleeding from hepatic parenchyma or adjacent vessels), bile leakage and cholangitis. The other complications such as pneumothorax resulting from pleural violation, bilio-pleural fistula and perforation of adjacent abdominal

structures including the gallbladder and large bowel, are described less frequently. Percutaneous dilatation is less effective (52%) than surgical treatment (89%). According to the literature, the radiological approach is also associated with a higher number of complications (35%) than surgical management (25%). Most frequently, it is recommended in very difficult cases of very high, hilar biliary strictures or in the treatment of very small diameter bile ducts^[12,13,31,40].

Endoscopic dilatation associated with insertion of biliary prosthesis during ERCP investigation is the most frequently used non-surgical method in the treatment of IBDI. According to the literature, the success of endoscopic (72%) and surgical (83%) management of IBDI is comparable. Frequency of complications in both treatment methods is also comparable (35% *vs* 26%). The common complications of endoscopic techniques regarding placement of biliary prostheses include cholangitis, pancreatitis, prosthesis occlusion, migration, dislodgement and perforation of the bile duct. Endoscopic treatment is recommended as initial treatment of benign biliary strictures, in patients with biliary fistula or when surgical treatment is not warranted^[12,13,41].

Surgical treatment of IBDI

The goal of surgical treatment is to reconstruct the bile duct to allow proper bile flow to the alimentary tract. In order to achieve this goal, many techniques are used. There are contradictory reports on the effectiveness of bile duct reconstruction methods in the literature. The following operations have been reported for surgical treatment of IBDI: Roux-en-Y HJ, end-to-end ductal biliary anastomosis (EE), ChD, Lahey HJ, jejunal interposition hepaticoduodenostomy, Blumgart (Hepp) anastomosis, Heinecke-Mikulicz biliary plastic reconstruction and Smith mucosal graft^[11,18,42-46].

Various surgical techniques including immediate surgical repair: In the case of recognition of IBDI during laparoscopic cholecystectomy, immediate cholangiography and conversion to an open procedure in order to define the extent of the injury are required. The injury should be repaired by an experienced hepatobiliary surgeon. If this is impossible, a patient should be transferred to a hepatobiliary surgery referral center after adequate drainage of the subhepatic region. Bile ducts of diameter less than 2-3 mm without communication with a main biliary tract, should be ligated in order to avoid postoperative bile leak leading to development of biloma and abscess in the subhepatic region. Bile ducts of diameter more than 3-4 mm should be repaired, not ligated, because they drain a wider hepatic area. Interruption of common hepatic duct or common bile duct continuity can be repaired by immediate tension-free EE with or without a T tube, using absorbable sutures. Security of the immediately repaired bile duct with a T tube is controversial. According to the literature, in liver transplantation, EE over a T tube is

associated with a significantly higher stricture rate than choledochostomy without a T tube (25% *vs* 11%). If the bile duct loss is too long and immediate and EE is not possible without tension, Roux-en-Y HJ is recommended^[5,12,13,25,31,47].

Surgical reconstructions: A number of reconstructions are used in surgical treatment of IBDI. There are a few conditions for proper healing of each biliary anastomosis. The anastomosed edges should be healthy, without inflammation, ischemia or fibrosis. The anastomosis should be tension-free and properly vascularized. It should be performed in a single layer with absorbable sutures^[25,38].

Currently, Roux-en-Y HJ is the most frequently performed surgical reconstruction of IBDI. In this surgical technique, a proximal common hepatic duct is identified and prepared and the distal common bile duct is sutured. End-to-side or end-to-end HJ is performed in a single layer using interrupted absorbable polydioxanone (PDS 4-0 or 5-0) sutures^[48,49]. Most authors prefer HJ because of the lower number of postoperative anastomosis strictures. According to Terblanche *et al*^[45], HJ is effective in 90% of cases. However, after this reconstruction, bile flow into the alimentary tract is not physiological, because the duodenum and upper part of the jejunum are excluded from bile passage. Physiological conditions within the proximal gastrointestinal tract are changed as a result of duodenal exclusion from bile passage. An altered bile pathway is a cause of disturbances in the release of gastrointestinal hormones^[48-50]. There is a hypothesis that in patients with HJ, the bile bypass induces gastric hypersecretion leading to a pH change secondary to altered bile synthesis and release of gastrin. A higher number of duodenal ulcers is observed in patients with HJ, which may be associated with a loss of the neutralizing effect of the bile, including bicarbonates and secondary gastric hypersecretion^[51]. Laboratory investigations revealed increased gastrin and glucagon-like immunoreactivity plasma levels and decreased triglycerides, gastric inhibitory polypeptide and insulin plasma levels in patients with HJ^[51]. An altered pathway of bile flow is also a cause of disturbance in fat metabolism in patients undergoing HJ^[51,52]. Moreover, the total surface of absorption in these patients is also decreased as a result of exclusion of the duodenum and upper jejunum from the passage of food. This hypothesis was supported by a study performed in our center. We compared early and long term results of two surgical reconstructions of IBDI: Roux-en-Y HJ and EE. The study showed a significantly lower weight gain in patients undergoing HJ in comparison to patients following physiological EE^[49]. The other disadvantage of HJ is a lack of ability to control endoscopic examination and endoscopic dilatation of the strictured biliary anastomosis. In order to resolve this problem, a longer jejunal loop (jejunostomy) is prepared and sutured to the abdominal subcutaneous tissue in the right subcostal region. Jejunostomy can be open or closed with the possibility of opening in a case of biliary

anastomosis stricture, which should be endoscopically dilated. Jejunostomy is associated with bile loss of about 40 mL/d^[53].

EE is a physiological biliary reconstruction^[49,54]. In this type of reconstruction, extensive mobilization of the duodenum with the pancreatic head through the Kocher maneuver, excision of the bile duct stricture, and refreshment of the proximal and distal stumps should be performed. Anastomosis is performed in a single layer with interrupted absorbable PDS 4-0 or 5-0 sutures^[49]. This reconstruction is not recommended by most authors because of the higher number of anastomosis strictures in comparison with HJ. We recommend EE first, because in some patients, extensive mobilization of the duodenum with the pancreatic head by the Kocher maneuver allows tension-free anastomosis after the extensive bile duct length loss. Excision of the bile duct stricture, dissection and refreshing of the proximal and distal stumps as far as the tissues are healthy and without inflammation, and the use of non-traumatic, monofilament-interrupted 5-0 sutures allows the achievement of good long term results. Use of an internal Y tube conducting the right and left hepatic ducts into the duodenum through the EE and the papilla of Vater also allows the proper healing of this anastomosis. In our department, this reconstruction was performed when the bile duct loss was from 0.5 to 4 cm. It allowed the achievement of very good long term results with effectiveness comparable to HJ. Establishing a physiological bile pathway allows proper digestion and absorption, which causes a greater weight gain in patients following EE, as noted in our study^[49]. Another essential advantage of EE is the possibility of control of the endoscopic examination in these patients. Fewer early complications are observed after EE than HJ, which is associated with opening of the alimentary tract and a higher number of anastomoses (biliary-enteric and entero-enteric)^[49].

Other biliary reconstruction methods are used less frequently. ChD is actually a rarely performed operation recommended by some authors only in cases of injury within the distal portion of the common bile duct. It guarantees physiological bile flow into the duodenum and anastomosis endoscopic control, as well as being technically easier. It is recommended in some cases of distal strictures, when use of the jejunal loop, as a result of numerous adhesions, is impossible. It should be performed on the large common bile duct (> 15 mm diameter) because the postoperative strictures are more frequent within the narrow duct. ChD should be created between the duodenum and the distal common bile duct in order to decrease the risk of so-called sump syndrome noted in 0.14%-3.3% of cases in the literature. Following ChD, recurrent ascending cholangitis because of bile reflux is noted in 0%-4% of patients^[11,51]. A higher rate of bile duct cancer in patients with ChD in comparison to HJ was noted by Tocchi *et al*^[55] during a 30-year observation period (7.6% *vs* 1.9%).

Jejunal interposition hepaticoduodenostomy, using 25-35 cm of the jejunal loop, is performed in some

surgical centers. This reconstruction includes three types of anastomosis (biliary-enteric, enteric-duodenal and entero-enteric). Biliary-enteric anastomosis is performed in a single layer with interrupted absorbable 5-0 sutures and enteric-duodenal anastomosis in a single layer with interrupted or continuous absorbable 4-0 sutures. The advantage of this reconstruction is physiological bile flow into the duodenum, which prevents duodenal ulcers caused by changes in the neurohormonal axis within the upper alimentary tract^[10,56].

The repair of hilar IBDI requires special surgical techniques. In the past, the so-called "mucosal graft technique" described by Smith in the 1960s was performed^[57,58]. This reconstruction involves creating a mucosal dome of jejunum (by removing a seromuscular patch) near the end of the Roux-en-Y loop through which a straight rubber tube is passed *via* hepatic ducts and through the liver parenchyma. This technique is based on the hypothesis that the jejunal mucosa grafts to the biliary epithelium, and a mucosa-to-mucosa anastomosis is created. Short-term results were good, but in the long term a high number of anastomosis strictures was observed. Therefore, currently, not Smith but the Blumgart-Hepp technique is used in reconstruction of hilar IBDI. In this technique, the dorsal surface of the left hepatic duct is placed parallel to the quadrate hepatic lobe; dissection and opening of the left hepatic duct longitudinally allows creation of a wide anastomosis of 1-3 cm in diameter^[11,25,57-60].

Biliary drainage: There are several methods of biliary drainage securing the anastomosis: external T tube, external Y tube, Rodney Smith drainage and internal Y tube. External T drainage involves using a typical Kehr tube with insertion of its short branches into the bile duct and passage of its long branch through the abdominal wall to the outside. Y drainage involves insertion of short branches of the Kehr tube into both right and left hepatic ducts, splinting of the anastomosis and passage of its long branch through the jejunal loop and abdominal wall to the outside (external Y drainage) or into the duodenum by the papilla of Vater (internal Y drainage). An external T or Y tube is removed percutaneously and an internal Y tube is removed endoscopically. Most frequently, external T drainage is used in biliary-enteric anastomosis and internal Y drainage in EE. In Rodney Smith drainage, two straight rubber tubes splinting the biliary-enteric anastomosis are passed *via* the hepatic ducts, through the liver parenchyma and through the abdominal wall to the outside. This drainage type is used in high intrahilar biliary-enteric anastomosis. In the past, it was used in the Smith "mucosal graft technique"^[54,58-60].

The use and duration of biliary drainage is still controversial. The advantage of biliary drainage is limitation of the inflammation and fibrosis occurring after the surgical procedure. In the opinion of some authors, the presence of the biliary tube prevents anastomosis stricture^[61]. The disadvantage of biliary drainage is a higher risk of postoperative complications^[62]. Mercado *et al*^[63]

Table 3 Terblanche clinical classification for assessment of long-term results of surgical bile duct repair

Grade	Result	
I	Excellent	No biliary symptoms with normal liver function
II	Good	Transitory symptoms, currently no symptoms and normal liver function
III	Fair	Clearly related symptoms requiring medical therapy and/or deteriorating liver function
IV	Poor	Recurrent stricture requiring correction, or related death

recommend using transanastomotic stents when there is a thin bile duct less than 4 mm in diameter, and when there is inflammation within the ductal anastomosed edges which makes proper healing of the anastomosis questionable. The duration of drainage is also controversial. According to most authors, the optimal length of time for biliary drainage is about 3 mo. Investigations showed that longer durations of biliary drainage do not provide any advantage^[18,64].

RESULTS OF SURGICAL TREATMENT OF IBDI

Short-term results and early complications

According to most authors, the early postoperative morbidity rate is 20%-30% and mortality rate 0%-2%^[31,42,44]. The most frequent early complication is wound infection, which is described in 8%-17.7%^[32,48,60,65]. Other complications reported in the literature are the following: bile collection, intra-abdominal abscess, biliary-enteric anastomosis dehiscence, biliary fistula, cholangitis, peritonitis, eventration, pneumonia, circulatory insufficiency, intra-abdominal bleeding, sepsis, infection of the urinary tract, pneumothorax, acute pancreatitis, thrombosis and embolic complications, diarrhea, ileus and multi-organ insufficiency^[18,32,42,66].

Long term results

Assessment of long term results is the most important in surgical treatment of IBDI. Proof of successful surgical treatment is the absence of biliary anastomosis stricture. In referral centers, a successful outcome after surgical repair of IBDI is observed in 70%-90% of patients^[3,5,45]. Two-thirds (65%) of recurrent biliary strictures develop within 2-3 years after the reconstruction, 80% within 5 years, and 90% within 7 years. Recurrent strictures 10 years after the surgical procedure are also described in the literature^[3,31,67]. A satisfactory length of follow-up, which is necessary in order to assess the long term results of the repair procedure, is 2-5 years^[18,31,64]. Some authors recommend 10 or 20 years of observation^[43,45].

There are a number of classifications in order to assess the long term outcomes of bile duct surgical repairs. In our opinion, the Terblanche clinical grading (1990) is the most useful classification. It is based on clinical biliary symptomatology and is presented in Table 3^[45]. Other less frequently used classifications by Nielubowicz *et al*^[68]

(1973), Lygidakis *et al*^[69] (1986), Muñoz *et al*^[70] (1990) and McDonald *et al*^[66] (1995) are described in the literature.

CONCLUSION

Surgical procedures performed within the biliary tract are very common. The incidence of IBDI has increased recently, and has been associated with increased use of laparoscopic cholecystectomy worldwide. It is essential to be careful in the proper visualization of the surgical area and the identification of structures before ligation or transection in order to decrease the risk of bile duct injuries during surgery. When biliary injury develops, early recognition and appropriate treatment are most important. Early and correct treatment allows avoidance of serious complications in patients with IBDI. Following bile duct repair, patients require long term and careful postoperative observation because of the possibility of biliary anastomosis stricture.

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What is artificial endocrine pancreas? Mechanism and history

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Received: July 3, 2009 Revised: August 13, 2009

Accepted: August 20, 2009

Published online: September 7, 2009

Abstract

The artificial endocrine pancreas is a feedback control instrument that regulates insulin delivery on a minute-by-minute basis according to measured blood glucose levels. Only one type of bedside-type artificial endocrine pancreas is now available in Japan: STG-22 (Nikkiso Co. Ltd., Japan). In the insulin infusion algorithm, insulin is infused on the basis of its proportional and derivative actions, to blood glucose concentrations with a constant time delay. The bedside-type artificial endocrine pancreas has been proven to be useful not only as a therapeutic tool for diabetes mellitus, but also as an elegant research tool for investigating the pathophysiology of the disease, by using the euglycemic hyperinsulinemic glucose clamp technique. The wearable type of closed-loop system has been developed recently. The breakthrough is the establishment of a needle-type glucose sensor. The development of closed-loop glycemic control systems that enable long-term physiological regulation has focused on implantable devices. Much effort has been expended to realize these devices.

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Key words: Diabetes mellitus; Artificial pancreas; Blood glucose; Insulin; Infusion

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Nishida K, Shimoda S, Ichinose K, Araki E, Shichiri M. What is artificial endocrine pancreas? Mechanism and history. *World J Gastroenterol* 2009; 15(33): 4105-4110 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4105.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4105>

INTRODUCTION

The strategy used in the therapy of diabetes mellitus can be divided into three periods. First, doctors attempted to prevent acute metabolic derangements and prolong the lifespan of patients through use of insulin (discovered in 1921 by Banting and Best) and through the introduction of oral hypoglycemic agents. Second, in association with prolongation of lifespan, an increase in chronic complications, especially microangiopathy, was observed. To counter these, the techniques of hemodialysis or kidney transplantation were established. The application of photocoagulation for preventing the blindness caused by diabetic retinopathy became popular. We are now in the third stage, in which we are attempting to prevent the onset of these chronic complications. Many retrospective studies and recently performed prospective studies have revealed that strict glycemic regulation is essential to prevent the onset of microangiopathy^[1-3].

HISTORY OF ARTIFICIAL ENDOCRINE PANCREAS

As early as 1959, Professor E Perry McCullagh, an endocrinologist at The Cleveland Clinic, demonstrated the concept of an implantable artificial endocrine pancreas. The closed-loop regulatory system, which consisted of a glucose monitoring device, transmitter, and insulin syringe, was looked upon as the future treatment device for diabetes mellitus.

The development of an artificial endocrine pancreas to substitute for the diseased pancreatic β cell function

has been attempted widely. Albisser *et al*^[4] in Toronto in 1974 and Shichiri *et al*^[5] in Osaka in 1975 succeeded with the clinical use of an artificial endocrine pancreas that consisted of an autoanalyzer for blood glucose determination, a minicomputer system, and a pump driving system. Next, the size of the whole system was reduced, which created a bedside-type artificial endocrine pancreas; Biostator (Miles Laboratory Inc., Elkhart, IN, USA) was developed (no longer available)^[6] and another type was developed by the Osaka University group^[7]. These devices have been used clinically on a short-term basis, and have a good reputation as elegant research tools to study the pathophysiology of diabetes mellitus.

BEDSIDE-TYPE ARTIFICIAL ENDOCRINE PANCREAS

Principle of the system

The artificial endocrine pancreas is a device that is composed of a sensor, computer and set of pumps. These components are connected in such a way as to form a closed loop for the subjects. The system is shown in Figure 1. By means of an indwelling dual-lumen catheter, venous blood is drawn into an analyzer that is modified for continuous blood glucose measurement. The computer receives the electrical signals generated by the glucose analyzer and interprets these in accordance with its internal algorithms that are programmed with specific parameters. In turn, the computer instructs one pump to delivery insulin, the amount varying according to the level of the blood glucose and its rate of change. Similarly, glucose or glucagon may be administered by another pump in a counter-regulatory manner when hypoglycemia tends to occur.

Glucose sensor

In our first artificial endocrine pancreas system, continuous glucose measurement was conducted with a Technicon AutoAnalyzer II using a modification of the glucose oxidase method^[5-7]. However, to minimize the blood sampling volume and to make the whole system smaller, a glucose sensor for continuous glucose monitoring of the whole blood was developed by combining glucose oxidase membrane with an electrode that measures hydrogen peroxide, one of the reaction products, polarographically. A key component of a low-noise blood glucose sensor with long-term stability is its membrane, therefore, hydrophilic Cuprophane 100 pmol/L with a pore size of 3 nm, was applied to cover the immobilized glucose oxidase (Figure 2).

Intravenous insulin infusion algorithm

To develop an insulin infusion algorithm, mathematical models and a computer algorithm are required. By applying the mechanical control theory, Albisser *et al*^[4] have proposed a set of relationships to translate information about blood glucose levels into rates of delivery of insulin

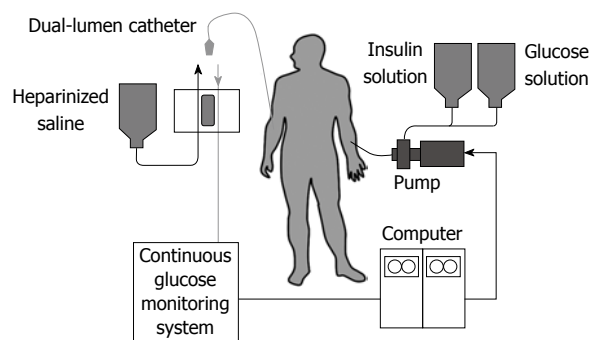


Figure 1 Schematic diagram of the bedside-type artificial endocrine pancreas.

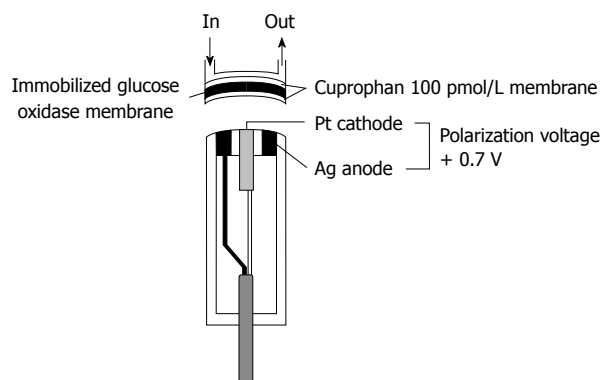


Figure 2 Structure of a glucose sensor of the bedside-type artificial endocrine pancreas. The immobilized glucose oxidase was covered with hydrophilic Cuprophane 100 pmol/L.

and glucagon (or glucose). In this algorithm, the rate of insulin infusion is regulated by the proportional (static) and derivative (dynamic) control mechanisms, which provides for single-phase insulin release, as well as a biphasic response to measured blood glucose concentrations. However, glucose infusion is necessary to prevent severe hypoglycemia caused by excessive insulin infusion in response to a hyperglycemic state.

It is well known that, in rat islets perfused with glucose, a biphasic response of insulin secretion is observed. With the aid of a control theory, we assume that insulin secretion responds not only to the glucose concentration itself, but also to the rate of change in glucose concentration. In other words, against the stepwise input of glucose concentration, an initial rapid insulin secretion is achieved by the derivative action and a second milder increase in secretion is achieved by the proportional action as an output. This relationship has been simulated successfully by using the transfer function with the first-order delay in both proportional and derivative action as shown in Figure 3^[8]. By applying this principle, an insulin infusion algorithm has been developed.

The characteristics of our insulin infusion algorithm are as follows: (1) the amount of insulin infusion is small enough to keep or mimic the physiological plasma insulin concentration; and (2) glucagon infusion is

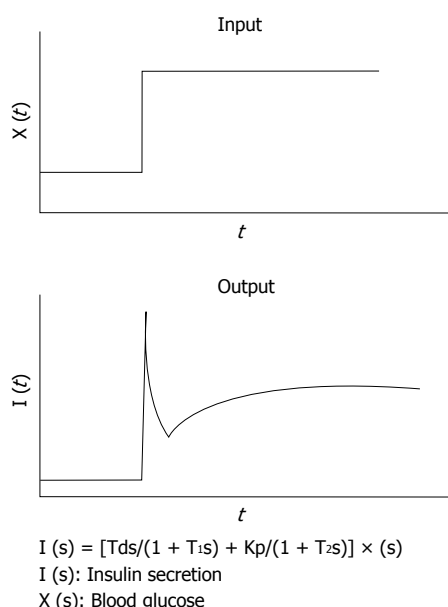


Figure 3 Mathematical model for insulin infusion algorithm. The stepwise input of glucose concentration $X(t)$ and biphasic response of insulin secretion $I(t)$ are depicted. The relationship between input and output is expressed in the transfer function. $I(s)$, $X(s)$ and $dX(s)$ are the Laplace transformed form of $I(t)$, $X(t)$ and $dX(t)/dt$, respectively. T_1 and T_2 are the first order delay in response.

not necessarily needed because the negative derivative action of blood glucose concentration reduces the insulin infusion rate when blood glucose is falling. The details of the computer algorithm have been described previously^[6,8-10].

Self-adaptive control algorithm for compensating insulin sensitivity changes

Even though the insulin secretory dynamics of healthy subjects are accomplished in patients with diabetes whose insulin sensitivity is low or super-normal, it is necessary to change manually the parameters for deciding insulin infusion rates for the adaptive control of blood glucose. Therefore, a computer algorithm for self-adaptive control has been established. Firstly, under the blood glucose regulation with artificial endocrine pancreas, the real rate of change in blood glucose concentration in each subject is calculated, and the difference between this and the projected rate of change in blood glucose concentration is assumed to be the index of insulin sensitivity. Secondly, according to the calculated insulin sensitivity, the computer automatically changes the parameters that regulate the insulin infusion rate^[11].

Glucose infusion algorithm

A counter-regulatory system might be useful and safe for the prevention of hypoglycemia caused by increased endogenous insulin secretion, and the change in insulin sensitivity that is observed frequently during insulin treatment with the artificial endocrine pancreas in patients with diabetes. In the glucose infusion algorithm, glucose is infused on the basis of proportional and

derivative actions of blood glucose concentration with a time delay constant between blood withdrawal and initiation of glucose infusion^[10,12].

Clinical application of bedside-type artificial endocrine pancreas

Only one type of bedside-type artificial endocrine pancreas is now available in Japan: STG-22 (Nikkiso Co. Ltd., Japan; Figure 4). Using this system, perfect blood glucose regulation with physiological plasma insulin profiles can be obtained in patients with diabetes. At present, clinical applications of the bedside-type artificial endocrine pancreas on a short-term basis include: blood glucose control in diabetic coma or diabetic ketoacidosis^[13], during surgery^[14-16], delivery^[17], during hemodialysis in diabetic nephropathy^[18], the prediction of the insulin requirement^[19], and blood glucose control in a hypoglycemic state such as in the case of insulinoma^[20].

In addition, by using this system, the euglycemic hyperinsulinemic glucose clamp study for the determination of insulin sensitivity in patients with diabetes is now applied widely^[21,22]. The euglycemic hyperinsulinemic clamp study was performed using an artificial pancreas according to the method of DeFronzo *et al.*^[23]. In brief, insulin is infused in a continuous fashion at a rate of 1.12-1.50 mU/kg per minute, after the priming insulin infusion during the first 10 min of the clamp at the same doses. Blood glucose levels were determined every 5 min during the 2-h clamp study, and euglycemia (5.0 mmol/L) was maintained by infusion of variable amounts of 10%-20% glucose solution. The total-body glucose disposal rate was evaluated as the mean of the glucose infusion rate during the last 30 min of the clamp. The insulin resistance index by the clamp was calculated by dividing the mean glucose infusion rate by the steady-state plasma insulin levels during the last 30 min of the clamp. The Artificial Organs Registry in Japan shows that the clinical applications of this bedside-type artificial endocrine pancreas have been increasing over time. The cumulative number of cases, including clinical and experimental applications, reached 14418 from 1983 to 2002. The number of applications was 465 in 2002 (29 for blood glucose control, 341 for laboratory and clinical research, and 95 for animal experiments) (Figure 5).

TREND IN THE DEVELOPMENT OF ARTIFICIAL ENDOCRINE PANCREAS

Wearable artificial endocrine pancreas

The ultimate goal of the development of the artificial endocrine pancreas is to achieve long-term strict glyce-mic regulation. In 1982, we succeeded in miniaturizing a glucose monitoring system to a needle-type, which consisted of a platinum anode and a silver cathode (0.4 mm in diameter and 2 cm in length). The electrode loaded with 0.6 V polarographic voltage measures hydrogen

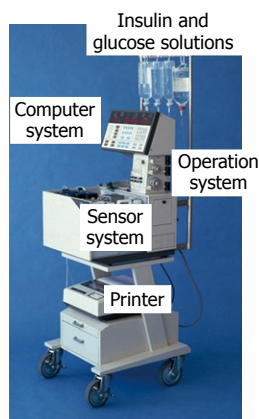


Figure 4 Bedside-type artificial endocrine pancreas (STG-22, Nikkiso Co. Ltd. Japan).

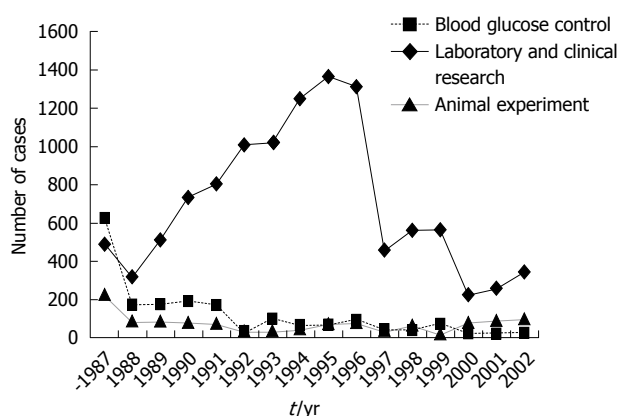


Figure 5 Number of cases to whom the bedside-type artificial endocrine pancreas had been applied in Japan. Artificial Organs Registry Report in Japan, 2002.

peroxide produced. It has been demonstrated that this sensor possesses excellent sensor characteristics suitable for application *in vivo*. We then developed a wearable type artificial endocrine pancreas, which consisted of a needle-type glucose sensor, microcomputer system, insulin and glucagon infusion pump systems, and a battery. The total system was packed into a small unit (12 cm × 15 cm × 6 cm in size and 400 g in weight)^[24-26]. However, the major obstacle in extending the term of glycemic control is the lack of a stable and reliable glucose sensor.

To obtain stable and reliable measurement of subcutaneous tissue glucose concentrations for at least 4 d, we have developed two types of glucose sensors: a miniaturized extracorporeal glucose monitoring system based on a microdialysis sampling method^[27]; and a ferrocene-mediated needle-type glucose sensor covered with biocompatible membrane^[28]. We have also reported that subcutaneous tissue glucose concentration was monitored continuously using these systems for 4 d. Furthermore, we have developed a newly designed wearable type artificial endocrine pancreas (12.6 cm × 2.9 cm × 9.6 cm in size and 250 g in weight) (KAP-003; Nikkiso, Tokyo, Japan), which consists of a glucose sensing system, microcomputer system, syringe pump for insulin infusion, and a battery^[29] (Figure 6).



Figure 6 Newly designed wearable artificial endocrine pancreas. The total system is packed into a small unit (12.6 cm × 2.9 cm × 9.6 cm) weighing 250 g (KAP-003, Nikkiso). A glucose sensor is placed in the subcutaneous tissue and measures glucose concentration continuously. The blood glucose concentration (BG, in mg/dL) and the insulin infusion rate (IIR, in mU/min) appear every minute on a large LCD display.

Implantable artificial endocrine pancreas

The trend in the development of an artificial endocrine pancreas is now from a wearable to an implantable one. However, many problems remain to be solved for each part of the devices. Technology derived from the development of various implantable artificial organs will be beneficial for the accomplishment of the long-term clinical application of the devices.

Our study has suggested that although closed-loop portal and peripheral venous insulin delivery systems are equally effective in terms of blood glucose control and insulin requirements, portal insulin delivery is superior to peripheral delivery in maintaining more appropriate hepatic glucose handling and physiological insulin profiles. These results indicate that the portal vein is the most suitable insulin delivery route for the implantable artificial endocrine pancreas^[30,31].

With regard to the metabolic efficacy and insulin requirement, intraportal insulin therapy is expected to be more effective than intraperitoneal insulin therapy. However, because the technique of placing an insulin catheter into the portal vein in humans is associated with severe invasion and high risks, such as infection and catheter thrombosis, there have been few reports of applying intraportal insulin therapy to patients with diabetes. Recently, there have been several reports of new methods that overcome these problems^[32,33]. Thus, with technical improvement, it should be possible to safely infuse insulin intraportally.

The application of a one-chip microcomputer could make the processor system smaller. The technology derived in the process of developing the clinical applications of implantable insulin infusion pumps has contributed to the completion of the effector implantation. Implantation of the entire system requires a small and powerful long-life battery. A transcutaneous energy transmission system is one of the candidates for this. As a stable and reliable long-life implantable glucose sensor is not yet available, it is not practical to implant

all these parts of these apparatus intracorporeally. It would be adequate to implant the computer and infusion pumps and retain the glucose sensor extracorporeally to regulate insulin infusion rates by telemetric means. In the near future, an implantable artificial endocrine pancreas with a telecommunications system will be available for the treatment of diabetes mellitus.

CONCLUSION

Successful glycemic control in patients with diabetes using the artificial pancreas emphasizes the importance of continuous glycemic monitoring for strict glycemic control. However, the major obstacle for extending the term of glycemic control in patients with diabetes is the development of an implantable, high-precision glucose sensor for tissue glucose determination. A needle-type glucose sensor, which is a miniature hydrogen peroxide electrode covered by membrane with biological activity, can be implanted easily and is exchangeable. The sensor has the *in vitro* and *in vivo* characteristics suitable for continuous tissue glucose monitoring. A wearable artificial endocrine pancreas, which incorporates a needle-type glucose sensor, has been devised and has regulated glycemia physiologically in patients with diabetes for > 6 d. Further improvements in sensor design, especially in membranes with biocompatibility, might reduce the host reactions to the sensor implanted in tissue and thus extend its biological life.

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Kazuhiro Hanazaki, MD, Professor and Chairman, Series Editor

Perioperative intensive insulin therapy using artificial endocrine pancreas in patients undergoing pancreatectomy

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Author contributions: Maeda H, Okabayashi T and Hanazaki K contributed equally to this work; Hanazaki K designed the research; Maeda H, Okabayashi T, Yatabe T and Yamashita K performed the research; Maeda H, Okabayashi T and Hanazaki K analyzed the data; Maeda H, Okabayashi T and Hanazaki K wrote the paper.

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Received: April 23, 2009 Revised: July 2, 2009

Accepted: July 9, 2009

Published online: September 7, 2009

and safe, without the occurrence of hypoglycemic episodes, even in patients after total pancreatectomy. Considering the increasing requirement for tight perioperative glycemic control and the recognized risk of hypoglycemia, we propose the use of an artificial endocrine pancreas that is able to monitor continuously blood glucose concentrations with proven accuracy, and administer automatically substances to return blood glucose concentration to the optimal narrow range.

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Key words: Blood glucose; Diabetes mellitus; Hyperglycemia; Pancreas; Artificial; Pancreatectomy

Peer reviewer: Dr. Ursula M Gehling, Department of Hepatobiliary Surgery and Visc, University Hospital Hamburg-Eppendorf, Martinistrasse 52, Hamburg 20246, Germany

Maeda H, Okabayashi T, Yatabe T, Yamashita K, Hanazaki K. Perioperative intensive insulin therapy using artificial endocrine pancreas in patients undergoing pancreatectomy. *World J Gastroenterol* 2009; 15(33): 4111-4115 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4111.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4111>

Abstract

Perioperative glycemic control is important for reducing postoperative infectious complications. However, clinical trials have shown that efforts to maintain normoglycemia in intensive care unit patients result in deviation of glucose levels from the optimal range, and frequent attacks of hypoglycemia. Tight glycemic control is even more challenging in those undergoing pancreatic resection. Removal of lesions and surrounding normal pancreatic tissue often cause hormone deficiencies that lead to the destruction of glucose homeostasis, which is termed pancreatogenic diabetes. Pancreatogenic diabetes is characterized by the occurrence of hyperglycemia and iatrogenic severe hypoglycemia, which adversely effects patient recovery. Postoperatively, a variety of factors including surgical stress, inflammatory cytokines, sympathomimetic drug therapy, and aggressive nutritional support can also affect glycemic control. This review discusses the endocrine aspects of pancreatic resection and highlights postoperative glycemic control using a closed-loop system or artificial pancreas. In previous experiments, we have demonstrated the reliability of the artificial pancreas in dogs with total pancreatectomy, and its postoperative clinical use has been shown to be effective

INTRODUCTION

Under normal conditions, blood glucose homeostasis is regulated by hepatic and/or pancreatic metabolism^[1-3]. The role of the hepatocyte in producing glucose in the fasting and stressed state, or for postprandial glucose uptake is critical for metabolic homeostasis^[1,2]. These functions depend largely on three circulating glucoregulatory hormones that are secreted by the pancreas: insulin, glucagon, and pancreatic polypeptide (PP)^[1-3]. After pancreatectomy, insufficiency or deficiency of these hormones causes glucose intolerance, a form of secondary diabetes mellitus termed pancreatogenic diabetes^[4-7]. The appropriate method for glycemic control in pancreatogenic diabetes after pancreatectomy has yet to be established because of the instability of blood glucose levels, especially in patients after total pancreatectomy. In this Topic Highlight, we review the problem of pancreatogenic diabetes in patients who have undergone pancreatectomy. In addition, as a solution

to pancreatogenic diabetes, we demonstrate the safety and usefulness of tight blood glucose control using an artificial endocrine pancreas, following proximal, distal and total pancreatectomy.

PANCREATOGENIC DIABETES

Pancreatogenic diabetes is characterized by diabetes with frequent episodes of iatrogenic hypoglycemia and hyperglycemia^[8], which is referred to as brittle diabetes (Figure 1). This is true particularly for patients after total or subtotal pancreatectomy. The condition is difficult to treat because of a paradoxical combination of enhanced peripheral insulin sensitivity and decreased hepatic insulin sensitivity, in addition to decreased glucagon secretion^[8-12]. Patients become hyperglycemic because of unsuppressed hepatic glucose production, when insulin replacement is insufficient. In contrast, patients become hypoglycemic when insulin replacement is barely excessive, as a result of the enhanced peripheral insulin sensitivity and a deficiency of pancreatic glucagon secretion^[10].

Shortly after surgical resection of the pancreas, 8%-23% of patients develop pancreatogenic diabetes, which increases to 40%-50% during follow-up^[5-7]. With the increasing incidence of pancreatectomy^[13,14], pancreatogenic diabetes as a sequela to surgical resection is an urgent medical issue.

ACTIONS OF THE GLUCOREGULATORY HORMONES

Insulin

Insulin-secreting β cells are distributed evenly throughout the pancreas. Insulin decreases serum glucose concentration through suppressing hepatic gluconeogenesis, glycogenolysis and facilitating hepatic glycogen synthesis^[1,2]. Insulin receptors are expressed on nearly every cell throughout the body and insulin decreases blood glucose concentration through facilitating glucose uptake into insulin-receptor-bearing cells^[10]. In addition to the glucoregulatory effect, insulin also has an anti-inflammatory effect^[15,16].

Glucagon

Glucagon-secreting α cells are located predominantly in the body and tail of the pancreas. Together with the β cells, the function of α cells is essential for maintaining the remarkable consistency of glucose levels under various states of supply and demand^[17]. During fasting, glucagon is responsible for maintaining adequate glucose production in hepatocytes by stimulating glycogenolysis and gluconeogenesis. Thus, glucagon works as a counter-regulatory mechanism for hypoglycemia. Surgical resection that causes reduction of α cells may be associated with severe hypoglycemic episodes, especially after total or distal pancreatectomy.

PP

PP-secreting cells are located mainly in the ventral pancreatic head and uncinate process^[18-20]. Studies on a

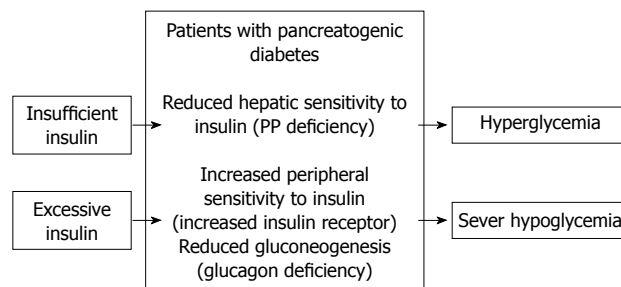


Figure 1 Pathophysiology of pancreatogenic diabetes according to published reports^[10-14].

canine model of chronic pancreatitis with accompanying PP deficiency have demonstrated hepatic resistance to insulin and inappropriate hepatic glucose production, despite physiological levels of insulin^[21]. Kono *et al*^[22] have shown that with co-infusion of PP in total pancreatectomized dogs, smaller amounts of insulin infusion are required to normalize glucose levels. Earlier studies have also demonstrated that PP reverses abnormal glucose production after pancreatectomy^[11]. Therefore, PP deficiency may serve as a potentially reversible pathophysiological factor that contributes to altered glucose metabolism^[8] following proximal or total pancreatectomy.

GLUCOSE CONTROL AFTER PANCREATECTOMY

Diabetes mellitus is a well-established risk factor for postoperative infectious complications^[23-25], and much effort has focused on preoperative glycemic control. However, after van den Berghe *et al*^[26] reported that tight glycemic control had a beneficial impact on the mortality rate of patients admitted to the intensive care unit (ICU), increased attention has been paid to postoperative glycemic control. Hyperglycemia itself, even in non-diabetic patients, has also been reported to be associated with an adverse outcome in surgical patients^[27].

Despite this increasing attention to postoperative glycemic control, tight control of blood glucose levels is still not practiced widely, because of the difficulty of keeping glucose levels within the aimed-for range, and frequent occurrences of hypoglycemia^[28,29]. Therefore, we recommend strongly the use of an artificial endocrine pancreas equipped with the ability to monitor accurately and control automatically blood glucose levels efficiently, safely and in a hands-free manner^[22,30-34].

EXPERIMENTAL STUDY

The initial aim of this experimental study was to investigate the effect of PP infusion on insulin requirements after total pancreatectomy in dogs^[22]. However, the study also demonstrated clearly the usefulness and safety of the artificial pancreas in controlling blood glucose after total pancreatectomy^[22]. We have used STG-22 (Nikkiso Co. Ltd., Tokyo, Japan) which is a bedside-type artificial

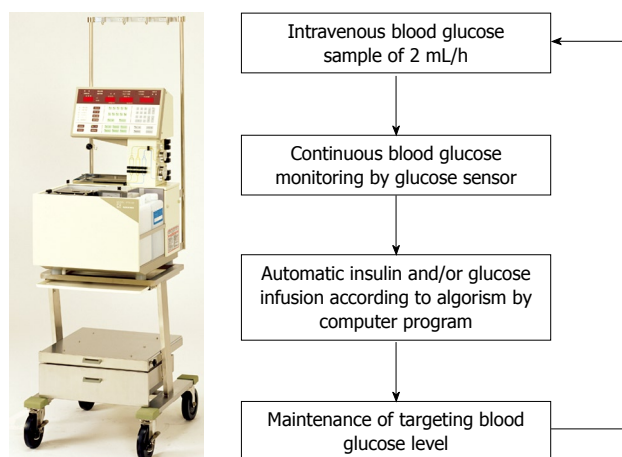


Figure 2 STG-22, a bedside-type artificial endocrine pancreas system with closed-loop.

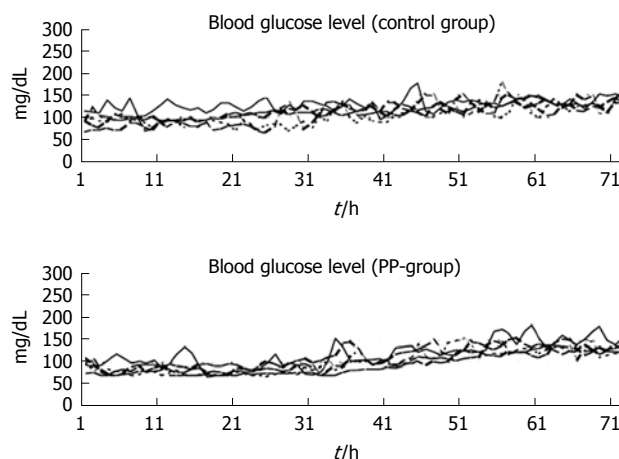


Figure 3 Continuous blood glucose levels over 72 h in 10 dogs after total pancreatectomy^[22].

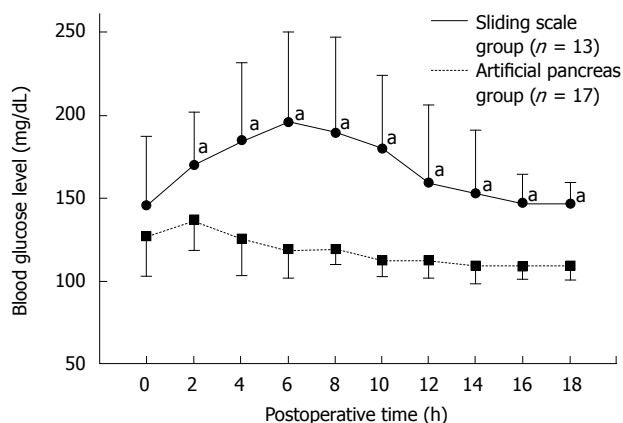


Figure 4 Postoperative blood glucose levels in the sliding scale ($n = 13$) and artificial pancreas ($n = 17$) groups during the first 18 h following pancreatectomy^[38]. Significant statistical difference ($^aP < 0.05$).

endocrine pancreas^[34]. The Nikkiso Company developed the STG-22 unit in 1984 as an artificial endocrine pancreas, which was only a single device with a closed-loop system (Figure 2). Peripheral venous blood for glucose monitoring was sampled continuously at 2 mL/h. STG-22 is capable of measuring continuously the blood glucose level with its glucose sensor, and automatically infuses insulin and/or glucose to adjust the blood glucose level in accordance with a target glucose value, which is the so called closed-loop system^[34]. Ten dogs underwent total pancreatectomy. Following surgery, five dogs were supported solely by the artificial pancreas for 72 h, while the other five were supported by the artificial pancreas plus an infusion of bovine PP at 2 pmol/kg per minute. Mean blood glucose levels and insulin requirements were compared between the two groups. In all 10 dogs, the blood glucose concentration was controlled tightly at a mean level of 110 ± 4 mg/dL, and there was no difference in mean blood glucose level between the two groups (Figure 3). The insulin requirement in the group treated with PP was 90.0 ± 20.8 mU/kg for the first day and 562.7 ± 126.5 mU/kg for the second day. This requirement was significantly less than that of the group without PP: 445.0 ± 151.9 mU/kg

for the first day and 1007.7 ± 144.9 mU/kg for the second day. During the operation of the artificial pancreas, there were no serious complications, such as catheter thrombosis, migration, infection, or episodes of hypo/hyperglycemia.

CLINICAL STUDY

In addition to endocrine insufficiency after pancreatic surgery, other factors such as surgical stress, inflammatory proteins^[35], sympathomimetic drug therapy^[3], and aggressive nutritional support can also make glycemic control difficult. To date, only one study has focused on tight postoperative glycemic control in patients undergoing pancreatic resection^[36]. Thirty patients with pancreatic neoplasia were divided prospectively into two groups: sliding scale ($n = 13$) and artificial pancreas ($n = 17$) groups. Blood glucose concentrations were monitored continuously by an artificial pancreas and glycemic control was achieved using the sliding scale method or the artificial pancreas itself.

In the sliding scale group, postoperative blood glucose levels rose steadily, and reached a maximum value of approximately 200 mg/dL between 4 and 6 h after pancreatectomy. In the artificial pancreas group, blood glucose levels decreased gradually, and reached the target range (80-110 mg/dL) by 6 h postoperatively (Figure 4). Total insulin administered per patient during the first 18 h after surgery was significantly higher in the artificial pancreas group (107 ± 109 IU) compared to the sliding scale group (8 ± 6 IU; $P < 0.01$). No hypoglycemia was recorded in either group. This study demonstrated the usefulness of the artificial pancreas for the first time in a clinical setting, using a number of cases and a prospective design.

TOTAL PANCREATECTOMY AND POSTOPERATIVE GLYCEMIC CONTROL

Total pancreatectomy results in an extreme form of pancreatogenic diabetes. The blood glucose concentration

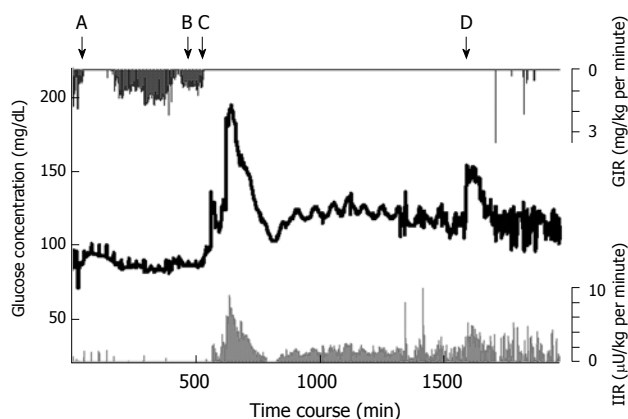


Figure 5 Blood glucose concentration during and after total pancreatectomy in a middle-aged female patient. Blood glucose is shown by the black line. The insulin infusion rate (light grey line) increased according to the increase in glucose concentration. A: Beginning of surgery; B: Wound closure; C: Beginning of parenteral nutrition in ICU; D: Beginning of combined enteral and parenteral nutrition; GIR: Glucose infusion rate; IIR: Insulin infusion rate.

goes up and down within a short duration, and iatrogenic hypoglycemia frequently affects prognosis. The patient described here was a middle-aged woman who underwent total pancreatectomy, and during the acute postoperative phase, received catecholamines to maintain blood pressure, and nutritional support of 25 kcal/kg per day soon after transfer from the operating theatre to the ICU.

Her blood glucose concentration profile is shown in Figure 5. An artificial pancreas (STG-22) was operated for 1994 min, automatic and manual calibration was performed seven times, and each calibration took approximately 8 min. Failure of blood withdrawal occurred seven times, which was a total of 20 min (data during calibration and failure of blood withdrawal was eliminated from the data analysis). Blood sample failures were resolved easily by trained ICU nurses.

The glucose concentration was stable during surgery and immediately after completion of total pancreatectomy (A to B in Figure 5). The glucose concentration increased with the beginning of parenteral nutrition in the ICU, and immediate administration of insulin suppressed the severe hyperglycemia. With the reduction in glucose concentration, the insulin infusion rate decreased to avoid hypoglycemia (the glucose surge after C in Figure 5). Her glucose concentration increased again with the beginning of enteral nutrition (Figure 4D), followed by increased insulin infusion. Total insulin administered during the operation of STG-22 was 96 IU. Her blood glucose concentration was 80-139 mg/dL for 91.3% of the time (Figure 6). No episode of hypoglycemia, defined as a blood glucose concentration < 40 mg/dL, occurred. The artificial pancreas was shown to be safe and efficient in the patient after total pancreatectomy.

FUTURE DIRECTIONS

Recently, the Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation (NICE-SUGAR) study has reported that glycemic control for critically ill patients in the ICU

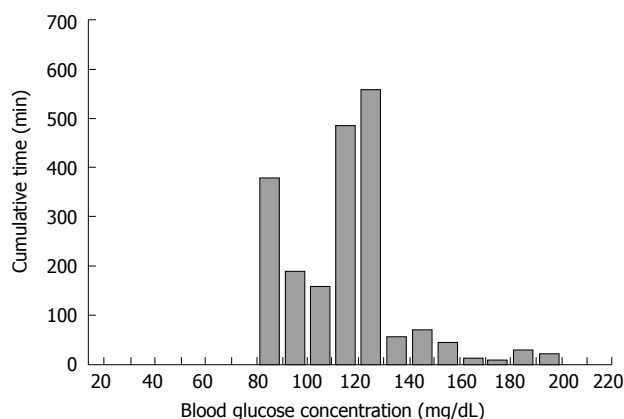


Figure 6 Histogram depicting the range and cumulative time for the blood glucose concentration of a middle-aged female who underwent total pancreatectomy.

results in beneficial effects on survival when the blood glucose concentration is targeted at ≤ 180 mg/dL^[37]. However, when limited to the surgical ICU setting, tight glycemic control also provides benefits for postoperative patients, according to a recent meta-analysis which included the results of the NICE-SUGAR study^[38]. In light of current knowledge, a prospective randomized control trial using the artificial endocrine pancreas is needed urgently to compare different ranges of blood glucose concentrations after major surgery. Such a study would ascertain the optimal range for blood glucose concentrations without hypoglycemia, which presumably weakens the potency of tight glycemic control.

In the future, along with efforts to reduce postoperative complications after pancreatectomy by improving surgical methods, drainage and patient selection, glycemic control should receive more attention, especially during the early postoperative period. Undoubtedly, it is very important to find out the optimal range for blood glucose levels and the optimal caloric intake shortly after pancreatectomy, and develop more sophisticated mechanical devices to control blood glucose.

CONCLUSION

Pancreatectomy often results in pancreatogenic diabetes for which insulin treatment is known to be difficult. To reduce the postoperative infectious complications related to hyperglycemia and iatrogenic hypoglycemia caused by conventional blood glucose control, perioperative use of an artificial pancreas is recommended as an ideal method for tight glycemic control after pancreatic surgery. The operation of an artificial endocrine pancreas is still somewhat resource-intensive and expensive, but if mortality and morbidity are reduced, it is worthwhile.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, Series Editor

Perioperative insulin therapy using a closed-loop artificial endocrine pancreas after hepatic resection

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Received: April 23, 2009 Revised: July 6, 2009

Accepted: July 13, 2009

Published online: September 7, 2009

Abstract

Postoperative hyperglycemia is common in critically ill patients, even in those without a prior history of diabetes mellitus. It is well known that hyperglycemia induced by surgical stress often results in dysregulation of liver metabolism and immune function, impairing postoperative recovery. Current evidence suggests that maintaining normoglycemia postoperatively improves surgical outcome and reduces the mortality and morbidity of critically ill patients. On the basis of these observations, several large randomized controlled studies were designed to evaluate the benefit of postoperative tight glycemic control with intensive insulin therapy. However, intensive insulin therapy carries the risk of hypoglycemia, which is linked to serious neurological events. Recently, we demonstrated that perioperative tight glycemic control in surgical patients could be achieved safely using a closed-loop glycemic control system and that this decreased both

the incidence of infection at the site of the surgical incision, without the appearance of hypoglycemia, and actual hospital costs. Here, we review the benefits and requirements of perioperative intensive insulin therapy using a closed-loop artificial endocrine pancreas system in hepatectomized patients. This novel intensive insulin therapy is safe and effectively improves surgical outcome after hepatic resection.

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Key words: Artificial pancreas; Hepatic resection; Hyperglycemia; Intensive insulin therapy; Surgical site infection

Peer reviewers: Giulio Marchesini, Professor, Department of Internal Medicine and Gastroenterology, "Alma Mater Studiorum" University of Bologna, Policlinico S. Orsola, Via Massarenti 9, Bologna 40138, Italy; Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico II University Medical School, VIA S. PANSINI, 5, Naples 80131, Italy

Okabayashi T, Maeda H, Sun ZL, Montgomery RA, Nishimori I, Hanazaki K. Perioperative insulin therapy using a closed-loop artificial endocrine pancreas after hepatic resection. *World J Gastroenterol* 2009; 15(33): 4116-4121 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4116.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4116>

INTRODUCTION

Anatomically, the liver is situated downstream from the pancreas. It is a primary site for the metabolism of pancreatic hormones, such as insulin and glucagon, which have a central role in the regulation of peripheral blood glucose levels^[1]. In addition, the liver is positioned downstream from the gut, from which it absorbs a large amount of ingested glucose, and is involved in glycogenolysis and gluconeogenesis. Accordingly, reduced liver function following hepatic resection using the Pringle procedure^[2] may result in metabolic disturbances of the pancreatic hormones and glucose intolerance.

Hyperglycemia has a deleterious effect on cells that passively take up glucose independent of insulin, including hepatocytes, alveolar cells, endothelial cells, neurons, and immune cells. Postoperative hyperglycemia is common in critically ill patients, even in those without a prior history of diabetes mellitus^[3-5]. It is well known that hyperglycemia induced by surgical stress often results in dysregulation of liver metabolism and immune function, leading to impaired postoperative recovery^[6,7]. Thus, the prevention of glucose toxicity in the mitochondrial compartment is important^[8].

In this Topic Highlight, we review the benefits and requirements of tight glycemic control in hepatic surgery, with a focus on postoperative infection control. We suggest that perioperative intensive insulin therapy using a closed-loop artificial endocrine pancreas system is safe and effectively improves surgical outcome after hepatic resection.

CURRENT STATE OF TIGHT GLYCEMIC CONTROL IN CRITICALLY ILL PATIENTS

In large randomized trials in which the use of tight blood glucose control (80-110 mg/dL) with intensive insulin therapy was compared with standard blood glucose control (< 200 mg/dL) in surgical intensive care unit (ICU) patients, strict control of postoperative blood glucose levels was shown to significantly reduce patient mortality and morbidity^[9,10]. In addition, postoperative hyperglycemia has been shown to be associated with an increased risk of surgical site infection (SSI)^[11,12]. Current evidence suggests that maintaining normoglycemia postoperatively improves surgical outcome and reduces mortality and morbidity in critically ill patients^[8,11,12]. These observations led to several short-lived multicenter randomized controlled studies designed to evaluate the benefit of tight glycemic control with intensive insulin therapy^[13,14]. The main reason for the early cessation of these clinical trials was the high incidence of hypoglycemia (10%-17%) induced by the intensive insulin therapy^[15,16], which could not be prevented because of technical limitations at that time^[17-19]. However, the subsequent development of accurate continuous blood glucose monitoring devices and closed-loop systems for computer-assisted blood glucose control in the ICU will likely reduce the incidence of hypoglycemia in these situations^[8].

For the reasons described above, achieving tight glycemic control with intensive insulin therapy has come under increased scrutiny in the management of patients in the surgical ICU. Recently, we demonstrated in two retrospective studies and in one randomized clinical trial that perioperative tight glycemic control using a closed-loop glycemic control system for patients undergoing liver resection was safe and effective in decreasing the incidence of SSI without increasing the risk of hypoglycemia.

INSULIN THERAPY IN THE SURGICAL ICU

In all three studies, perioperative blood glucose concentrations were monitored continuously using the STG-22 system, developed by Nikkiso Co. (Tokyo, Japan). Patients were divided into two groups: one in which glucose levels were controlled by manual injection of insulin according to the commonly used sliding-scale method (SS group)^[20,21], and a second group in which programmed infusions of insulin were administered as determined by the control algorithm of a closed-loop artificial endocrine system (AP group).

Conventional insulin therapy using the sliding-scale method

Blood glucose levels in patients in the SS group were monitored continuously by the artificial pancreas and patients were checked routinely by nursing staff every 2 h. Blood glucose levels in these patients were controlled by subcutaneous injections of regular human insulin, with the dose determined by the sliding-scale method and the target blood glucose level to avoid hypoglycemia set at 150-200 mg/dL^[20,21].

Novel insulin therapy using a closed-loop artificial endocrine pancreas system

The STG-22 unit was developed in 1984 by Nikkiso Co. as a closed-loop artificial endocrine pancreas system. The STG-22 system is a reliable and accurate device that measures blood glucose concentrations continuously and is comparable to the ABL 800FLEX machine (Radiometer Medical ApS, Brønshøj, Denmark) recommended by the National Committee for Clinical Laboratory Standards^[22,23]. The STG-22 closed-loop glycemic control system is composed of a glucose sensor for the detection and/or monitoring of glucose and pumps for the infusion of appropriate amounts of insulin or glucose^[24,25]. The insulin and glucose pumps are regulated by a computer on the basis of target blood glucose values that are defined prior to initiation of the system. The STG-22 maintains stable blood glucose concentrations by automatic infusion of regular insulin or glucose into the circulation^[24,25]. In the ICU, peripheral blood was sampled continuously at 2 mL/h over the first 18 h postoperatively to monitor glucose levels. In addition, the STG-22 was used to evaluate the patients' insulin requirements.

Statistical analysis

Continuous variables are presented as the mean \pm SD. Dichotomous variables are presented as both absolute numbers and percentages. Data were analyzed using Student's *t*-test (two-tailed), with dichotomous variables analyzed by the χ^2 test (two-tailed) or Fisher's exact test (two-tailed). *P* < 0.05 was considered significant. All analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

RETROSPECTIVE STUDIES

The benefits of using a closed-loop glycemic control system in patients after hepatectomy were investigated in two retrospective studies. The aim of the first study was to evaluate the usefulness of the closed-loop system in providing continuous monitoring and strict control of postoperative blood glucose levels in patients after hepatic resection^[25]. The aim of the second study was to identify, using multivariate analysis, risk factors and predictors of SSI, as well as how to prevent the development of SSI, in a consecutive series of patients undergoing hepatic resection for liver disease in a single institution.

In the first study^[25], postoperative blood glucose levels increased initially in the SS group, reaching a plateau of approximately 250 mg/dL between 4 and 7 h after hepatectomy. Thereafter, blood glucose levels decreased, returning to normal within 16 h after surgery. In the AP group, blood glucose decreased gradually, reaching target levels (90-110 mg/dL) within 12 h after surgery. Total insulin administered per patient during the first 16 h after surgery was significantly higher in the AP group compared with the SS group (183 ± 188 IU *vs* 8 ± 7 IU, respectively, $P < 0.001$). These data suggest that the sliding-scale method is not as effective as the closed-loop artificial endocrine system in preventing hyperglycemia resulting from disturbed glucose metabolism following liver resection.

In the second study^[26], the association between SSI and various clinical parameters was investigated in 152 patients following hepatic resection. The incidence of SSI in these patients was 14.5%. Multivariate analysis identified four independent parameters that were correlated with the occurrence of SSI, namely (1) body mass index > 23.6 kg/m², (2) estimated blood loss volume > 810 mL, (3) the presence of postoperative bile leak organ/space SSI; and (4) use of the sliding-scale method for postoperative glucose control. No SSI was observed after liver resection in patients in whom postoperative blood glucose levels were controlled by an artificial pancreas. The results of this second retrospective study demonstrated that a lack of postoperative glycemic control is associated with a significantly higher incidence of postoperative infectious complications and a longer period of hospitalization.

PROSPECTIVE RANDOMIZED CLINICAL TRIAL

A prospective randomized trial was conducted in patients undergoing hepatic resection to evaluate the postoperative condition of the patients and the effects of a closed-loop artificial pancreas on tight glycemic control during intensive insulin therapy after hepatectomy^[27]. Patients were randomly assigned to receive intensive insulin therapy using a closed-loop glycemic control system (i.e. an artificial pancreas; target

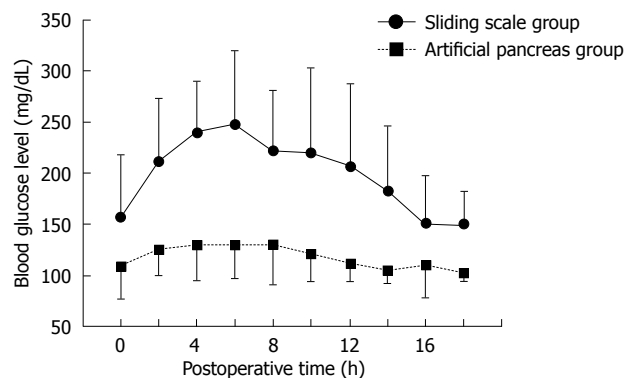


Figure 1 Postoperative blood glucose levels in hepatectomized patients in whom blood glucose was controlled using either the sliding-scale method or a closed-loop artificial endocrine pancreas system (Okabayashi et al^[27] *Diabetes Care* 2009).

blood glucose 80-110 mg/dL; AP group) or conventional insulin therapy using the sliding-scale method (target blood glucose 150-200 mg/dL; SS group). Perioperative blood glucose levels were monitored continuously in both groups using a closed-loop system. Neither group experienced hypoglycemia (blood glucose < 40 mg/dL). Although perioperative blood glucose levels in the AP group were close to 100 mg/dL, those in the SS group were > 150 mg/dL, which is the same as in our first retrospective study^[25] (Figure 1). The incidence of SSI was significantly lower in the AP than in the SS group (2.3% *vs* 18.2%, respectively, $P = 0.030$), the duration of hospitalization was significantly shorter for patients in the AP group compared with the SS group (14.3 ± 5.9 d *vs* 18.7 ± 11.7 d, respectively, $P = 0.049$). The impact on hospital costs is one of the most frequently discussed consequences of SSI within a clinical setting. A few studies have presented data on the incidence and costs of infection for the hospital and post-discharge periods. As there were no incidences of post-discharge SSI in the current study, the costs-of-illness during hospitalization were incorporated into the overall costs. Total hospital costs were significantly lower for patients in the AP group than in the SS group ($16\,407 \pm 5\,284$ \$ *vs* $21\,879 \pm 15\,784$ \$, respectively, $P = 0.047$). There is evidence suggesting that SSI prolongs the length of hospitalization for patients undergoing cardiac surgery, caesarean section, orthopedic surgery and general surgery, and increases the total costs of a patient's treatment^[28]. These results support the notion that intensive insulin therapy using a closed-loop glycemic control system after hepatic resection results in the maintenance of near normoglycemia, contributing to a reduction in both the incidence of SSI and total hospital costs per patient due to a decreased duration of hospitalization. Thus, the closed-loop system promises more effective and safer intensive insulin therapy in hepatectomized patients. An important question that has arisen from this study is how to choose candidates for intensive insulin therapy using a closed-loop system. This important issue must be addressed in future studies.

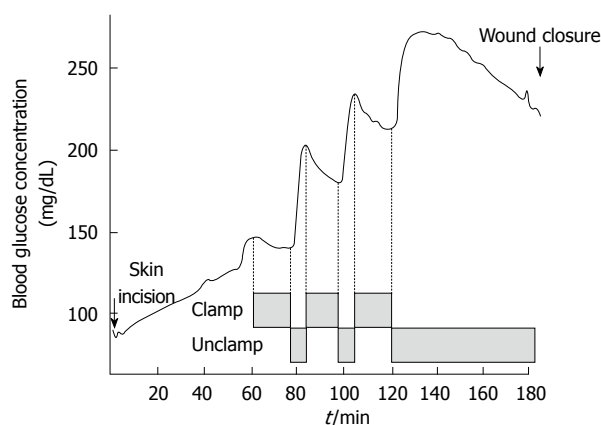


Figure 2 Typical trend of blood glucose concentrations during the Pringle maneuver for hepatic resection (Maeda *et al*^[32] *Am J Surg* 2009).

PROBLEMS ASSOCIATED WITH INTENSIVE INSULIN THERAPY IN LIVER SURGERY

The possible risks of targeting normoglycemia include intraoperative hyperglycemia, hypoglycemia, and the need for perioperative parenteral nutrition.

The Pringle maneuver was introduced in liver surgery to reduce hepatic hemorrhage^[2] and is now widely used in hepatic resections to control intraoperative bleeding^[29-31]. This inflow-occlusion technique involves total compression of the hepatoduodenal ligament, generally by clamping it for 15 min during hepatic parenchymal resections, followed by 5 min of unclamping^[31,32]. The clamping-unclamping procedure is repeated until the hepatic resection is complete. Glucose concentrations just before the first Pringle maneuver were significantly higher than baseline, but decreased gradually with the first clamping of the hepatoduodenal ligament. After unclamping of the hepatoduodenal ligament, there was an immediate and marked increase in glucose levels. The decrease and subsequent increase in glucose levels are seen with following rounds of clamping and unclamping of the hepatoduodenal ligament. After the surgery is completed, glucose concentrations gradually decline (Figure 2)^[32]. In future studies, we will determine whether the rapid fluctuations in blood glucose levels during the Pringle maneuver should be controlled.

The most feared risk associated with intensive insulin therapy is postoperative hypoglycemia, which may cause convulsions, coma, and brain damage, as well as cardiac arrhythmias^[15]. In ICU studies, the risk of severe hypoglycemia (glucose < 40 mg/dL) has been shown to increase from 5.0% to 18.7% with intensive insulin therapy^[10,15,16]. Van den Berghe^[8] suggested that clinical outcome by intensive insulin therapy (targeting blood glucose level of 80-110 mg/dL) was more effective for reducing hospital mortality and morbidity in critically ill adult patients compared with moderate intensive insulin therapy (targeting blood glucose level of 110-150 mg/dL). However, a large international randomized trial in 2009

showed that a blood glucose target of less than 180 mg/dL resulted in lower mortality than a target of 81-108 mg/dL^[16]. Furthermore, contrary to this report^[8], a recent meta-analysis^[15] did not support the benefits of intensive insulin therapy that it was not associated with significantly reduced hospital mortality but was associated with an increased risk of hypoglycemia. These randomized trials had several issues as follows: (1) trials were not blinded, (2) unusually high mortality in the usual care group, (3) parenteral nutrition (different administration of energy at the ICU), and (4) a markedly increased risk of hypoglycemia. To address this issue on the focus of a more effective blood glucose control method under impartial conditions without hypoglycemia, we are constructing a prospective randomized comparison study between intensive insulin therapy and moderate intensive insulin therapy using a closed-loop artificial endocrine pancreas. There were no occurrences of hypoglycemia during intensive insulin therapy using a closed-loop glycemic control system in our series of studies. In our experience of more than 200 patients in the surgical and medical ICU, we have not seen hypoglycemia develop in any patient when intensive insulin therapy is administered using a closed-loop glycemic control system (data not shown).

Enteral feeding was started as soon as possible after patients were hemodynamically stable. However, if the energy intake target could not be achieved, parenteral feeding was initiated to compensate for the deficit. In our series, all patients were given parenteral nutrition following surgery^[33], with the total caloric requirement calculated according to the Harris-Benedict equation^[34]. Based on the results of our studies, maintaining an adequate calorie level, and controlling postoperative glucose levels with insulin therapy contributed to a reduction in the incidence of SSI. Moreover, the duration of hospitalization after liver surgery was reduced in patients who received perioperative tight glycemic control with intensive insulin therapy, and it is likely that this is related to a reduction in postoperative complications due to infection (SSI). In our studies, tight glycemic control was maintained for 18 h in patients in the surgical ICU after liver resection, and excellent glucose control was achieved without hypoglycemia using the closed-loop system.

Another outcome of our studies was that a brief period of glycemic control impacted on the incidence of SSI. Bacterial growth curves for *Escherichia coli*, *Streptococcus*, *Proteus*, *Staphylococcus* and *Pseudomonas*, among others, indicate that under optimum conditions the greatest growth occurs between 2 and 18 h^[35]. We strongly believe that by instigating perioperative tight glycemic control for a brief period (at least 18 h) after surgery, postoperative infectious morbidity is decreased.

FUTURE DIRECTIONS

Clearly, we support a recent report that suggests that the development of accurate, continuous blood glucose

monitoring devices (preferably closed-loop systems) for computer-assisted blood glucose control in the ICU will help prevent hypoglycemia^[17]. However, using currently available technology, tight glucose control (i.e. targeting blood glucose levels of 80-110 mg/dL) using a closed-loop system in the surgical ICU was not achieved in 100% of cases, with the reported range being 60%-100%. Thus, further studies are needed to determine a better algorithm with which more accurate tight glucose control can be achieved. Regardless, we believe that by using the closed-loop glycemic control system during intensive insulin therapy in the ICU, the incidence of hypoglycemia and/or problems of nutritional support are reduced, as is the burden on nursing staff that is normally associated with the requisite frequent monitoring of blood glucose levels.

CONCLUSION

Intensive insulin therapy using a closed-loop artificial endocrine pancreas system during hepatic resection not only maintained near normoglycemia, but also contributed to a reduction in the rate of SSI and a decrease in total hospital costs due to shortened hospitalization. The closed-loop glycemic control system promises to revolutionize intensive insulin therapy for patients with disturbed glucose metabolism after liver resection.

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TOPIC HIGHLIGHT

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Relationship between perioperative glycemic control and postoperative infections

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Received: May 5, 2009 Revised: May 21, 2009

Accepted: May 28, 2009

Published online: September 7, 2009

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Hanazaki K, Maeda H, Okabayashi T. Relationship between perioperative glycemic control and postoperative infections. *World J Gastroenterol* 2009; 15(33): 4122-4125 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4122.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4122>

Abstract

Perioperative hyperglycemia in critically ill surgery patients increases the risk of postoperative infection (POI), which is a common, and often costly, surgical complication. Hyperglycemia is associated with abnormalities in leukocyte function, including granulocyte adherence, impaired phagocytosis, delayed chemotaxis, and depressed bactericidal capacity. These leukocyte deficiencies are the cause of infection and improve with tight glycemic control, which leads to fewer POIs in critically ill surgical patients. Tight glycemic control, such as intensive insulin therapy, has a risk of hypoglycemia. In addition, the optimal targeted blood glucose range to reduce POI remains unknown. Since 2006, we have investigated tight perioperative blood glucose control using a closed-loop artificial endocrine pancreas system, to reduce POI and to avoid hypoglycemia. In this Topic Highlight, we review the relationship between perioperative glycemic control and POI, including the use of the artificial pancreas.

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Key words: Glycemic control; Surgical site infection; Artificial pancreas; Insulin therapy; Glucose toxicity

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INTRODUCTION

Recent evidence suggests that perioperative hyperglycemia is the main risk factor for the development of postoperative infection (POI)^[1,2]. Intensive glucose control^[3-7] leads to fewer POIs in critically ill surgical patients^[1]. However, tight glycemic control^[8-12] such as intensive insulin therapy (IIT)^[3-7] has a risk of hypoglycemia. In addition, the optimal target range for blood glucose to reduce POI remains unknown^[1,2,13-15]. We recently demonstrated that tight perioperative glycemic control can be achieved by using an artificial endocrine pancreas^[16,17] for surgical patients, and that it was a safe and effective treatment for decreasing the incidence of POI, without increasing the risk of hypoglycemia^[18-22].

In this Topic Highlight, we review the relationship between perioperative glycemic control and POI, including the use of an artificial pancreas.

RELATIONSHIP BETWEEN PERIOPERATIVE HYPERGLYCEMIA AND POI

Perioperative hyperglycemia in critically ill surgery patients increases the risk of POI, which is a common, and often costly, surgical complication^[1,2,18-22]. It is well known that diabetic patients are at higher risk of postoperative complications, including POI, than non-diabetic surgery patients. Indeed, hyperglycemia correlates positively with POI in diabetic surgical patients^[23] and with a poor prognosis following stroke or head injury^[24,25]. POI is exacerbated by perioperative hyperglycemia in critically ill surgery patients^[1].

Strict glycemic control decreases the risk of infection

and improves other outcomes for trauma, cardiac, and critically ill non-diabetic surgery patients. Ramos *et al*^[1] associated postoperative hyperglycemia with an increased risk of 30 d postoperative infectious complications and a longer hospital stay, independent of diabetic status. On the basis of these findings, they recommended evaluation of the possible benefits of postoperative glycemic control in general surgical patients^[1].

Hyperglycemia is also associated with a sustained decrease in polymorphonuclear leukocyte function^[26]. Abnormalities in leukocyte function^[27] have been identified that are caused by the hyperglycemic state. These include abnormalities in granulocyte adherence^[28], impaired phagocytosis^[29], delayed chemotaxis^[30], and depressed bactericidal capacity^[29,31]. The degree of hyperglycemia that has been shown to impair phagocytic function is as low as 200 mg/dL^[32]. These leukocyte deficiencies appear to improve with tight glycemic control^[33]. Together with the demonstrated role of perioperative hyperglycemia in POI, it is clear that tight glycemic control should improve the clinical outcome for all surgical patients.

Insulin displays a potent and acute anti-inflammatory effect by inhibiting the tissue factor, plasminogen activator inhibitor-1^[34], and the intranuclear nuclear factor κ B (NF κ B)^[35]. Insulin plays a vital role, not only in blood glucose control, but also as an anti-inflammatory and anti-oxidant agent. Insulin suppresses the proinflammatory effects of NF κ B, activator protein 1, early growth response 1, and high mobility group box 1^[34-36], as well as inhibiting nicotinamide hypoxanthine dinucleotide oxidase action to reduce reactive oxygen species production^[34,35].

IIT has been reported to reduce infection rates after neurosurgery; therefore, this approach might be an appropriate blood glucose control method to prevent POI^[37]. However, clinical evidence is still needed to confirm the efficacy of IIT for strict blood glucose control in preventing POI. Although improved control of blood glucose fluxes have high potential to improve survival and decrease morbidity in surgical patients, the association between perioperative blood glucose range and the incidence of POI remains unclear in the majority of surgical patients due to lack of evidence. Further studies, including prospective randomized controlled trials, are necessary to clarify this issue.

THE OPTIMAL TARGET RANGE FOR BLOOD GLUCOSE TO PREVENT POI IN SURGICAL PATIENTS

Good long-term glycemic control is strongly associated with significantly fewer POIs in diverse surgical populations^[38]. Zerr *et al*^[39] reported that elevated blood glucose levels of more than 200 mg/dL in diabetic patients are associated with a higher incidence of deep sternal wound infection. Use of perioperative glycemic control in the range of 150-200 mg/dL significantly reduced POI in diabetic patients undergoing open heart surgery^[40].

Van den Berghe *et al*^[3] demonstrated that tight glycemic control (IIT) to maintain blood glucose levels in the target range of 80-110 mg/dL improved morbidity and mortality in the surgical intensive care unit (ICU). However, a recent large study by NICE-SUGAR Study Investigators^[15] completely denied the effectiveness of IIT shown by Van den Berghe *et al*^[3]. Contrary to Van den Berghe's first trial^[3], IIT increased mortality among adults in the ICU and a blood glucose target less than 180 mg/dL (most frequent blood glucose value of 142 mg/dL) resulted in lower mortality than did a target of 81-108 mg/dL (most frequent blood glucose value of 107 mg/dL)^[15]. Recent meta-analysis^[41] including NICE-SUGAR^[15], concluded that IIT significantly increased the risk of hypoglycemia and conferred no overall mortality benefit among critically ill patients; however, this therapy might be beneficial to patients admitted to a surgical ICU. Some patients might benefit from IIT, although the characteristics of such patients remain to be clearly defined, as does the effect of different blood glucose algorithms, the method of measuring blood glucose, and the influence of nutritional strategies^[41]. Unfortunately, however, the optimal targeted blood glucose range to prevent POI remains unclear, especially in the intraoperative targeted blood glucose zone. IIT is also a risk factor for hypoglycemia (≤ 40 mg/dL)^[3,4,12-15], which is linked to serious neurological events. Hypoglycemia is the major potential harm of tight glucose control. Tight glycemic control without hypoglycemia will be required in the future.

TIGHT PERIOPERATIVE GLYCEMIC CONTROL USING A CLOSED-LOOP ARTIFICIAL ENDOCRINE PANCREAS SYSTEM

Since 2006, we have been developing a closed-loop artificial pancreas system^[16-20] (STG-22, Nikkiso Co. Ltd., Tokyo) to achieve perioperative glycemic control and prevent POI in general surgery, without the risk of hypoglycemia associated with tight glycemic control.

Firstly, we confirmed that the STG-22^[21,22] could reliably and accurately measure blood glucose concentration similarly to the ABL 800FLEX machine (Radiometer Medical Aps, Brønshøj, Denmark) recommended by the National Committee for Clinical Laboratory^[21,22]. This closed-loop glycemic control system maintained stable blood concentrations by the automatic infusion of regular insulin and/or glucose into the circulation^[18].

Secondly, we performed two prospective randomized clinical trials and found that tight perioperative glycemic control using a closed-loop artificial pancreas system (STG-22, Nikkiso Co. Ltd., Tokyo) decreased surgical site infection (SSI) in patients who underwent pancreatectomy^[19] or hepatectomy^[20]. In our reports, perioperative blood glucose levels were continuously monitored using an artificial endocrine pancreas^[16-20] and glucose levels were controlled using either the sliding scale method^[42] (SS group: targeted blood glucose zone

of 150-200 mg/dL) or the artificial pancreas (AP group: targeted blood glucose zone of 80-110 mg/dL). In our study of patients who had undergone a pancreatectomy, the AP group, maintained near-normal glycemia, whereas the blood glucose levels of the SS group plateaued at approximately 200 mg/dL between 4 and 6 h after pancreatectomy^[19]. In addition, the incidence of SSI in the AP group (0%) was significantly lower than that of the SS group (about 30%). In our study of patients who had undergone a hepatectomy^[20], the AP group again maintained near-normal glycemic control and a reduction in the incidence of SSI. As a consequence, the length of stay and cost of hospitalization was reduced. It is worth noting that there were no incidents of hypoglycemia using a closed-loop artificial pancreas system, despite maintaining blood glucose levels similar to those obtained with IIT. Our results support the conclusions of previous reports^[1,38-40] that glycemic control could be a simple intervention to decrease the risk of infectious complications resulting from hyperglycemia after surgery.

Thirdly, to date, more than 200 general surgeries (hepatic resection, pancreatic resection, esophageal resection, and emergency operations) have been performed under tight perioperative glycemic control using an artificial pancreas (STG-22) with no incidence of hypoglycemia^[20]. To avoid hypoglycemia in tight glycemic control and improve POI in surgery, the closed-loop artificial endocrine pancreas system is an effective and safe means. Furthermore, novel perioperative glycemic control using an artificial pancreas markedly improved the labor burden on nursing staff, and hence reduced concerns about hypoglycemia^[43].

Finally, because an ideal comparative study of tight glycemic control should be carried out under conditions without risk of hypoglycemia, blood glucose control using a closed-loop artificial pancreas system might be beneficial for the detection of the optimal target range for blood glucose in surgical patients.

CONCLUSION

Perioperative hyperglycemia increases the risk of POI. Therefore, perioperative glycemic control in patients undergoing surgery is beneficial to reduce POI. As a closed-loop artificial pancreas system is able to perform tight glycemic control without increasing the risk of hypoglycemia, it might be a safe and useful blood glucose control system in critically ill surgical patients. At present, however, the perioperative optimal target range for blood glucose to reduce POI remains uncertain. Further studies are needed to address this issue.

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TOPIC HIGHLIGHT

Kazuhiro Hanazaki, MD, Professor and Chairman, Series Editor

Intraoperative glycemic control procedures and the use of an artificial pancreas

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Received: May 4, 2009 Revised: July 24, 2009
Accepted: July 31, 2009
Published online: September 7, 2009

Abstract

Strict intraoperative glycemic control can significantly decrease the incidence of postoperative infection; however, anesthesiologists must carefully control blood glucose levels as well as properly manage the respiratory and cardiovascular systems. However, standard blood glucose measurement systems and insulin dosing algorithms, which are necessary for achieving strict glycemic control, have not yet been developed. An artificial pancreas (STG-22™; Nikkiso Co., Tokyo, Japan) is considered a highly accurate blood glucose monitoring system capable of closed-loop control of blood glucose. The device has, however, many problems to be addressed since it is a large and expensive system with little versatility, and it requires a large amount of blood to be collected. Therefore, the development of less invasive and inexpensive systems with future technological progress is greatly anticipated.

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Key words: Strict glycemic control; Artificial pancreas; Anesthesiologist; Sliding scale

Peer reviewer: Christa Buechler, PhD, Regensburg University Medical Center, Internal Medicine I, Franz Josef Strauss Allee 11, 93042 Regensburg, Germany

Yamashita K, Yatabe T. Intraoperative glycemic control procedures and the use of an artificial pancreas. *World J*

Gastroenterol 2009; 15(33): 4126-4131 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4126.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4126>

INTRODUCTION

Management of the respiratory and cardiovascular systems is the most important task of an anesthesiologist. Traditionally, the purpose of intraoperative glycemic control is to prevent three major problems: hypoglycemia, ketoacidosis, and hyperglycemia-associated osmotic diuresis (dehydration). So far, particular attention has been given only to the case of abnormal hyperglycemia. On the other hand, postoperative glycemic control appears to influence a patient's prognosis, and as a result, this has drawn attention to intraoperative glycemic control^[1,2]. In this report, the recent concept of glycemic control, problems of glycemic control procedures and effects of intraoperative glycemic control on a patient's prognosis are reviewed based on recent publications.

NEW CONCEPT OF GLYCEMIC CONTROL

The concept of glycemic control in the care of critically ill patients largely changed after van den Bergh *et al*^[3] published a report in 2001. They comparatively investigated two groups of patients admitted to a surgical intensive care unit. In the first group, insulin control was instituted when the blood glucose level exceeded 100 mg/dL in order to maintain the blood glucose level in the target range from 80 to 110 mg/dL (strict glycemic control group). In the second group, insulin control was instituted if the blood glucose level exceeded 215 mg/dL in order to maintain the blood glucose level in the target range from 180 to 200 mg/dL (conventional glycemic control group). The results of insulin control were remarkable. The mortality rate of patients during the stay in the intensive care unit was 4.6% for the strict glycemic control group *vs* 8.0% for the conventional treatment group. The mortality rate among patients who remained in the intensive care unit for five or more days was 10.6% for the strict glycemic control group *vs* 20.2% for the conventional treatment group. The in-hospital mortality rate was reduced by 3.7% by strict

glycemic control. However, the test protocol has been questioned since there is a large discrepancy between the actual mortality rate of 26.3% in the examined group and the mortality rate predicted by APACHE II score (median score 7). To address this criticism, a study was conducted in a medical intensive care unit and results were reported in 2006^[4]. This study, where long-term prognosis following a similar glycemic control was monitored for one year or longer, showed the benefits of strict glycemic control in patients who remained in the intensive care unit for three or more days, but not in those who stayed for a shorter period. The mortality rate of the strict glycemic control group was 41.5% compared with the 50.9% of the conventional treatment group. However, the underlying detailed mechanism was not clarified and various basic and clinical studies to clarify this mechanism are presently ongoing.

DOES INTRAOPERATIVE GLYCEMIC CONTROL INFLUENCE PATIENT PROGNOSIS?

To date, there have been no randomized controlled trials to assess the effects of intraoperative glycemic control on patient prognosis. However, it is likely that intraoperative hyperglycemia decreases immunocompetence and increases the incidence of wound infection^[5-10]. Various types of anesthetic agents also alter blood glucose level^[11]. Furthermore, it has been reported that the incidence of wound infection is markedly high among hyperglycemic patients whose blood glucose level was reported to be 149 mg/dL or above intraoperatively^[12,13]. Moreover, every 18 mg/dL increase in blood glucose level from 110 mg/dL is reported to increase the risk of wound infection by 17%^[14]. Based on these results, intraoperative glycemic control that maintains blood glucose at a level not higher than 150 mg/dL is considered necessary. On the other hand, intraoperative glycemic control reportedly increases the risks of hypoglycemia due to unstable actions of insulin owing to hypothermia and impaired tissue perfusion^[15,16]. Therefore, the establishment of concrete glycemic control procedures is eagerly anticipated, and the development of a continuous intraoperative blood glucose monitoring system is considered very important.

CURRENT STATUS OF GLYCEMIC CONTROL PROCEDURES

As mentioned above, concrete glycemic control procedures for achieving strict glycemic control have not yet been established. The detailed procedures described by van den Bergh *et al.*^[3] in 2001 were not very clear. According to another line of thought, full-time staff can be designated to carry out frequent blood glucose measurements in order to achieve glycemic control. While this validates this line of thought, it can not

be executed in general practice. To perform accurate and safe glycemic control, the following elements are considered important: (1) frequency of blood glucose measurements, (2) accuracy of blood glucose measurement results, (3) accuracy of the insulin dosing algorithm, and (4) blood collection volume.

FREQUENCY OF BLOOD GLUCOSE MEASUREMENT

As for the frequency of blood glucose measurement, continuous monitoring is preferable to intermittent measurements. Two types of system capable of continuous blood glucose monitoring are currently used. One measures glucose concentration in the intracellular substance obtained by directly inserting an electrode subcutaneously, and the other collects venous blood continuously for blood glucose monitoring^[17,18]. The former system is based on the near proportional relation between glucose concentration in the intracellular substance and blood glucose level. However, the measurement stability of this type of system is in question because rapid changes in blood glucose level are not readily reflected in the intracellular substance. On the other hand, an artificial pancreas (STG-22TM) was developed by Nikkiso (Tokyo, Japan) and this device is considered the only system in the world representing the latter type of system, which collects venous blood continuously and performs blood glucose monitoring. Furthermore, this system carries out direct measurements of blood glucose and can therefore show rapid changes in blood glucose level (Figures 1 and 2). Although STG-22TM is expected to serve as a standard blood glucose monitoring system, it cannot be used for all patients because of its high cost. Generally, intermittent blood glucose measuring systems are highly versatile and can be used in any facility. However, many difficulties are encountered in their use for strict glycemic control, mostly attributed to increased workloads from frequent measurements and large measurement errors^[19]. It is considered that the achievement of strict glycemic control is currently supported by the efforts and dedication of medical staff as well as through proper education^[18].

PROBLEMS ENCOUNTERED REGARDING THE VOLUME OF BLOOD COLLECTED

To achieve successful strict glycemic control, blood glucose measurement must be performed at least every hour. Since around 0.5 mL of blood is required for a single blood glucose measurement, a total of 12 mL of blood in point-of-care blood glucose testing must be collected daily. When blood glucose control is unstable, more frequent measurements are needed and approximately 20 mL of blood must be collected daily solely for blood glucose measurement. This becomes a considerable burden for patients with anemia and for

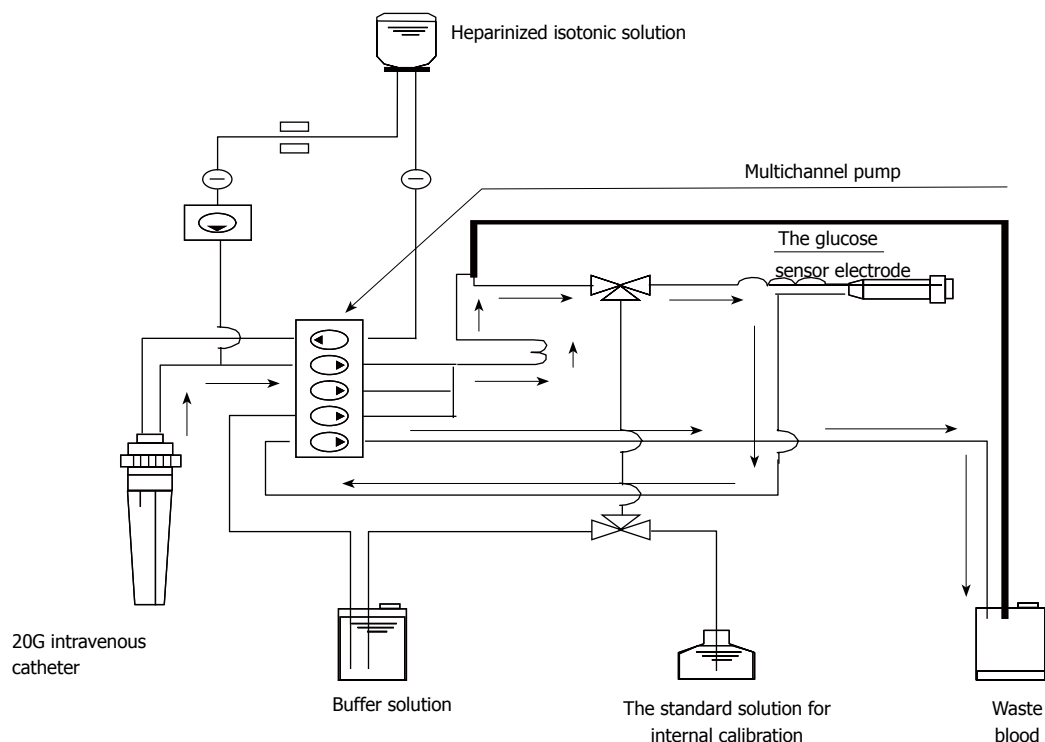


Figure 1 The whole circuit of the STG-22™. Arrows indicate the direction of blood sampling.

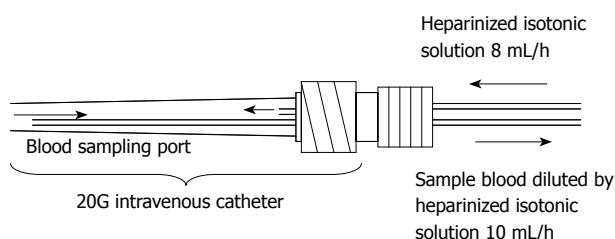


Figure 2 This figure shows the dual lumen catheter technique.

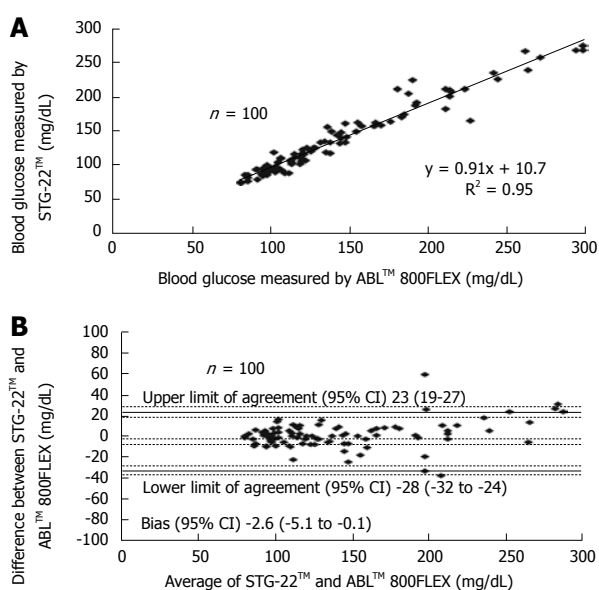


Figure 3 Scatter plot of individual blood glucose levels measured by both STG-22™ and ABL™ 800FLEX during surgery^[20].

glucose measurement technologies requiring no blood collection is greatly anticipated.

ACCURACY OF BLOOD GLUCOSE MEASUREMENT RESULTS

Measurement errors become an issue during strict glycemic control for maintaining blood glucose at a level between 80 and 110 mg/dL. According to the standards established by the International Organization for Standardization, the errors defined for glucose concentrations of 4.1 mmol (74 mg/dL) or above must not be greater than 20%, and those for glucose concentrations of 4.1 mmol (74 mg/dL) or below must not be greater than ± 0.8 mmol (14 mg/dL), and these values are not guaranteed by the accuracy of portable blood glucose measurement systems. STG-22™, which is currently being used for a clinical study by our group, is a highly accurate and reliable system. Its measurement errors are below 21% during intraoperative measurement^[20] (Figure 3) and not more than 15% during postoperative measurements^[21] (Figures 4-6). These findings suggest that STG-22™ can sufficiently perform closed loop control of blood glucose based on measurement results.

ACCURACY OF INSULIN DOSING ALGORITHMS

Sliding scale dosing of insulin, described previously as a special feature, is not described here. A program that calculates insulin dosage based on the blood glucose level, rate of its change and target blood glucose level

pediatric patients, and thus the development of blood

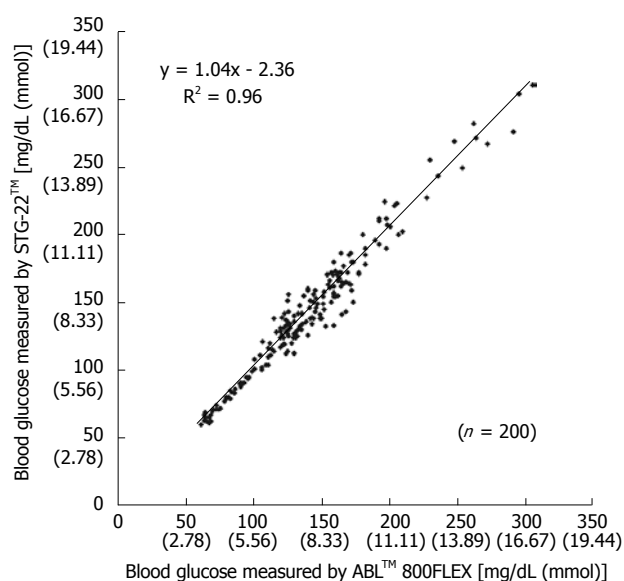


Figure 4 Scatter plot of individual blood glucose levels measured by both STG-22™ and ABL™ 800FLEX in post-surgical patients^[21].

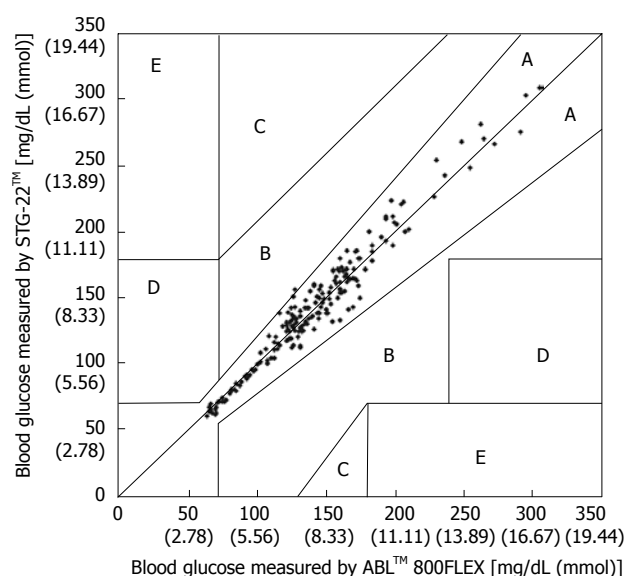


Figure 5 Error grid analysis for evaluation of blood glucose measured by STG-22™ compared with those measured by ABL™ 800FLEX. Zone A, B: Accurate or acceptable; Zone C: Unnecessary corrections that could lead to a poor outcome; Zone D: Dangerous failure to detect and treat; Zone E: 'Erroneous treatment'^[21].

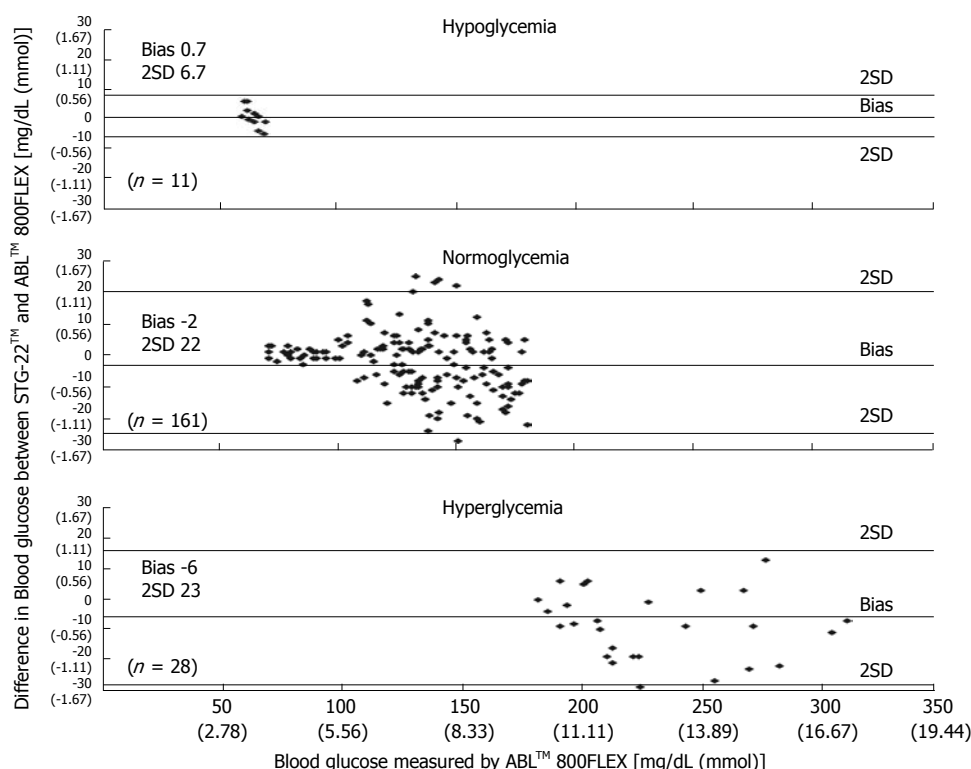


Figure 6 Bland-Altman plot of blood glucose measurements from STG-22™ and ABL™ 800FLEX^[21].

is expected to be used widely in clinical applications, since such a program has been reported to decrease the incidence of hypoglycemia by approximately 0.3%^[22]. On the other hand, STG-22™ is the only system equipped with a closed-loop algorithm that enables continuous measurement of blood glucose levels and provides updated results. This system utilizes Shichiri's algorithm, which calculates insulin dosage on the basis

of the fact that glucose-stimulated insulin secretion is based on the proportional-derivative action^[23]. The results of our previous studies have proven that use of STG-22™ achieves good postoperative glycemic control among patients who received surgical treatments^[24,25]. Here, we aim to further evaluate the applicability of this system to intraoperative glycemic control.

NEW BLOOD GLUCOSE MEASUREMENT TECHNOLOGY

A continuous noninvasive blood glucose monitoring system is an ideal instrument for strict glycemic control. Pasic *et al*^[26] previously developed a blood glucose measurement system by combining microdialysis and an optic fiber, and they succeeded in performing continuous *in vitro* measurement for 3 d. Maruo *et al*^[27] have also developed a near-infrared based blood glucose monitoring system, and its application to patients in the intensive care unit showed a good outcome. However, these systems present disadvantages in terms of convenience and accuracy, making strict glycemic control difficult. Further improvements to these systems are needed.

NEW INSULIN DOSING TECHNOLOGIES

Recently, an insulin inhalant has been approved in the US and Europe. In terms of drug assimilation, the lung possesses a large surface area for drug absorption. Moreover, the rate of solute absorption is fast in the alveolar epithelium, and mucosal clearance in the alveoli is slower than that in the bronchi. Therefore, the lung is a very suitable route for administering high-molecular-weight insulin. Because of the reasons mentioned above, the lung can therefore possibly be used as a new route for administering drugs when peripheral circulation is impaired. In support of this, Barnett *et al*^[28] have reported that long-term inhaled insulin therapy has no adverse effects on pulmonary function and thus its wide application is expected in the future.

CONCLUSION

A highly accurate continuous blood glucose monitoring system and closed-loop control of blood glucose are essential for anesthesiologists to achieve strict intraoperative glycemic control. STG-22TM is the only system that performs both functions; however, it requires 50 mL of blood per day for continuous blood glucose monitoring, and the collection of such a volume is invasive and remains laborious. With technological progress, the development of blood glucose monitoring systems with improved convenience, accuracy and minimal invasiveness are anticipated in the future.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Yin DH

TOPIC HIGHLIGHT

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Blood glucose control in patients with severe sepsis and septic shock

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Received: May 7, 2009 Revised: June 6, 2009

Accepted: June 13, 2009

Published online: September 7, 2009

Abstract

The main pathophysiological feature of sepsis is the uncontrollable activation of both pro- and anti-inflammatory responses arising from the overwhelming production of mediators such as pro- and anti-inflammatory cytokines. Such an uncontrollable inflammatory response would cause many kinds of metabolic derangements. One such metabolic derangement is hyperglycemia. Accordingly, control of hyperglycemia in sepsis is considered to be a very effective therapeutic approach. However, despite the initial enthusiasm, recent studies reported that tight glycemic control with intensive insulin therapy failed to show a beneficial effect on mortality of patients with severe sepsis and septic shock. One of the main reasons for this disappointing result is the incidence of harmful hypoglycemia during intensive insulin therapy. Therefore, avoidance of hypoglycemia during intensive insulin therapy may be a key issue in effective tight glycemic control. It is generally accepted that glycemic control aimed at a blood glucose level of 80-100 mg/dL, as initially proposed by van den Berghe, seems to be too tight and that such a level of tight glycemic control puts septic patients at increased risk of hypoglycemia. Therefore, now many researchers suggest less strict glycemic control with a target blood glucose level of 140-180 mg/dL. Also specific targeting of glycemic control in diabetic patients should be considered. Since there is a significant

correlation between success rate of glycemic control and the degree of hypercytokinemia in septic patients, some countermeasures to hypercytokinemia may be an important aspect of successful glycemic control. Thus, in future, use of an artificial pancreas to avoid hypoglycemia during insulin therapy, special consideration of septic diabetic patients, and control of hypercytokinemia should be considered for more effective glycemic control in patients with severe sepsis and septic shock.

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Key words: Blood glucose; Diabetes mellitus; Insulin; Hypercytokinemia; Inflammation mediators

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Hirasawa H, Oda S, Nakamura M. Blood glucose control in patients with severe sepsis and septic shock. *World J Gastroenterol* 2009; 15(33): 4132-4136 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4132.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4132>

INTRODUCTION

There are many pathophysiological changes during severe sepsis and septic shock, and one of the most striking is metabolic derangement. Among the metabolic changes, hyperglycemia is the most important^[1,2]. Accordingly therapeutic approaches to hyperglycemia in the management of severe sepsis and septic shock have had much attention. Intensive insulin therapy became popular in the intensive care unit (ICU) after Van den Berghe's research reporting its effectiveness on glycemic control^[3,4]. However, a recent large-scale randomized trial indicated that such glycemic control is not effective in reducing ICU mortality and that glycemic control with intensive insulin therapy increases the risk of hypoglycemia, and complications arising from hypoglycemia^[5]. Therefore, in this paper we will discuss the effectiveness of intensive insulin therapy in the ICU and the future perspectives on tight glycemic control from the viewpoint of the correlation between inflammatory hypercytokinemia and hyperglycemia.

METABOLIC CHANGE IN SEPSIS

Recent advances in molecular biology have contributed to the tremendous progress in understanding the pathophysiology of sepsis. Now it is widely accepted that the main features of sepsis are the uncontrollable activation of not only pro-inflammatory, but also anti-inflammatory responses, because of overwhelming production of pro-inflammatory and anti-inflammatory mediators^[6-8]. Such overwhelming production of mediators causes many pathological changes in vital organs and systems including metabolic changes^[9]. One such metabolic change is hyperglycemia arising from muscle glycolysis and lipolysis, and subsequent gluconeogenesis and glycolysis in the liver^[9-12]. The other feature of metabolic change in sepsis is hyperlactatemia due to glycolysis in muscle caused by counterregulatory hormones and cytokines, sometimes referred to as the "lactate shuttle"^[9,13]. Hyperglycemia in critical illness, such as severe sepsis, is not only a marker of severity of illness and the predictor of poor outcome^[1,2], but also has many kinds of adverse effects on vital organs. One such adverse effect on the innate immune system impairs the ability of the host to combat infection, resulting in reduced neutrophil activity such as chemotaxis, formation of reactive oxygen species, and phagocytosis of bacteria despite accelerated diapedesis of leukocytes into peripheral tissue, as well as specific alterations in cytokine patterns, with increased concentrations of the early proinflammatory cytokines, tumor necrosis factor- α and interleukin (IL)-6, and a reduction of endothelial nitric oxide formation^[14]. Recently it has also been reported that the variability of the glucose level in blood is independently associated with hospital mortality in septic patients^[15,16] and that severity of sepsis has a strong effect on glycemic variability in blood^[13].

Without question, treatment of severe sepsis and septic shock starts with control of the infection source. Antibiotics, drainage of abscesses, and operations to control the source, when indicated, are essential in the initial treatment^[17]. Furthermore, hemodynamic stabilization is also very important for the initial treatment of such patients^[18].

On the other hand, an epoch-making paper by Van den Berghe in 2001 reported that tight glycemic control with intensive insulin therapy in the surgical ICU, to control the blood glucose level between 80 and 110 mg/dL, resulted in improvement in survival and a shortened length of hospital stay^[3]. The same authors reported later that intensive insulin therapy reduced morbidity but not mortality in the medical ICU^[4]. These studies^[3,4] led the influential guidelines for the management of severe sepsis and septic shock to recommend tight glycemic control as one of the most important therapeutic approaches^[19]. Since the publication of the guidelines, tight glycemic control in ICU patients has become popular and it is now one of the standard clinical practices in the ICU. This recommendation remained the same in the revised version of the guidelines published in 2008^[20].

CLINICAL EFFECT OF GLYCEMIC CONTROL IN SEPTIC PATIENTS

Since the publication of the papers by Van den Berghe^[3,4], indicating that tight glycemic control between 80 and 110 mg/dL with intensive insulin therapy reduces morbidity and mortality among critically ill patients in the surgical ICU, and that the intensive insulin therapy significantly reduces morbidity but not mortality among all patients in the medical ICU, many secondary clinical trials on tight glycemic control have been carried out. A meta-analysis and systematic reviews on tight glycemic control were also published. In a review, the Van den Berghe group reconfirmed that maintaining strict normoglycemia with the use of intensive insulin improves the outcome of critically ill patients^[21]. They also published a paper in which they concluded that intensive insulin therapy reduced mortality of all medical/surgical ICU patients, except those with a prior history of diabetes, that intensive insulin therapy did not cause harm, and that a blood glucose target < 110 mg/dL was most effective, but also carried the highest risk of hypoglycemia^[22].

On the other hand, Brunkhorst and the SepNet group from Germany published a paper which found that use of intensive insulin therapy placed critically ill patients with sepsis at increased risk for serious adverse events related to hypoglycemia, without showing any benefit, and they stopped the trial for safety reasons^[23]. Treggiari and colleagues also showed that a policy of intensive insulin therapy in a group of ICU patients was not associated with a decrease in hospital mortality, and they concluded that further study was needed prior to widespread implementation of intensive insulin therapy in critically ill patients^[24]. The meta-analysis by Wiener and colleagues published in JAMA concluded that, in critically ill adult patients, tight glucose control is not associated with significantly reduced hospital mortality, but that it is associated with an increase risk of hypoglycemia^[25]. Finally, the long-awaited result of the NICE-SUGAR (Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulator) trial was published recently. This concluded that intensive glucose control increased mortality among adults in the ICU and that a blood glucose target of 180 mg/dL or less resulted in lower mortality than did a target of 81-108 mg/dL^[5]. Furthermore it was also pointed out that intensive insulin therapy increased the labor requirement of nursing staff in the ICU^[26]. Now the statement by Merz and Finfer, that each ICU should define a blood glucose range which can be achieved without causing a significant increase in severe hypoglycemia, and which fits within the constraints of their nursing and economic resources sounds fair and acceptable^[26]. They also concluded that the upper limit of glucose control should currently be 140-180 mg/dL^[26]. Preiser also suggested that a blood glucose range of 80-110 mg/dL may not be normal or desirable and that lowering of blood glucose by intensive insulin therapy can induce a shortage in the provision of glucose, the predominant source of energy useable by the

myocardium during ischemia, and neuroglycopenia in cases of cerebral injury^[27].

MECHANISM OF BENEFICIAL OR HARMFUL EFFECTS OF TIGHT GLYCEMIC CONTROL

There are many papers which illustrate the mechanism of the beneficial effect of tight glycemic control. Wade tell us that hyperglycemia can alter cytokine production and phagocytosis both by means of hyperosmotic stress and by mechanisms other than hyperosmolality^[28]. Egi from Bellomo's group suggested that variability of glucose concentration is a significant independent predictor of ICU and hospital mortality, and that decreasing the variability of blood glucose concentration might be an important aspect of glucose management^[29]. The question whether intensive insulin therapy *per se* or a lowered glucose level by intensive insulin therapy is the main mechanism of the beneficial effect of tight glycemic control with intensive insulin therapy has not yet been answered.

On the other hand, Jeschke *et al*^[30] reported that insulin therapy improves the systemic inflammatory reaction to severe trauma. Vanhorebeek from Van den Berghe's group reported that protection of hepatocyte mitochondrial ultrastructure and function is one of the mechanisms of the beneficial effect of strict blood glucose control with insulin in critically ill patients^[31]. Another researcher from Van den Berghe's group also reported that intensive insulin therapy prevented critical polyneuropathy/myopathy and the necessity for treatment with prolonged mechanical ventilation^[32,33]. Dugo and colleagues showed, in an experimental study, that the inhibitory effect of insulin on the activity of glycogen synthase kinase-3 β , contributed to the protective effect of insulin against organ injury/dysfunction caused by excessive systemic inflammation, independently of any effects on blood glucose^[34]. Another possible mechanism of the beneficial effect of intensive insulin therapy or tight glycemic control is through RAGE (receptor of advanced glycation end product). Now RAGE is considered to play an important role in the pathophysiology of severe sepsis and septic shock. It is possible that tight glycemic control can reduce the production of AGE (advanced glycation end product), and that tight glycemic control can thereby reduce the inflammatory response mediated through AGE and RAGE interaction^[35]. This could be another mechanism of the beneficial effect of tight glycemic control in severe sepsis.

There are many papers reporting that hypoglycemia during intensive insulin therapy is the main reason why intensive insulin therapy cannot show a beneficial effect on mortality in severe sepsis and septic shock. Waeschle *et al*^[16] showed that the risk of hypoglycemia with intensive insulin therapy is very high among patients with severe sepsis and septic shock. The meta-analysis mentioned above on the benefit and risks of

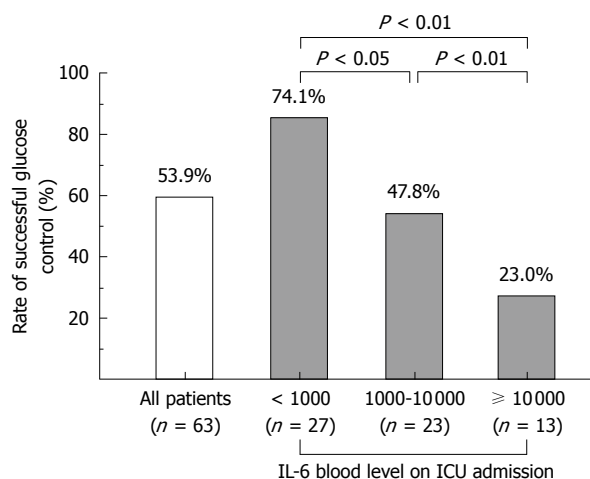


Figure 1 Correlation between IL-6 blood level on ICU admission and success rate of blood glucose control among patients with severe sepsis and septic shock. The blood glucose level is targeted to be between 110 and 150 mg/dL with intensive insulin therapy.

tight glucose control in critically ill adults by Wiener^[24], indicated that intensive insulin therapy increased the risk of hypoglycemia among critically ill patients including those with severe sepsis and septic shock. Krinsley and Grover indicated that even a single episode of severe hypoglycemia was independently associated with increased risk of mortality and therefore that safe implementation of tight glycemic control requires appropriate monitoring to reduce the risk of this complication^[36]. They proposed to move beyond tight glucose control to safe effective glucose control avoiding hypoglycemia^[37]. The mechanism by which hypoglycemia increased mortality in severe sepsis and septic shock has not yet been fully elucidated. However, brain damage because of an energy deficit in the brain through hypoglycemia is possibly one mechanism^[38].

FUTURE PERSPECTIVES ON TIGHT GLYCEMIC CONTROL

It is now clear that to benefit from tight glycemic control, we should avoid hypoglycemia. On the other hand it has also become clear that it is not so easy to keep the blood glucose level within the targeted range even though the targeted range is not very tight such as between 150 and 180 mg/dL in patients with severe sepsis and septic shock.

In our ICU, we routinely check IL-6 blood levels in every patient every day. The reason why we specifically measure IL-6 is not that we think IL-6 is the most important proinflammatory cytokine, but rather that IL-6 is the most easily measurable cytokine because of its relatively high blood level and relatively long half-time in the blood^[39]. Since blood levels of many kinds of cytokines change synergistically in sepsis, we do not need to measure blood levels of various cytokines to make a diagnosis of hypercytokinemia but we can measure only one of the most easily measurable cytokines to make the diagnosis of hypercytokinemia in

sepsis. It is reported that if a patient has an IL-6 level of more than 1000 pg/mL, this patient can be diagnosed with systemic inflammatory response syndrome or hypercytokinemia^[40].

We applied tight glycemic control with a target blood glucose level of 110-150 mg/dL in patients with severe sepsis and septic shock. The overall success rate for tight glycemic control was only 53.9%. However when we subgrouped the patients according to the IL-6 blood level on ICU admission, we found that the success rate of tight glycemic control was relatively high in subgroups whose IL-6 blood level on ICU admission was lower than 1000 pg/mL. On the other hand, the success rate of tight glycemic control was very low among the patients whose initial IL-6 blood level in the ICU was higher than 10000 pg/mL as shown in Figure 1. These data indicate that hypercytokinemia correlates with hyperglycemia in sepsis and that countermeasures to hypercytokinemia in sepsis would be one of the key factors for successful glycemic control. Recently, we published a paper showing the efficacy of continuous hemodiafiltration (CHDF) with a cytokine-adsorbing hemofilter made from polymethyl methacrylate (PMMA) membrane in patients with septic shock^[41]. We found that, in septic patients with severe hypercytokinemia, blood glucose control became easier once we lowered the blood level of cytokines with PMMA-CHDF.

Since one of the reasons why tight glycemic control could not show a beneficial effect on critically ill patients is hypoglycemia during intensive insulin therapy, avoidance of hypoglycemia should always be considered^[36,37]. For this purpose an artificial pancreas has promising potential^[42]. On the other hand, a pharmacological dose of a steroid is recommended in the guidelines for the management of severe sepsis and septic shock^[18,19]. However, steroid administration may create some difficulties in glycemic control in septic patients^[43]. An artificial pancreas may be most effective on such patients with severe sepsis and septic shock receiving steroid therapy.

Another important issue of tight glycemic control in critically ill patients, including those with severe sepsis and septic shock, is whether tight glycemic control would also be effective in critically ill diabetics. It is proposed that precisely defined target glucose levels, treatment intervention and the avoidance of hypoglycemic episodes during insulin therapy should be studied before the widespread application of tight glycemic control in critically ill diabetic patients^[44]. Egi and colleagues reported that, unlike nondiabetic patients, diabetic patients showed no clear association between hyperglycemia during the ICU stay and mortality, and there were markedly lower odds ratios of death at all levels of hyperglycemia suggesting that hyperglycemia may have different biological and/or clinical implications in critically patients with diabetes mellitus^[45].

As mentioned above, it has not yet been fully elucidated whether intensive insulin therapy or normoglycemia with intensive insulin therapy really has the beneficial effect on severely septic patients. However, if normoglycemia is the key mechanism of tight glycemic control with

intensive insulin therapy, modulation of glucose use and gluconeogenesis in sepsis with adrenergic β receptor blockade is one of the future approaches in this area^[46].

CONCLUSION

It is now suggested that tight glycemic control with a target blood glucose level of 90-110 mg/dL does not improve clinical outcome and that less strict glycemic control with a target blood glucose level of 140-180 mg/dL is more effective. Also specific targeting of glycemic control in diabetic patients should be considered. Since there is a significant correlation between success rate of glycemic control and the degree of hypercytokinemia in septic patients, some countermeasures to hypercytokinemia may be an important aspect of successful glycemic control. Thus, in future, use of an artificial pancreas to avoid hypoglycemia during insulin therapy, special consideration of septic diabetic patients, and control of hypercytokinemia should be considered for more effective glycemic control in patients with severe sepsis and septic shock.

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Problems associated with glucose toxicity: Role of hyperglycemia-induced oxidative stress

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Received: May 26, 2009 Revised: June 27, 2009

Accepted: July 4, 2009

Published online: September 7, 2009

stress. *World J Gastroenterol* 2009; 15(33): 4137-4142 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4137.asp>
DOI: <http://dx.doi.org/10.3748/wjg.15.4137>

Abstract

Glucose homeostasis deficiency leads to a chronic increase in blood glucose concentration. In contrast to physiological glucose concentration, chronic super-physiological glucose concentration negatively affects a large number of organs and tissues. Glucose toxicity means a decrease in insulin secretion and an increase in insulin resistance due to chronic hyperglycemia. It is now generally accepted that glucose toxicity is involved in the worsening of diabetes by affecting the secretion of β -cells. Several mechanisms have been proposed to explain the adverse effects of hyperglycemia. It was found that persistent hyperglycemia caused the functional decline of neutrophils. Infection is thus the main problem resulting from glucose toxicity in the acute phase. In other words, continued hyperglycemia is a life-threatening risk factor, not only in the chronic but also the acute phase, and it becomes a risk factor for infection, particularly in the perioperative period.

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Key words: Glucose toxicity; Diabetes; Complication; Surgery; Surgical site infection; Emergency medicine; Critical care medicine

Peer reviewer: Christa Buechler, PhD, Regensburg University Medical Center, Internal Medicine I, Franz Josef Strauss Allee 11, 93042 Regensburg, Germany

Kawahito S, Kitahata H, Oshita S. Problems associated with glucose toxicity: Role of hyperglycemia-induced oxidative

INTRODUCTION

Chronic hyperglycemia is a characteristic of the diabetic condition, while glucose toxicity is the main cause of diabetic complications, which are often observed only several years after the beginning of the illness^[1]. Glucose toxicity, in its narrow sense, can indicate a clinical condition where control of diabetes in particular is poor, since hyperglycemia itself reduces the insulin secretion capacity of pancreatic β -cells, and the resultant increase in insulin resistance leads to further hyperglycemia. This vicious circle finally leads to the total incapacity of β -cells to secrete insulin^[2,3].

On the other hand, acute hyperglycemia, similar to chronic hyperglycemia, is known to cause injury to many organs. Hyperglycemia in the acute phase causes the functional decline of neutrophils, and is a risk factor that causes infection in the perioperative period. In the first half of this review, we will present an introduction to the various mechanisms known to be involved in the control of glucose homeostasis and in the development of glucose toxicity. In the latter half, diabetic complications (chronic and acute) and implications for the fields of surgery, emergency and critical care medicine will be presented and discussed.

GLUCOSE HOMEOSTASIS

Glucose homeostasis is maintained by the highly coordinated interaction of three physiologic processes: insulin secretion, tissue glucose uptake and hepatic glucose production. In this way, the body tries to maintain a constant supply of glucose for cells by keeping glucose concentration in the blood constant. Normal glucose homeostasis represents the balance between intake (glucose absorption from the gut), tissue utilization (glycolysis, pentose phosphate pathway activity, tricarboxylic acid cycle activity, glycogen synthesis) and endogenous production (glycogenolysis and gluconeogenesis)^[4]. The most important metabolic fuels are glucose and fatty acids. Glucose is preferentially used by brain and muscles,

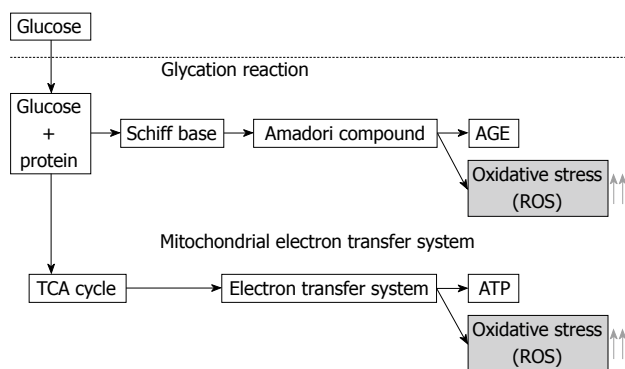


Figure 1 Increase in oxidative stress in the diabetic state. Acceleration of glycation response and the intramitochondrial electron transfer system was detected in the diabetic state, causing oxidative stress as the responses accelerated. Black arrows indicate pathway, grey arrows indicate increase or decrease. AGE: Advanced glycosylation end products; ROS: Reactive oxygen species; TCA cycle: Tricarboxylic acid cycle; ATP: Adenosine tri-phosphate.

and to ensure a continuous supply of glucose to the brain and other tissues, metabolic fuels are stored for use in time of need. Glucose homeostasis is controlled primarily by the anabolic hormone insulin and also by some insulin-like growth factors^[5]. Several catabolic hormones (glucagons, catecholamines, cortisol, growth hormone, and adrenocorticotrophic hormone) may antagonize the action of insulin and are known as anti-insulin or counter-regulatory hormones^[6].

It is often found that critically ill patients incur hyperglycemia because of insulin resistance even if it is not complicated by diabetes^[7]. When severe stress occurs, insulin resistance and an insulin secretion decrease result from the response to stress by the neuroendocrine system because secretion of anti-insulin hormones is enhanced. This leads to enhancement of glucogenesis in the liver, of lipolysis in adipose tissue, and of protein catabolism in skeletal muscle. This is known as surgical diabetes. Patients with diabetes are more susceptible to stress and easily develop exacerbation of diabetes, resulting in an increase in the incidence of complications.

MECHANISM OF GLUCOSE TOXICITY

It has been found that oxidative stress is associated with the molecular mechanism of the decreased insulin biosynthesis and secretion, which is the main etiology of glucose toxicity. Because pancreatic islet cells show extremely weak manifestation of antioxidative enzymes^[8,9], it is thought that the pancreas may be more susceptible to oxidative stress than other tissues and organs.

Pathway of oxidative stress production

Metabolic reactions continuously produce reactive oxygen species (ROS), such as superoxides (O_2^-), hydroxyl radicals (OH^\cdot), peroxy radicals (ROO^\cdot) or nitric oxide. ROS are involved in a diversity of biological phenomena, such as inflammation, carcinogenesis, aging, and atherosclerosis. However, several antioxidant enzymes help to maintain low levels of ROS. Oxidative stress corresponds

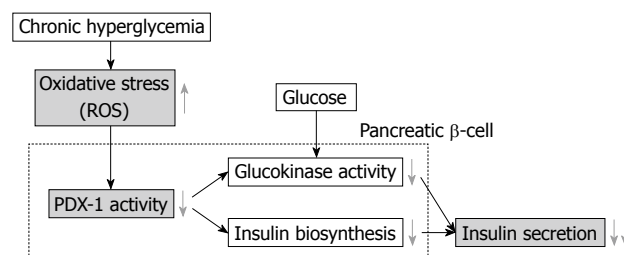


Figure 2 The mechanism of insulin secretion reduction due to glucose toxicity. DNA binding capacity of PDX-1 decreases as a result of oxidative stress caused by hyperglycemia, while insulin biosynthesis and secretion also decrease. Black arrows indicate pathway, grey arrows indicate increase or decrease. PDX-1: Pancreatic duodenal homeobox-1.

to the overproduction of ROS that can damage cellular components, such as lipids, proteins or DNA^[10]. There are strong indications that oxidative stress may be a key event in diabetic complications^[11,12]. It is reported that 8-hydroxy-2-deoxyguanosine (8-OHdG), which is an indicator of oxidative damage of DNA, increases in patients with type 2 diabetes mellitus^[13], and that 8-OHdG, 4-hydroxy-2-nonenal and heme oxygenase-1, all oxidative stress markers, increase in the pancreatic islet cells of type 2 diabetes mellitus animal models^[14,15]. Generation of ROS in diabetes seems to be directly linked to chronic hyperglycemia. Various pathways are thought to be involved in the increase in oxidative stress in a hyperglycemic state (Figure 1). With the first pathway, more oxidative stress is caused by hyperglycemia because a non-enzymatic glycosylation reaction (glycation) is enhanced in the hyperglycemic state. ROS are then generated by the Amadori compound, which is an intermediate metabolite, before leading to the production of metabolites, known as advanced glycosylation end products (AGE) as the result of a glycation reaction^[16]. Another pathway involves the mitochondrial electron transfer system, which also becomes a source of oxidative stress. This system is located in the mitochondrial inner membrane, where Adenosine tri-phosphate is produced as an important organic energy source. In this electron transfer system, water molecules are generated by deoxidation of four of the electrons of oxygen molecules, ROS is produced as an intermediate product in this process, and some of the ROS leak out from this system. Part of the oxygen used for this process is produced as superoxide anions even under physiological conditions, and their production increases in the hyperglycemic state^[17]. Furthermore, the hexosamine pathway also becomes a source of oxidative stress. It was found that glucosamine, which is an intermediate metabolite in this process, also brings about oxidative stress^[18]. Because this hexosamine pathway is enhanced in the diabetic state, the oxidative stress thus generated will increase.

Reduction of insulin biosynthesis and secretion by oxidative stress

Figure 2 shows the mechanism of the reduction of insulin secretion by oxidative stress. It is known that biosynthesis

of insulin decreases when pancreatic β -cells are exposed to chronic hyperglycemia in animal models of type 2 diabetes mellitus, and a similar phenomenon was induced by oxidative stress caused in the diabetic state. In other words, promoter activity of the insulin gene and mRNA expression decrease, and insulin gene expression is thus inhibited, when the β -cell line and isolated pancreatic islet cells are exposed to oxidative stress. It was also found that the DNA binding capacity of Pancreatic duodenal homeobox-1 (PDX-1), which is a very important transcription factor for insulin genes, decreases^[18,19]. Finally, as for the control of glucokinase transcription by PDX-1, it is reported the promoter activity, manifestation, and the enzyme activity of glucokinase decreases with oxidative stress^[20]. In fact, the use of antioxidant drugs resulted in an improvement of insulin secretion capacity as well as an increase in insulin mRNA expression^[21]. On the other hand, it was also found that oxidative stress and activation of the c-Jun N-terminal kinase pathway are involved in a decline in insulin biosynthesis and secretion due to chronic hyperglycemia^[22].

Increase in insulin resistance caused by oxidative stress

Glucotoxicity not only affects the secretion of pancreatic hormones but also participates in insulin resistance of insulin-sensitive tissues, which include liver, skeletal muscle, and adipose tissue. Insulin resistance has been shown to be present before the onset of chronic hyperglycemia, although the latter may contribute to aggravation of the diabetic state by increasing insulin resistance^[23].

Oxidative stress is also strongly suspected to be involved in chronic hyperglycemia-induced insulin resistance^[24]. Indeed, it is known that incubation of primary adipocyte cells with chronic high glucose concentration can induce oxidative stress^[25]. Moreover, it was demonstrated that oxidative stress induces insulin resistance in the 3T3-L1 adipocyte cell line by inhibiting the translocation of Glut 4 to the plasma membrane^[26]. Finally, it was found that oxidative stress can induce insulin resistance in intact rat muscle^[27].

GLUCOSE TOXICITY AND DIABETIC COMPLICATIONS

The prevalence of diabetes mellitus is increasing worldwide at an alarming rate due to population growth, obesity, sedentary life style and aging. Consequently, diabetic complications are also on the increase. Prevention and treatment of complications are considered to be most important for general care of diabetic patients. The basic causes of complications include tissue metabolism disorders caused by chronic hyperglycemia, which results in damage to many organs. The main diabetic complications are listed in Table 1. They are divided into chronic and acute complications based on the disease course.

Chronic complications

When metabolic disorders due to diabetes continue

Table 1 Types of diabetic complications

Chronic complications
Microvascular diseases: retinopathy, neuropathy, nephropathy
Macrovascular diseases: aortic sclerosis, stroke, myocardial infarction, angina pectoris, obstructive peripheral vascular disease, <i>etc</i>
Others: cataract, dermatopathy, hypertension, osteopenia, osteomalacia, arthropathy, soft tissue fibromatosis, <i>etc</i>
Acute complications
Diabetic coma: ketoacidotic coma, non-ketotic hyperosmolar coma, lactic acidosis
Acute infection: bacterial, mycotic, viral, <i>etc</i>

for many years, vascular tissue is affected the most. Chronic complications are divided into microvascular diseases that are specific to and common in diabetes and macrovascular diseases that are not specific but frequent and thus important for a prognosis. Vascular endothelial cell function in the blood vessels of diabetic patients is impaired, and many basic research endeavors have demonstrated that vasodilative reaction is also impaired^[28,29]. Hyperglycemia-induced oxidative stress is also involved in the development of both macrovascular and microvascular diabetic complications^[30].

Chronic hyperglycemia can induce microvascular complications such as retinopathy, neuropathy or nephropathy^[31]. The retina is highly sensitive to oxidative stress since it has higher oxygen uptake and glucose oxidation than any other tissue^[32]. Studies of diabetic rat retina and retinal cells incubated with a high-concentration of glucose have shown that the concentration of superoxides is elevated^[33]. It has been demonstrated in animal models that oxidative stress is not only involved in the development of retinopathy but also in the persistence of the pathology after normalization of glucose concentration, probably as the result of persistent ROS^[34]. Oxidative stress is also strongly suspected to be involved in the development of diabetic neuropathy^[35]. Several studies have shown the capacity of antioxidant enzymes to prevent or reverse the toxic effect of chronic hyperglycemia in the nerves^[36]. Moreover, oxidative stress may contribute to the pathogenesis of diabetic nephropathy since the presence of high concentrations of mitochondrial oxidative stress markers has been demonstrated in the urine and kidneys of diabetic rats^[37].

Chronic hyperglycemia can also induce macrovascular complications. Cardiovascular complications are the most prevalent cause of death in diabetic patients. Moreover, it has been clearly shown that chronic hyperglycemia during diabetic and pre-diabetic states is linked to an increased risk for the development of cardiovascular diseases^[38]. Long-term incubation of macrovessels with high-concentration glucose was found to strongly increase the risk of cardiovascular, cerebrovascular and peripheral arterial diseases^[39]. Activation of protein kinase C by hyperglycemia is thought to play a central role in vascular complications since it leads to: (1) modification

of contractile protein function, (2) an increase in the activity of nitric oxide synthase, and (3) activation of the angiotensin-converting enzyme (ACE). Activation of ACE has been linked with apoptosis and necrosis of cardiomyocytes and endothelial cells^[40]. The importance of ACE in cardiovascular disease development was confirmed by studies showing the inhibition of ACE can protect against cardiovascular diseases^[41]. Finally, protein glycation is another factor probably involved in the development of cardiovascular diseases^[42].

Acute complications

Hyperglycemia can exacerbate a number of perioperative problems, including cardiac, neurologic, and infectious complications. In general, most outcomes tend to improve with treatment of hyperglycemia^[43]. Diabetic coma is a form of consciousness disturbance that is characteristic of diabetes in acute complications. Diabetic coma has three categories, ketoacidotic coma due to hyperglycemia, non-ketotic hyperosmolar coma without ketoacidosis, and, rarely, lactic acidosis.

Infection in acute complications is a clinical condition that is not specific to but can easily become complicated in diabetic states. Diabetic patients have reduced immune function and enhanced bactericidal activity, so that special attention is required since the infection focus expands much faster than in non-diabetic patients. This becomes a problem particularly in the fields of surgery, emergency and critical care medicine.

Association between hyperglycemia and infection

It has been confirmed that perioperative appropriate glycemic control promotes wound healing. Perioperative infectious complications, including surgical site infection, represent serious postoperative complications. It is well known that, compared with non-diabetic patients, diabetic patients suffer from an increased incidence of perioperative infections, especially surgical site infection. It was reported that patients with preoperative elevation of HbA1c levels show a significantly higher incidence of surgical site infection than patients with normal HbA1c levels^[44,45].

Recent basic researches have found that the functional decline of neutrophils is caused by a hyperglycemic state^[46], and that the mechanism of this decline includes increased adhesive capacity and diminished chemotaxis, phagocytic activity and bactericidal capacity^[47,48]. Neutrophilic function is reduced in proportion to an increase in the blood glucose level, and 200 mg/dL is assumed to be the threshold of neutrophil dysfunction. Furnary *et al*^[49] reported that the incidence of deep sternal wound infection decreased from 2.0% to 0.8% in a patient group whose blood glucose level was kept below 200 mg/dL by insulin administration, and there are other reports of reduced infectious risk due to strict glycemic control^[50]. Maintenance of a perioperative hyperglycemic state, since the stress response is a risk factor of postoperative infection, is extremely important for postoperative infection prophylaxis.

CONCLUSION

Several recent clinical studies have demonstrated the efficacy of strict glycemic control for reducing the mortality rate of post-operative or emergency patients^[51-53]. It was thought that perioperative strict glycemic control was essential to relieve a perioperative inflammatory response and improve patient outcome. However, despite frequent blood glucose testing, it has been shown that intensive insulin therapy is sometimes difficult to perform when using sliding-scale manual insulin injection. Consequently, hypoglycemic events could not be avoided during intensive insulin therapy with intermittent blood glucose sampling. It has therefore been suggested that continuous blood glucose monitoring would be beneficial for maintaining target blood glucose levels^[54,55]. We also believe that it is necessary to establish more accurate glycemic control methods because, in view of the global increase in diabetes, it is expected that glycemic control in surgical and emergency settings will become increasingly important.

Recently the usefulness of a closed-loop system (artificial endocrine pancreas), which provides continuous monitoring and strict control of blood glucose, was reported. STG-22™ (Nikkiso, Tokyo, Japan) is a novel artificial endocrine pancreas with a closed-loop glycemic control system that provides continuous blood glucose monitoring through a glucose sensor electrode and subsequent automatic insulin and glucose infusion to maintain appropriate blood glucose levels^[54-57]. The usefulness of a closed-loop system providing continuous monitoring and strict control of post-operative blood glucose in patients after hepatic resection was also reported^[58]. Accurate and continuous blood glucose monitoring and close glycemic control may be possible with an artificial endocrine pancreas. Establishment of a new perioperative blood glucose control method with the aid of an artificial endocrine pancreas is urgently needed.

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S- Editor Li LF **L- Editor** Lutze M **E- Editor** Zheng XM

Molecular determinants of the profibrogenic effects of endothelin-1 in pancreatic stellate cells

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Supported by A grant from the Deutsche Forschungsgemeinschaft (Ja 819/3-2)

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Received: June 30, 2009 Revised: July 27, 2009

Accepted: August 3, 2009

Published online: September 7, 2009

Abstract

AIM: To gain molecular insights into the expression and functions of endothelin-1 (ET-1) in pancreatic stellate cells (PSC).

METHODS: PSCs were isolated from rat pancreas tissue, cultured, and stimulated with ET-1 or other extracellular mediators. Cell proliferation was assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine into DNA and cell migration was studied in a transwell chamber assay. Gene expression at the level of mRNA was quantified by real-time Polymerase chain reaction. Expression and phosphorylation of proteins were monitored by immunoblotting, applying an infrared imaging technology. ET-1 levels in cell culture supernatants were determined by an enzyme immunometric assay. To study DNA binding of individual transcription factors, electrophoretic mobility shift assays were performed.

RESULTS: Among several mediators tested, transforming growth factor- β 1 and tumour necrosis factor- α displayed the strongest stimulatory effects on ET-1 secretion. The cytokines induced binding of Smad3 and NF- κ B, respectively, to oligonucleotides derived from the ET-1 promoter, implicating both transcription factors in the induction of ET-1 gene expression. In accordance with previous studies, ET-1 was found to stimulate migration but not proliferation of PSC. Stimulation of ET-1 receptors led to the activation of two distinct mitogen-activated protein kinases, p38 and

extracellular signal-regulated kinases (ERK)1/2, as well as the transcription factor activator protein-1. At the mRNA level, enhanced expression of the PSC activation marker, α -smooth muscle actin and two proinflammatory cytokines, interleukin (IL)-1 β and IL-6, was observed.

CONCLUSION: This study provides novel lines of evidence for profibrogenic and proinflammatory actions of ET-1 in the pancreas, encouraging further studies with ET-1 inhibitors in chronic pancreatitis.

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Key words: Chronic pancreatitis; Endothelin-1; Fibrosis; Pancreatic stellate cells

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Jonitz A, Fitzner B, Jaster R. Molecular determinants of the profibrogenic effects of endothelin-1 in pancreatic stellate cells. *World J Gastroenterol* 2009; 15(33): 4143-4149 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4143.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4143>

INTRODUCTION

Pancreatic fibrosis represents a key feature of chronic pancreatitis and pancreatic cancer. Fibrosis not only accompanies the tumour, but plays an active role in its progression^[1,2]. The cellular and molecular basis of pancreatic fibrogenesis has therefore attracted significant interest in pancreatologists in recent years. In 1998, pancreatic stellate cells (PSC) were identified as the main source of extracellular matrix (ECM) proteins in the diseased pancreas^[3,4]. In response to profibrogenic stimuli, PSC undergo phenotypic changes termed activation, which include proliferative activity, expression of myofibroblastic markers [such as α -smooth muscle actin (α -SMA)], and enhanced synthesis of ECM components. The activation process is also triggered when PSC are isolated from healthy pancreas and when cultured in cell culture dishes. Numerous extracellular mediators and intracellular pathways of PSC activation have been described since 1998, which are summarized in recent reviews^[2,5]. Thus, platelet-derived growth factor

(PDGF) has been identified as a strong PSC mitogen, while transforming growth factor (TGF)- β 1 is considered the most relevant stimulator of ECM synthesis^[6,7]. In addition, proinflammatory cytokines, including tumour necrosis factor (TNF)- α , and ethanol metabolites, have been implicated in pancreatic fibrogenesis^[7-9]. Despite many efforts, however, little progress has been made so far with respect to the development of therapeutic strategies to interrupt fibrogenesis in the context of chronic pancreatitis and pancreatic cancer. One reason is the lack of efficient antifibrotic drugs that are applicable in patients and not in experimental settings only.

Recent studies on fibroproliferative disorders in different organs have indicated an antifibrotic effect of endothelin (ET)-receptor antagonists^[10]. Using bosentan, a clinically available dual ET-receptor antagonist targeting the two receptor types ET_{RA} and ET_{RB}, we recently observed inhibition of key functions of activated PSC, including proliferation and collagen synthesis^[11]. When bosentan was applied to rats with chronic pancreatitis, at least a tendency towards a diminished disease progression was observed in a subgroup of animals with less severe disease. Based on the results of these experiments, we were further interested in the molecular role of the ET-1/ET receptor axis in the process of stellate cell activation.

In the present study, we have analyzed the biological and molecular effects of ET-1 in PSC and addressed the question of how ET-1 expression in PSC is regulated. The experiments were built on earlier reports by our group and others, which had suggested an autocrine loop of ET-1. Specifically, ET-1 was previously shown to activate extracellular signal-regulated kinases (ERK) 1 and 2, and to enhance PSC contraction and migration^[11-13]. Our novel data deciphers ET-1 signalling in greater detail, and identifies, for the first time, target genes of ET-1 in PSC. Interestingly, these genes include not only α -SMA (which is directly linked to the activation process), but also two proinflammatory cytokines, interleukin (IL)-1 β and IL-6. Furthermore, our investigations revealed TGF- β 1 and TNF- α as potent inducers of ET-1 expression in PSC. Together, the data suggest that ET-1 is an important player in a network of proinflammatory and profibrogenic mediators that fosters interactions between inflammatory cells and PSC in a vicious cycle of inflammation and fibrosis.

MATERIALS AND METHODS

Materials

Iscove's modified Dulbecco's medium (IMDM) and all supplements for cell culture were obtained from Biochrom (Berlin, Germany), Nycodenz from Nycomed (Oslo, Norway), the rat-specific ET-1 enzyme immunometric assay (EIA) kit from Biotrend (Köln, Germany), and the 5-bromo-2'-deoxyuridine (BrdU) labelling and detection enzyme-linked immunosorbent assay kit as well as the polynucleotide kinase from Roche Diagnostics (Mannheim, Germany). Human connective tissue growth factor (CTGF) was delivered by EMP Genetech (Ingolstadt, Germany),

and recombinant cytokines [rat interferon (IFN)- γ and PDGF, human TNF- α and TGF- β 1] by R&D Systems (Minneapolis, MN, USA). TRIzol and all reagents used for reverse transcription and TaqmanTM real-time polymerase chain reaction (PCR) were from Applied Biosystems (Foster City, CA, USA). PVDF membrane was supplied by Millipore (Schwalbach, Germany), protein- and phospho-protein specific antibodies to ERK1/2 and p38 (all raised in rabbits) by New England BioLabs (Frankfurt, Germany), and Odyssey[®] blocking buffer, stripping buffer and secondary antibodies for immunoblotting by LI-COR (Bad Homburg, Germany). Immunoglobulins used in gel shift assays were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), DNA oligonucleotides from BioTeZ (Berlin, Germany), and [γ -³²P] ATP from Hartmann Analytic (Braunschweig, Germany). Collagenase, ET-1, tissue culture dishes (Corning plasticware), carboxyfluorescein succinimidyl ester (CFSE), as well as standard laboratory chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

PSC were isolated from the pancreas of male LEW.1W inbred rats by collagenase digestion of the organ and Nycodenz (120 g/L) density gradient centrifugation essentially as previously described^[14]. The cells were resuspended in IMDM supplemented with 17% foetal calf serum (FCS), 10 mL/L non-essential amino acids (dilution of a 100 \times stock solution), 10⁵ U/L penicillin and 100 mg/L streptomycin, and cultured at 37°C in a 5% CO₂ humidified atmosphere. All experiments were performed with cells that were passaged only once. Therefore, PSCs in primary culture were grown to subconfluency, harvested by trypsinization (usually, on day seven after isolation), and recultured at equal seeding densities.

Quantification of DNA synthesis

Cell proliferation was assessed by measuring incorporation of BrdU into newly synthesized DNA. Therefore, cells growing in 96-well plates were treated under serum-free conditions with ET-1 as indicated. At the time of ET-1 application, BrdU labelling was initiated by adding labelling solution at a final concentration of 10 μ mol/L (in the culture medium). Twenty-four hours later, labelling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

Cell migration assay

Serum-starved PSC were labelled with the fluorescence dye CFSE, which was dissolved in dimethyl sulfoxide and applied at a final concentration of 333 μ mol/L (chosen based on the results of assay optimization). The cells were incubated with the dye for 20 min in darkness at room temperature. Migration assays were performed under serum-free conditions in 24-transwell plates using inserts with a pore size of 8 μ m. Therefore, labelled cells were seeded at a density of 50 000 cells per well into the upper chamber, while ET-1 was added to the medium of the lower chamber as indicated. After an incubation period of 24 h, cells in the transwell inserts were removed by medium aspiration and mechanical

detachment from the upper side of the membrane. Cells adhering to the lower side of the filter or the bottom of the lower chamber were harvested by trypsinization and transferred into 96-well plates. Fluorescence intensity was then measured using a fluorescence microplate reader ($\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 517 \text{ nm}$).

Immunoblotting

Protein extracts from equal numbers of PSCs (pretreated as indicated) were prepared and subjected to immunoblot analysis as previously described^[14]. After electrophoresis, proteins were blotted onto PVDF membrane. The filters were rinsed in phosphate-buffered saline (PBS) and blocked for 1 h using Odyssey® Blocking Buffer, before primary antibodies were added to the blocking solution. One hour later, the blots were washed four times for 5 min in PBS containing 0.1% Tween 20 (PBS-T), and exposed to secondary antibody (IRDye® 800CW conjugated goat anti-rabbit IgG) for 1 h. Subsequently, the membranes were washed again (PBS-T, three times for 5 min), rinsed in PBS and scanned at a wavelength of 800 nm using an Odyssey® Infrared Imaging System. Signal intensities were quantified by means of the Odyssey® software version 3.0. Prior to reprobing with additional primary antibodies, the blots were treated with stripping buffer according to the instructions of the manufacturer.

Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Total RNA from cells pretreated as indicated was isolated with TRIzol reagent according to the manufacturer's instructions. Next, RNA was reverse transcribed into cDNA by means of TaqMan™ Reverse Transcription Reagents and random hexamer priming. Relative quantification of target cDNA levels by real-time PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using TaqMan™ Universal PCR Master Mix and the following Assay-on-Demand™ rat gene-specific fluorescently labelled TaqMan™ MGB probes: Rn00573960_g1 (CTGF), Rn00561129_m1 (ET-1), Rn00580432_m1 (IL-1 β), Rn00561420_m1 (IL-6), Rn00572010_m1 (TGF- β 1), and Rn01527838_g1 [hypoxanthine phosphoribosyl transferase (HPRT), used as house-keeping control gene]. The Custom TaqMan Gene Expression Assay specific for rat α -SMA (GenBank Accession Number: X06801) has been described previously^[11]. Following the guidelines of the manufacturer, PCR was performed under the following conditions: 95°C for 10 min, 50 cycles of 15 s at 95°C, 1 min at 60°C. The reactions were performed in triplicate, and repeated six times with independent samples. Relative expression of each mRNA compared with HPRT was calculated according to the equation $\Delta\text{Ct} = \text{Ct}_{\text{target}} - \text{Ct}_{\text{HPRT}}$. The relative amount of target mRNA in control cells and cells treated as indicated was expressed as $2^{-(\Delta\Delta\text{Ct})}$, where $\Delta\Delta\text{Ct}_{\text{treatment}} = \Delta\text{Ct}_{\text{ET-1}} - \Delta\text{Ct}_{\text{control}}$.

ET-1 polypeptide quantification

ET-1 polypeptide concentrations in PSC culture supernatant

were determined by EIA. Therefore, cells were seeded at a density of 50000 cells per well into 24-well plates. After an overnight incubation, medium was replenished and cells were exposed to different mediators as indicated. 48 h later, supernatants were collected and stored at -80°C until they were subjected to EIA analysis. The assay was performed according to the instructions of the manufacturer. FCS of the culture medium was also analyzed and found to contain no detectable amounts of ET-1 (data not shown).

Electrophoretic mobility shift assays (EMSA)

PSC growing in six-well plates were incubated for 16 h in FCS-free culture medium, before they were treated as indicated. Nuclear extracts were prepared as previously described^[14,15]. For EMSA experiments, nuclear proteins of 10^5 cells were incubated with double-stranded oligonucleotides which were end-labelled with [γ -³²P] ATP by polynucleotide kinase. The sequence of the activator protein (AP)-1 probe was 5'-CGCTTGA TGACTCAGCCGATC-3' (consensus binding motif underlined). To study inducible protein binding to the ET-1 promoter, the following probes derived from the rat promoter sequence^[16] (accession number: S76970) were applied: 5'-GATTGTCAGACGGCGGGCGTCTGCCTCTGAAG-3' (corresponding to bases -196 to -165), and 5'-AGCCGTGATTTCCTCTAGAGC-3' (-163 to -144). The first oligonucleotide contains two consensus motifs for Smad transcription factors (underlined), which in the homologous region of the human ET-1 promoter have been identified as core sequences within a TGF- β response element^[17]. The underlined bases of the second oligonucleotide correspond to a putative, although imperfect NF- κ B/c-Rel binding site. Binding reaction and supershift analysis (initiated by adding 0.5 μ g antibody) were performed as described^[14,15]. Protein-DNA complexes were analyzed by electrophoretic separation on a 6% non-denaturing polyacrylamide gel. Dried gels were exposed to X-ray film.

Statistical analysis

Results are expressed as mean \pm SE for the indicated number of separate cultures per experimental protocol. Statistical significance was analyzed using Wilcoxon's rank sum test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of ET-1 on PSC functions and gene expression

Initial studies dealt with the biological effects of ET-1 in PSC. In accordance with previous reports^[12,13], ET-1 stimulated migration but had no effect on PSC proliferation (Figure 1). Next, we analyzed regulation of gene expression by ET-1, using real-time PCR. Interestingly, ET-1 significantly enhanced expression of α -SMA, suggesting direct stimulation of myofibroblastic differentiation (Figure 2). Furthermore, we focussed on cytokines and growth factors that have previously been implicated in autocrine or paracrine maintenance and

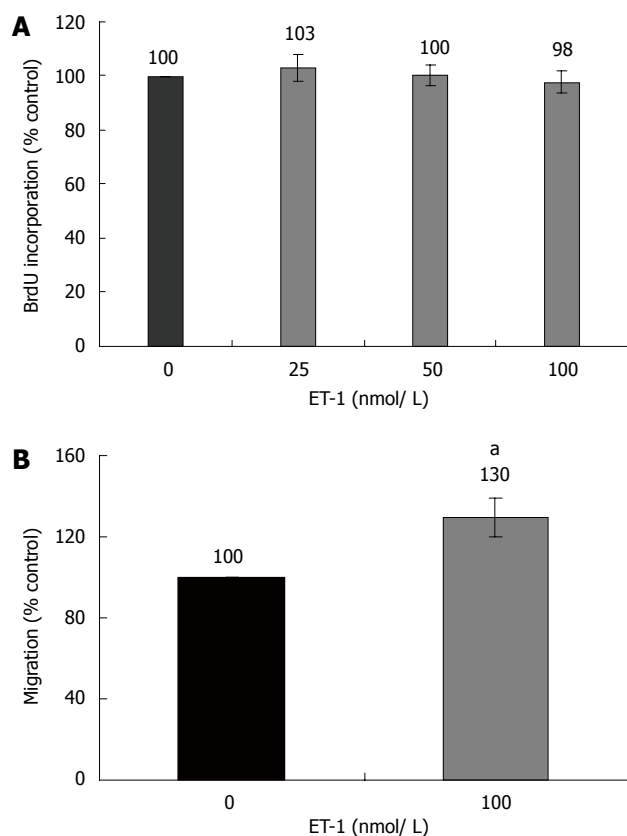


Figure 1 Effects of ET-1 on PSC proliferation and migration. A: PSCs growing in 96-well plates were treated, under FCS-free conditions, with ET-1 at the indicated concentrations for 24 h. Cell proliferation was assessed with the BrdU DNA-incorporation assay. One hundred percent BrdU incorporation corresponds to untreated PSCs; B: CFSE-labelled cells were seeded into the upper chamber of 24-transwell plates, whereas ET-1 (100 nmol/L) was added to the lower chamber as indicated. Cell migration under FCS-free conditions was analyzed as described in the "Materials and Methods" section. One hundred percent cell migration corresponds to the intensity of the fluorescence signal received from untreated PSCs. Data in (A) and (B) are presented as mean \pm SE ($n \geq 6$ separate cultures); ^a $P < 0.05$ vs control cultures.

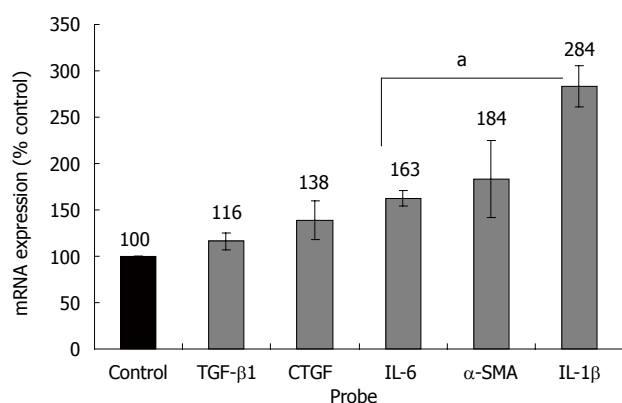


Figure 2 Effects of ET-1 on PSC gene expression. PSC growing in 6-well plates were starved of serum for 1 h before they were stimulated with ET-1 (100 nmol/L) as indicated. The mRNA expression of TGF-β1, CTGF, IL-6, α-SMA, IL-1β and the housekeeping gene HPRT was analyzed by real time PCR, and relative amounts of target mRNA were calculated. One hundred percent mRNA expression of each gene corresponds to untreated PSC. Data of 6 independent experiments (with triplicate samples) were used to calculate mean \pm SE; ^a $P < 0.05$ vs control cultures.

enhancement of PSC activation^[6-8,18,19]. ET-1 significantly increased expression of two proinflammatory mediators,

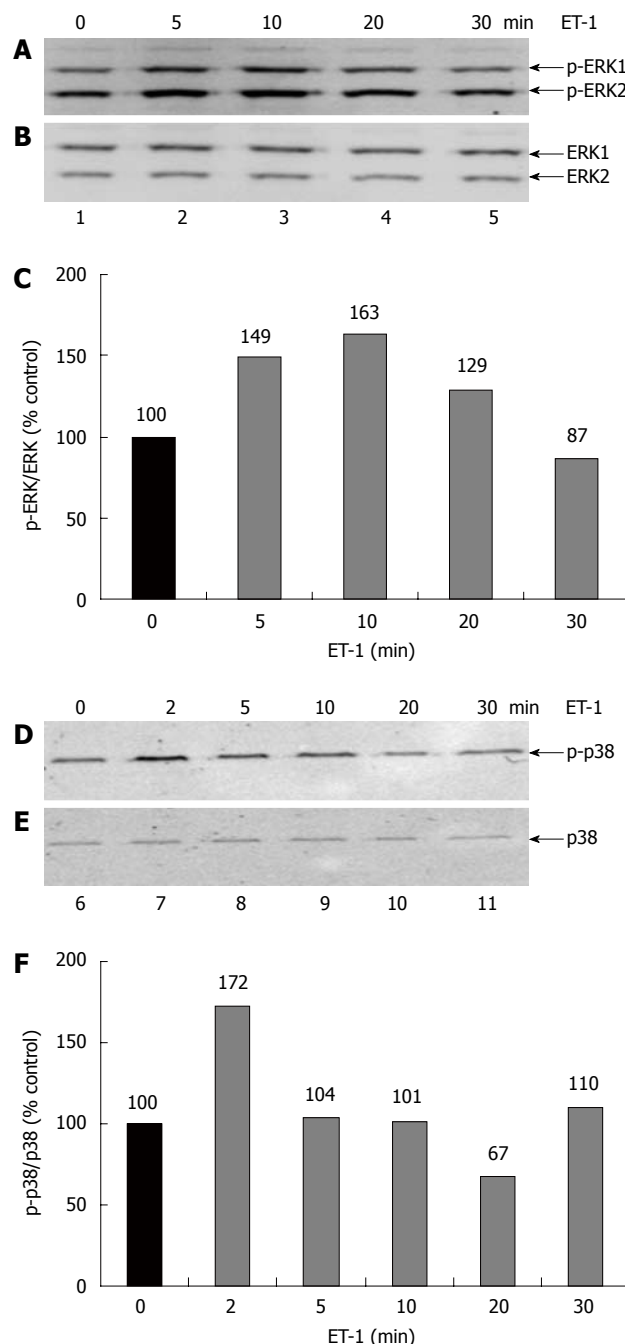


Figure 3 ET-1 induces phosphorylation of ERK1/2 and p38. PSC were starved of serum for 16 h before they were stimulated with ET-1 (100 nmol/L) for the indicated periods of time (A, D). ERK1/2 and p38 phosphorylation were analyzed by immunoblotting (B, E). Reprobing of the blots with anti-ERK1/2 and anti-p38 protein-specific antibodies revealed no systematic differences in the ERK1/2 and p38 amount among the samples (C, F). Fluorescence signal intensities of phospho (p)-ERK1/2, ERK1/2 protein, phospho (p)-p38 and p38 protein were quantified using Odyssey[®] software version 3.0. Subsequently, the ratios of phospho-ERK/ERK protein (C) and phospho-p38/p38 (F) protein were calculated. A ratio of one hundred percent corresponds to unstimulated control cultures. The data shown are representative of three independent experiments.

IL-1β and IL-6, but not of TGF-β1 and CTGF, two potent inducers of collagen synthesis.

Transduction of the ET-1 signal in PSC

Extending our previous pilot study^[11], we observed that ET-1 induced a rapid and transient phosphorylation of two

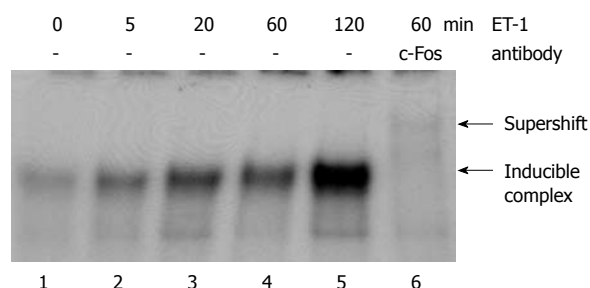


Figure 4 ET-1 activates DNA binding of AP-1. Serum-starved PSC growing in 6-well plates were incubated with ET-1 (100 nmol/L) for the indicated periods of time. Nuclear extracts were subjected to EMSA analysis using a [32 P]-labelled oligonucleotide with an AP-1 binding motif. Supershift analysis was performed by incubating the binding reaction with a c-Fos-specific antibody. Shifted complex and supershifted band (lane 6, faint since antibody binding may diminish binding of c-Fos to the oligonucleotide) are pointed out by arrows. Results are representative of three independent experiments.

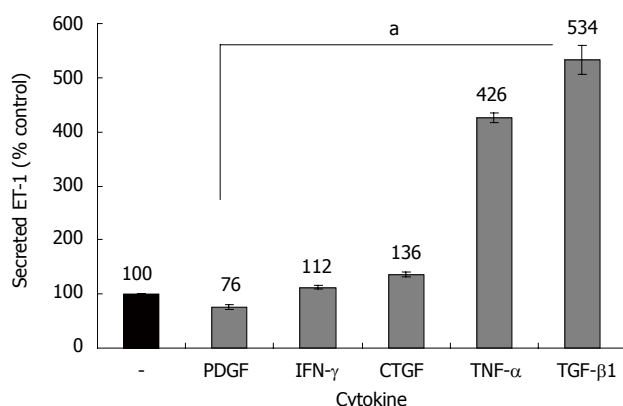


Figure 5 Regulation of ET-1 expression in PSC. PSC growing in 24-well plates were treated with PDGF (10 μ g/L), IFN- γ (100 μ g/L), CTGF (2 mg/L), TNF- α (10 μ g/L), and TGF β 1 (5 μ g/L) for 48 h as indicated. Afterwards, supernatants were collected and subjected to EIA analysis of the ET-1 content. Data from six separate cultures were used to calculate mean \pm SE; $^aP < 0.05$ vs control cultures.

distinct types of mitogen-activated protein (MAP) kinases, ERK1/2 and p38 (Figure 3). EMSA experiments revealed that ET-1 stimulation strongly increased DNA binding of the transcription factor complex AP-1 (Figure 4). As indicated by the results of a supershift analysis, the DNA/protein complex contained the AP-1 subunit c-Fos (Figure 4, lane 6). ET-1 stimulation of PSC was also associated with some enhancement of the DNA binding of NF- κ B. The relevance of this finding, however, remained uncertain since activation of NF- κ B was quite weak (data not shown).

Regulation of ET-1 gene expression

In the course of the studies, four mediators involved in PSC activation (PDGF, CTGF, TNF- α and TGF- β 1)^[2] as well as one antagonist, the antiproliferative and antifibrotic cytokine IFN- γ ^[20], were tested regarding their effects on ET-1 synthesis in PSC (Figure 5). TGF- β 1 and TNF- α strongly stimulated secretion of ET-1 by the cells, whereas PDGF displayed the opposite effect. After application of CTGF or IFN- γ , statistically significant but nevertheless small increases of ET-1 levels in culture supernatants were observed.

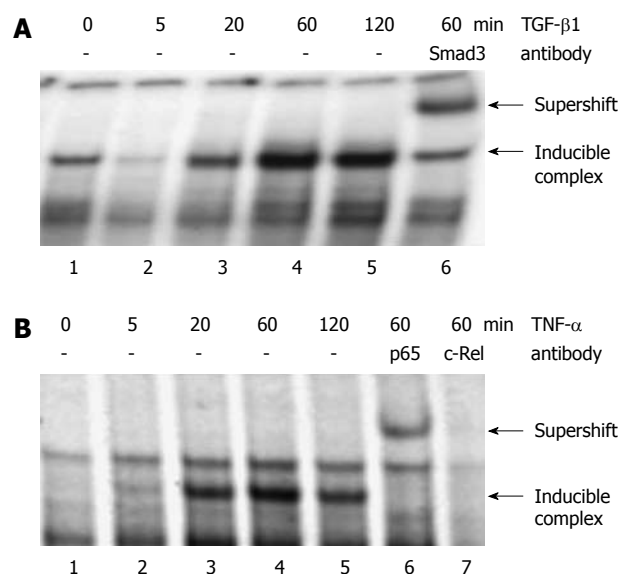


Figure 6 Effects of TGF- β 1 and TNF- α on protein binding to the ET-1 promoter. Serum-starved PSC growing in 6-well plates were incubated with (A) TGF- β 1 (5 μ g/L) and (B) TNF- α (10 μ g/L) for the indicated periods of time. Nuclear extracts were subjected to EMSA analysis using [32 P]-labelled oligonucleotides derived from the ET-1 promoter as described in the "Materials and Methods" section. Supershift analysis was performed by incubating binding reactions with the indicated protein-specific antibodies. Inducible complexes and supershifted bands (lane 6 in A and B) are pointed out by arrows. Addition of c-Rel antibody (B, lane 7) weakened protein binding to the oligonucleotide, suggesting the presence of the c-Rel protein in the complex (competitive binding of antibody and oligonucleotide to c-Rel). Results in (A) and (B) are representative of three independent experiments.

To study the molecular mechanisms underlying the effects of TGF- β 1 and TNF- α , EMSA experiments using oligonucleotides derived from the rat ET-1 promoter were performed. TGF- β 1 induced the binding of a transcription factor complex containing Smad3 to a DNA sequence that corresponds to the TGF- β response element of the human ET-1 gene^[17] (Figure 6A). TNF- α stimulation of PSC was associated with enhanced protein binding to a putative NF- κ B site in the ET-1 promoter. Antibodies to the NF- κ B subunits p65RelA and c-Rel shifted the protein/DNA complex or weakened DNA/protein interaction, suggesting the presence of both proteins (Figure 6B).

DISCUSSION

PSC are the principal effector cells in pancreatic fibrosis^[2,21]. Progressive replacement of pancreatic parenchyma by connective tissue promotes the development of exocrine and endocrine organ failure in the course of chronic pancreatitis. Furthermore, paracrine interactions between stroma cells and pancreatic cancer cells have been implicated in accelerated tumour growth and resistance to chemotherapeutics^[22-24]. PSC are therefore considered attractive targets for the adjuvant treatment of pancreatic cancer based on inhibition of fibrogenesis. One important prerequisite for such an antifibrotic therapy is to understand the molecular processes underlying PSC activation in pancreatitis and cancer.

We and others have recently shown that ET-1 acts on PSCs in an autocrine fashion^[11-13]. In agreement with a previous study^[13], we have now found that application of ET-1 to PSC cultures promotes migration. Furthermore, our novel data indicate that ET-1 directly fosters exhibition of an activated, myofibroblastic phenotype, since α -SMA expression in PSC was enhanced. As previously suggested by Masamune *et al*^[13], exogenous ET-1 did not stimulate cell proliferation. Nevertheless, the dual-specific ET receptor antagonist bosentan significantly inhibits PSC growth^[11], suggesting a contribution of endogenous ET-1 to the induction of mitogenesis. Together, these findings clearly point to a profibrogenic role of the ET-1/ET receptor axis in PSC. Additional support for this conclusion comes from our studies at the molecular level: ET-1 induced an activation of the MAP kinases ERK1/2, p38 and the transcription factor AP-1. All these signalling molecules have previously been identified as key components of the intracellular network triggering PSC activation^[14,15,25]. Furthermore, ET-1 significantly stimulated expression of two cytokines that have previously been suggested as autocrine enhancers of PSC activation, IL-1 β and IL-6^[18,19]. Since both cytokines are well-established pro-inflammatory mediators, our data also implicate, for the first time, ET-1 in the enhancement of local inflammatory reactions in the pancreas.

In the present study, experiments aimed at elucidating regulation of ET-1 gene expression in PSC revealed that TGF- β 1 and TNF- α strongly enhanced the release of ET-1 by culture-activated PSC. Based on our molecular studies, Smad transcription factors and NF- κ B are likely to be involved in the induction of ET-1 expression by TGF- β 1 and TNF- α , respectively. Although both cytokines have previously been shown to stimulate ET-1 synthesis in other types of cells^[17,26], this finding is interesting since it suggests the existence of a regulatory network in which TGF- β 1 and TNF- α exert their biological effects on stellate cells in part through the ET-1/ET receptor axis. Given that ET-1 induces expression of IL-1 β /IL-6, and TGF- β 1 is secreted by PSC^[27], a vicious cycle may develop which leads to enhanced inflammation and progressive fibrosis.

Unexpectedly, IFN- γ displayed a small stimulatory effect on ET-1 release by PSC, although it inhibited expression of the ET-1 gene in a cell line of immortalized PSC^[28]. Here and in the case of PDGF, which diminished ET-1 synthesis, the underlying molecular mechanisms warrant further investigation.

As indicated by the results of our previous study^[11], interruption of autocrine and paracrine loops at the level of ET-1 action represents a suitable approach to target activated PSC. However, the antifibrotic efficiency of the ET receptor antagonist bosentan in our rat model of severe chronic pancreatitis^[29] was quite limited. Based on our previous and novel data, we therefore suggest that in future preclinical studies ET-1 receptor antagonists should be combined with drugs interfering with a different critical step of fibrogenesis and inflammation, such as transduction of profibrogenic signals in PSC.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Mrs. Helga Schulze and Mrs. Katja Bergmann.

COMMENTS

Background

Fibrosis is a key feature of chronic pancreatitis and pancreatic cancer. The extensive deposition of extracellular matrix (ECM) proteins fosters the development of an exocrine and endocrine organ insufficiency, and accelerates progression of the tumour. Pancreatic stellate cells (PSC) are the principal effector cells in pancreatic fibrosis. They are activated by profibrogenic stimuli, which include, for example, cytokines and ethanol metabolites. So far, there are no specific therapies available to interfere with dysregulated fibrogenesis in the diseased pancreas.

Research frontiers

The molecular mechanisms underlying induction and maintenance of PSC activation are incompletely understood. This deficit hampers to some degree the development of therapeutic approaches aimed at the inhibition of pancreatic fibrosis in pancreatitis and cancer.

Innovations and breakthroughs

The results of this study provide molecular insights into endothelin-1 (ET-1) action in PSC. The data indicate that ET-1 stimulates exhibition of an activated stellate cell phenotype. The intracellular signalling molecules found to be activated by ET-1, ERK1/2, p38 and AP-1, have previously been linked to the process of stellate cell activation. With IL-1 β and IL-6, two proinflammatory cytokines were identified as target genes of ET-1, suggesting for the first time a direct link between inflammation and fibrosis at the level of ET-1 action. In addition, studies on the regulation of ET-1 expression revealed that transforming growth factor- β 1 and tumour necrosis factor- α strongly stimulate ET-1 secretion by PSC. Together, these data suggest that ET-1 is part of a network of proinflammatory and profibrogenic mediators that fosters interactions between inflammatory cells and PSC, ultimately enhancing inflammation and fibrosis.

Applications

The results encourage further studies aimed at the inhibition of fibrogenesis by targeting the ET-1/ET receptor axis. The authors suggest that combinations of ET receptor antagonists with drugs interfering with independent steps of stellate cell activation should be tested in animal models of pancreatic fibrosis.

Terminology

Pancreatic fibrosis refers to the process of progressive replacement of pancreatic tissue by connective tissue. PSC are fibroblast-like cells that produce most of the ECM in the diseased organ. ET-1 is a polypeptide originally identified as an endothelial cell-derived hormone with vasoconstrictive activity. Additional cellular origins and functions of ET-1 have been described.

Peer review

The authors revealed alteration of several factors involved in inflammation and fibrogenesis focusing on ET-1 action in isolated PSC. The study is well designed and provided some interesting results of scientific value, implying future therapeutic strategy using ET-1 receptor antagonists against chronic pancreatitis.

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S- Editor Tian L L- Editor Webster JR E- Editor Ma WH

ORIGINAL ARTICLES

Measuring Ca^{2+} influxes of TRPC1-dependent Ca^{2+} channels in HL-7702 cells with Non-invasive Micro-test Technique

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Supported by The National Natural Science Foundation of China, No. 30270532 and No. 30670774; Tsinghua-Yue-Yuen Medical Science Foundation, No. 20240000531 and No. 20240000547

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Received: May 8, 2009 Revised: July 21, 2009

Accepted: July 28, 2009

Published online: September 7, 2009

CONCLUSION: In HL-7702 cells, there is a type of TRPC1-dependent Ca^{2+} channel, which could be detected *via* NMT and inhibited by La^{3+} .

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Key words: Non-invasive Micro-test Technique; Ca^{2+} channels; Transient Receptor Potential Canonical 1; Gene expression; HL-7702 cells

Peer reviewers: Zong-Jie Cui, PhD, Professor, Institute of Cell Biology, Beijing Normal University, 19 Xijiekou Wai Street, Beijing 100875, China; You-Yong Lu, Professor, Beijing Molecular Oncology Laboratory, Peking University School of Oncology and Beijing Institute for Cancer Research, #52, Fucheng Road, Haidian District, Beijing 100036, China

Zhang ZY, Wang WJ, Pan LJ, Xu Y, Zhang ZM. Measuring Ca^{2+} influxes of TRPC1-dependent Ca^{2+} channels in HL-7702 cells with Non-invasive Micro-test Technique. *World J Gastroenterol* 2009; 15(33): 4150-4155 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4150.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4150>

Abstract

AIM: To explore the possibility of using the Non-invasive Micro-test Technique (NMT) to investigate the role of Transient Receptor Potential Canonical 1 (TRPC1) in regulating Ca^{2+} influxes in HL-7702 cells, a normal human liver cell line.

METHODS: Net Ca^{2+} fluxes were measured with NMT, a technology that can obtain dynamic information of specific/selective ionic/molecular activities on material surfaces, non-invasively. The expression levels of TRPC1 were increased by liposomal transfection, whose effectiveness was evaluated by Western-blotting and single cell reverse transcription-polymerase chain reaction.

RESULTS: Ca^{2+} influxes could be elicited by adding 1 mmol/L CaCl_2 to the test solution of HL-7702 cells. They were enhanced by addition of 20 $\mu\text{mol/L}$ noradrenalin and inhibited by 100 $\mu\text{mol/L}$ LaCl_3 (a non-selective Ca^{2+} channel blocker); 5 $\mu\text{mol/L}$ nifedipine did not induce any change. Overexpression of TRPC1 caused increased Ca^{2+} influx. Five micromoles per liter nifedipine did not inhibit this elevation, whereas 100 $\mu\text{mol/L}$ LaCl_3 did.

INTRODUCTION

Changes in the concentration of Ca^{2+} in the cytoplasmic space play a central role in intracellular signaling pathways in liver cells, including glucose, fatty acid, amino acid and xenobiotic metabolism, bile acid secretion, protein synthesis and secretion, the movement of lysosomes and other vesicles, the cell cycle and cell proliferation, and apoptosis and necrosis^[1-4]. In earlier reports, it has been shown that ligand-gated, store-operated, receptor-activated, and stretch-activated Ca^{2+} -permeable channels are expressed in hepatocytes and in liver cell lines. No voltage-operated Ca^{2+} channels (VOCCs) have been detected^[5-8]. There is increasing evidence that members of the canonical subgroup of Transient Receptor Potential (TRP) proteins constitute tetramers of both receptor-activated and store-operated Ca^{2+} channels (SOCs)^[9-11], and Transient Receptor Potential Canonical 1 (TRPC1) is considered as one of the most likely candidates in forming Ca^{2+} channels in mammalian cells^[12-15].

The Non-invasive Micro-test Technique (NMT) was developed in the late 20th century, and is a new

technology for obtaining dynamic information on specific ionic/molecular activities on material surfaces, non-invasively. This technique incorporates different temporal and spatial resolution domains from other traditional methods, and its 3-dimensional measurement capability enables us to observe the physiological characteristics of biological phenomena that would be difficult or even impossible with other techniques^[16]. To date, Ca^{2+} , H^+ , K^+ , Cl^- , NO^- , Mg^{2+} , Cd^{2+} , Al^{3+} , and O_2 have been detected as sensors for ionic/molecular species.

In the present study, we used NMT to study the Ca^{2+} influxes elicited by extracellular elevations of Ca^{2+} concentration, and the inhibitory effects of several Ca^{2+} channel blockers, to investigate the role of TRPC1 in regulating Ca^{2+} fluxes in HL-7702 cells.

MATERIALS AND METHODS

Materials

Nifedipine, noradrenalin, protease inhibitor Cocktail, Fast Red TR, Naphthol AS-MX phosphate, and Calcium Ionophore I [Cocktail A were bought from the Sigma-Aldrich Company (Catalog Number: 21048)]. Lipofectamine 2000 was purchased from Invitrogen. The TRPC1 polyclonal antibody was acquired from the Abnova Company. Peroxidase-conjugated secondary antibody was obtained from the Beijing Zhongshan Golden Bridge Co.. All the other reagents were of reagent grade.

Cell culture, plasmid construction and transfection

The human liver cell line, HL-7702, bought from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, was maintained in RPMI-1640 containing 10% FBS, 1% Penicillin, and Streptomycin. Plasmid pBS-T-TRPC1 was constructed and verified as previously described^[17]. HL-7702 cells were grown to 75%-80% confluence in 35 mm dishes in advance and transfection was carried out with 1 $\mu\text{g}/\text{mL}$ of the recombinant plasmid and Lipofectamine 2000 according to the manufacturer's protocol.

Measurements of extracellular Ca^{2+} influxes

Measurements of net influxes of Ca^{2+} were performed using NMT (BIO-001A, Younger USA Sci. & Tech. Co., Amherst, MA, USA; Applicable Electronics Inc., Forestdale, MA, USA; and ScienceWares Inc., East Falmouth, MA, USA). The electrode was controlled to move with an excursion of 10 μm at a programmable frequency in the range of 0.3-0.5 Hz; this minimized mixing of the bathing saline.

To construct the microelectrodes, borosilicate micropipettes (2-4 μm aperture, XYPG120-2, Xuyue (Beijing) Science and Technology Co., Ltd., Beijing, China) were silanized with tributylchlorosilane and the tips filled with Calcium Ionophore I - Cocktail A. An Ag/AgCl wire electrode holder (XYEH01-1) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. Only electrodes with Nernstian slopes > 25 mV were used. Ca^{2+} fluxes were calculated by Fick's law of

diffusion: $J_0 = -[D \times (dC/dX)]$ where J_0 represents the net Ca^{2+} flux (in $\mu\text{mol}/\text{cm}$ per second), D is the self-diffusion coefficient for Ca^{2+} (in cm^2/s), dC is the difference value of Ca^{2+} concentrations between the two positions, and dX is the 10 μm excursion over which the electrode moved in our experiments. Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner, and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software.

Single cell reverse transcription-polymerase chain reaction (RT-PCR)

Single cell RT-PCR was performed to determine whether the cells measured by NMT were successfully transfected, using a previously described method, with some modifications^[18]. Directly after the Ca^{2+} influx assay, the contents of tested cell were aspirated into a microelectrode. The tip of the electrode was then broken in a PCR tube and stored at -80°C until use. Reverse transcription was carried out using a kit from TIANGEN (Beijing, China) according to the manufacturer's instructions. The first PCR was performed using specific primers (Forward: GCAATGATACCTTCCATTTCGTTTC; Reverse: CGATGCACTAGGCAGCAGATC) and the following conditions: pre-denaturation at 94°C for 3 min; followed by 30 cycles of denaturation at 94°C for 30 s and annealing at 60°C for 30 s, then synthesis at 72°C for 60 s; the last step was extension at 72°C for 5 min. After the first PCR, 1 μL of the reaction products was used as the template for a secondary PCR with the same conditions as above and 25 cycles. The predicted size of the TRPC1 amplicons were 455 bp and reaction products were confirmed and analyzed by agarose gel electrophoresis.

Western blotting

Total proteins were obtained from cultured cells by using lysis buffer (35 mmol/L Tris at pH 7.4, 0.4 mmol/L EDTA, 10 mmol/L MgCl_2 , 0.1% protease inhibitor Cocktail). For western blotting analysis, 20 μg proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto to polyvinylidene difluoride membranes for 2 h at 20 V. The membranes were then blocked for 2 h with blocking solution (5% bovine serum albumin) and probed with anti-TRPC1 antibodies. The primary antibodies were incubated for 1 h at room temperature and, after washing, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. Finally, the proteins on the membranes were dyed by staining solution containing Fast Red TR and Naphthol AS-MX phosphate. Immunoblots were then scanned to obtain images.

Statistical analysis

Data were expressed as mean \pm SD of n cells from at least six cell culture dishes. The statistical significance of diversities between means was determined using the DUNNET t -test. A value of $P < 0.05$ was considered significant.

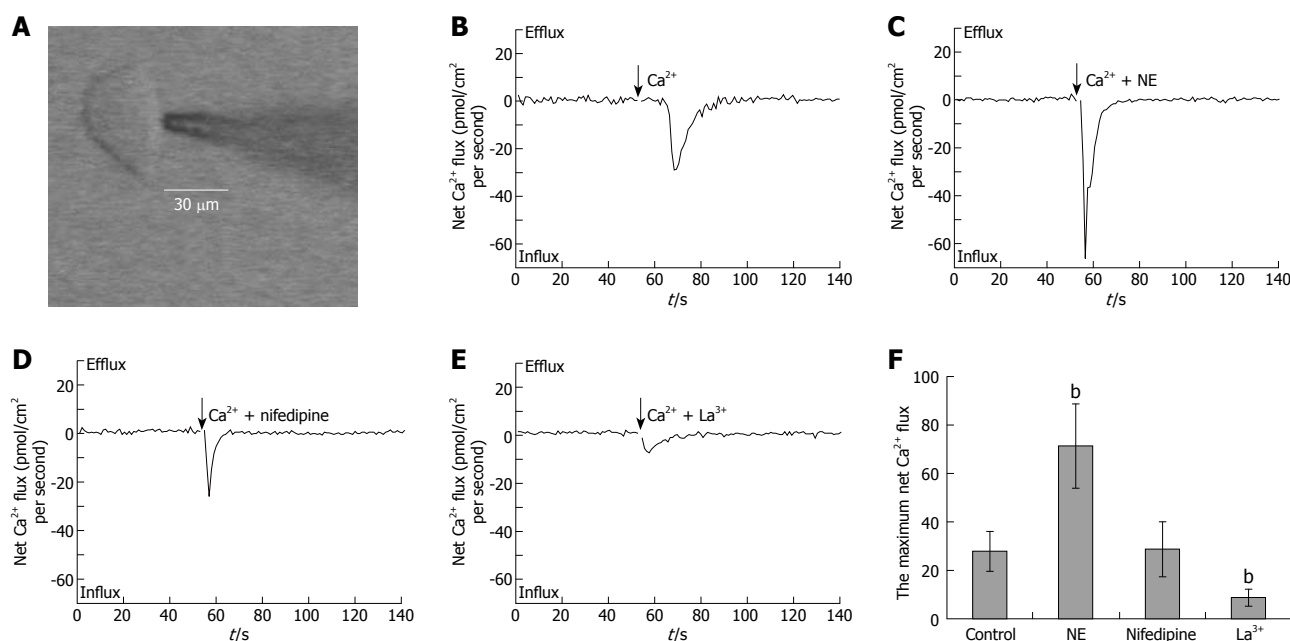


Figure 1 The Ca^{2+} fluxes of HL-7702 cells. A: A screen-printed picture of a cell measured by NMT; B: Net Ca^{2+} fluxes of a HL-7702 cell. The maximum values were 28.2 ± 8.2 pmol/cm² per second ($n = 6$); C: Net Ca^{2+} fluxes of a noradrenalin-treated HL-7702 cell. The maximum values were 71.7 ± 17.5 pmol/cm² per second ($n = 6$); D: Net Ca^{2+} fluxes of a nifedipine-treated HL-7702 cell. The maximum values were 29.0 ± 11.3 pmol/cm² per second ($n = 6$); E: Net Ca^{2+} fluxes of a La^{3+} -treated HL-7702 cell. The maximum values were 9.0 ± 3.5 pmol/cm² per second ($n = 6$); F: Bar graph of the maximum net Ca^{2+} influxes in four groups. $^bP < 0.01$, control group vs noradrenalin-treated or La^{3+} -treated group.

RESULTS

The Ca^{2+} influxes of HL-7702 cells were measured by NMT

Before the experiment, 35 mm dishes with pre-dispersed normal HL-7702 cells were perfused with test solution containing (in mmol/L): 2.3 NaHCO_3 , 27 Na_2SO_4 , 9.7 KCl, 61.1 MgCl_2 , and 366.7 NaCl. The Ca^{2+} influxes were then measured by NMT. The background noise was recorded for three min before 1 mmol/L CaCl_2 was added to elicit an inward Ca^{2+} current. As shown in Figure 1A, the Ca^{2+} selective microelectrode moved between two positions close to the tested cells constantly to acquire experimental data. Net Ca^{2+} fluxes are depicted in Figure 1B, a giant wave trough emerged shortly after CaCl_2 was added.

Effects of noradrenalin, nifedipine and La^{3+} on Ca^{2+} influxes of HL-7702 cells

To further identify the property of the Ca^{2+} influxes of HL-7702 cells, three drugs were selected as tools in the following experiments. After background noise was recorded for three min, a Ca^{2+} channel agonist, noradrenalin (20 $\mu\text{mol/L}$), or two types of inhibitors, nifedipine (5 $\mu\text{mol/L}$) or LaCl_3 (100 $\mu\text{mol/L}$) were added into the test solution together with 1 mmol/L CaCl_2 . The effects of the three drugs on Ca^{2+} influxes are shown in Figure 1C-E. Net Ca^{2+} fluxes were significantly influenced by noradrenalin and La^{3+} ; however, nifedipine did not induce any change. A bar graph of the maximum net Ca^{2+} fluxes in the four groups (three drug-treated groups and control group) is depicted in Figure 1F; these experiments were repeated six times ($n = 6$).

Effects of TRPC1-transfection on Ca^{2+} influxes of HL-7702 cells

When TRPC1-transfected HL-7702 cells were measured by NMT, the maximum net Ca^{2+} influxes increased to 48.9 ± 6.4 pmol/cm² per second ($n = 6$) and a deeper wave trough was observed (Figure 2A). The bar graph shown in Figure 2B shows that the statistical difference between the TRPC1-transfected group and the control group was significant ($P < 0.01$). Single cell RT-PCR and western blotting were performed after NMT experiments. The results of agarose gel electrophoresis and SDS-PAGE are shown in Figure 2C and D, respectively. They demonstrated that TRPC1-expressions in tested cells were elevated after transfection.

Effects of nifedipine and La^{3+} on Ca^{2+} influxes of TRPC1-transfected HL-7702 cells

TRPC1-transfection induced increases in Ca^{2+} influxes. To investigate the effects of nifedipine and La^{3+} on these increased Ca^{2+} influxes, the two drugs were added into the test solution together with 1 mmol/L CaCl_2 . As shown in Figure 3A-C, the maximum net Ca^{2+} fluxes maintained an average of 44.0 ± 5.7 pmol/cm² per second ($n = 6$) when 5 $\mu\text{mol/L}$ nifedipine was applied, whereas 100 $\mu\text{mol/L}$ LaCl_3 made reduced the maximum value to 7.6 ± 1.9 pmol/cm² per second ($n = 6$). A significant statistical difference only existed between the TRPC1 + La^{3+} -treated group and the TRPC1-transfected group ($P < 0.01$). Single cell RT-PCR and western blotting were performed after NMT experiments to confirm the increased TRPC1 expression (data not shown).

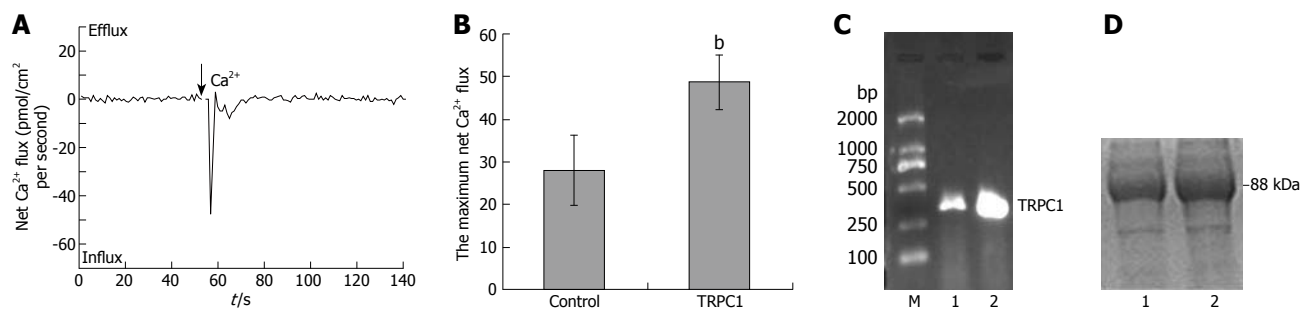


Figure 2 TRPC1-transfection influences Ca^{2+} influxes of HL-7702 cells. A: Net Ca^{2+} fluxes of a TRPC1-transfected cell. The maximum values were 48.9 ± 6.4 pmol/cm² per second ($n = 6$); B: Bar graph of the maximum net Ca^{2+} fluxes in the control group and the TRPC1-transfected group. The statistical difference between the two groups was significant ($^bP < 0.01$); C: Single cell RT-PCR products from cultured HL-7702 cells and TRPC1-transfected cells using primers for human TRPC1 (455 bp); lane M: DNA marker, lane 1: TRPC1 amplified from HL-7702 cells, lane 2: TRPC1 amplified from transfected cells; D: TRPC1 protein was detected in cultured HL-7702 cells and TRPC1-transfected cells using western-blotting analysis; lane 1: TRPC1 protein extracted from HL-7702 cells, lane 2: TRPC1 protein from transfected cells. The molecular mass of TRPC1 is 88 kDa.

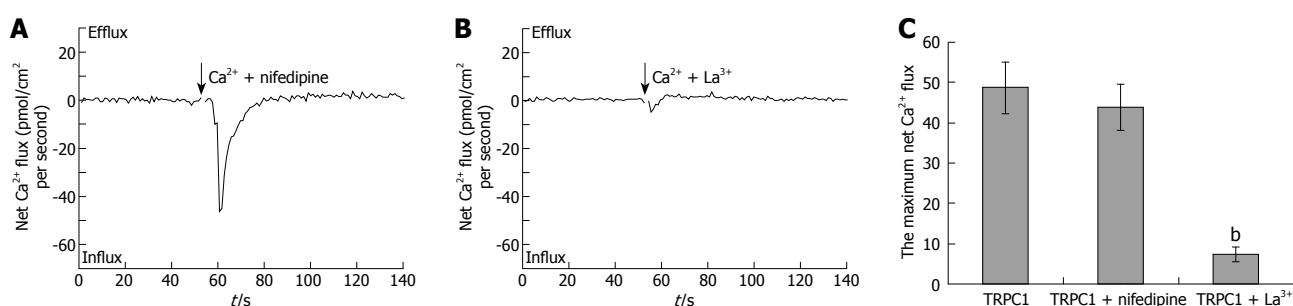


Figure 3 Nifedipine and La^{3+} influence Ca^{2+} influxes of TRPC1-transfected HL-7702 cells. A: Net Ca^{2+} fluxes of a TRPC1 + nifedipine-treated cell. The maximum values were 44.0 ± 5.7 pmol/cm² per second ($n = 6$); B: Net Ca^{2+} fluxes of a TRPC1 + La^{3+} -treated cell. The maximum values were 7.6 ± 1.9 pmol/cm² per second ($n = 6$); C: Bar graph of the maximum net Ca^{2+} fluxes in the TRPC1-transfected group, TRPC1 + nifedipine-treated group, and TRPC1 + La^{3+} -treated group. A significant statistical difference only existed between the TRPC1-transfected group and the TRPC1 + La^{3+} -treated group ($^bP < 0.01$).

DISCUSSION

Initially, we detected Ca^{2+} influxes in HL-7702 cells using NMT when 1 mmol/L CaCl_2 was added to the test solution and the extracellular Ca^{2+} concentration markedly changed. Ca^{2+} influx could be influenced by two drugs, noradrenalin, and LaCl_3 ; however nifedipine did not induce any change.

As a known neurotransmitter, noradrenalin can act through α_1 -adrenoceptors to activate phospholipase C. This generates inositol 1,4,5-trisphosphate (IP_3) within the cell, which in turn mediates the rise of Ca^{2+} concentration by release from intracellular stores^[19,20] and opening of receptor operated Ca^{2+} entry at the plasma membrane^[21-23]. Thus, the increase in Ca^{2+} influx induced by noradrenalin together with 1 mmol/L external CaCl_2 could be explained if receptor operated Ca^{2+} channels played a central role in these experiments. On the other hand, Ca^{2+} influxes were prominently inhibited by La^{3+} . Analogs often effect their action through competitive inhibition with their common receptors or channels. La^{3+} has a similar size of ionic radius to that of Ca^{2+} , which enables La^{3+} to compete with Ca^{2+} , which makes La^{3+} a non-selective Ca^{2+} channel blocker. In addition, a study showed that La^{3+} could inhibit both influx and efflux of Ca^{2+} in lacrimal cells^[24], which was consistent with the present study. Most importantly, nifedipine, as an antagonist of L-type Ca^{2+} channels, did not induce any change in Ca^{2+} influxes, which would indicate that

no VOCCs existed or that transmembrane Ca^{2+} influxes elicited by external Ca^{2+} did not pass through VOCCs in HL-7702 cells.

TRPC1 is one of seven members of the TRPC sub-family of non-selective cation channels and is expressed in a wide variety of cell types and tissues^[25-27]. It is likely that TRPC1 plays a significant part in intracellular Ca^{2+} homeostasis. In salivary gland cells, the current through the endogenous SOCs was the same as the membrane current which was activated by the depletion of intracellular Ca^{2+} stores in cells in which TRPC1 was ectopically expressed and the endogenous SOCs were decreased by transfection with antisense TRPC1^[28]. In kidney epithelial cells, a new receptor-operated channel formed by heteromeric assembly of TRPP2 and TRPC1 subunits was discovered^[29]. In T cells, intracellular Ca^{2+} elevation induced by $\Delta 9$ -tetrahydrocannabinol was attributable entirely to extracellular Ca^{2+} influxes, which were not dependent on store depletion, but mediated through TRPC1 channels^[30]. The results of the second part of our study do not conflict with these reports. In HL-7702 cells, overexpression of TRPC1 causes an increase of Ca^{2+} influxes induced by adding external Ca^{2+} , and the non-selective Ca^{2+} channel blocker, La^{3+} , can attenuate this elevation. In summary, there is a TRPC1-dependent Ca^{2+} -type channel(s), either receptor-activated or store-operated present in HL-7702 cells, which can be inhibited by La^{3+} .

Taken together, NMT is a powerful tool for ion

channel research, which has been effectively applied in various systems^[31-33]. We used NMT to explore the properties of Ca^{2+} channels, and found that there was a TRPC1-dependent Ca^{2+} -type channel(s), which could be detected *via* NMT and inhibited by La^{3+} , in HL-7702 cells.

COMMENTS

Background

Ca^{2+} plays an important role in intracellular signaling pathways and Transient Receptor Potential Canonical 1 (TRPC1) is considered as one of the most likely candidates in forming Ca^{2+} channels in mammalian cells. As a technology to obtain dynamic information of specific ionic/molecular activities on material surfaces non-invasively, Non-invasive Micro-test Technique (NMT) is being increasingly applied to study characters of ion channels.

Research frontiers

TRPC1 has been verified as a molecular candidate or a regulator of Ca^{2+} channels in several mammalian cells. However, the role of TRPC1 in normal human liver cells has not been elucidated. In this study, the authors demonstrate that a TRPC1-dependent Ca^{2+} -type channel(s) exists in HL-7702 cells, using NMT, a non-invasive technique.

Innovations and breakthroughs

This is the first study to investigate the role of TRPC1 in regulating Ca^{2+} channels in normal human liver cells. Furthermore, measuring Ca^{2+} influxes was performed non-invasively, which cannot be accomplished with other traditional techniques.

Applications

Cytoplasmic Ca^{2+} overloading might cause damage to liver cells. By understanding the role of TRPC1 in mediating extracellular Ca^{2+} influxes, this study might represent a future strategy for preventing or treating diseases induced by dysfunctions of Ca^{2+} channels in the clinic, such as hepatic ischemia-reperfusion injury.

Terminology

The canonical transient receptor potential (TRPC) channel subfamily consists of seven mammalian cation channels and is expressed in almost every tissue, including the liver. The TRPC1 channel is permeable to Ca^{2+} and is the most likely candidate for receptor-operated Ca^{2+} channels. In addition, TRPC1 also plays a dominant role in mediating store-operated Ca^{2+} channels in many types of cells.

Peer review

The authors recorded Ca^{2+} influxes elicited by adding external Ca^{2+} into a test solution in several different conditions. It revealed that there is a TRPC1-dependent Ca^{2+} -type channel(s), which can be detected *via* NMT, in normal human liver cells. This is an interesting study.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Lin YP



BRIEF ARTICLES

Effect of early preoperative 5-fluorouracil on the integrity of colonic anastomoses in rats

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Supported by Haydarpasa Numune Education and Research Hospital

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Received: April 15, 2009 Revised: July 28, 2009

Accepted: August 4, 2009

Published online: September 7, 2009

Abstract

AIM: To determine the effect of chemotherapy on wound healing by giving early preoperative 5-fluorouracil (5-FU) to rats with colonic anastomoses.

METHODS: Sixty Albino-Wistar male rats (median weight, 235 g) were used in this study. The rats were fed with standard laboratory food and given tap water *ad libitum*. The animals were divided into three groups: Group 1: Control group (chemotherapy was not administered), Group 2: Intraperitoneally (IP) administered 5-FU group (chemotherapy was administered IP to animals at a dose of 20 mg/kg daily during the 5 d preceding surgery), Group 3: Intravenously (IV) administered 5-FU group. Chemotherapy was administered *via* the penil vein, using the same dosing scheme and duration as the second group. After a 3-d rest to minimize the side effects of chemotherapy, both groups underwent surgery. One centimeter of colon was resected 2 cm proximally from the peritoneal reflection, then

sutured intermittently and subsequently end-to-end anastomosed. In each group, half the animals were given anaesthesia on the 3rd postoperative (PO) day and the other half on the 7th PO day, for *in vivo* analytic procedures. The abdominal incisions in the rats were dissected, all the new and old anastomotic segments were clearly seen and bursting pressures of each anastomotic segment, tissue hydroxyproline levels and DNA content were determined to assess the histologic tissue repair process.

RESULTS: When the IV group was compared with the IP group, bursting pressures of the anastomotic segments on the 3rd and 7th PO days, were found to be significantly decreased, hydroxyproline levels at the anastomotic segment on the 7th PO day were significantly decreased ($P < 0.01$).

CONCLUSION: In this study, we conclude that early preoperative 5-FU, administered IV, negatively affects wound healing. However, IP administered 5-FU does not negatively affect wound healing.

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Key words: 5-fluorouracil; Neoadjuvant therapy; Rats; Wound healing

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Ozel L, Ozel MS, Toros AB, Kara M, Ozkan KS, Tellioglu G, Krand O, Koyuturk M, Berber I. Effect of early preoperative 5-fluorouracil on the integrity of colonic anastomoses in rats. *World J Gastroenterol* 2009; 15(33): 4156-4162 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4156.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4156>

INTRODUCTION

Colorectal cancer is a common malignancy in most developed countries worldwide^[1]. Metastasis frequently occurs before clinical detection of the primary tumour. Despite the advances in surgical techniques, this characteristic of the malignancy prevents a significant

improvement in cure rates for colorectal cancers^[2]. While cancer therapy was limited to surgery in the past, nowadays therapy includes the combination of surgical radiotherapy, chemotherapy, hormonal and biological therapies^[3,4]. 5-fluorouracil (5-FU) treatment has been accepted as standard chemotherapy for colorectal cancers for a long time. Recently, high recurrence rates, the presence of distant metastasis, the possibility of complete resection, and the removal of circulating tumour cells after curative resection of colorectal cancers constitute a new therapeutic approach known as neo-adjuvant chemotherapy^[5-7]. While the integrity of anastomoses after colorectal cancer resection is an important parameter on mortality and morbidity, the effects of preoperative chemotherapy on wound healing and anastomoses are also important and have not been clearly outlined.

This study investigates the effects of early preoperative administration of 5-FU, given intravenously (IV) and intra-peritoneally (IP), on wound healing in colon anastomoses.

MATERIALS AND METHODS

Sixty male Wistar-Albino rats weighing between 225 and 315 g were used in this study. All rats were clinically healthy and were fed with standard laboratory food and water. The animals were numbered at the beginning of the study and weighed every day during the study. There were three groups: Group I, a control group ($n = 20$); group II which received 5-FU IP ($n = 20$); group III which received 5-FU IV ($n = 20$). The study was approved by the local Ethics Committee of Haydarpaşa Numune Hospital.

Drug administration

When pilot studies and related literature were taken into account, a dose of 20 mg/kg 5-FU was calculated to be the maximum non-lethal dose^[8]. Chemotherapy was not administered to the control group. The second group was administered 5-FU IP at a dose of 20 mg/kg in saline at a concentration of 5 mg/mL each day prior to surgery. The third group was given 5-FU *via* the penile vein at a dose of 20 mg/kg in saline at a concentration of 2 mg/mL each day for 5 d before surgery. Both groups underwent surgery on the 3rd d after chemotherapy to reduce the adverse effects of chemotherapy.

Operative procedure

The same operative procedure was performed in all groups by the same surgeon. 10 mg/kg of ketamine was given subcutaneously to rats under ether anaesthesia. After shaving the frontal abdominal wall, this area was cleaned with povidone iodine and covered with sterile cloths. The abdomen was entered through a 3 cm mid-line incision, 1 cm of colon 2 cm proximal of the peritoneal reflection was resected, and a side-to-side anastomosis was made using ten intermittent sutures with 6/0 polypropylene (Ethicon). Muscles of the front abdominal wall and skin were closed by continuous suture with 3/0 silk. Half the

animals in each group were anaesthetized again either on day 3 or 7 after surgery for *in vivo* analytic procedures. The animals were then killed by haemorrhage for *in vitro* analytic procedures.

Analytic procedures

After making an abdominal incision, macroscopic evaluations of the anastomotic segment were performed. Adhesions surrounding the anastomoses were not cut, and bursting pressure was measured for every anastomotic segment during the internal passage of 200 mL/h saline. For this purpose, a 10/0 silicon catheter was passed *via* the anus to 2 cm distal of the anastomosis and the colon was ligated with silk suture above the catheter. The colon was cut 3 cm proximal to the anastomotic segment. The catheter, which had its end fixed to a standard sphygmomanometer (Petaş, Turkey), was moved 1 cm to the anastomotic segment from the cut end of the colon and the colon was ligated with silk suture around the catheter. The perfusator (Becton Dickinson, France) was maintained at a speed of 200 mL/h and saline was given continuously through the catheter situated in the anus. Increased pressure on the sphygmomanometer was observed. Pressure values of the first leakage from the anastomotic segments, when increased pressure on the sphygmomanometer stopped and the time of falling pressure were recorded as bursting pressures. These bursting pressures were recorded in mmHg for each animal.

After measuring the anastomotic pressures and just before the animals were sacrificed, 5 mL blood samples were taken from the inferior vena cava to determine white blood cell, haemoglobin and platelet counts.

Anastomotic segments were isolated from the surrounding tissues. One cm of colonic segment, including the anastomotic area was resected and was longitudinally separated into two parts. One of the segments was frozen at -45°C for hydroxyproline measurements (hydroxyproline was used as a marker of collagen content) which were calculated as nanograms per gram of tissue. Anastomotic hydroxyproline content was measured by spectrophotometric determination using the method described by Bergman *et al*^[9]. The other segment was embedded in paraffin for DNA content measurement and histological assay. Tissue DNA content was determined by flow cytometry using Mod-Fit Ver 5.01 software^[10].

Tissue sections from routinely embedded paraffin blocks were stained with hematoxylin-eosin and examined by light microscopy. Histological examination was evaluated using the criteria determined by de Roy van Zuidewijn *et al*^[11]. Slides were evaluated twice by the same observer in a blind fashion. Granulation tissue was evaluated as; 1-low; 2-medium; 3-high (intense) and histological parameter scores related to muscular tissue were evaluated as; 1-negative; 2-medium; 3-complete. According to a seven-point scale, mucosal re-epithelization scores were as follows; 1-negative, 2-little-one line cubic, 3-lot-one line cubic, 4-nearly one line cubic, 5-finished-one line cubic, 6-one line glandular, 7-normal glandular mucosa.

Table 1 Leukocyte, platelet and haemoglobin values of blood samples in each group (mean \pm SE)

Groups	Leukocytes ($10^9/L$)		Platelets ($10^9/L$)		Haemoglobin (g/dL)	
	3rd d	7th d	3rd d	7th d	3rd d	7th d
Control	9.0 \pm 3.04	7.6 \pm 3.4	1117 \pm 169	1270 \pm 229	11 \pm 1.7	12.6 \pm 1.2
IP	9.1 \pm 3.3	9.7 \pm 2.4	808 \pm 203	1228 \pm 194	10.5 \pm 1.7	11.9 \pm 0.45
IV	7.4 \pm 4.7	15.9 \pm 5.2	736 \pm 243	1542 \pm 289	9.6 \pm 1.13	11.16 \pm 1.9

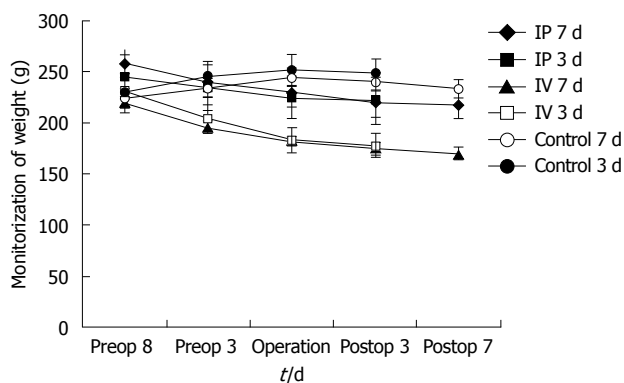


Figure 1 Weights of rats during experimental procedure. Preop: Pre-operation; Postop: Post-operation.

Statistical analysis

All data are presented as means \pm SE. Non-parametric Kruskal Wallis variant analysis was used for multiple group comparisons of statistical analysis and subgroup comparisons were performed with Dunn's test. Pair group analysis was evaluated using Mann-Whitney *U* test.

RESULTS

During the experimental procedure, two animals died on the 3rd and 7th postoperative (PO) days in the IV group. The cause of death was leakage from the anastomotic site. No mortality was observed in the control and IP groups. Dead rats were replaced with new animals.

Weight measurements

Rats were weighed at the beginning of the study, during the chemotherapy period and in the postoperative period. Weight loss was observed in all animals in the chemotherapy groups (IP and IV) compared with baseline values, whereas control animals gained weight during the preoperative period. Animals in the control group also lost weight during the PO period. Weight loss in the rats is shown in Figure 1. On the 3rd PO day, the IV 5-FU group weighed significantly less than the control group ($P < 0.01$) and the IP group ($P < 0.01$). Weight loss in the IV 5-FU group on the 7th PO day was found to be significantly lower than the control group ($P < 0.01$) and the IP group ($P < 0.001$).

Hematological effects

Haemoglobin, white blood cell and platelet counts in the blood samples taken on the 3rd and 7th PO days after chemotherapy are shown in Table 1. There was no statistically significant difference between the groups for

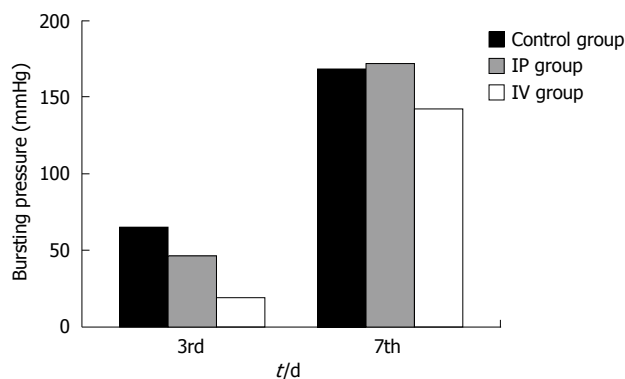


Figure 2 Bursting pressure values of experimental groups on postoperative 3rd and 7th d.

haemoglobin counts on the 3rd and 7th PO days. On the 3rd PO day in the IV 5-FU group, the platelet count was found to be significantly lower than the control group ($P < 0.05$).

Bursting pressure

Bursting pressures were measured at anastomotic segments on the 3rd and 7th PO days (Figure 2). Bursting pressure values were significantly lower than the control ($P < 0.01$) and IP group on ($P < 0.05$) the 3rd PO day in the IV 5-FU group. On the 7th PO day, bursting pressure values in the IV and IP groups were not significantly different from the control group. However, bursting pressure values in the IV 5-FU group were significantly lower when compared with the IP group ($P < 0.01$).

Tissue hydroxyproline

Anastomotic hydroxyproline values were statistically significantly higher in the IP group compared to the control and IV group on the 3rd PO day ($P < 0.01$). There were no statistically significant differences between the IV and control groups on PO day 3, regarding hydroxyproline levels. Hydroxyproline levels in the IP group were the highest and anastomotic hydroxyproline levels in the IV group were significantly lower than the IP group on PO day 7 ($P < 0.01$) (Figure 3).

DNA analysis

DNA contents in the anastomotic segments were evaluated using a flow cytometer technique in this study^[10]. When the anastomotic segments were examined, a decrease in cell proliferation was noted on the 3rd PO day in the IV group, when compared to the control group, and on the 7th PO day between the control and the IP group. However, this decrease was not statistically significant.

Table 2 Histological scores of anastomotic colon healing in all groups on the 3rd and 7th postoperative days (mean \pm SE)

Groups	3rd d			7th d		
	Control	IP	IV	Control	IP	IV
Granulation	1.66 \pm 0.70	2.14 \pm 0.89	1.42 \pm 0.53	2.66 \pm 0.50	2.71 \pm 0.48	2.57 \pm 0.53
Re-epithelization	1.55 \pm 1.66	2.71 \pm 2.21	1.00 \pm 0.00	4.77 \pm 1.20	5.28 \pm 0.95	2.42 \pm 2.14
Muscular tissue	1.00 \pm 0.00	1.00 \pm 0.00	1.00 \pm 0.00	1.22 \pm 0.44	1.42 \pm 0.44	1.14 \pm 0.37

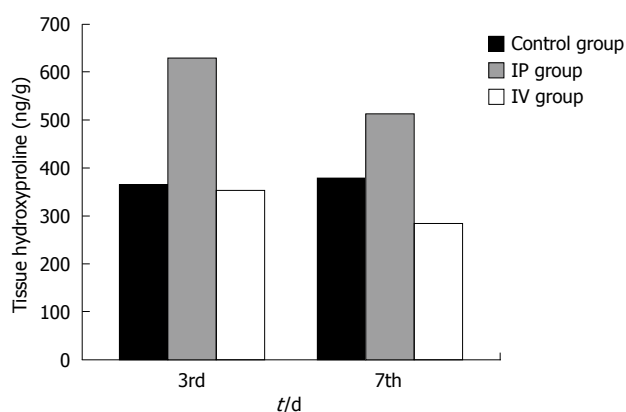


Figure 3 Hydroxyproline levels in anastomotic segment.

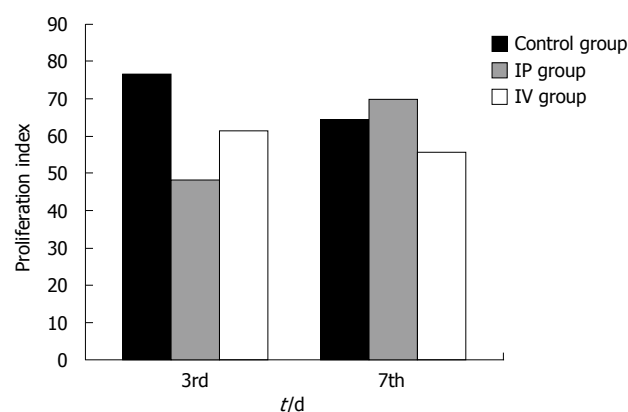


Figure 4 Proliferative cell rates of anastomotic segments.

The tissue proliferation index of anastomotic segments is shown in Figure 4.

Histological analysis

Scores for the histological parameters in the groups are given in Table 2. The best granulation tissue was observed on the 3rd and 7th d in the IP 5-FU group. On the 3rd d of re-epithelization, there was no statistically significant difference between the control and both 5-FU administered groups. However, on the 7th d there was a statistically significant difference between the control and the IV 5-FU group ($P < 0.05$), and between the IP 5-FU and IV 5-FU groups ($P < 0.05$). On multiple comparisons between the groups, the statistically significant difference was maintained ($P < 0.05$). Muscle tissue formation was not complete on the 3rd and 7th d and related values were not statistically different between the groups.

DISCUSSION

Colorectal cancer is the second most frequent type of cancer in industrialized countries. Despite improvements in surgical techniques, almost half of patients with colorectal cancer will eventually die of recurrent disease.

When colon cancer recurs after surgical resection, local disease is found in 25%-40% of patients, peritoneal implants in 12%-28% of patients, and liver metastases in 40% of patients. Hepatic and peritoneal metastases have been reported to be the most frequent failure patterns in resected colorectal cancer patients^[12,13].

Solomon *et al.*^[14] reported on the incidence of colorectal cancer cells on peritoneal surfaces. Overall, 15% of patients had positive cytology for cancer cells in the peritoneal or bowel surface. Stage II and III disease is treated with wide surgical resection in combination with

adjuvant or neo-adjuvant chemoradiation. The combined modality of chemotherapy and surgery increases overall survival and the disease-free period^[15-18]. 5-FU was first reported to be effective for colorectal cancer in the 1950s. For more than 10 years, 5-FU was the only adjuvant drug given as a single agent. Since its introduction, 5-FU has remained the cornerstone of adjuvant treatment for colorectal cancer. A number of clinical and experimental data are available on the effects of 5-FU, either alone or in combination with other chemotherapeutic agents, on wound healing^[19-22].

In the adjuvant therapy of colorectal cancers, 5-FU was used alone in prolonged systemic regimens or in combination with other agents^[22-25]. Fundamentals of preoperative chemotherapy were based on the results of Cole *et al.*^[5]. According to these results, numerous malignant cells pass into the peripheral circulation during surgical manipulations for localized carcinomas. Preoperatively administered cytotoxic agents may allow tumour resectability and decrease the incidence of distant or local metastases^[26].

5-FU can be injected IV and hepatic concentrations can also be achieved intraportally and IP^[22,27,28]. In addition, intravenous administration may not allow sufficient penetration in the abdominal cavity. This could be the reason why chemotherapeutic agents may not be effective enough to eliminate micrometastases, especially at the resection site and peritoneal surfaces, which are high risk sites for local recurrence. When the drug is administered *via* the intraperitoneal route, high local and hepatic concentrations can be achieved^[28]. Local peritoneal recurrence and haematological toxicity were lower when 5-FU was administered IP. A prospective trial showed that 5-FU administered IP reduced peritoneal failure significantly more than intravenous 5-FU^[29]. There

is an increased interest in the use of intraperitoneal chemotherapy with 5-FU to treat advanced colon cancer. Randomised clinical trials have reported a reduction in local recurrence rate with either intraperitoneal 5-FU alone or combined with intravenous 5-FU^[30].

Toxic side effects are the major dose-limiting factors in chemotherapy. In patients with advanced primary colon cancer, a significantly higher dose of 5-FU can be given by the intraperitoneal route than by the intravenous route^[29]. We used the intraperitoneal approach to deliver high concentrations of 5-FU into the peritoneal cavity without increasing the risk of systemic toxicity. In addition, previous clinical and experimental studies have shown that immediate postoperative 5-FU given IP has no adverse effect on outcome^[8].

In 1994, Kelsen *et al*^[31] reported a phase I trial of postoperative intraperitoneal floxuridine and leucovorin plus systemic 5-FU and levamisole after resection of high-risk colon cancer. Intraperitoneal therapy appeared to be well tolerated, with no substantial increase in postoperative morbidity and no operative mortality.

In 1998, Scheithauer *et al*^[30] accepted 241 patients with resected stage III or high-risk stage II colon cancer into a trial, comparing intravenous 5-FU and levamisole given for a period of 6 mo with a treatment program consisting of leucovorin plus 5-FU given IV and IP. In patients with stage III disease, a significant improvement in disease-free survival and overall survival rates was observed using the systemic plus intraperitoneal treatment, with an estimated 43% reduction in mortality rate. Intraperitoneal and systemic chemotherapy markedly reduced local regional recurrences^[30].

It is stated that the highest non-lethal dose of 5-FU is 20 mg/kg for rats and this was the dose administered in our previous trial^[11]. The 500 mg/m² human dose is equal to the animal dose used in this study. A 3 d interval between the last day of chemotherapy and the surgical procedure was approved to reduce the strong side effects of chemotherapy^[6,22].

Weight reductions were detected both during 5-FU injections and after surgery. The weight of the rats was recorded during the 5-FU injection period and days after the operation. All rats lost weight compared with baseline values during 5-FU administration and after the operation, which was statistically significant in the IV group. These results were concordant with reports from the literature^[6,8,32]. Weight loss after 5-FU is related to anastomotic healing.

The burst pressure values of the anastomotic segments were measured *in vivo* on the 3rd and 7th PO days in rats receiving and not receiving 5-FU in this study, similar to that in the study by Kuzu *et al*^[6]. Anastomotic burst pressures have been measured *in vitro* in most previous similar reports^[8,22]. We think that *in-vivo* measurements reflect the clinical status much better. The bursting sites we found in this study are in accordance with those in other studies which reported that the anastomosis is the most common bursting point^[22,33]. On the 3rd

PO day, bursting pressure values for the IV 5-FU group were found to be significantly lower than the control and IP groups. On the 7th PO day, bursting pressure values in the IV 5-FU group were significantly lower than the IP group. Similar results have been found in previously reported studies^[22,32,33].

Graf *et al*^[32] reported that early postoperative 5-FU administration had a negative impact on the bursting strength of colonic anastomosis.

5-FU administered preoperatively may also have a negative influence on the ability of fibroblasts to proliferate and synthesize collagen. Reduced collagen synthesis can lead to anastomotic dehiscence^[22].

Collagens which guarantee tissue continuity in the tissue repair period contain high proportions of glycine, proline and hydroxyproline. Tissue hydroxyproline levels are important parameters in the tissue repair process^[34,35]. Some studies have found that 5-FU decreases the hydroxyproline content of wounds^[19,21]. There were significant differences in hydroxyproline levels at the anastomotic segments on both the 3rd and 7th PO days in the IV group compared to the IP group. The decrease in collagen content was correspondant with the decrease in bursting pressures.

It is already known that the chemotherapeutic agent 5-FU inhibits DNA synthesis and cell proliferation, by affecting the cell cycle^[36]. Based on this mechanism of action, the effect of chemotherapy on cell proliferation at the anastomotic segment was evaluated by DNA analysis. An obvious decrease in cell proliferation was found at the anastomotic segment of the IV chemotherapy group, compared with the IP and control groups on the 7th PO day. This decrease was not statistically significant. The effects of 5-FU on cancer kinetics determined by DNA analysis, was reported to stress the importance of neo-adjuvant chemotherapy. It was thought that available parameters could be used to determine the anti-tumour effects of 5-FU^[37]. The DNA proliferation index can be used as a parameter to determine the effect of chemotherapy on tissue repair; however, further clinical and experimental studies are required.

We looked at histological aspects of the colon during a 7-d period. Although there were no statistically significant differences between the granulation tissues on the 3rd and 7th PO days in our study, the decrease in re-epithelization, as a sign of a mucosal improvement, was statistically significant in the IV group compared with the control and IP groups. According to the results of de Roy van Zuidewijn *et al*^[11], completion of colonic muscular tissue repair takes about 21 d. Consistent with our study, muscular repairs were not complete in any of the groups at the 3rd and 7th d of the early tissue repair period, and no difference was found between the control and the experimental groups. Our histological findings suggested that IP 5-FU administration may be preferred over IV administration which was in accordance with our previous results.

All anastomoses were examined macroscopically during the second laparotomy, before the bursting pressure

measurements. Intra-abdominal adhesions were not classified but the integrity of anastomoses, the existence of perianastomotic abscess or peritonitis and the formation of adhesions were less frequent in the IP group, when compared to the control and IV groups.

5-FU derivatives inhibit DNA synthesis by inhibiting thymidylate synthetase, and reduce the biological activity of RNA in both human cells and growing bacteria^[38,39].

When applied locally, 5-FU seems to behave like an antibiotic. This confirms certain studies which implied an increasing bactericidal effect when antimicrobial drugs and antineoplastic drugs were administered together. Nyhlén *et al.*^[40] reported that against two of the three tested strains of *Staphylococcus epidermidis*, the combination of ciprofloxacin and 5-FU resulted in a synergistic prolongation of the postantibiotic effect (PAE) in comparison with the PAE induced by the drugs alone. However, these results need to be confirmed clinically.

In conclusion, our results demonstrate that, early preoperative IV administration of 5-FU has a negative effect on anastomotic healing of the colon. However, the IP route of 5-FU administration has no adverse effect on the healing process of colon anastomoses and burst pressure. This study was performed on healthy animals and may not reflect the exact situation in the case of malignant tumors.

COMMENTS

Background

For cancers of the large bowel, multi-institutional trials have demonstrated a significant reduction in mortality with adjuvant chemotherapy compared with surgery alone. 5-fluorouracil (5-FU) was therefore used as a neoadjuvant therapy in the present study where it had no adverse effect on anastomotic healing and burst pressure.

Research frontiers

5-FU is the mainstay of systemic treatment for colorectal cancer. Intravenous administration may not allow sufficient penetration in the abdominal cavity. This may be the reason why chemotherapeutic agents are not effective enough to kill all micrometastases, especially at the resection site and peritoneal surfaces. Because of these theoretical advantages, neoadjuvant intraperitoneal chemotherapy may be a new treatment option for colorectal cancer. This study investigated the effect of preoperative 5-FU on the healing of colorectal anastomoses in the rat.

Innovations and breakthroughs

5-FU can be injected not only intravenously, but also intraportally and intraperitoneally (IP). When administered by the intraperitoneal route, high local and hepatic concentrations can be achieved. Whether 5-FU compromises wound healing is still controversial. Previous clinical and experimental studies have shown that immediate postoperative 5-FU given IP has no adverse effect on outcome. Therefore, 5-FU was used as neoadjuvant therapy in the present study.

Applications

This study may represent a future strategy for neoadjuvant chemotherapy in colonic cancer.

Terminology

5-FU is the most widely used chemotherapeutic agent in the adjuvant treatment of colon cancer. 5-FU acts during synthesis by interfering with normal pathways. 5-FU might also have a negative influence on the ability of fibroblasts to proliferate and synthesize collagen. Reduced collagen synthesis could lead to anastomotic dehiscence.

Peer review

It is an interesting topic for the readers of *WJG*. The authors described pre- and postoperative variables after two methods of 5-FU administration.

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S- Editor Li LF L- Editor Webster JR E- Editor Zheng XM

Fentanyl inhibits glucose-stimulated insulin release from β -cells in rat pancreatic islets

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Received: April 22, 2009 Revised: July 7, 2009

Accepted: July 14, 2009

Published online: September 7, 2009

Abstract

AIM: To explore the effects of fentanyl on insulin release from freshly isolated rat pancreatic islets in static culture.

METHODS: Islets were isolated from the pancreas of mature Sprague Dawley rats by common bile duct intraductal collagenase V digestion and were purified by discontinuous Ficoll density gradient centrifugation. The islets were divided into four groups according to the fentanyl concentration: control group (0 ng/mL), group I (0.3 ng/mL), group II (3.0 ng/mL), and group III (30 ng/mL). In each group, the islets were co-cultured for 48 h with drugs under static conditions with fentanyl alone, fentanyl + 0.1 μ g/mL naloxone or fentanyl + 1.0 μ g/mL naloxone. Cell viability was assessed by the MTT assay. Insulin release in response to low and high concentrations (2.8 mmol/L and 16.7 mmol/L, respectively) of glucose was investigated and electron microscopy morphological assessment was performed.

RESULTS: Low- and high-glucose-stimulated insulin release in the control group was significantly higher

than in groups II and III (62.33 ± 9.67 μ IU vs 47.75 ± 8.47 μ IU, 39.67 ± 6.18 μ IU and 125.5 ± 22.04 μ IU vs 96.17 ± 14.17 μ IU, 75.17 ± 13.57 μ IU, respectively, $P < 0.01$) and was lowest in group III ($P < 0.01$). After adding 1 μ g/mL naloxone, insulin release in groups II and III was not different from the control group. Electron microscopy studies showed that the islets were damaged by 30 ng/mL fentanyl.

CONCLUSION: Fentanyl inhibited glucose-stimulated insulin release from rat islets, which could be prevented by naloxone. Higher concentrations of fentanyl significantly damaged β -cells of rat islets.

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Key words: Fentanyl; Inhibition; Insulin release; Islets

Peer reviewer: Ming Li, Associate Professor, Tulane University Health Sciences Center, 1430 Tulane Ave S1-83, New Orleans 70112, United States

Qian TL, Wang XH, Liu S, Ma L, Lu Y. Fentanyl inhibits glucose-stimulated insulin release from β -cells in rat pancreatic islets. *World J Gastroenterol* 2009; 15(33): 4163-4169 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4163.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4163>

INTRODUCTION

In recent years, anesthesiologists and surgeons have had to manage an increasing number of diabetic patients. A concern for the anesthesiologist is how to control blood glucose and protect islet function in the clinical setting. Because the risk of surgery and anesthesia is higher for these patients, it is particularly important to maintain whole-body glucose homeostasis during the perioperative period. It is well known that many drugs used during surgery for anesthesia or pain relief have effects on pancreatic islets, for example, one study has demonstrated that β -endorphin, an endogenous opioid peptide, can inhibit insulin secretion^[1]. Therefore, when administering drugs to diabetic patients, the surgeons and anesthesiologists must consider the protection of islet function in these patients. Fentanyl was first synthesized 60 years ago and is currently the most popular opioid used in the preoperative period because of its safety and efficacy. In addition,

fentanyl transdermal patches have been developed to manage chronic pain associated with diabetic neuropathy. These patches have been designed to provide continuous, rate-controlled systemic delivery of the fentanyl base for up to 72 h^[2,3].

The minimum effective plasma concentration of fentanyl is 0.63 ng/mL after intravenous administration and the therapeutic plasma concentration is 1-2 ng/mL. However, the plasma concentration often exceeds 3 ng/mL because many practitioners prefer to administer high doses of fentanyl^[4,5]. In most cancer cases secondary to disease progression, the initial median transdermal fentanyl dose is generally 60-70 µg/h (release rate), which increases to about 170 µg/h over time^[6]. Furthermore, a small proportion of patients require doses of between 400 and 1000 µg/h in the latter stages of therapy and the mean treatment period is often longer than 50 d. These large doses are associated with high plasma concentrations (up to 14.5 ng/mL) which are linearly related to the dose^[7,8]. Furthermore, fentanyl has been commonly used for patients undergoing cardiac operations because high doses of fentanyl can stabilize the cardiovascular circulatory system during operations. After administration of 50-100 µg/kg fentanyl, the plasma concentration of fentanyl in these patients is commonly above 20-30 ng/mL^[9]. However, high plasma concentrations of fentanyl are associated with clinical side effects such as nausea and vomiting, constipation, skin itching and respiratory depression. So far, the potential effects of fentanyl on pancreatic islet β-cells remain unknown.

Because some opioid receptor agonists affect insulin release^[10,11], it would be expected that high-doses of fentanyl would have an effect on islet insulin secretion. Studies have suggested that some opiates inhibit insulin secretion^[12]. In mouse pancreatic islets incubated under static conditions, glucose-stimulated insulin release is inhibited by β-endorphin and endomorphin-1, endogenous opioid receptor agonists. This inhibition could be prevented by naloxone, an opioid receptor antagonist^[13]. Therefore, we hypothesized that fentanyl would affect insulin release. Therefore, we investigated the effect of fentanyl on rat islets in a static culture model and believe the results have important implications on the use of fentanyl in clinical situations, particularly in people with diabetes.

MATERIALS AND METHODS

Animals

Male SD rats weighing 250-300 g were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences and housed under constant conditions of temperature (20-22°C) and artificial lighting (12-h light-dark cycle) before taking part in the study. The study was carried out in accordance with the Guidelines for Animal Experimentation, Tongji University of Shanghai, China, and all the tests were approved by the Animal Experimentation Committee of Tongji University of Shanghai.

Islet preparation

After male adult rats were anesthetized with 50-75 mg/kg sodium pentobarbital, the abdominal wall was cut open and 10 mL of Hank's buffered saline solution (HBSS) containing collagenase V 1.0-1.2 mg/mL (Sigma Chemical Co., St Louis, MO, USA) was injected into the common bile duct of the rat. The pancreas, which was swollen with the digestion solution, was quickly excised and immersed into a plastic culture bottle containing HBSS and incubated with shaking for 13-15 min at 37°C. The digested suspension was obtained by filtering the suspension through a 0.5-mm metal mesh and washed with HBSS containing 2% bovine serum albumin (BSA). A total of 300-400 islets were obtained from each rat by discontinuous Ficoll density gradient centrifugation (density: 1.100, 1.077) (Ficoll, Sigma Chemical Co., St Louis, MO, USA). After being washed with HBSS containing 2% BSA, the islets were cultured for 24 h with 5% CO₂ and collected for further tests^[14]. The islets were identified by dithizone (Sigma, USA) staining. Cells stained red under light microscopy were considered to be islets^[15]. The combination dyes acridine orange (AO) and propidium iodide (PI) (AO: 0.67 µmol/L; PI: 75 µmol/L) were applied to differentiate between viable and non-viable whole islets; the dyes stained living cells green and dead cells red using minimal background fluorescence^[16].

Groups

The islets were divided into four groups according to the fentanyl concentration used: control group (0 ng/mL), group I (0.3 ng/mL), group II (3.0 ng/mL), and group III (30 ng/mL). In each group, the islets were co-cultured for 48 h with drugs as follows: fentanyl alone, fentanyl + 0.1 µg/mL naloxone and fentanyl + 1.0 µg/mL naloxone. There were 12 wells in each group with 30 IEQ (islet equivalent, the diameter of an islet of 150-200 µm equates to 1.7 IEQ) islets/well. An additional two groups were co-cultured with naloxone alone at concentrations of 0.1 or 1.0 µg/mL.

Islet viability

The effect of fentanyl on cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, USA) assay to display a direct correlation with cell metabolism. After co-culturing with fentanyl for 48 h, 100 µL MTT solution was added to each well to a final concentration of 0.5 mg/mL per well and the plates were cultured for 4 h at 37°C. The supernatant was then removed by the addition of DMSO to each well to dissolve the deposition, and the optical density (OD) disparity was read at 490 nm using a spectrophotometer microplate reader (Labsystems, Finland). The inhibition of islet cells caused by the different concentrations of fentanyl was calculated using the formula: inhibition rate (%) = $(1 - \text{OD}_{\text{drug exposure}} / \text{OD}_{\text{control}}) \times 100$ ^[17,18].

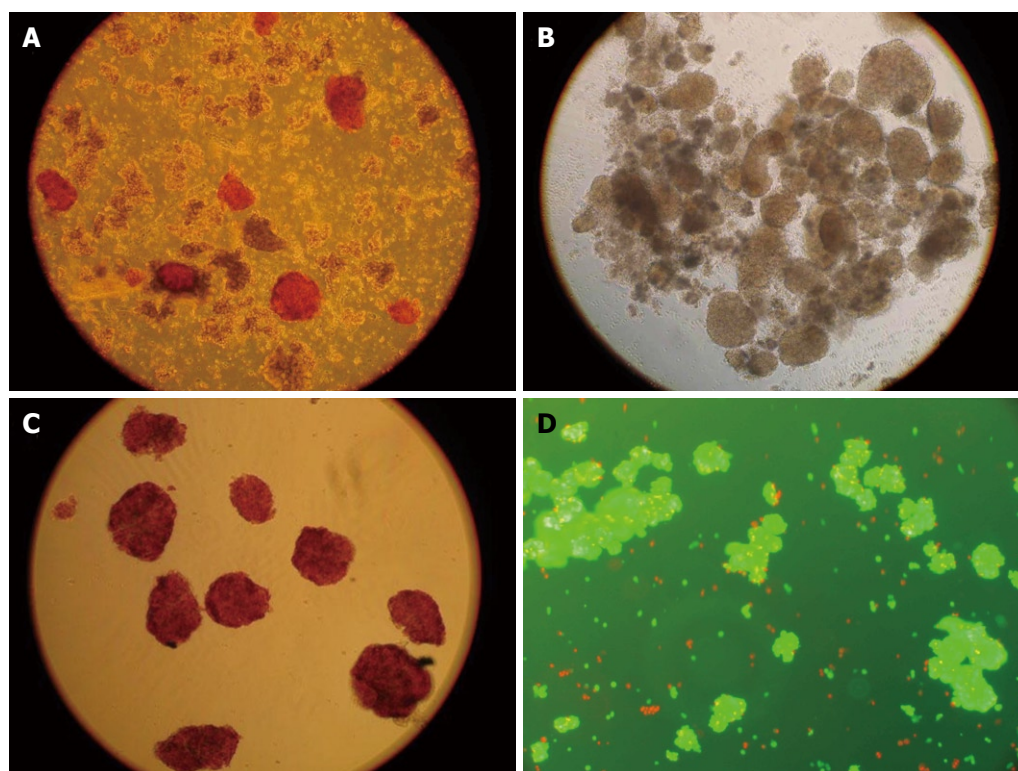


Figure 1 The procedure of islets preparation. A: The islets were stained red by DTZ in digested pancreatic tissue; B: Pure islets isolated from digested pancreatic tissue; C: Pure islets stained by DTZ; D: The dye AO-PI stained living cells green and dead cells red in minimal background fluorescence ($\times 100$).

Glucose-stimulated insulin release assay in static culture

After incubating with the drugs for 48 h, the islets were washed with serum-free and glucose-free medium twice. The insulin release stimulation test was performed by first incubating the islets in low (2.8 mmol/L) and then high (16.7 mmol/L) concentrations of glucose in static culture medium for 1 h each. Supernatant from each well was collected and the insulin level was determined using a rat insulin radioimmunoassay kit (Linco Research, Inc, St Charles, MO, USA). The stimulation index (SI) was calculated by dividing the value of high glucose-stimulated secretion by the value of low glucose-stimulated secretion^[19,20].

Electron microscopy studies

After co-culturing with fentanyl for 48 h, the islets in the control group and those in group III were cut into thin sections and mounted on slides. Samples were stained with 2% uranyl acetate and lead citrate. The sections were viewed and photographed on a JEOL JEM-1230 transmission electron microscope (Jeol Ltd., Japan) at 80 kV.

Statistical analysis

Statistical and graphic analyses were performed with SPSS 13.0 software. Differences between groups were evaluated with one-way ANOVA or Kruskal-Wallis *H* tests, as appropriate, and the differences between two groups were analyzed with the LSD test or Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant.

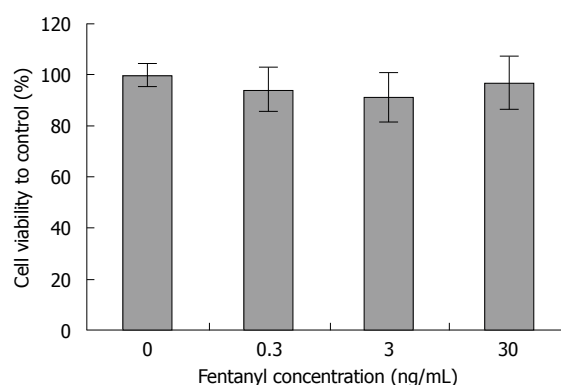


Figure 2 The cell viability after co-culture with fentanyl. The viability measured by MTT was 100% in the control group. The viability of islets exposed to 0.3, 3 and 30 ng/mL fentanyl was 94.3%, 91.3% and 96.9%, respectively. There was no difference between groups. The data represent means \pm SE.

RESULTS

Islet viability assessment

In this study, the rat islets were isolated from the rat pancreas with good quality. The viability was about 90% and the purification rate was about 95% (Figure 1). Fentanyl had no effect on cell viability (Figure 2).

Insulin release test

Fentanyl significantly inhibited the low and high concentration glucose-stimulated insulin release in a concentration-dependent manner ($P < 0.01$), and the insulin release was lowest at the concentration of 30 ng/mL ($P < 0.01$). After adding 0.1 $\mu\text{g/mL}$ naloxone, fentanyl still significantly inhibited glucose-stimulated insulin release ($P < 0.01$). However, after adding 1 $\mu\text{g/mL}$ naloxone,

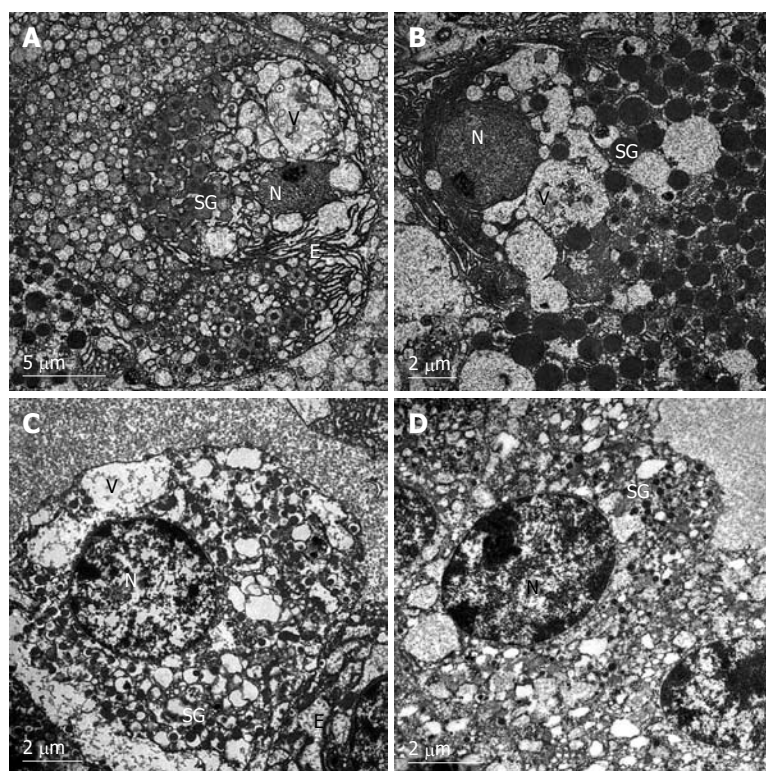


Figure 3 Electron micrographs of β -cells in rat pancreatic islets of control group. N: Nucleus; SG: Secretory granule; V: Vesicle; E: Endoplasmic reticulum. The typical β -cells had an abundance of cytoplasmic granules and endoplasmic reticulum. The electron density of the granules increased when the granules were maturing in the vesicles. The mature secretory granules displayed a highly electron-dense core surrounded by a wide electron-lucent halo. The granules had a space between the core and the membrane. The vesicles showed a normal round outline (A, $\times 6000$). There were many nascent granules in the β -cells; subsequent maturation involved further condensation of the matrix constituents and a reduction in granule diameter (B, $\times 8000$).

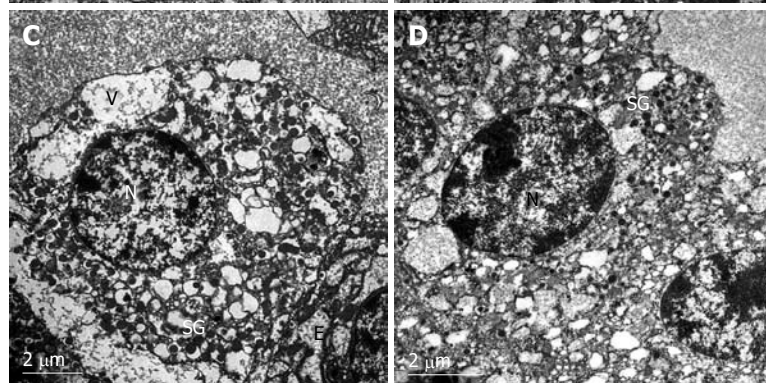


Figure 4 Electron micrographs of rat pancreatic islet β -cells after exposure to 30 ng/mL fentanyl for 48 h. N: Nucleus; SG: Secretory granules; V: Vesicle; E: Endoplasmic reticulum. Chromatin margination and severe cytoplasmic vacuolization and degeneration were observed in the islets. Large and small vacuoles are present in the cytoplasm. The secretory granules and endoplasmic reticulum were much smaller than in the control group (A, $\times 10000$). In addition, endoplasmic reticulum and normal vesicles were almost never observed (B, $\times 12000$).

Table 1 The glucose-stimulated insulin release (μ IU) in response to fentanyl with different concentrations of naloxone (mean \pm SD, $n = 12$)

	Fentanyl alone		Fentanyl + 0.1 μ g/mL naloxone		Fentanyl + 1 μ g/mL naloxone	
	Low glucose	High glucose	Low glucose	High glucose	Low glucose	High glucose
Control (0 ng/mL)	62.33 \pm 9.67	125.5 \pm 22.04	62.33 \pm 9.67	125.5 \pm 22.04	62.33 \pm 9.67	125.5 \pm 22.04
I (0.3 ng/mL)	54.75 \pm 5.93 ^a	118.17 \pm 16.81	54.33 \pm 8.99	110.4 \pm 15.69	61.5 \pm 8.13	126.75 \pm 16.48
II (3 ng/mL)	47.75 \pm 8.47 ^b	96.17 \pm 14.17 ^b	45.92 \pm 7.63 ^b	88.25 \pm 11.22 ^d	59.75 \pm 8.42	118.33 \pm 21.09
III (30 ng/mL)	39.67 \pm 6.18 ^b	75.17 \pm 13.57 ^b	36.33 \pm 5.79 ^b	68.67 \pm 11.99 ^d	61.08 \pm 8.07	126.0 \pm 15.54
Naloxone			61.67 \pm 9.16	120.3 \pm 18.04	60.67 \pm 9.15	123.0 \pm 20.89

^a $P < 0.05$, ^b $P < 0.01$ vs control group (Mann-Whitney U test); ^d $P < 0.01$ vs control group (LSD test). Low- and high-glucose-stimulated insulin release in the 3 and 30 ng/mL fentanyl groups was significantly lower than in the control group ($P < 0.01$), and was lowest with 30 ng/mL fentanyl ($P < 0.01$). After adding 0.1 μ g/mL naloxone, insulin secretion in the 3 and 30 ng/mL fentanyl groups remained significantly lower than the control group ($P < 0.01$) and was lowest with 30 ng/mL fentanyl ($P < 0.01$).

the insulin release showed no difference from that of controls. Naloxone had no effect on insulin release (Table 1). There was no difference in SI between any of the groups (Table 2).

Evaluation of electron microscopy studies

Electron microscopy of β -cells in rat islets from the control group showed no pathological changes (Figure 3). After exposure to 30 ng/mL fentanyl for 48 h, the β -cells were in a poor morphological condition, and exhibited chromatin margination and severe cytoplasmic vacuolization and degeneration (Figure 4).

DISCUSSION

Many studies have focused on islet isolation, application and function in the diabetes field. Many factors affect the isolation of rat pancreatic islets; therefore, the yields and function of the isolated islets vary considerably,

Table 2 Stimulation index at different concentrations of fentanyl and naloxone (mean \pm SD, $n = 12$)

	Fentanyl	Fentanyl + 0.1 μ g/mL naloxone	Fentanyl + 1 μ g/mL naloxone
Control (0 ng/mL)	2.01 \pm 0.21	2.02 \pm 0.21	2.02 \pm 0.21
I (0.3 ng/mL)	2.17 \pm 0.36	2.07 \pm 0.35	2.08 \pm 0.26
II (3 ng/mL)	2.04 \pm 0.26	1.95 \pm 0.23	1.99 \pm 0.34
III (30 ng/mL)	1.89 \pm 0.16	1.89 \pm 0.19	2.08 \pm 0.27
Naloxone		1.97 \pm 0.29	2.04 \pm 0.28

despite the introduction of novel or improved methods. Large variations in yields and function have been found, even if the same sources of collagenase are used^[21]. In our study, the islets were isolated from the rat pancreas with good quality (viability rate was about 90% and purification rate was about 95%). Generally, we obtained 300-400 islets from each rat. This indicates that our method is efficient and provides high yields of islets.

The high yields and favorable function of the isolated islets were attributed to the pancreatic tissue being infused and fully digested. Low yields of islets typically result from insufficient digestion; therefore, we suggest that the digestion time and collagenase concentration should be optimized precisely for islet isolation.

Over the last two decades, despite the development of more potent, safer, faster onset, and shorter- and longer-lasting opioids, fentanyl has remained the mainstay of anesthesiologists and Certified Registered Nurse Anesthetists in the perioperative period, and for physicians involved in pain management. Because diabetic patients undergo higher risk procedures during the period of operation and anesthesia, anesthesiologists and surgeons should protect islet function and try to minimize any harmful medical effects on islets during the procedure. Although some studies have suggested that some opiates inhibit insulin secretion, it was unknown whether fentanyl has a similar effect^[12].

Before our current study, a number of studies have demonstrated direct effects of endogenous and selective opioid receptor agonists on insulin release^[10,11]. Some opioid receptor agonists, such as methadone, were found to improve multiple-low-dose streptozotocin-induced type 1 diabetes in mice, which suggests that the opioid receptor agonists improve insulin release *in vivo*^[22]. However, other studies have suggested that some opiates inhibit insulin secretion^[12]. β -endorphin seems to inhibit glucose-induced insulin secretion but, conversely, an excitatory effect has been reported in many studies^[11,23]. These observations have yielded conflicting results with marked variations between species. Therefore, it could not be hypothesized whether fentanyl inhibits insulin release^[13].

Our results clearly demonstrate that fentanyl inhibited glucose-stimulated insulin release from islets *in vitro*. Electron microscopy studies showed that the cells in the control group exhibited good viability but, after the cells were exposed to 30 ng/mL fentanyl for 48 h, chromatin margination and severe cytoplasmic vacuolization were observed, indicating that the cells were in a poor condition. Thus, our test suggests that the islets were injured by exposure to fentanyl at the concentrations tested.

We know that glucose uptake by tissues is as likely to play a decisive role as does the release of insulin. In the presence of insulin, opioid receptors in the pancreas have been reported to regulate plasma glucose and the activation of mu-opioid receptors, which could increase the utilization of glucose in peripheral tissue to lower the plasma glucose^[24,25]. *In vivo*, a potent opiate was shown to lower glucose levels by enhancing peripheral glucose utilization without improving insulin release^[26]. Also, serum glucose levels are responsible for the altered potency of mu-opioid agonists only during the early stages of diabetes^[27]. Thus, the plasma glucose-lowering response induced by mu-opioid receptor activation *in vivo* is not attributed to improved insulin release.

Endogenous opioid receptors are expressed in endocrine pancreas and studies have indicated that endogenous opioid peptides and selective opiate receptor agonists suppress insulin release^[28]. Thus, the effect of fentanyl on insulin release might be related to opiate receptor activation. Activation of the opioid receptor initiates a cascade of events that result in an array of biological effects, which inhibit insulin release^[29,30]. It seems that fentanyl-induced mu-opioid receptor activation leads to the inhibition of glucose-stimulated insulin release. This inhibitory effect could be reversed by high doses of naloxone, an opioid receptor antagonist. Therefore, the activation of opioid receptors induced by fentanyl appears to be a route by which insulin release is inhibited.

There are two processes that take place during insulin secretion: hormone biosynthesis and release. Many drugs affect insulin secretion, although the exact mechanism varies. Many drugs have different effects on either of the two processes. For example, long-chain fatty acids powerfully increase insulin release and inhibit glucose-stimulated pro-insulin biosynthesis^[31,32]. In the present study, it is unknown which of these processes are affected by fentanyl. Because fentanyl may be involved in both insulin release and biosynthesis^[33,34], the role of opioid receptor activation on insulin secretion requires further investigation.

There are certain limitations in our experiment. The pharmacology of the mu receptor signaling pathway involves G-protein coupling which, when activated, closes Ca^{2+} channels and opens K^{+} channels, resulting in a decline in intracellular Ca^{2+} levels and hyperpolarization; all of which are short-term effects. The magnitude of the insulin response to glucose is related not only to the absolute level of glucose but also to the rate of change in the glucose level. There are two phases of insulin release. An acute increase in glucose level elicits a rapid and transient secretion of insulin, called the first or acute-phase response, which subsides within 10 min. The second phase response begins when glucose levels increase slowly and progressively for up to 4 h^[35]. As in many other studies, we measured the insulin release over 60 min in static culture after incubating in low (2.8 mmol/L) and then high (16.7 mmol/L) glucose concentrations^[19,20,36]. Therefore, the effect of fentanyl on glucose-stimulated insulin release in rat islets in dynamic culture also needs to be investigated. In addition, perfusion of islets with glucose provides a dynamic profile of the characteristics of glucose-stimulated insulin release, which could be used to fully determine the effect of fentanyl on the inhibition of insulin release, and the ability of the cells to down-regulate insulin secretion after a fentanyl challenge are a focus of our future studies.

In conclusion, fentanyl inhibited glucose-stimulated insulin release from islets, and the inhibition could be reversed by naloxone. Because islet function varies between animal species, translation of our results to the

clinical setting will necessitate further studies. However, our results indicate the potential for fentanyl to inhibit insulin release; thus, it is necessary to test the plasma insulin level and the islet function regularly in patients treated with fentanyl, particularly patients treated with fentanyl for a long term and/or with high doses of fentanyl. Further studies are needed to explore the exact mechanism by which fentanyl affects islet insulin release.

COMMENTS

Background

Fentanyl citrate is a potent synthetic narcotic analgesic extensively used for anesthesia and analgesia in the operating room and intensive care unit. In recent years, anesthesiologists and surgeons have had to manage an increasing number of diabetic patients. However, the effects of fentanyl on the glucose-stimulated insulin secretion capacity of rat β -cells *in vitro* has remained unclear. Although some studies have suggested that some opiates inhibit insulin secretion, it could not be concluded whether fentanyl has a similar effect, because the earlier studies yielded conflicting results, with marked species variation. Therefore, this study investigated the effects of fentanyl on rat islets in static culture.

Research frontiers

This study is a novel field in diabetes investigation that has been largely overlooked. The research team used freshly isolated islets to study the effects of the opiate fentanyl on insulin release, which is an important subject for anesthesiologists and surgeons. In this study, the results demonstrated that fentanyl inhibited glucose-stimulated insulin release in rat islets and higher concentrations of fentanyl significantly damaged rat islets.

Innovations and breakthroughs

The study of effects of anesthetic and analgesic drugs on pancreatic islets is a novel field in diabetes management. This is the first study to report that fentanyl inhibits the release of insulin from rat islets. These findings would be helpful for clinicians who administer fentanyl.

Applications

The results may stimulate further investigation of diabetes management in the anesthesia field. These findings suggest that it is important to regularly test the plasma insulin level and islet function in patients treated with fentanyl for the long term and/or at high doses, and could help to develop novel approaches to help people with diabetes to maintain whole-body glucose homeostasis during the perioperative period.

Peer review

The author has used rat islets as a model to study the effect of the mu-opioid agonist fentanyl on glucose-stimulated insulin release. Generally, this is a topic that has been overlooked in the past, thus this study is important and unique. Also, the islet preparation and EM technology were good.

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S- Editor Tian L L- Editor Logan S E- Editor Zheng XM

BRIEF ARTICLES

Indicators of prognosis after liver transplantation in Chinese hepatocellular carcinoma patients

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Received: March 23, 2009 Revised: July 1, 2009

Accepted: July 8, 2009

Published online: September 7, 2009

Abstract

AIM: To identify prognostic factors of patients with hepatocellular carcinoma (HCC), who were treated by orthotopic liver transplantation (OLT).

METHODS: From January 2000 to October 2006, 165 patients with HCC underwent OLT. Various clinicopathological risk factors for actuarial and recurrence-free survival were identified using the Kaplan-Meier method with the log-rank test. The Cox proportional hazards model was used to identify independently predictive factors for actuarial and recurrence-free survival, which were used to propose new selection criteria. We compared the outcome of the subgroup patients meeting different criteria. Survival analysis was performed using the Kaplan-Meier method with the log-rank test.

RESULTS: The median follow-up was 13.0 mo (2.8-69.5 mo). Overall, 1-, 2-, 3- and 5-year actuarial survival was 73.3%, 45.6%, 35.4% and 32.1%, respectively. One-, 2-, 3- and 5-year overall recurrence-free survival was 67.0%, 44.3%, 34.5% and 34.5%, respectively. In univariate analysis, number of tumors, total tumor size, lobar distribution, differentiation, macrovascular invasion, microvascular invasion, capsulation

of the tumor, and lymph node metastasis were found to be associated significantly with actuarial and tumor-free survival. By means of using the multivariate Cox proportional hazards model, total tumor size and macrovascular invasion were found to be independent predictors of actuarial and tumor-free survival. When the selection criteria were expanded into the proposed criteria, there was no significant difference in 1-, 2-, 3- and 5-year actuarial and tumor-free survival of the 49 patients who met the proposed criteria (97.6%, 82.8%, 82.8% and 82.8%, and 90.7%, 82.8%, 68.8% and 68.8%, respectively) compared with that of patients who met the Milan or University of California, San Francisco (UCSF) criteria.

CONCLUSION: Macrovascular invasion and total tumor diameter are the strongest prognostic factors. The proposed criteria do not adversely affect the outcome of liver transplantation for HCC, compared with the Milan or UCSF criteria.

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Key words: Hepatocellular carcinoma; Prognosis; Liver transplantation; Outcome assessment; Survival

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Li J, Yan LN, Yang J, Chen ZY, Li B, Zeng Y, Wen TF, Zhao JC, Wang WT, Yang JY, Xu MQ, Ma YK. Indicators of prognosis after liver transplantation in Chinese hepatocellular carcinoma patients. *World J Gastroenterol* 2009; 15(33): 4170-4176 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4170.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4170>

INTRODUCTION

In the 1980s, outcomes for orthotopic liver transplantation (OLT) for hepatocellular carcinoma (HCC) were discouraging. There were high recurrence rates and a low patient survival of 30% at 3 years^[1]. After the Milan criteria (single tumor up to 5 cm or up to three tumors up to 3 cm) were introduced by Mazzaferro *et al*^[2] in the

early 1990s, recurrence rate fell to 8%, and tumor-free patient survival at 4 years was 83%. With worldwide adoption of these criteria, 5-year survival rates rose to 60%-80%^[3-5].

Recently, there have been numerous proposals for expanding the Milan criteria. The University of California, San Francisco (UCSF) proposed an expansion of transplantation criteria^[6]. The expanded criteria includes having a single tumor < 6.5 cm in diameter, or having no more than three tumors, the largest of which is < 4.5 cm in diameter, and with a total tumor diameter of < 8 cm. Using these criteria, 1- and 5-year recurrence-free survival rates were 90% and 75.2%, respectively. Marsh *et al*^[7] have studied 407 cases who underwent OLT at the University of Pittsburgh between 1981 and 2002, and found that patients who exceeded the Milan criteria had a 49.7% recurrence-free survival rate at 5 years. Todo *et al*^[8] have reported that patients with unresectable HCC who underwent living donor liver transplantation under the expanded indication criteria had 3-year survival and disease-free survival rates of 60.4% and 52.6%, respectively. In Kyoto^[9], the only OLT exclusion criteria are for patients with extrahepatic metastasis or macroscopic vascular invasion, and there are no restrictions based upon on the number or size of tumors. There was no difference in the 4-year survival rate in the Kyoto study between patients with HCC who fit (66%) or do not fit (60%) the Milan criteria. Therefore, it has been suggested that there are differences in OLT criteria between the East and West, which may be correlated with race or combinations of underlying hepatic diseases.

Worldwide, 55% of HCC cases and deaths occur in China^[10]. Over 90% of HCC patients in China are also infected with hepatitis B virus (HBV). These rates are different from those in Europe, America, and even Japan. To explore these issues, we evaluated the OLT outcomes for HCC patients at the Liver Transplantation Center of the West China Hospital, Chengdu, China. We aimed to analyze the effect of HCC prognostic factors on actuarial and recurrence-free survival after OLT, and to reevaluate the prediction criteria that are the basis for OLT selection.

MATERIALS AND METHODS

Patients

Between January 2000 and October 2006, a total of 424 OLTs were performed at the Liver Transplantation Center of the West China Hospital. Among these, 165 (38.9%) patients were diagnosed with HCC, and 148 underwent follow-up. Follow-up excluded 17 patients who died of complications during the 3-mo period after the operation. Of the 148 HCC patients, 146 were diagnosed with HCC before liver transplantation. In two patients, HCC was found incidentally by pathological examination of the explanted liver after transplantation. Preoperative clinical data of the 148 HCC patients are

Table 1 Clinical data for 148 patients

	n (%)
Sex	
Male	133 (89.9)
Female	15 (10.1)
Age (yr)	Range 17-68 (median 45)
Etiology	
Hepatitis B	137 (92.6)
Hepatitis B, C	2 (1.4)
Alcoholic	1 (0.7)
Idiopathic	8 (5.4)
Pre-transplantation AFP (ng/mL)	
≤ 400	60 (40.5)
> 400	88 (59.5)
Child-Pugh Class	
A	76 (51.4)
B	57 (38.5)
C	15 (10.1)
Meld score	
< 14	107 (72.3)
≥ 14	41 (27.2)
Pre-transplantation therapy	
Positive	47 (31.7)
Negative	101 (68.2)
Tumor stage	pTNM
I	27 (18.2)
II	40 (27.0)
IIIa	66 (44.6)
IIIb	10 (6.8)
IIIc	5 (3.4)

shown in Table 1. Liver transplantation was considered for patients with HCC if the tumor was determined to be unresectable because of its location, or because of concomitant liver disease and HCC without extrahepatic spread.

Diagnosis

Preoperative diagnosis of HCC was made using artery high-flow perfusion and imaging examinations, which showed intrahepatic tumors. Examinations included at least two of the following three methods: ultrasound, contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI). Macroscopic vascular invasion was observed using Doppler ultrasonography and contrast-enhanced CT before operation, and was validated through pathological examination of the impaired liver after operation. Diagnosis of HCC was confirmed by fine-needle aspiration cytology or biopsy of all known tumors. Pretransplant studies in patients with HCC included abdominal, thoracic, and head CT scans and bone scintigraphy to rule out extrahepatic tumor spread.

Follow-up

The patients were followed for tumor recurrence with CT scans and α -fetoprotein level (AFP) every 6 mo for 5 years. Annually thereafter, or as the clinical situation dictated, suspicious lesions in the liver or lung were biopsied. Bone lesions were not biopsied routinely but were observed for bone pain and progression of growth. A rising AFP level alone was not taken to be confirmatory

of tumor recurrence, but the date of recurrence was taken as the time that the AFP level began to rise once tumor recurrence had been confirmed.

Immunosuppression and antiviral protocols

Immunosuppression consisted of cyclosporine or tacrolimus and corticosteroids, with or without azathioprine and mycophenolate. In cases in which acute rejection was suspected, a liver biopsy was performed, and steroid pulse therapy was conducted after the rejection diagnosis was made. The steroids were withdrawn 3-6 mo after surgery to minimize the risk of tumor recurrence^[8,11]. Lamivudine was administered to hepatitis-B-surface-antigen-positive patients before and after surgery, and hepatitis B immune globulin was administered to HBV-DNA-positive patients before, during and after surgery.

Statistical analysis

Cumulative survival time was calculated from the date of transplantation to the date of death, from the date of transplantation to the date of final follow-up, or from the date of transplantation to the date of loss to follow-up. The latter two conditions were tabulated from censored data. Tumor-free survival (TFS) time was calculated from the date of transplantation to the date when tumor recurrence and metastases were discovered, from the date of transplantation to the date of final follow-up, or from the date of transplantation to the date of loss to follow-up. The latter two conditions were also tabulated from censored data. Statistical analysis software (SPSS 13.0) was used for data processing and analysis. The Kaplan-Meier method was used to calculate the cumulative survival rate (CSR), TFS rate, and to present the corresponding survival curves in graphical form. The log-rank test was used to compare the differences between groups. The univariate Cox proportional hazard regression model was used to analyze each factor that might have influenced liver transplantation prognosis in HCC patients, and to identify factors with statistical significance. The multivariate Cox proportional hazard regression model was used to analyze and confirm the independent prognostic factors for OLT in HCC patients. Statistical significance was defined as $P < 0.05$.

RESULTS

Pathological characteristics of explanted liver

The pathological data on HCC tumors and potential indicators are shown in Table 2. In general, OLT was offered when liver function was impaired, or when the HCC become unresectable. HCC was diagnosed before transplantation in 148 patients, and in two, we identified an incidental tumor during pathological examination of the explanted liver.

Survival

The median follow-up time for the 148 patients was 13.0 mo (2.8-69.5 mo). The follow-up time of 126 of these patients was ≥ 6 mo. Eighty-one patients survived,

Table 2 Pathological data from 148 HCC patients

Characteristics	n (%)
Tumor	
≤ 3	101 (68.2)
> 3	47 (31.8)
Total tumor size (cm)	
≤ 5	24 (16.2)
5-9	30 (20.9)
> 9	94 (63.5)
Lobar distribution	
Unilobar	80 (54.1)
Bilobar	68 (45.9)
Differentiation	
Well	35 (23.6)
Moderate	100 (67.6)
Poor	13 (8.8)
Vascular invasion	
None	47 (31.8)
Micro	49 (33.1)
Macro	52 (35.1)
Lymph node status	
Negative	143 (96.6)
Positive	5 (3.4)
Cirrhosis	
Negative	13 (8.8)
Positive	135 (91.2)

with a median follow-up of 15.5 mo (2.8-69.5 mo). Sixty-two patients died, with a median follow-up of 11.0 mo (3.0-38.0 mo). Five patients were lost to follow-up, with a median follow-up of 16.0 mo (14.0-23.0 mo).

The mean total cumulative survival time of the entire group was 33.6 mo (95% CI: 27.5-39.6 mo). The CSR was 73.3% at 12 mo, 45.6% at 24 mo, 35.4% at 36 mo, and 32.1% at 60 mo. The average TFS time of the entire group was 32.8 mo (95% CI: 26.4-39.1 mo). The TFS rate was 67.0% at 12 mo, 44.3% at 24 mo, 34.5% at 36 mo, and 34.5% at 60 mo.

Predictors of actuarial survival

Based on the results of the univariate Cox regression model analysis, the following 10 variables significantly affected CSR: (1) age; (2) largest tumor size; (3) total tumor size; (4) tumor number; (5) bilobar disease; (6) macrovascular invasion; (7) microvascular invasion; (8) lymph nodes positive; (9) differentiation; and (10) capsule invasion. The above 10 variables were analyzed using the multivariate Cox proportional hazard regression model and the stepwise regression method, to identify the independent factors that influenced total survival rate. The results showed that total tumor size and macrovascular invasion were the two risk factors that affected total survival rate.

Predictors of recurrence-free survival

According to the results of the univariate Cox regression model analysis (Table 3), the following eight variables significantly affected tumor-free survival rate: (1) total tumor size; (2) tumor number; (3) bilobar tumor; (4) tumor differentiation; (5) macrovascular invasion; (6) microvascular invasion; (7) tumor capsular invasion;

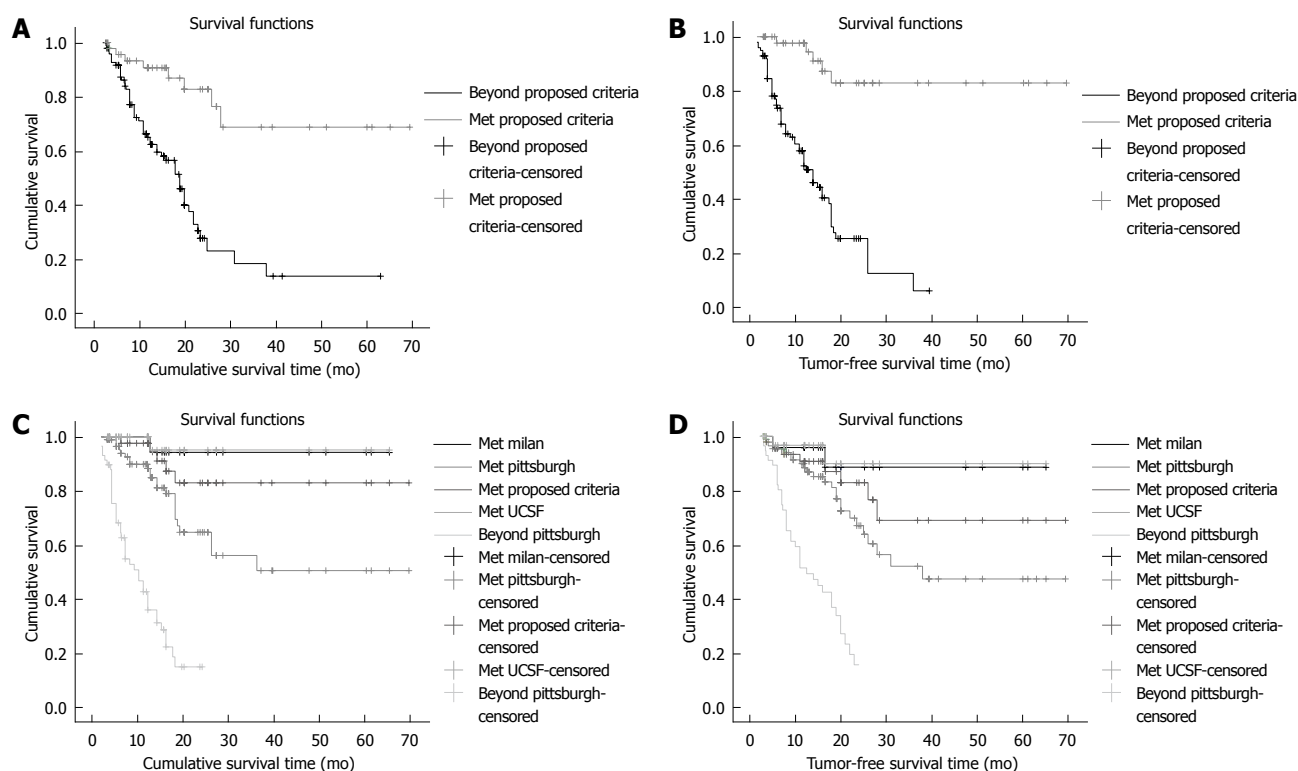


Figure 1 Proposed prognostic criteria. A: CSR for proposed criteria. Log-Rank test: $P < 0.001$; B: TFS rate for proposed criteria. Log-Rank test: $P < 0.001$; C: Comparison of CSR for different criteria; D: Comparison of TFS rate for different criteria.

Table 3 Univariate analysis of all variables that significantly affected tumor-free survival

Predictors	Relative risk	95% CI	P
Total tumor size (≤ 9 cm vs > 9 cm)	2.291	1.654-3.173	0.000
Tumor number (≤ 4 vs > 4)	1.127	1.052-1.207	0.003
Bilobar disease	2.206	1.327-3.666	0.002
Macrovascular invasion	2.951	2.160-4.032	0.000
Microvascular invasion	4.479	2.126-9.436	0.000
Positive lymph nodes	12.472	4.457-34.898	0.000
Differentiation	1.641	1.014-2.654	0.044
Capsule invasion	1.462	1.092-1.957	0.011

and (8) lymph nodes positive. The above eight variables were analyzed using the multivariate Cox proportional hazard regression model and the stepwise regression method, to identify independent factors that influenced the TFS rate. The results showed that total tumor size, macrovascular invasion and lymph node status were the independent predictors of TFS.

Proposed criteria

Macrovascular invasion and total tumor size were prognostic factors that independently influenced CSR and TFS. Our proposed criteria for OLT selection included patients with total tumor size ≤ 9 cm and who were without macrovascular invasion or extrahepatic metastases, regardless of the number of tumor lesions.

For the cases which conformed to the proposed criteria ($n = 49$), the total survival rate at 1, 2, 3 and 5 years was 97.6%, 82.8%, 82.8% and 82.8%, respectively

(Figure 1A). The average survival time was 53.7 mo (43.8-63.6 mo). Among these 49 patients, 18 (16.3%) died by the end of follow-up, and seven (9.5%) died of recurrence. There were 41 cases of TFS (83.7%). The average TFS rate at 1, 2, 3 and 5 years was 90.7%, 82.8%, 68.8% and 68.8%, respectively (Figure 1B). The average TFS time was 60.0 mo (52.3-67.7 mo).

Comparisons of the CSR and the TFS at 1, 2, 3 and 5 years for different patient subgroups are shown in Figure 1C and D. CSR and TFS were similar in patients who met the proposed criteria and Milan criteria ($P = 0.321$, $P = 0.331$), and UCSF criteria ($P = 0.229$, $P = 0.257$). However, the patient number was increased more in the proposed criteria ($n = 49$), than the Milan ($n = 24$) or UCSF ($n = 33$) criteria. What's more CSR was similar in patients who met the proposed criteria and the Pittsburgh criteria ($P = 0.158$). Although the patient number was greater in the Pittsburgh ($n = 90$) than the proposed criteria, the TFS for the Pittsburgh criteria was lower than that for the proposed criteria ($P = 0.027$).

DISCUSSION

Numerous studies have shown that tumor size is an important prognostic factor for liver transplantation in patients with HCC^[8,12,13]. Bismuth *et al*^[12] have found that HCC patients with two or fewer tumors, each with a diameter ≤ 3 cm, had an 83% 3-year CSR and TFS following liver transplantation and hepatectomy. Roayaie *et al*^[14] have reported that HCC patients with tumor diameters > 7 cm (12 cases) and 5-7 cm (32 cases) had 5-year tumor-free rates of 34% and 55%, respectively,

following transplantation. Tumor diameter > 7 cm and the presence of vascular invasion were correlated with HCC recurrence. In this study, total tumor size was shown to influence prognosis in the univariate Cox regression model analysis, and was shown to be an independent prognostic factor in the multivariate analysis. Compared to largest tumor size, tumor number can better predict the OLT prognosis in HCC patients. Univariate Cox regression analysis showed that the 2-year CSR and TFS rates were 73.5% and 75.6%, respectively. These rates were significantly different compared to those in cases where total tumor size was > 9 cm. Tumor number and size are believed to have a combined effect on HCC recurrence probability, and this effect is taken into consideration during selection of HCC OLT recipients in many institutions^[2,6]. The total tumor size in the present study also reflects a combined effect of tumor number and size.

Many other studies have shown that macrovascular invasion is a primary factor that influences prognosis after OLT in HCC patients^[12,13,15-18]. Shetty *et al*^[18] have concluded that macrovascular invasion and AFP levels were prognostic factors that influenced CSR and TFS. Moreover, microvascular invasion had no significant influence on prognosis. Bismuth *et al*^[12] have reported that 10 of 60 (16.7%) HCC patients who underwent OLT had tumor thrombus in the main trunk of the portal vein, and had a 3-year survival rate of only 20%. Thus, they proposed that tumor thrombus in the main trunk of the portal vein was a major risk factor for OLT. Other studies have proposed that when HCC is accompanied by vascular invasion, tumor cells are more likely to be present in the circulation, rather than being restricted to the liver^[19,20]. While circulating tumor cells may not develop into distant metastases, immunosuppressive treatment after OLT may increase the possibility of tumor recurrence. Currently, most transplantation centers advocate exclusion of HCC patients with tumor thrombus in the main trunk or right and left branches of the portal vein, for these reasons. In the present study, multivariate Cox regression analysis showed that macrovascular invasion was an independent risk factor that affected CSR and TFS. In the 52 cases with macrovascular invasion, the recurrence rate was as high as 71.2% during follow-up. The 1-year CSR was 53.5%, and TFS was 14.4%. The 2-year CSR and TFS were 34.2% and 12.9%, respectively. Therefore, OLT patients with macrovascular invasion had poor long-term prognosis.

Univariate analysis identified hepatic lobar distribution, differentiation, and capsule invasion as prognostic factors that influenced CSR and TFS in the present study. However, multivariate analysis showed that only total tumor size and macrovascular invasion were independent prognostic factors that influenced CSR and TFS. Therefore, hepatic lobar distribution, differentiation, and capsule invasion may have a relationship with total tumor size or macrovascular invasion. We believe that these three indicators may reflect malignant tumor

invasion to a certain degree, since HCC patients who have bilobar distribution, low differentiation or capsular (or non-capsular) tumor invasion have a relatively high incidence of tumor recurrence and metastasis.

Our data showed that age was related significantly to CSR by univariate Cox regression analysis, with a relative risk of 0.963 (95% CI: 0.936-0.991). The younger the onset of HCC, the greater the possibility of tumor recurrence was after liver transplantation. An early age of HCC onset may lead to greater malignancy and faster disease development. An understanding of this mechanism requires further exploration.

Positive lymph nodes and distant metastasis are considered to be absolute contraindications for liver transplantation in HCC patients^[21]. Marsh *et al*^[22] have found that the average TFS time for 231 HCC liver transplantation cases with negative local lymph nodes was 140.6 ± 6.8 mo. The TFS time was significantly less, 5.3 ± 1.0 mo, for six cases with positive local lymph nodes. In the present study, five HCC cases (3.4%) had positive lymph nodes in the porta hepatis. Four of these patients died, and all five had tumor recurrence. The average survival time of these five patients was only 7.0 mo, and the average TFS time was 4.2 mo, in accordance with previous studies. Detection of lymph nodes can be observed in HCC patients with hepatitis, who have enlarged, inflammatory lymph nodes. Therefore, careful observations should be conducted during surgery, and all enlarged lymph nodes should be sent to the pathology department for intraoperative frozen-section examination. Liver transplantation should not be carried out in patients with confirmed positive lymph nodes.

China has the greatest incidence of liver cancer worldwide^[10]. Each year, over half of liver cancer detections and deaths occur in China. Liver cancer (mainly HCC) has been the primary indication for liver transplantation. It is worth noting that the hepatic disease background and epidemiology of HCC is unique to China and is considerably different from that in Europe and America. Therefore, prognostic factors for liver transplantation in Chinese HCC patients are likely different from those in other countries. It is therefore necessary to explore the novel criteria for HCC liver transplantation in China.

Here, we studied independent prognostic factors, and developed proposed criteria for liver transplantation in HCC patients. The proposed criteria are as follows: total tumor size ≤ 9 cm, and there should be no macrovascular invasion, positive lymph nodes or extrahepatic metastases, regardless of tumor number and distribution. According to the proposed criteria, we screened HCC patients with liver transplantation. The postoperative 2- and 5-year CSR was 82.8% and 68.8%, respectively, using our proposed criteria. There was no significant difference between the proposed and the Milan criteria (both 88.5%) ($P = 0.321$). Using the proposed criteria, the 2- and 5-year TFS rates were both 82.8%.

Compared to the Milan criteria (both rates 94.1%),

there was no significant difference ($P = 0.331$). We compared the HCC cases that fitted the proposed criteria (49 cases) and those that exceeded the criteria (99 cases) (Figure 1A and B). The 2- and 5-year CSR of the cases that fitted the proposed criteria were 82.8% and 68.8%, respectively, while the CSR for cases exceeding the criteria were 27.9% and 14.0%, respectively ($P < 0.001$). The 1- and 2-year TFS of the cases that fitted the proposed criteria were 97.6% and 82.8%, respectively, while the TFS for cases exceeding the criteria were 52.5% and 25.7%, respectively ($P < 0.001$). Use of our proposed criteria included 16.9% more cases than the Milan criteria and 10.8% more cases than the UCSF criteria. In this study, we also analyzed cases that fitted the proposed criteria, but exceeded the Milan criteria (25 cases). Our results showed that the 1-, 2- and 5-year CSRs were 85.2%, 77.4% and 48.4%, respectively, and the corresponding TFS rates were 95.0%, 70.4% and 70.4%, respectively. It is agreed generally in the field of transplantation that HCC patients whose 5-year, post-transplantation, survival rate is $\geq 50\%$ qualify as transplant candidates^[23,24]. The present study showed that the 5-year CSR and TFS of the HCC patients were 68.8% and 82.8%, respectively. The 5-year CSR and TFS of the HCC patients that fitted our proposed criteria but exceeded the Milan criteria (25 cases) were 48.4% and 70.4%, respectively. The CSR and TFS rate curves of these patients were not significantly different compared to those of patients who fitted the Milan criteria ($P = 0.105$ and $P = 0.115$, respectively).

In summary, we suggest that the proposed criteria function to predict the prognosis of liver transplantation in patients with HCC. The proposed criteria allow us to increase the range of indicators for HCC patients in need of liver transplantation, and decrease the exclusion rate, and they allow more patients to receive therapeutic liver transplantation in China.

COMMENTS

Background

In the 1980s, the outcome of orthotopic liver transplantation (OLT) for hepatocellular carcinoma (HCC) was discouraging. After the Milan criteria (single tumor up to 5 cm, or up to three tumors up to 3 cm) were introduced by Mazzaferro *et al* in the early 1990s, recurrence rate fell to 8%, and tumor-free patient survival at 4 years was 83%. The Milan criteria have long been considered to be the classical criteria for selection of patients with HCC for liver transplantation. Recently, there have been numerous proposals for expanding the Milan criteria, such as the University of California, San Francisco (UCSF) criteria.

Research frontiers

The Milan criteria have long been considered to be the classical criteria for selection of patients with HCC for liver transplantation. China has the greatest incidence of liver cancer worldwide. Each year, over half of liver cancer detections and deaths occur in China. A substantial proportion of adult Living donor liver transplantation patients not fulfilling the Milan or UCSF criteria have been found to survive longer than expected after transplantation. Therefore, it seems reasonable to attempt further reduction of unnecessary dropouts arising from the strict application of narrow selection criteria.

Innovations and breakthroughs

In the present study, the authors identified various clinicopathological risk factors for actuarial and recurrence-free survival of 165 patients with HCC who

underwent OLT using the Kaplan-Meier method with the log-rank test. They found that macrovascular invasion and total tumor diameter, as assessed on explanted liver, were the strongest prognostic factors.

Peer review

The authors reviewed their single-center experience with 148 OLTs for HCC and proposed a new prognostic score.

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S- Editor Li LF L- Editor Kerr C E- Editor Zheng XM

Overexpression of polo-like kinase1 predicts a poor prognosis in hepatocellular carcinoma patients

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Received: June 6, 2009 Revised: August 5, 2009

Accepted: August 12, 2009

Published online: September 7, 2009

Key words: Hepatocellular carcinoma; Immunohistochemistry; Reverse transcription-polymerase chain reaction; Survival analysis; Polo-like kinase 1

Peer reviewer: Naofumi Mukaida, MD, PhD, Chairperson and Professor, Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan

He ZL, Zheng H, Lin H, Miao XY, Zhong DW. Overexpression of polo-like kinase1 predicts a poor prognosis in hepatocellular carcinoma patients. *World J Gastroenterol* 2009; 15(33): 4177-4182 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4177.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4177>

Abstract

AIM: To elucidate the role of overexpressed polo-like kinase1 (PLK1) in hepatocellular carcinoma (HCC).

METHODS: We prospectively collected clinicopathological, immunohistochemical and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) data from 135 HCC patients undergoing successful hepatectomy. The correlations between PLK1 mRNA expression and clinicopathologic variables were analyzed by Mann-Whitney *U* test. Prognostic factors were identified by univariate and multivariate analyses.

RESULTS: Immunohistochemical results showed overexpression of PLK1 was mainly found in tumor tissues compared with tumor-free tissue. A similar mRNA result was obtained by semi-quantitative RT-PCR. A total of 111 samples were positive for PLK1 mRNA expression. The positive expression was correlated with venous invasion, tumor nodules and Edmondson grade. Furthermore, 1, 3, 5-year survival rates in the positive expression group were significantly lower than the negative control group. Multivariate analysis showed that positive PLK1 expression was an independent risk factor for HCC.

CONCLUSION: PLK1 could be a potential biomarker for diagnosis and therapy for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the deadliest of all cancers, ranking third among all cancer-related mortalities^[1]. Surgical resection plays a major role in the treatment of HCC. However, less than 30% of HCC patients are surgical candidates^[2] due to limiting factors such as severe impairment of hepatic functional reserve, bilobar tumor distribution and extra-hepatic metastasis. No single-agent or combination chemotherapy regimen has been found to be particularly effective in HCC^[3]. Locoregional treatment is not the first choice for HCC^[4] and is reserved for non-surgical candidates.

The study of carcinogenesis mechanisms may provide new treatment regimens for cancer. Many key carcinogenetic pathways, such as increased angiogenesis, aberrant signal transduction and dysregulated cell cycle control, appear to be involved in tumor development^[5]. As a cell cycle control kinase, polo-like kinase1 (PLK1) and its overexpression are highly associated with many human cancers, including bladder^[6], breast^[7-10], colorectal^[11,12], endometrial^[13,14], esophageal^[15-17], gastric^[18-21], glioma^[22], hepatoblastoma^[23], hepatocellular carcinoma^[24], head and neck^[25], leukemia and lymphoma^[26,27], melanoma^[28,29], non-small-cell lung^[30], ovarian^[31], papillary^[32], pancreatic^[33,34], prostate^[35] and thyroid cancer^[36]. However, up to now, as far as we know, there are few studies available on the overexpression of PLK in HCC. The objective of our study was to understand the relevance of PLK1 expression in HCC.

MATERIALS AND METHODS

Patients

There were 135 successful hepatectomy procedures for HCC performed from January 2003 to September 2008 in our department. None of these patients were given preoperative transarterial chemoembolization as a neoadjuvant treatment, but they all regularly received postoperative chemoembolization by hepatic artery infusion. All specimens from 135 cases were collected, immediately frozen in liquid nitrogen and subsequently stored at -70°C for reverse transcription-polymerase chain reaction (RT-PCR). Five cases of liver regenerating nodule samples were obtained from patients with liver cirrhosis as controls. The clinicopathologic variables were recorded in detail including gender, age, liver cirrhosis, hepatitis B surface antigen, α -fetoprotein (AFP), venous invasion, Edmondson stage, tumor size (cm) and number of tumor nodules. Two expert pathologists who were blinded to the other results of the study, scored the HCC samples. The study protocol was approved by the Ethics Committee of Central South University, and written informed consent was provided by all participants prior to initiation of the study.

Follow-up

All patients were involved in our follow-up system, and were reviewed at 1-2 mo intervals. Routine post-operative medical examinations were carried out every 2 mo, including liver function, serum AFP level, B-ultrasound and CT. Follow-up ranged from 3-62 mo and ended on December 31, 2008. The median follow-up time was 18 mo.

Semi-quantitative RT-PCR

Total RNA was extracted from tumor and tumor-free tissues using the TRIzol reagent (Gibco BRL) according to the manufacturer's instructions (Gibco BRL). cDNA synthesis performed using the reverse transcription system. The primers were as follows: PLK1, 5'-GATTCCACGGCTTTTTCGAG-3', 5'-CCCACACAGGGTCTTCTTCC-3' (product size, 296 bp); β -actin: 5'-CGCGAGAA GATGACCCAGAT-3', 5'-GCACTGTGTTGGCGTACAGG-3' (product size, 550 bp). The following PCR cycling parameters were employed: at 95°C for 5 min, followed by 35 cycles at 95°C for 45 s, 56°C for 1 min, at 72°C for 1 min and then 72°C for 7 min. The PCR products were resolved on a 1.5% agarose gel. All experiments were carried out in triplicate.

Immunohistochemical tissue slides

Immunohistochemical reaction against PLK1 (BD Transduction, a monoclonal mouse antibody) was performed in 5 μm paraffin sections. Negative controls were processed without primary antibody. For antigen retrieval, deparaffinized slides were placed in 0.01 mol/L sodium citrate buffer, pH 6.0 and boiled for 5 min in a pressure cooker. Then, slides were allowed to cool down for an additional 5 min in the same buffer. After

several rinses in TBS and pre-treatment with blocking reagent (Dako, Glostrup, Denmark) for 5 min, slides were incubated with primary antibody diluted 1:500 (PLK1) and 1:50 (Ki-67) in antibody diluent solution (Zymed, San Francisco, CA, USA) for 20 min at room temperature and then at 4°C overnight. After slides were washed in TBS, The slides were visualized using Aquatex (Merck, Gernsheim, Germany).

Statistical analysis

Quantitative values were presented as mean \pm SD or median (range). Independent Student's *t*-test was used to compare PLK1 mRNA expression in HCC and non-cancer samples. The Mann-Whitney *U* test was used for correlations between PLK1 mRNA expression and clinicopathologic variables. The Kaplan-Meier method was employed to calculate survival and the log-rank test to compare survival among two patient groups. Cox regression was adopted for multivariate analysis of prognostic predictors. The statistical software package SPSS16.0 (SPSS Inc, Chicago, IL) was applied for all analyses. A statistically significant *P* value was defined as < 0.05 .

RESULTS

Immunohistochemical analysis of PLK1 protein expression

The pattern of PLK1 protein expression was examined by means of immunohistochemical analysis. Overexpression was detected in tumor tissues, especially in cytoplasm, compared with the tumor-free tissue. A more varied morphology of the cells was reflected by high expression in the cytoplasm. (Figure 1A and B). Tumor tissues had sharp margins under the microscope (Figure 1B and D). No or sporadic expression was observed in adjacent normal tissue or regenerating nodules (Figure 1C and D).

Expression of PLK1 mRNA in HCC

To compare the expression levels of PLK1 mRNA between neoplasm, adjacent normal tissue in the experimental group and regenerating nodules in the control group, total RNA was extracted for RT-PCR. The results indicate that HCC tissues expressed a significantly higher level of PLK1 mRNA than adjacent normal tissue and regenerating nodule (0.53 ± 0.05 vs 0.23 ± 0.04 and 0.20 ± 0.02 , respectively, $P < 0.01$). When regenerating nodules were compared with adjacent normal tissue, the mRNA levels of regenerating nodules were slightly elevated, but the difference was not statistically significant (0.23 ± 0.04 vs 0.20 ± 0.02 , $P > 0.05$) (Figure 2).

There were 111 samples (111/135, 82.22%) in which the OD values of PLK1 mRNA in HCC tissues were higher than those of adjacent normal tissue ($P < 0.05$). These were named the PLK1 positive group. The remaining 24 samples (24/135, 17.78%) were named the PLK1 negative group. There were statistically significant differences in PLK1 mRNA levels between the positive and negative groups ($P = 0.003$).

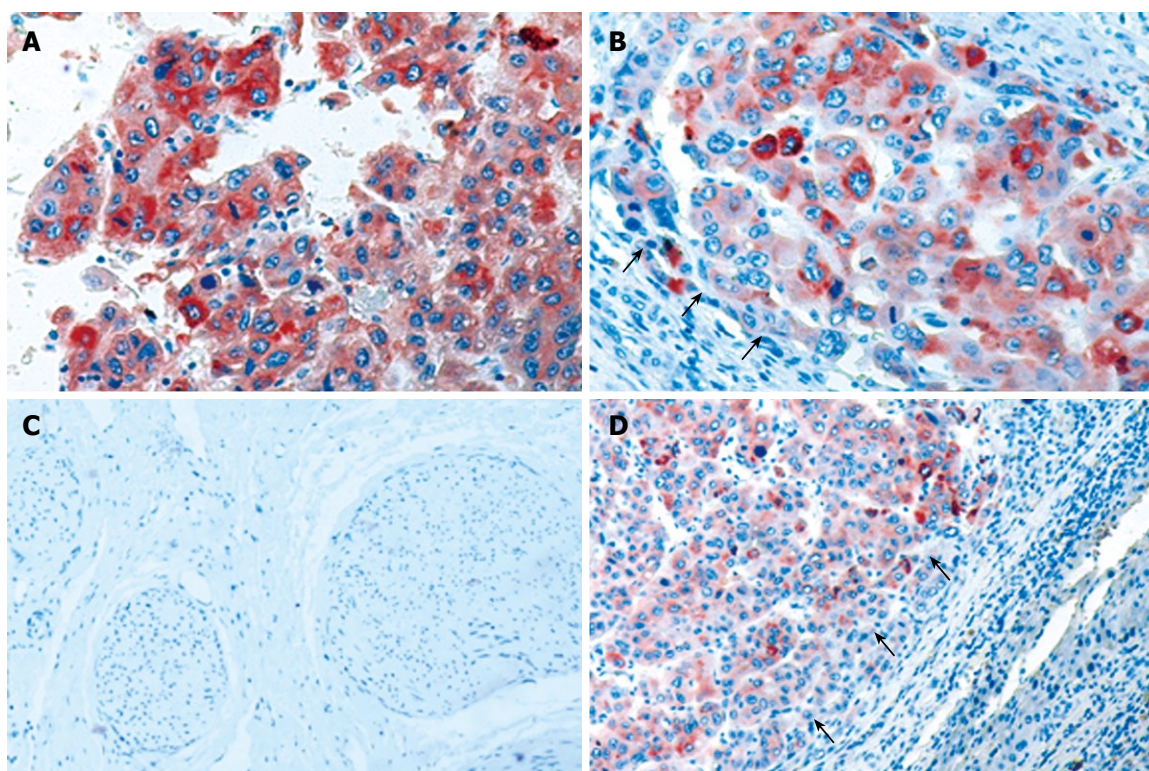


Figure 1 Expression of PLK1 in HCC tissue specimens. A: Expression of PLK1 in HCC tissue, especially in cytoplasm; B, D: Tumor tissue margins seen under the microscope (black arrows); C: Liver regenerating nodule sample; D: Adjacent normal tissue examined for PLK1 expression. Original magnifications, A and B $\times 200$; C and D $\times 100$.

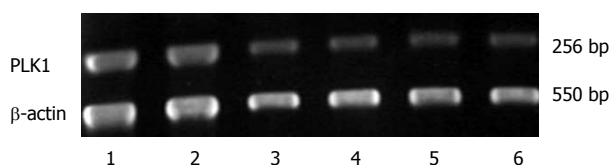


Figure 2 Expression of PLK mRNA in HCC, adjacent normal and regenerating nodule tissues. Lanes 1 and 2, HCC tissues; Lanes 3 and 4, adjacent normal tissue; Lanes 5 and 6, regenerating nodule tissues. RT-PCR for β -actin was used to monitor the quality of the RNA sample. RT-PCR was performed in triplicate.

Correlations between PLK1 positive expression group and clinicopathologic variables

The relationship between PLK1 positive expression groups and clinicopathologic data was analyzed by statistical software (Table 1). By the Mann-Whitney *U* test, the positive expression group with multiple tumor nodules had values significantly higher than that with solitary tumor nodules ($P = 0.002$). The high Edmondson-Steiner grade (III, IV) in the positive expression group was also significantly stronger than those of low grade tumors (I, II) ($P = 0.022$). Furthermore, venous invasion was significantly correlated with the positive expression group ($P = 0.042$). However, this study also showed that positive expression had no significant relationship with gender, age, liver cirrhosis, HBsAg, AFP or tumor size ($P > 0.05$) (Table 1).

Survival analysis of prognostic factors

As mentioned above, 135 cases of HCCs were divided

into positive and negative expression groups. The Kaplan-Meier method was employed to analyze the correlation of PLK1 expression level and the prognosis of HCC patients. Our results indicated that the positive expression group correlated with a shorter survival time than the negative group. The median survival time was 22.53 mo *vs* 60.88 mo. In addition, the 1, 3 and 5 year survival rates for the patients with positive and negative expression in HCC were 91.3%, 67.1% and 50.3% and 67.8%, 42.3% and 20.9%, respectively. The overall survival rates in the two groups was significantly different ($P = 0.003$, log-rank test) (Figure 3). At the same time, all the clinicopathologic variables were also analyzed with the Kaplan-Meier method. The results showed that a high Edmondson grade, venous invasion and multiple tumor nodules were correlated with a poor prognosis in HCC while the other clinicopathologic variables did not provide any independent prognostic information (Table 2).

By multivariable Cox regression analysis, PLK1 mRNA positive expression (RR, 3.507; 95% CI: 1.386-8.874, $P = 0.008$), high Edmondson grade (RR, 1.929; 95% CI: 1.069-3.482, $P = 0.029$), multiple tumor nodules (RR, 2.377; 95% CI: 1.384-4.082, $P = 0.002$) and venous invasion (RR, 4.848; 95% CI: 2.649-8.871, $P < 0.001$) were found to be independent prognostic factors for survival (Table 3).

DISCUSSION

Polo-like kinase 1 (PLK1) belongs to a family of

Table 1 Correlations between PLK1 mRNA expression and clinicopathologic variables by Mann-Whitney *U* test

Factors	<i>n</i>	PLK1 mRNA		<i>P</i>
		Positive	Negative	
Gender				
Male	114	93	21	0.650
Female	21	18	3	
Age (yr)				
≤ 40	65	53	12	0.842
> 40	70	58	12	
Liver cirrhosis				
Yes	109	93	16	0.055
No	26	18	8	
HBsAg				
Positive	96	80	16	0.598
Negative	39	31	8	
AFP (ng/mL)				
≤ 400	51	45	6	0.156
> 400	84	66	18	
Venous invasion				
+ (cases)	53	48	5	0.042
- (cases)	82	63	19	
Edmondson stage				
I - II	56	41	15	0.022
III-IV	79	70	9	
Tumor size (cm)				
≤ 5.0	36	28	8	0.417
> 5.0	99	83	16	
Tumor nodule				
Single tumor nodule	77	70	7	0.002
Multiple tumor nodule	58	41	17	

HBsAg: Hepatitis B surface antigen; AFP: α -fetoprotein.

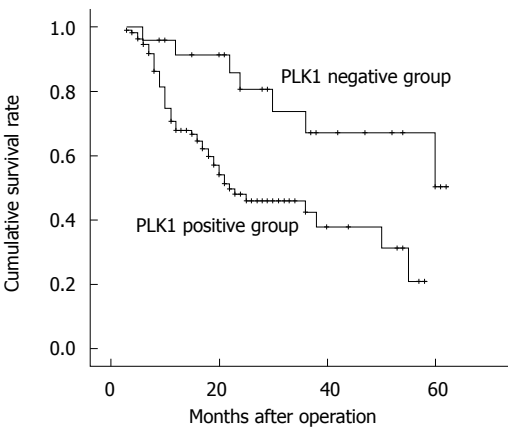


Figure 3 Log-rank test shows that HCC patients in the positive PLK1 mRNA expression group had a lower survival than those in the negative group.

conserved serine/threonine kinases involved in multiple mitotic processes^[37], including functional maturation of centrosomes, establishment of the bipolar spindle^[38], chromosome segregation^[39] and response to DNA damage^[40]. PLK1 is essential in the G₂/M-phase transition. The enzyme is able to activate CDC25c which in turn activates the CDC2/cyclin B1 complex leading to the import of cyclin B1 into the nucleus^[41], and it is also able to phosphorylate cyclin B1 directly^[42]. Moreover, PLK1 has been implicated in the regulation of anaphase-promoting complex/cyclosome^[43]. Interestingly, overexpression of PLK1 has been associated with tumor

Table 2 Correlation of several clinicopathological factors and of PLK1 mRNA expression group with patient survival (log-rank test)

Factors	<i>P</i> -value
Gender	0.155
Age	0.125
Liver cirrhosis	0.746
HBsAg	0.766
AFP	0.416
Venous invasion	< 0.001
Edmondson stage	0.001
Tumor size (cm)	0.183
Tumor nodule	0.018
PLK1 expression	0.003

Table 3 Multivariate survival analysis by Cox's proportional-hazard model for PLK1

	Relative risk	95.0% CI	<i>P</i> -value
Venous invasion	4.848	2.649-8.871	< 0.001
High Edmondson stage	1.929	1.069-3.482	0.029
Multiple tumor nodule	2.377	1.384-4.082	0.002
PLK1 positive expression	3.507	1.386-8.874	0.008

development and can serve as a prognostic marker for some cancers^[12,20,31]. However, few previous reports have examined the possible role of PLK1 in HCC.

In our study, overexpression of PLK1 by immunohistochemical analysis was detected in tumor tissues, while no or sporadic expression were observed in adjacent normal tissue or regenerating nodules. Further investigation showed that the degree of PLK1 mRNA expression was also higher in HCC tissues than in adjacent normal tissues and regenerating nodules; elevated mRNA levels of PLK1 were detected in 82.22% of tumor samples. These data indicate that overexpression of PLK1 was a frequent event in hepatocellular carcinoma.

In investigating the association between PLK1 expression and clinicopathological data, PLK1 positive expression was correlated with multiple tumor nodules, high Edmondson-Steiner grade (III, IV) and venous invasion; 1, 3 and 5 year survival rates of the positive expression group were 67.8%, 42.3% and 20.9%, respectively, lower than the negative group ($P < 0.01$); multivariate analysis showed that PLK1 was an independent prognostic factor. Thus, overexpression of PLK1 predicts a poor prognosis in HCC patients.

PLK1 is well known to be involved in cell proliferation. Was the PLK1 overexpression the cause of tumor formation or the consequence of high mitotic index during tumor cell proliferation? Others studies have shown that the overexpression of PLK1 in NIH3T3 leads to tumor formation^[44], and knockdown of PLK1 causes inhibition of growth and induction of apoptosis in human esophageal cancer cells^[17]. Furthermore, PLK1 was inhibited by DNA damage in the G₂ phase of mitosis. When the conserved threonine residue in the T-loop was changed to aspartic acid, expression of these mutants

was found to override the G2 arrest induced by DNA damage^[45]. In our studies, the level of PLK1 mRNA expression in HCC was obviously higher than in regenerating nodules, which also indicted that PLK1 might be a “proto-oncogene” for HCC.

In summary, this is the first attempt to clarify the relation of PLK1 expression and HCC. Our results indicate that high PLK1 expression predicts a poor prognosis in HCC patients. The data further suggest that PLK1 may serve as a potential biomarker for HCC. However, this is only a preliminary step. Further studies are warranted to understand the overexpression mechanism of PLK1 and to develop effective targeted interventions as a therapy for HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the deadliest of all cancers with as yet incompletely elucidated causes. As a cell cycle control kinase, polo-like kinase1 (PLK1) and its overexpression are highly associated with many human cancers. However, few studies are available on the overexpression of PLK1 in HCC.

Research frontiers

PLK1 is involved in cell proliferation. Its overexpression is associated with many human cancers. It is unclear whether the overexpression was the cause of tumor formation or the consequence of a high mitotic index during tumor cell proliferation. The trigger mechanisms of PLK1 overexpression in tumor formation are also unknown. Effective targeted interventions against PLK1 overexpression may be possible strategies as a therapy for HCC. All these questions are hotspots in the research field related to the article.

Innovations and breakthroughs

As a preliminary study of the relation between overexpression of PLK1 and HCC, it was found that overexpression of PLK1 was mainly found in tumor tissues compared with tumor-free tissue by immunohistochemical tissue slides and semi-quantitative reverse transcription-polymerase chain reaction analysis. In all HCC patients involved in the follow-up system, the survival rates in the PLK1 positive expression group were significantly lower than the negative control group. Theses results indicate that PLK1 could be a potential biomarker for diagnosis and therapy for HCC.

Applications

Overexpression of PLK1 is correlated with tumors. However the mechanism remains unclear. Their studies find overexpression of PLK1 in HCC, analyze the relationship between the prognosis of HCC and the overexpression, and provide the theoretical basis for targeted intervention against PLK1 as a means of treatment of HCC.

Terminology

PLK1: PLK1 is an important regulator of cell cycle progression during M-phase, involved in the assembly and dynamics of the mitotic spindle apparatus and in the activation and inactivation of CDK/cyclin complexes.

Peer review

This is a good descriptive study in which authors correlated polo-like kinase 1 expression in human HCC with clinicopathological findings. The data are very informative and deserve publication in the journal. The results are interesting and suggest that PLK1 could be a potential biomarker for diagnosis and therapy for HCC.

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S- Editor Tian L L- Editor O'Neill M E- Editor Ma WH

Expression and clinical significance of S100A2 and p63 in esophageal carcinoma

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Received: March 4, 2009 Revised: August 2, 2009

Accepted: August 9, 2009

Published online: September 7, 2009

igation into the combined expression of S100A2 and p63 may be helpful in early diagnosis and in evaluating the prognosis of ESCC.

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Key words: Immunohistochemistry; *In situ* hybridization; Esophageal carcinoma; p63; S100A2

Peer reviewer: Sabine Mihm, Professor, Department of Gastroenterology, Georg-August-Universität, Robert-Koch-Str.40, Göttingen D-37099, Germany

Cao LY, Yin Y, Li H, Jiang Y, Zhang HF. Expression and clinical significance of S100A2 and p63 in esophageal carcinoma. *World J Gastroenterol* 2009; 15(33): 4183-4188 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4183.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4183>

Abstract

AIM: To investigate the expression and clinical significance of S100A2 mRNA and protein, p63 protein in esophageal squamous cell carcinoma (ESCC) and their roles in carcinogenesis and progression of esophageal carcinoma (EC).

METHODS: Immunohistochemical staining (S-P method) for S100A2 and p63 protein were performed in 40 samples of ESCC and 40 samples of normal esophageal mucosa. *In situ* hybridization (ISH) was used to detect the expression of S100A2 mRNA.

RESULTS: Expression of S100A2 mRNA in ESCC was positive in 77.5% of samples, which was lower than that in normal mucosa (100%) by ISH ($P = 0.002$). The expression level of S100A2 mRNA was closely related to differentiation and node-metastasis ($P = 0.012$, $P = 0.008$). Expression of S100A2 protein was positive in 72.5% of ESCC samples and expression of p63 protein was positive in 37.5% of ESCC samples, and was lower than that in normal mucosa (100%) ($P = 0.000$). The expression of S100A2 protein was correlated with the differentiation and node-metastasis ($P = 0.007$, $P = 0.001$), but no relationship was observed between the expression of p63 protein and clinical pathological manifestations. S100A2 protein was positively correlated with the expression of S100A2 mRNA, and negatively associated with the expression of p63 protein ($P = 0.000$, $P = 0.002$).

CONCLUSION: S100A2 and p63 protein both play important roles in the carcinogenesis of ESCC. An inves-

INTRODUCTION

Esophageal carcinoma (EC) is one of the most common malignant diseases in China and has a poor survival rate. The carcinogenesis and development of EC is a complex process referring to multiple factors, stages and genovariations, and to numerous changes in genes and proteins at the molecular level. However, the biological roles of these changes in esophageal carcinogenesis are still vague. Up to now, no specific tumor marker for EC has been identified, and there are no biomarkers for use in screening, early diagnosis and judgment of the biological behaviour of EC.

As a tumor suppressor gene, *S100A2* is located on human chromosome 1q21^[1] and its encoding protein is a calcium-binding protein constituted by 97 amino acids^[2]. As a member of the S100 family, S100A2 is individual compared with other members of this family. Firstly, S100A2 protein is predominantly located in the nucleus rather than the cytosol like other S100 proteins. Moreover, *S100A2* is down-regulated in several tumors and may play a role in inhibiting tumor initiation or in suppressing tumor cell growth, whereas other members of the S100 family are up-regulated in tumors and may play a promotional role in carcinogenesis. Therefore, *S100A2* is considered a candidate tumor-suppressor gene^[3].

p63 gene, a new member of the *p53* gene family, was identified and named in 1998 by Yang *et al*^[4]. *p63* gene is located on human chromosome 3q27-29 and expresses

at least six protein isoforms, which can be divided into two groups -TAp63 and Δ Np63^[5]. Among them, the transcription level of Δ Np63 α is the highest. TAp63 is able to activate the transcription of specific target genes and induce cell cycle arrest and apoptosis. Similar to p53, TAp63 has anti-oncogene activity. Δ Np63 is unable to activate transcription and inhibit transcription activation by both p53 and TAp63. Δ Np63 has anti-apoptosis and proto-oncogene activity^[6,7].

Normally, *p63* is mainly expressed in the basal lamina of many epithelial tissues and plays an important role in initiating epithelial stratification during development and in maintaining the proliferative potential of basal keratinocytes in mature epidermis. Recently, *p63* gene has been studied in the fields of tumorigenesis, cell apoptosis and tissue growth.

At present, few studies have been performed on the expression and relationship of *S100A2* and *p63* in EC. In this study, *in situ* hybridization (ISH) used to identify *S100A2* mRNA and immunohistochemical staining (S-P method) used to identify S100A2 and p63 protein, were performed in 40 samples of esophageal squamous cell carcinoma (ESCC) and 40 samples of normal esophageal mucosa. The purpose of this study was to investigate the expression of S100A2 mRNA, S100A2 protein and p63 protein in EC, and their relationship with clinical pathological features, and to explore their roles in the carcinogenesis and prognosis of EC.

MATERIALS AND METHODS

Patients and samples

Forty specimens of ESCC and matched adjacent normal mucosa were obtained from the Department of Pathology, First Affiliated Hospital, Anhui Medical University between 2005 and 2006. None of the patients had been treated with radiotherapy or chemotherapy prior to surgery. Samples were taken from tumor tissue without hemorrhage or putrescence, whereas the matched normal mucosa samples were taken from the surgical cutting edge, which was approximately 3-5 cm from the cancerous lesion. The clinical diagnosis in all 40 patients was confirmed by histological examination after surgery.

ISH and immunohistochemistry

ISH reagents were purchased from Boster Co. (Wuhan, China) and Zhong Shan Co. (Beijing, China). A digoxigenin-labeled oligonucleotide probe was used. The probe sequence of S100A2 is described as: 5'-TGATGTGCAGTTCTCTGGAGCAGGCGCTGGCTGTG-3'; 5'-ACTGTCATGTGCAATGACTTCTTCCAGGGCTGCCC-3'. ISH for S100A2 was performed as follows: The tissue sections were treated with 3% hydrogen peroxide and 10% pepsin (diluted with 3% citric acid), respectively, after deparaffinization and rehydration. The sections were prehybridized at 37°C for 4 h with a prehybridization solution (Boster Co., China). Next, the sections were incubated in 100 μ L hybridization solution/section containing 1 μ L of denatured probe and 400 μ L dilution of oligonucleotide probe (Boster Co., China) at 43°C for 16-20 h. The slides

were washed at 37°C in $2 \times$ SSC (5 min, three times), $0.5 \times$ SSC (5 min, three times) and $0.2 \times$ SSC (5 min, three times), respectively. During the color reaction procedure, the slides were incubated in sheep serum at 37°C for 30 min and then incubated with mouse antidigoxigenin antibody at 37°C for 60 min. After washing with PBS, the color was developed in DAB (3,3'-diaminobenzidine) (Zhongshan Co., China) for 15-30 min identified by occasional observation. Counterstaining of slides was then conducted with hematoxylin followed by a sealing procedure with neural gum.

S100A2 polyclonal antibody was purchased from Neomarker Co. (USA) and was used at a concentration of 1:100, and p63 monoclonal antibody was purchased from Maixin Co. (China). Immunohistochemistry for S100A2 and p63 were performed as follows: Deparaffinization and rehydration of sections (3×3 min with xylene; 3×2 min with 100% ethanol; 2 min with 95% ethanol, 2 min with 75% ethanol, and 2×1 min with distilled water), was performed using microwave repaired antigen. The samples were then washed 3×5 min with PBS. Endogenous peroxidases were blocked by soaking the slides in a solution of 3% H₂O₂ for 15 min at room temperature (RT). The samples were then washed 3×5 min with PBS, and 50 μ L of nonimmune animal blood serum was added to each section for 15 min at RT. The samples were shaken and excess PBS was wiped off. Primary antibody (50 μ L) was added to each section immediately, and incubated overnight at 4°C in a humidified chamber. The slides were washed 3×10 min in PBS. Fifty microliters of biotin labeling secondary antibody was added to each section for 15 min at 37°C, then the slides were washed 3×5 min with PBS. (ABC was made according to the Vector protocol 30 min before use (mix 5 mL PBS with two drops of solution A and two drops of solution B). The samples were incubated for 15 min at RT, then washed 3×5 min in PBS. Freshly prepared DAB (100 μ L) was added to the slides and the color change was observed approximately 5-20 min later. Coloration was stopped by flushing with water. The slides were then counterstained with hematoxylin followed by a sealing procedure with neural gum.

Normal epithelium showing strong S100A2 or a p63-positive section served as the positive control, and that without the antibody section served as the negative control.

ISH and immunohistochemistry evaluation

S100A2 mRNA and S100A2 protein were expressed in the nucleus or the nucleus and cytoplasm. The percentage of positive cells in each high power field was noted, and less than 10% of positive cells was considered negative and greater than 10% was considered positive. p63 protein was expressed in the nucleus. The grading of positive cells was as follows: no p63-positive cells was characterized as negative, less than 25% was weakly positive (+), 25%-75% was moderately positive (++), and greater than 75% was strongly positive (+++). The percentage of staining was estimated by two independent pathologists, respectively.

Table 1 Expression of S100A2 mRNA, S100A2 protein and p63 protein in ESCC and normal mucosa ($n = 40$) n (%)

	S100A2 mRNA		S100A2 protein	
	Negative	Positive	Negative	Positive
Normal mucosa	0	40 (100)	0	40 (100)
ESCC	9	31 (77.5) ^a	11	29 (72.5) ^a

^a $P < 0.01$ vs normal mucosa. ESCC: Esophageal squamous cell carcinoma.

Table 2 Relationship between the expression of S100A2 mRNA and S100A2 protein, and clinical pathological features in ESCC n (%)

	n	S100A2 mRNA		S100A2 protein	
		Negative	Positive	Negative	Positive
Gender					
Male	31	7	24 (77.4)	8	23 (74.2)
Female	9	2	7 (77.8)	3	6 (66.7)
Age (yr)					
< 62	21	4	17 (81)	4	17 (81)
≥ 62	19	5	14 (73.7)	7	12 (63.2)
Clinical stage					
I and II	31	5	26 (83.9)	6	25 (80.7)
III and IV	9	4	5 (55.6)	5	4 (44.4)
Tumor location					
Upper and middle segment	23	3	20 (87)	5	18 (78.3)
Inferior segment	17	6	11 (64.7)	6	11 (64.7)
Tumor size (cm)					
< 3.5	16	3	13 (81.3)	4	12 (75)
≥ 3.5	24	6	18 (75)	7	17 (70.8)
Depth of tumor invasion					
Not to serosa	20	4	16 (80)	4	16 (80)
To serosa	20	5	15 (75)	7	13 (75)
Degree of differentiation					
Well	15	1	14 (93.3)	2	13 (86.7)
Moderately	14	2	12 (85.7)	2	12 (85.7)
Poorly	11	6	5 (45.5) ^a	7	4 (36.4) ^b
Lymph node metastasis					
Negative	25	2	23 (92)	2	23 (92)
Positive	15	7	8 (53.3) ^d	9	6 (40) ^d

^a $P < 0.05$, ^b $P < 0.01$ vs the well and moderately differentiated group; ^d $P < 0.01$ vs the lymph node-negative group.

Statistical analysis

All data were analyzed with SPSS 13.0 software. Pearson's χ^2 test was used for data measurement. Using the Monte Carlo simulation method, the exact probability value was calculated for the data where the theoretical frequency was less than five. Non-parametric Spearman rank correlation analysis was used for correlation analysis of ranked data.

RESULTS

The expression of S100A2 mRNA and S100A2 protein in ESCC and normal mucosa

S100A2 mRNA and S100A2 protein were expressed in the nucleus or the nucleus and cytoplasm (Figure 1A and B, Figure 2A and B). All 40 samples of normal esophageal mucosa positively expressed S100A2 mRNA and

Table 3 Relationship between S100A2 mRNA and S100A2 protein in ESCC

S100A2 protein	S100A2 mRNA		Total
	+	-	
+	27	2	29
-	4	7	11
Total	31	9	40

$r = 0.607$, $P = 0.000$.

S100A2 protein. In 40 ESCC samples, 31 positively expressed S100A2 mRNA, and 29 expressed S100A2 protein. The positive rates of S100A2 mRNA and S100A2 protein in ESCC (77.5% and 72.5%, respectively) were significantly lower than that in normal mucosa (100% and 100%, respectively) ($P < 0.01$) (Table 1).

The relationship between expression of S100A2 mRNA and S100A2 protein and clinical pathological features in ESCC

S100A2 mRNA was positive in 93.3% (14/15), 85.7% (12/14), and 45.5% (5/11) in the well differentiated, moderately differentiated, and poorly differentiated groups, respectively. The differences were significant among the three groups ($P < 0.05$). Moreover, the expression level of S100A2 mRNA was significantly higher in the well and moderately differentiated groups than that in the poorly differentiated group ($P < 0.05$). The expression of S100A2 was positively correlated with node metastasis ($P < 0.01$). S100A2 protein was positive in 86.7% (13/15), 85.7% (12/14), and 36.4% (4/11) in the well differentiated, moderately differentiated, and poorly differentiated groups, respectively. The expression level of S100A2 protein was related to differentiation and lymph node metastasis. The expression of S100A2 mRNA and S100A2 protein was not correlated with gender, age, clinical stage, tumor location, tumor size, and depth of tumor invasion ($P > 0.05$) (Table 2).

Relationship between S100A2 mRNA and S100A2 protein in ESCC

A positive correlation was found between the expression of S100A2 mRNA and S100A2 protein ($r = 0.607$, $P < 0.001$). Of 40 ESCC samples, 27 expressed S100A2 mRNA and S100A2 protein at the same time, and seven cases did not express these parameters at the same time. Four cases expressed S100A2 mRNA but not S100A2 protein. Two cases did not express S100A2 mRNA but expressed S100A2 protein (Table 3).

The expression of p63 protein in ESCC and normal mucosa

p63 protein was expressed in the nucleus (Figure 3A and B). In 40 samples of normal esophageal mucosa, 10 expressed p63 but none were strongly positive. In 40 ESCC samples, 21 expressed p63. Among these, 15 cases were strongly positive. p63 protein was positive in 52.5% and

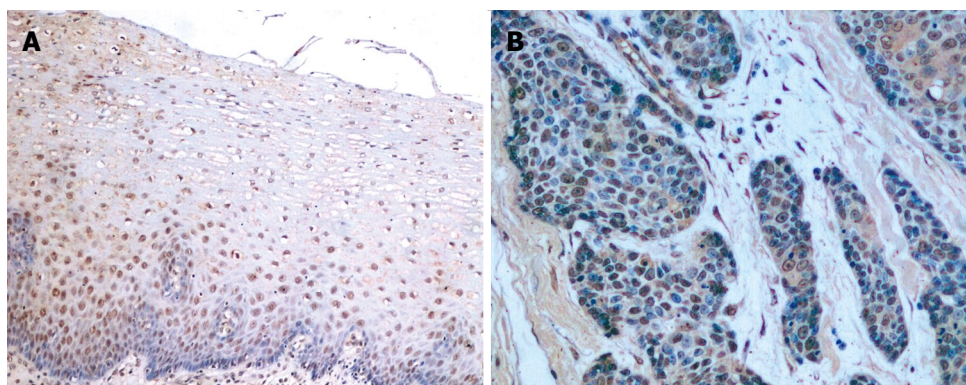


Figure 1 The expression of S100A2 mRNA. A: Normal mucosa (+) (ISH, $\times 100$); B: ESCC (+) (ISH, $\times 200$).

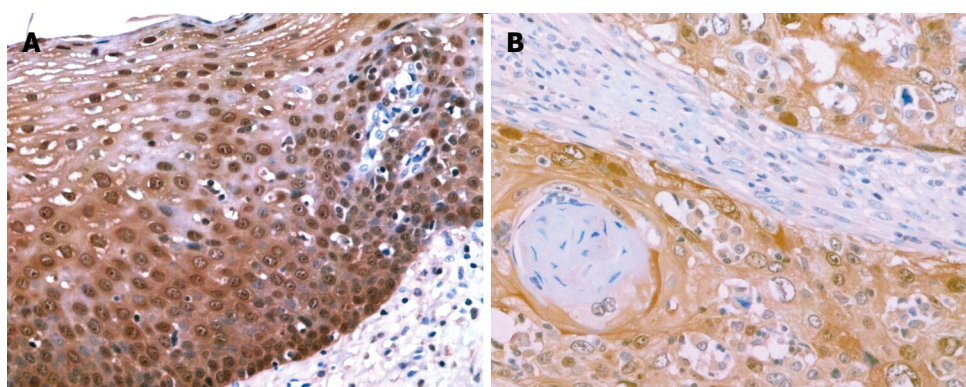


Figure 2 The expression of S100A2 protein. A: Normal mucosa (+) (S-P, $\times 200$); B: ESCC (+) (S-P, $\times 200$).

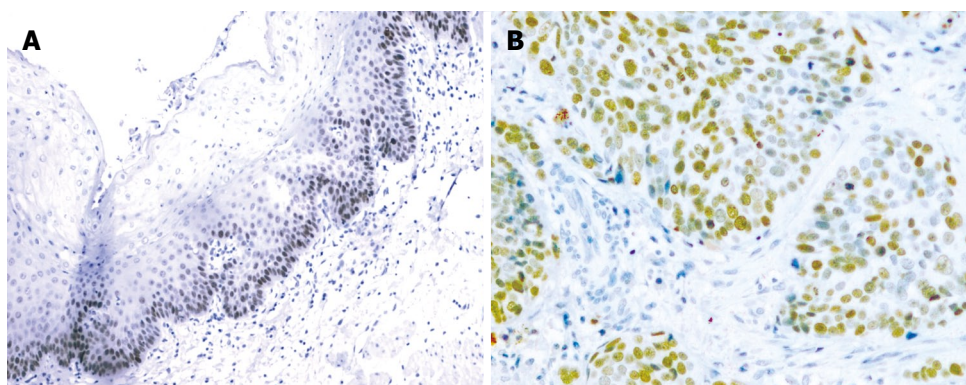


Figure 3 The expression of p63 protein. A: Normal mucosa (+) (S-P, $\times 100$); B: ESCC (+++) (S-P, $\times 200$).

Table 4 Expression of p63 protein in ESCC and normal mucosa

	<i>n</i>	p63 protein				Positive rate (%)	Strong positive rate (%)
		-	+	++	+++		
Normal mucosa	40	30	10	0	0	25.0	0.0
ESCC	40	19	2	4	15	52.5 ^a	37.5 ^b

^a $P < 0.05$, ^b $P < 0.001$ vs normal mucosa.

strongly positive in 37.5% of ESCC samples, and was higher than that in normal mucosa (25.0%) ($P < 0.05$, $P < 0.001$) (Table 4).

Relationship of p63 protein expression with clinical pathological features of ESCC

The expression of p63 protein was not correlated with gender, age, clinical stage, tumor location, tumor size,

depth of tumor invasion, degree of differentiation or lymph node metastasis ($P > 0.05$).

Relationship between S100A2 protein and p63 protein in ESCC

A negative correlation was found between the expression of S100A2 protein and p63 protein ($r = -0.474$, $P < 0.01$). Of 40 ESCC samples, 11 expressed S100A2 and p63 at the same time, and 1 did not express these parameters at the same time. 18 ESCC samples were S100A2-positive and p63-negative. 10 cases were S100A2-negative and p63-positive (Table 5).

DISCUSSION

Up to now, 21 genes encoding S100 calcium-binding proteins of the EF-hand type have been identified. Calcium ion (Ca^{2+}) plays an important role in the regulation of a number of cellular processes. The

Table 5 Relationship between S100A2 protein and p63 protein in ESCC

p63 protein	S100A2 protein		Total
	+	-	
+	11	10	21
-	18	1	19
Total	29	11	40

$r = -0.474$, $P = 0.002$.

second messenger role of Ca^{2+} is mediated, at least in part, by calcium-binding proteins which contain the EF-hand motif. Through modulating Ca^{2+} and interacting with the target proteins, S100 proteins have a multitude of biological functions *in vivo*. Many S100 genes were reported to be clustered in the epidermal differentiation complex in chromosome 1q21^[8-12]. This region is involved in epidermal differentiation and proliferation and is also frequently rearranged in tumors^[13]. Studies have shown that the S100 gene deregulated expression in human diseases, especially in tumors. Nowadays, S100 protein antibodies have widespread application for tumor diagnosis by immunohistochemistry.

As a member of the *S100* gene family, S100A2 is significantly downregulated in several malignant tumors, such as breast cancer^[14], melanoma^[15], prostatic carcinoma^[16], and pulmonary carcinoma^[17]. Moreover, S100A2 may be closely associated with the development and prognosis of tumors^[18].

Using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), Ji *et al.*^[19] examined the differential expression of *S100A2* and another 15 *S100* genes in 62 cases of ESCC *vs* the corresponding normal esophageal mucosa. Their results showed that the S100A2 gene was significantly downregulated ($P < 0.05$) in ESCC *vs* normal esophageal mucosa. Moreover, the deregulation of *S100A2* gene was significantly correlated with the deregulation of *S100A8*, *S100A14* and *S100P*. Kyriazanos *et al.*^[20] examined the clinical significance of *S100A2* expression in 116 resected specimens of ESCC using immunohistochemistry. Their results showed that S100A2 was positive in 49 cases (42.2%) and its expression was significantly higher in large and well differentiated tumors. Lymph node-positive tumors had a lower expression of S100A2 protein in comparison to the corresponding lymph node-negative equivalents in each of the T stages, but the difference was statistically significant only for T1b tumors. S100A2 status became an independent predictor of patient survival in lymph node-negative cases. Node-negative ESCC patients without S100A2 expression may be a high-risk group with poor survival and will need further attention to design appropriate adjuvant therapy.

We used ISH for the first time to detect the expression of *S100A2* mRNA and used immunohistochemical staining for S100A2 protein in 40 ESCC samples and 40 samples of normal esophageal mucosa. Our results showed that *S100A2* mRNA and S100A2 protein although positive in ESCC were both lower than that in

normal mucosa ($P < 0.01$). This indicated that S100A2 was downregulated in ESCC and that the *S100A2* gene is concerned with the carcinogenesis of EC. A positive correlation was found between the expression of *S100A2* mRNA and S100A2 protein ($P < 0.001$), which indicated that S100A2 protein expression is regulated mainly at the transcriptional level, and the decrease in S100A2 protein expression corresponded to the decrease in transcriptional activity. In addition, our findings on S100A2 mRNA are consistent with those of Ji *et al.*^[19]. We suggest that the positive rate in our study is higher than that in the study by Ji *et al.*^[19] due to the difference in case number (40 *vs* 62) and method (ISH *vs* RT-PCR).

Furthermore, we analyzed the relationship between S100A2 positive expression and the clinical pathological features of ESCC. We found that the expression level of S100A2 mRNA and S100A2 protein were both significantly higher in the well and moderately differentiated groups than in the poorly differentiated group ($P < 0.05$). These differences were also significant between the lymph node-positive group and the lymph node-negative group ($P < 0.01$), which is roughly consistent with the findings of Kyriazanos *et al.*^[20]. These results indicate that S100A2 plays an important role in tumor cell differentiation, and that S100A2 might be an important biomarker in the biological behaviour of EC.

A study on the course of EC, found that *p63* gene and its encoding protein played important roles in the early period of the physiological and pathological course of esophageal mucosa, compared with mutation of p53 which occurs in the last stage of cancerization lineage from metaplasia, atypical hyperplasia to adenocarcinoma. Glickman *et al.*^[21] detected p63 protein in EC by immunohistochemical staining. Their results showed that p63 protein was highly expressed in ESCC, but was not expressed in adenocarcinoma of the esophagus and colorectal cancer. This indicated that *p63* gene is upregulated in ESCC and is concerned with the development of ESCC.

We used immunohistochemical staining for p63 protein in 40 ESCC samples and 40 samples of normal esophageal mucosa. Our results showed that p63 protein in normal esophageal mucosa was positive in 25.0%, and the expression was weakly positive and localization in the basal cells or the bottom of the prickle cell layer of normal esophageal mucosa. This supports the view that p63 is mainly expressed in the corpus of many epithelial tissues, and plays an important role in the abstraction, differentiation and morphogenesis of many epithelial tissues^[22]. Moreover, our results showed that p63 protein has a high expression rate in the tissue of EC, which indicates that p63 is closely related to the carcinogenesis of EC. In contrast, we found that the expression of p63 protein was not correlated with the clinical pathological features of ESCC.

Our study showed that the expression of S100A2 protein was reduced and the expression of p63 protein was increased, and a negative correlation was observed between them ($P < 0.01$). This indicated that S100A2 protein and p63 protein may both play important roles in the carcinogenesis of EC. An investigation into the combined expression of S100A2 and p63 may be use-

ful in early diagnosis and in evaluating the prognosis of ESCC.

COMMENTS

Background

Esophageal carcinoma (EC) is one of the most common malignant diseases in China and has a poor survival rate. Up to now, no specific tumor marker of EC has been identified. Recently, *S100A2* has been considered a candidate tumor-suppressor gene, and *p63* gene has been studied in the fields of tumorigenesis, cell apoptosis and tissue growth.

Research frontiers

S100A2 is down-regulated in several tumors and may play a role in inhibiting tumor initiation or in suppressing tumor cell growth. Therefore, *S100A2* has been considered a candidate tumor-suppressor gene. *p63* gene, a new member of the *p53* gene family, is normally expressed in the basal lamina of many epithelial tissues and plays an important role in initiating epithelial stratification during development and in maintaining proliferative potential of basal keratinocytes in mature epidermis. Recently, *p63* gene has been studied in the fields of tumorigenesis, cell apoptosis and tissue growth.

Innovations and breakthroughs

At present, few studies have been carried out on the expression and the relationship of *S100A2* and *p63* in EC. In this study, *in situ* hybridization (ISH) to identify *S100A2* mRNA and immunohistochemical staining (S-P method) to identify *S100A2* and *p63* protein were performed in ESCC and normal esophageal mucosa samples.

Terminology

S100A2 gene is located on human chromosome 1q21 and its encoding protein is a calcium-binding protein constituted by 97 amino acids. *S100A2* protein is predominantly located in the nucleus rather than the cytosol like other *S100* proteins. *S100A2* is down-regulated in several tumors. Therefore, *S100A2* is considered a candidate tumor-suppressor gene.

Peer review

The study investigated *S100A2* and *p63* expression in EC tissue samples and paired normal mucosa samples. *S100A2* expression was analysed both on the mRNA and protein level by ISH and IH, respectively, *p63* expression was determined by IH. The authors described a decreased expression of *S100A2* and an increased expression of *p63* in tumor samples. Statistical analysis revealed an inverse correlation. This is a clear strength of this study.

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S- Editor Li LF L- Editor Webster JR E- Editor Ma WH

Dysplasia in perforated intestinal pneumatosis complicating a previous jejunio-ileal bypass: A cautionary note

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Received: July 31, 2009 **Revised:** August 4, 2009

Accepted: August 11, 2009

Published online: September 7, 2009

Abstract

We present the case of an elderly woman who developed a bowel perforation related to pneumatosis intestinalis, 33 years after a jejunio-ileal bypass for severe obesity. Final histological examination revealed the presence of dysplasia in the resected specimen. On the basis of our case and a review of the literature, we discuss the etiopathogenesis, the clinical aspects and the treatment of this rare condition.

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Key words: Acute abdomen; Pneumatosis intestinalis; Peritonitis; Obesity; Dysplasia

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Portolani N, Baiocchi GL, Gadaldi S, Fisogni S, Villanacci V. Dysplasia in perforated intestinal pneumatosis complicating a previous jejunio-ileal bypass: A cautionary note. *World J Gastroenterol* 2009; 15(33): 4189-4192 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4189.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4189>

INTRODUCTION

Pneumatosis intestinalis (PI) is an unusual intestinal

pathology characterized by a collection of multiple gas-filled cysts within the wall of the gastrointestinal tract. According to this general definition, PI may be seen in some critical events, including necrotizing enterocolitis in the newborn and ischemic bowel disease in the adult, but also in innocuous conditions which are associated with a wide spectrum of diseases and situations which do not require surgery^[1-7]. Benign causes are statistically more prevalent; PI may resolve spontaneously, although recurrent episodes are reported and the long term outcome of patients is often unclear because of the absence of an exhaustive imaging follow up^[1]. The lack of reliable data regarding the outcome predictors for patients with PI complicates management decisions.

A pneumoperitoneum and a pneumoretroperitoneum can be present regardless of causes and are generally considered a complication of PI. Peritonitis may be consequent, but this is not the rule and a lack of perforation is reported in the presence of mild clinical symptoms^[3]. These conditions are difficult to manage, taking into account that some clinical conditions, such as immunodepression or obesity (not infrequent in these patients) may mask the clinical relevance of pneumoperitoneum so delaying treatment. On the other hand, an automatic referral to surgery may result in an unnecessary laparotomy.

Herein we present a case of PI associated with jejunio-ileal bypass performed many years previously and complicated by a pneumoperitoneum in an obese patient. The histological data, showing a dysplasia of the mucosa, are subsequently discussed.

CASE REPORT

C.D., a 73-year old woman, having undergone an end-to-side jejunio-ileal bypass for severe obesity 33 years previously, was admitted to the Medical Division of our hospital complaining of abdominal pain, constipation and mild dyspnea lasting 2 d. Relevant history included chronic obstructive pulmonary disease without dyspnea at rest, postsurgical hypothyroidism and atrial fibrillation with necessity of continuous warfarin administration recorded; no significant signs of digestive impairment were present. Weight was 105 kg and BMI was 41. At admission, the patient was oliguric, hypotensive (85/55 mmHg) and hypoxemic (PCO₂ 65 mmHg, SpO₂ 90%). On physical examination the abdomen appeared distended, with



Figure 1 Unenhanced CT scan, showing the presence of multiple gas-filled parietal cysts and huge pneumoperitoneum.

diffuse pain and a mild rebound tenderness; no peristalsis at auscultation. Laboratory investigations revealed mild metabolic acidosis (pH 7.3, bicarbonate 15.4 mmol/L) with normal lactate value, leukocytosis (14 000/mL) and acute renal failure (creatinine 2.6 mg/dL); PCR was 459, albuminemia 2.7 g/dL. A marked reduction of prothrombin value ($< 10\%$, INR > 8) was noted, requiring plasma transfusion. Thorax radiography was negative for pneumothorax or pneumomediastinum. Computed tomography (CT) without intravenous contrast medium showed some gas-filled cysts within the wall of the jejunum, ileum and mesentery, associated with massive free intraperitoneal air (Figure 1). A rapid worsening of the patient's general condition was observed, leading to urgent surgical intervention; the patient was classed as ASA four according to the American Society of Anesthesiology. At laparotomy, pneumatosis affected only the defunctionalized jejunal loop (Figure 2), which was abnormally dilated and distally anastomized end-to-side to the sigmoid loop. The first and the last segment of the bypassed bowel, including the ileocolic anastomosis, were relatively healthy. A transmural perforation was present in the antimesenteric border of the bowel along the tract with pneumatosis, in a context of fibrinous peritonitis, without evidence of free biliary or enteric fluid. A segmental 90 cm-long ileal resection with end-to-end anastomosis was performed, including the entire unhealthy bowel. The bacteriologic study on the peritoneal fluid showed the presence of *Difteroides*.

The macroscopic pathological analysis of the resected specimen showed a thickened bowel wall in the presence of multiple cysts without fluid, with a thin distended mucosa and absence of folded structure (Figure 3A and B). At the histological evaluation, the cysts were located in the submucosa and subserosa, with giant cells lining the cyst walls (Figure 3C and D). The mucosa was severely thinned, in some areas with a single cellular layer, and pseudo-atrophy of villi alternated with inflammation and ulceration. In some areas the epithelium was characterized by mild cytological atypia indicating low dysplasia (Figure 4A). Immunohistochemical staining with p53 (Thermo Fisher Scientific, Fremont, CA, USA, dilution 1:100) and p16 (Kit CINtec® Histology, MTM Laboratory, Germany) confirmed the diffuse positivity of epithelial

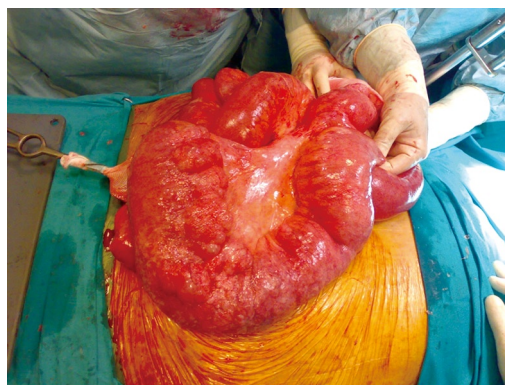


Figure 2 Intraoperative view of dilated and inflamed defunctionalized jejunal loop, with fibrous peritonitis. No clear evidence of enteric or biliary contamination was present.

cells in the focal mild dysplasia (Figure 4B and C). Marked flogistic reaction of the peritoneal serosa was reactive at the intestinal perforation.

The patient consequently became severely compromised during the whole surgical operation with prolonged hypotension and needed care in the intensive recovery room for 2 d. A progressive normalization of the septic signs and of renal function was then observed. Subsequently the patient's recovery was uneventful and she was discharged on post-operative day 14.

DISCUSSION

Pneumatosis intestinalis may occur in many different situations. The etiology actually favours iatrogenic conditions (surgery, endoscopy, enteric tube placement, positive pressure ventilation), immunodepression, collagen vascular disease and infectious agents^[2,4,5,7,8]. The jejunio-ileal bypass has accounted for some cases of PI; this condition is generally considered benign with a favourable outcome, supporting the concept that "surgical indication is not indicated under the usual circumstances"^[9]. The published experiences on this topic, generally in the form of single cases or small series of patients, refer to reports from the 1970s^[9-15], when this type of bariatric surgery was adopted in many centres. PI occurred any time during the first postoperative phase (mean delay 5 mo, range 1-20 mo)^[7] and patients recovered spontaneously in the majority of cases^[9,11].

Although responsible for a small number of cases, the "model" of jejunio-ileal bypass is well within the supposed pathogenetic mechanisms of PI, because these patients really present several predisposing factors. The first is ascribed to surgery alone with the consequent modification in bowel pressure^[16]. Another factor may be related to the marked obesity, as PI has been described in obese patients without a history of abdominal surgery^[9]. The type of reconstruction of bowel continuity also seems to play a central role; increased colonic gas secondary to fermentation of undigested lactose may reflux into the excluded segment of the small bowel. This occurs in particular when an ileum-sigmoid anastomosis has been performed, so favoring, because of abnormal

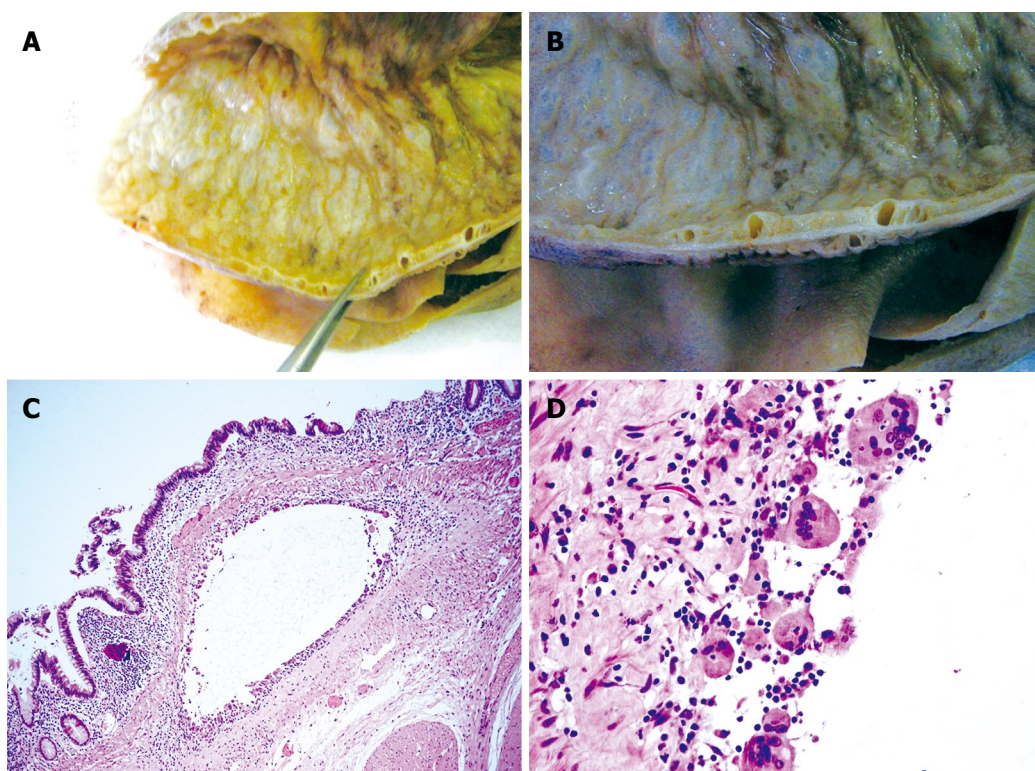


Figure 3 Pathological findings. A and B: Macroscopic appearance of surgical specimen with the typical cysts at different magnifications; C and D: Submucosal cysts (C, HE, × 20) lined by giant cells (D, HE, × 100).

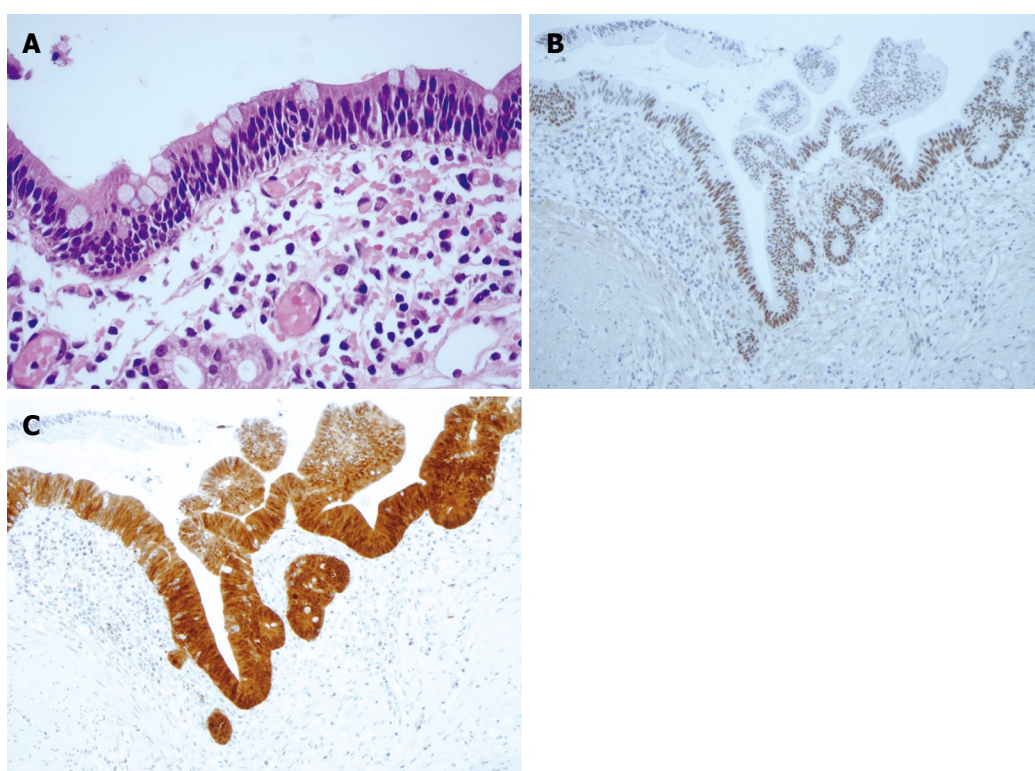


Figure 4 Histological appearance and immuno-histochemical staining. Focal area of mild dysplasia (HE, × 60) (A), Positivity for P53 (B) and P16 (C).

pressure, the dissolution of gas into the bowel wall. This mechanism may be augmented by the concomitant bacteria overgrowth, most likely anaerobic, in the bypassed small bowel segment, so assigning the etiology of PI a mechanical - infectious process^[12]. Our case tends to support this hypothesis. PI involved only the bypassed small bowel where the two mechanisms, high pressure and bacterial overgrowth, were more significant. An anastomosis had been built between the defunctionalized

ileum and the sigmoid, the excluded small bowel was significantly dilated, with a clear discrepancy of size compared with that of the functioning bowel, and it was edematous. Histology showed a severe mucosal injury with marked inflammation and signs of a remodeling reactive process.

Pneumoperitoneum is a complication of PI, but not an automatic indication for surgery. An increasing list of conditions relating to pneumoperitoneum without

peritonitis are now recognized^[17,18], PI being the most common. These cases have a benign progression without laparotomy^[17-22]. They are the prevalent situations where no transmural perforation exists: as a consequence, no peritoneal contamination results from the rupture of the intramural sterile blebs. According to these considerations, the demonstration of PI in a patient with pneumoperitoneum may allow us to avoid the need for a laparotomy^[19]. This assumption must be accepted with caution in our opinion. The presence of PI is not enough to consider pneumoperitoneum as a benign condition in every case, with a good spontaneous outcome. Surgical indication must rely above all on the clinical data; neither CT nor the amount of free air are reliable guides for therapy^[20]. A negative abdominal examination could exclude signs of peritonitis, but clinical signs may be minimal or absent, for example in immunodepressed patients, or difficult to discover, as in obese patients. These two situations clearly define an increased risk of a delayed diagnosis of peritonitis, which can present with an ominous course in these frail patients. In our patient, peritonitis was not clear at the time of admission owing to the marked obesity of the patient, but the general signs of septic compromise (hypotension, oliguria, leukocytosis, high PCR value) suggested the clinical relevance of the radiological images. Laparotomy became urgent within a few hours. A complete perforation was discovered in the defunctionalized bowel without extravasation of enteric content, but the presence of gram-positive bacteria in the peritoneal fluid together with the histological demonstration of inflamed peritoneal serosa confirmed the contamination from the intestinal lumen. In the context of a fibrinous peritonitis a simple suture of the perforation might be advised, but this was not our choice because the bowel appeared very dilated and inflamed. The possibility of saving the proximal and distal segments, which were macroscopically normal, directed us towards performing the resection of the pathological bowel.

The histological examination of the resected bowel showed obvious pathological aspects of the PI with typical submucosal cysts surrounded by granulomatous reaction; the peculiarity of the case is the discovery of focal areas of mild dysplasia confirmed by the immunohistochemical evaluation with p53 and p16. These findings have never been reported before. Owing to the rare indication for surgery in these patients, an exhaustive histological evaluation of the pathologic bowel is rare but generally marked by an intact, pale and sometimes transparent mucosa^[7]. These changes, together with the unusually long time between the intestinal bypass and the discovery of PI in our patient, support the hypothesis of a long standing remodeling process, promoting the concept of a preneoplastic condition. This possibility, together with the rare occurrence of malignant lymphomas developing in the excluded intestine^[23], leads us to advise a long term follow up. When surgery is indicated for this complication, complete resection of the tract with pneumatosis, if

technically possible in symptomatic patients, appears to us to be advisable.

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S- Editor Tian L L- Editor Logan S E- Editor Ma WH

Neuroendocrine carcinomas arising in ulcerative colitis: Coincidences or possible correlations?

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Received: June 7, 2009

Revised: August 6, 2009

Accepted: August 13, 2009

Published online: September 7, 2009

development of pancellular dysplasia involving epithelial, goblet, Paneth and neuroendocrine cells. It has yet to be established which IBD patients have a higher risk of developing NENs.

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Key words: Colorectal cancer; Large cell carcinoma; Neuroendocrine carcinoma; Small cell carcinoma

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Grassia R, Bodini P, Dizioli P, Staiano T, Iiritano E, Bianchi G, Buffoli F. Neuroendocrine carcinomas arising in ulcerative colitis: Coincidences or possible correlations? *World J Gastroenterol* 2009; 15(33): 4193-4195 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4193.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4193>

Abstract

Patients with inflammatory bowel disease (IBD) are at increased risk of colorectal malignancies. Adenocarcinoma is the commonest type of colorectal neoplasm associated with ulcerative colitis (UC) and Crohn's disease, but other types of epithelial and non-epithelial tumors have also been described in inflamed bowel. With regards to non-epithelial malignancies, lymphomas and sarcomas represent the largest group of tumors reported in association with IBD, especially in immunosuppressed patients. Carcinoids and in particular neuroendocrine neoplasms other than carcinoids (NENs) are rare tumors and are infrequently described in the setting of IBD. Thus, this association requires further investigation. We report two cases of neoplasms arising in mild left-sided UC with immunohistochemical staining for neuroendocrine markers: a large cell and a small cell neuroendocrine carcinoma of the rectum. The two patients were different in age (35 years *vs* 77 years) and disease duration (11 years *vs* 27 years), and both had never received immunosuppressant drugs. Although the patients underwent regular endoscopic and histological follow-up, the two neoplasms were locally advanced at diagnosis. One of the two patients developed multiple liver metastases and died 15 mo after diagnosis. These findings confirm the aggressiveness and the poor prognosis of NENs compared to colorectal adenocarcinoma. While carcinoids seem to be coincidentally associated with IBD, NENs may also arise in this setting. In fact, long-standing inflammation could be directly responsible for the

INTRODUCTION

It is well recognized that ulcerative colitis (UC) and Crohn's disease (CD) predispose to the development of colorectal adenocarcinoma (CRC). The duration and the anatomic extent of the disease have been shown to be independent risk factors for the development of CRC^[1]. Thus, cancer is infrequently encountered when disease duration is less than 8-10 years, thereafter the risk rises at approximately 0.5%-1% per year^[2]. Additional risk factors are the presence of primary sclerosing cholangitis and a family history of CRC. Younger age at diagnosis and the degree of endoscopic and histological activity may also play an important role^[1]. A comprehensive meta-analysis of all published studies reporting CRC risk in UC up to 2001 showed that the risk for any patients with colitis was 2% at 10 years, 8% at 20 years and 18% after 30 years of disease^[3].

Based on this evidence, guidelines for screening and surveillance of asymptomatic CRC in patients with inflammatory bowel disease (IBD) have been developed to detect either dysplasia or early cancer at a surgically curable stage, and to reduce CRC related mortality^[1,2].

While adenocarcinoma is the most common colorectal epithelial malignancy associated with UC

and CD, other types of carcinomas such as squamous cell carcinoma, small cell carcinoma and “hepatoid” carcinoma have been described in inflamed bowel. With regards to non-epithelial malignancies, lymphomas and sarcomas represent the largest group of tumors reported in association with IBD, especially in immunosuppressed patients^[4].

Carcinoid tumors and neuroendocrine neoplasms other than carcinoids (NENs) may also be associated with UC and CD of the colon based on the finding of increased numbers of neuroendocrine cells in inflamed mucosa, suggesting that long-standing inflammation is directly responsible for the development of this kind of neoplasia^[4].

NENs of the colon and rectum are rare tumors, and are infrequently described in the setting of IBD. Here we report two cases of NENs arising in UC.

CASE REPORT

Case 1

A 35-year-old man with an 11-year history of mild distal colitis was admitted due to constipation, proctorrhagia, tenesmus and perianal pain. Twelve months before admission, a surveillance colonoscopy with biopsy showed endoscopic remission of the inflammatory disease. His treatment consisted of oral mesalamine 2.4 g/d and he had never received immunosuppressant drugs.

A second colonoscopy indicated a rectal substenosis caused by a large ulcerated mass originating about 2 cm above the anorectal junction, with a proximal extension of about 4 cm.

Histological examination showed a high grade large cell neuroendocrine carcinoma. Immunohistochemical staining was positive for chromogranin, synaptophysin, cytokeratin 7 and chorioallantoic membrane 5.2. Laboratory findings including chromogranin A and neuron-specific enolase were negative.

A staging computed tomography scan and somatostatin receptors imaging were negative for further spread of the tumor. The patient underwent a low anterior colorectal resection and distal rectal mucosectomy with colo-anal anastomosis and loop ileostomy. A definitive pathological investigation confirmed the previous histological findings, and the proliferative marker Mib1/Ki67 was positive in about 50% of the neoplastic cells. The TNM stage was pT₃^{R1}pN₂^{17/30}M₀G3. He was treated with adjuvant radiotherapy and chemotherapy with cisplatin and etoposide. One year after surgery, multiple liver metastases were observed. The patient died 15 mo after diagnosis.

Case 2

A 77-year-old man presented with bloody diarrhea, anorectal pain, mild anemia and a 3 kg weight loss. The patient had a 27-year history of left-sided colitis with mild activity. Ten months before admission, a surveillance colonoscopy with biopsy showed mild left-sided inflammation. He was receiving oral mesalamine 2.4 g/d.

Another colonoscopy was performed, which showed

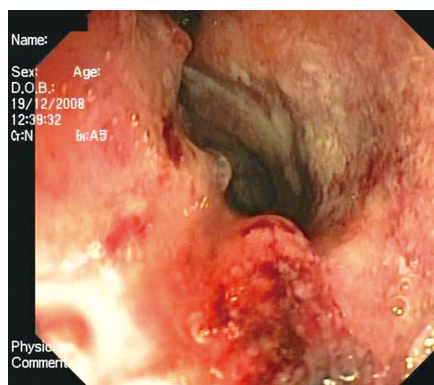


Figure 1 Small cell neuroendocrine carcinoma: endoscopic view.

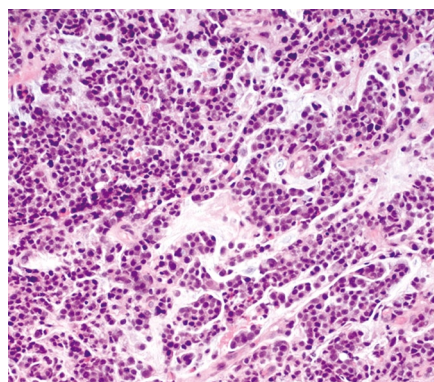


Figure 2 Cells with minimal cytoplasm, fusiform cell shape, finely granular chromatin, small, or absent, nucleoli (HE stain, original magnification × 20).

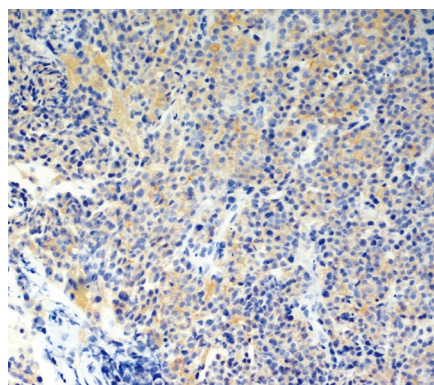


Figure 3 Immunohistochemical analysis reveals neuroendocrine differentiation with positive staining for synaptophysin (original magnification × 20).

a large circular neoplasm of about 5 cm in length, 3 cm above the anorectal junction (Figure 1). Histological examination with immunohistochemical analysis excluded adenocarcinoma. A tumor with neuroendocrine differentiation and strong staining for synaptophysin was observed and a high grade small cell neuroendocrine carcinoma was diagnosed (Figures 2 and 3). The proliferative marker Mib1/Ki67 was positive in about 90% of the neoplastic cells. MR scanning and somatostatin receptors imaging were negative for further neoplastic localizations. The patient is scheduled to receive neoadjuvant radiotherapy.

DISCUSSION

The reported incidence of NENs is between 0.1% and 3.9% of all colorectal malignancies. Bernick *et al.*^[5], at New York Memorial Sloan-Kettering Cancer Center, reported an incidence of 0.6% in colon and rectal cancers.

NENs are subdivided on the basis of cytological-histological features and immunohistochemical findings into small cell neuroendocrine carcinoma (SCNC) and large cell neuroendocrine carcinoma (LCNC)^[5]. The criteria for separating small cell from large cell variants are similar to those proposed for the classification of pulmonary neuroendocrine tumors^[6]. SCNCs resemble their pulmonary homonyms, having minimal cytoplasm, fusiform cell shape, fine granular chromatin, small or absent nucleoli, and nuclear moulding. LCNCs are characterized by cells with more cytoplasm, a round or polygonal cell shape, prominent nucleoli, and a coarser chromatin pattern.

While the diagnosis of SCNC does not necessitate immunohistochemical documentation of neuroendocrine differentiation, the diagnosis of LCNC requires positive immunohistochemical staining for one of the three neuroendocrine markers (chromogranin, synaptophysin and neuron-specific enolase) in at least 10% of tumor cells^[5].

Even though they are extremely rare, it is important to recognize NENs pathologically, since they have a particularly poor prognosis compared to colorectal adenocarcinoma. NENs shows a high rate of liver metastasis (50%) and the reported 1-year survival rate is about 40%^[5]. Thus, patients could benefit from treatment with alternative cytotoxic chemotherapeutic agents^[6].

There have been reports of neuroendocrine tumors associated with UC, but many of these tumors are carcinoids, often well-differentiated and clinical indolent, and are found incidentally at a different site from dysplastic areas after surgery for IBD^[7]. Greenstein *et al.*^[7] believe that this association is coincidental, particularly since there are no significant demographic or clinicopathological differences between the carcinoids that occur in patients with and without IBD. Furthermore, the same authors point out that neuroendocrine cell hyperplasia, probably due to a trophic role of these cells in epithelial re-growth, is not itself an established association with carcinoids.

Sigel *et al.*^[8] reported the only series of NENs other than carcinoids arising in 14 IBD patients (eight CD; six UC). Similar to our findings, they observed that all the tumors arose in areas involved by IBD, and six of the 14 neoplasms affected the rectum. The NENs were well differentiated in 11 cases and poorly differentiated in three cases; two of these three patients died at 3 and 11 mo after tumor excision.

These authors suggested that neuroendocrine differentiation, different to carcinoids, might evolve from multipotential cells in dysplastic epithelium. This was based on their findings that dysplasia was found in adjacent mucosa in more than one-third of cases^[8].

Our cases confirm the aggressive biological behavior and poor response to chemotherapy of NENs compared to colorectal adenocarcinoma. Both NENs in these patients arose in mild left-sided UC, however, the patients were different in age (35 years *vs* 77 years) and disease duration (11 years *vs* 27 years). This raises the question as to whether these carcinomas represent an incidental finding in the background of IBD or if there is a real, although rare, association between IBD and these tumors.

The hypothesis of a pancellular dysplasia involving epithelial, goblet, Paneth and neuroendocrine cells in chronic inflamed mucosa cannot be confirmed from the few published case reports^[8-11]. In addition, the differences in terms of duration, extension, histological and endoscopic activity of the underlying IBD in our and other reported cases^[8-11] do not allow the identification of a subgroup of patients at risk.

ACKNOWLEDGMENTS

The authors wish to thank Mrs Lidia Soncini for support in the English version.

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S- Editor Tian L L- Editor Webster JR E- Editor Ma WH



CASE REPORT

Successful interferon desensitization in a patient with chronic hepatitis C infection

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Received: February 26, 2009 Revised: July 13, 2009

Accepted: July 20, 2009

Published online: September 7, 2009

Gastroenterol 2009; 15(33): 4196-4198 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4196.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4196>

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is one of the leading known causes of chronic liver diseases, including cirrhosis and hepatocellular carcinoma^[1,2]. Risk factors associated with transmission of HCV include transfusion of infected blood products, injection drug use, employment in patient care or clinical laboratory work, exposure to an infected sex partner or household member, exposure to multiple sex partners, and low socioeconomic status^[3].

HCV has a positive-sense, single-stranded RNA genome that has been classified into six different genotypes from 1 to 6^[4,5]. The genotype determination is a relevant clinical practice, which not only helps predict the probability of sustained virological response (40%-45% for genotype 1 compared with 70%-80% for genotypes 2 and 3), but also is used routinely to determine duration of treatment (48 wk for genotypes 1 and 4 vs 24 wk for genotypes 2 and 3)^[6,7]. Various genotypes have distinct geographical distributions around the world^[8], and recent reports from Tehran and five cities from different locations in Iran, have shown that genotype 1a was predominant (47%), and 3a, 1b and 4 had a prevalence of 36%, 8% and 7%, respectively^[9].

Interferon, in various forms or combinations, is the only proven effective treatment for hepatitis C. It has a fundamental, irreplaceable role in the treatment of patients with HCV infection. Treatment of hepatitis C, even when absolutely necessary, is almost impossible when interferon cannot be administered for any reason.

Here, we report a patient with chronic HCV infection and advanced fibrosis who was unable to receive interferon because of systemic hypersensitivity. The patient was desensitized successfully through gradual incremental exposure, and HCV infection was eradicated after a complete course of treatment, with no further allergic reactions.

Abstract

Treatment of hepatitis C, even when absolutely necessary, is almost impossible when interferon cannot be administered for any reason. We report a 65-year-old patient with chronic hepatitis C virus (HCV) infection and fibrosis, who was unable to receive interferon because of systemic hypersensitivity. The patient was desensitized successfully through gradual incremental exposure to interferon, and HCV infection was eradicated after a complete course of treatment, with no further allergic reactions. This case report that describes successful eradication of hepatitis C in a patient with advanced liver disease after desensitization to interferon revealed that desensitization may not necessarily damage the therapeutic efficacy of the drug.

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Key words: Chronic hepatitis C; Desensitization; Drug hypersensitivity; Interferon $\alpha 2b$; Ribavirin

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Taghavi SA, Eshraghian A. Successful interferon desensitization in a patient with chronic hepatitis C infection. *World J*

CASE REPORT

The patient was a 65-year-old man who was referred to

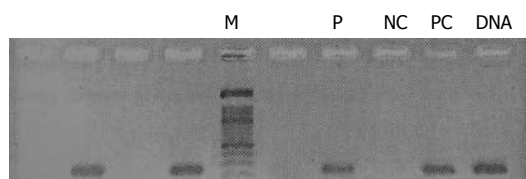


Figure 1 Reverse transcription polymerase chain reaction (RT-PCR) showing cDNA band of hepatitis C virus (HCV). P: Patient; PC: Positive control; NC: Negative control; M: Marker.

Table 1 Dosing protocol for the day 1

Dose number	Time from first dose (h)	Strength	Volume	Drug dosage (IU)
1	0	1/100000	0.1	3
2	1	1/100000	0.1	3
3	2	1/10000	0.1	30
4	3	1/10000	0.1	30
5	4	1/1000	0.1	300
6	5	1/1000	0.1	300
7	6	1/100	0.1	3000
8	7	1/100	0.1	3000
9	8	1/100	0.1	3000
10	9	1/100	0.1	3000
11	10	1/100	0.1	3000
12	11	1/100	0.1	3000
13	12	1/100	0.1	3000
14	13	1/100	0.1	3000
Cumulative dose				24666

our liver clinic with a positive anti-HCV antibody ELISA. The patient's main complaint was fatigue. Past medical history was positive for two coronary angioplasties for ischemic heart disease. No other medical diseases were present in his past history. General physical examination was normal except for mild hepatomegaly. Results of laboratory tests were within normal ranges except for a marginally low albumin level (3.4 g/dL) and mildly elevated aspartate aminotransferase (45-80 IU/mL) and alanine aminotransferase (46-78 IU/mL).

Reverse transcription polymerase chain reaction (RT-PCR) revealed a cDNA band that indicated the presence of HCV (Figure 1). Genotype-specific primers for HCV genotyping showed the virus to be of genotype 1b. Liver needle biopsy showed chronic hepatitis with moderate activity and advanced fibrosis (grade 9/18, stage 5/6).

The patient was selected for treatment with interferon plus ribavirin. The dosage of ribavirin was kept constant at 1200 mg/d throughout treatment. Since he was not able to afford the cost of pegylated interferon therapy, PDferon®, a brand of interferon α -2b produced in Iran (Pooyeshdarou Pharmaceutical Co., Tehran, Iran), was used.

After administration of the first dose of interferon α 2b, the patient developed generalized maculopapular rash with severe itching and low-grade fever. As a result, treatment was stopped, with a diagnosis of hypersensitivity to interferon.

Since there was no other choice for treatment of hepatitis C, after careful discussion with the patient about possible benefits and side effects, a decision was made to proceed with a course of desensitization. The

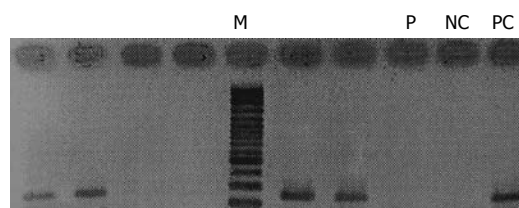


Figure 2 No evidence of cDNA band of HCV with RT-PCR after treatment with interferon plus ribavirin following desensitization. P: Patient; PC: Positive control; NC: Negative control; M: Marker.

Table 2 Dosing protocol for day 2

Dose number	Time from first dose (h)	Strength	Volume	Drug dosage (IU)
1	0	1/100	0.1	3000
2	1	1/100	0.1	3000
3	2	1/10	0.1	30000
4	3	1/10	0.1	30000
5	4	1/1	0.1	300000
6	5	1/1	0.1	300000
7	6	1/1	0.1	300000
8	7	1/1	0.1	300000
Cumulative dose				1266000

patient was kept in hospital during the first and second day of the treatment, with close observation of his vital signs. Resuscitation equipment was kept at the bedside. A routine blood count and chemistry were performed on days 1 and 2 and revealed normal results. A 1-mL vial of PDferon® that contained 3000000 IU was diluted to 1/100000 concentration, and 0.1 mL of the resulting solution was injected subcutaneously.

Desensitization started from 8 am on the first day and dose escalation continued until the sixth dose (14.00 h). The dose was kept the same during the afternoon and injections stopped at 21.00 h to allow for patient rest (Table 1). Desensitization restarted on the next day at the same time, with a 1/100 concentration (Table 2). From day 3 onwards, until the end of the first week, the drug was given at a dose of 1500000 IU/d in the morning, with a 1-h period of observation after the injections. From the second week until the end of the treatment (54 wk), interferon was given at a dosage of 3000000 IU every other day.

The only observed reaction during the treatment was a mild generalized pruritus at the start of day 2, with no accompanying rash or change in vital signs. These symptoms responded to intramuscular injection of diphenhydramine. After completing the treatment course, the patient recovered from HCV and nested RT-PCR revealed no cDNA band, which indicated eradication of HCV (Figure 2). RT-PCR was repeated another two times at 6-wk intervals, with the last one at 18 mo after the end of treatment. All turned out to be negative.

DISCUSSION

Since the patient had advanced liver fibrosis and there was no alternative treatment for hepatitis C, after extensive

discussion with the patient about the possible side effects, as well as alternatives, a decision was made to proceed with desensitization. The protocol was based on previous experience with desensitization to penicillin. This decision was reviewed by another independent gastroenterologist and the patient gave written informed consent.

Interferon products are used worldwide as an effective treatment for chronic hepatitis C. Although several potential antiviral drugs are in the pipeline, interferon is still the only proven effective treatment for this viral disease.

Considering the above facts, when a patient is in great need of antiviral therapy for hepatitis C (advanced fibrosis for instance) and has an allergic reaction to interferon, there are very limited, if any, treatment options available. Although desensitization through gradual exposure to incremental doses is an established option for treatment of allergic reaction to protein and non-protein drugs^[10], there are no reports of its use for desensitization to interferon. The main theoretical risk (beside the proven risk of anaphylaxis) with desensitization to protein drugs such as interferon is that it induces neutralizing antibodies, which potentially are directed towards the effective sites of the drug, which renders it ineffective, or at least reduces its therapeutic potential.

This case report describes successful eradication of hepatitis C in a patient with advanced liver disease, and is an indication that desensitization may not necessarily damage the therapeutic efficacy of the drug. Although in this case a regular (non-pegylated) interferon product was used, it may be expected that the same procedure can be applied to pegylated interferon products, since it is the protein component towards which hypersensitivity is usually directed.

Further studies including measurement and characterization of possible contributing antibodies are needed

before this method can be suggested as a standard protocol for interferon desensitization.

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S- Editor Tian L L- Editor Kerr C E- Editor Yin DH



Composite neuroendocrine and adenomatous carcinoma of the papilla of Vater

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Received: May 6, 2009 Revised: July 18, 2009

Accepted: July 25, 2009

Published online: September 7, 2009

Abstract

Malignant tumors of papilla are usually adenocarcinomas. We present a 67-year-old female who became icteric as result of a malignant tumor infiltrating the papilla of Vater. Histopathological assessment of surgically excised tumor showed both neuroendocrine and adenocarcinomatous features. To our knowledge, this is the seventh report of this rare neoplastic association in the duodenal periampullary region.

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Key words: Adenocarcinoid; Composite carcinoma; Carcinoma of Vater papilla; Carcinoid; Gastropancreatic neuroendocrine tumor

Peer reviewer: Dr. Gert De Hertogh, Morphology and Molecular Pathology, University Hospitals KULeuven, Leuven 3000, Belgium

Musialik JA, Kohut MJ, Marek T, Wodołański A, Hartleb M. Composite neuroendocrine and adenomatous carcinoma of the papilla of Vater. *World J Gastroenterol* 2009; 15(33): 4199-4200
Available from: URL: <http://www.wjgnet.com/1007-9327/15/4199.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4199>

INTRODUCTION

In adults the commonest causes of obstructive jaundice

are biliary stone disease, pancreatic cancer or chronic pancreatitis. A less frequent cause is a benign or malignant tumor arising from the papilla of Vater. Malignant tumors of papilla are usually adenocarcinomas. We present a patient who became icteric as a consequence of a malignant tumor infiltrating the papilla of Vater which histologically showed both neuroendocrine and adenocarcinomatous features.

CASE REPORT

A 67-year-old cholecystectomized woman was admitted to our department with symptoms of obstructive jaundice and cholangitis. Severe epigastric pain, fever and jaundice developed 1 d before presentation. The serum bilirubin level was 5.3 mg/dL, white cell blood count was $20.4 \times 10^3/\mu\text{L}$ and serum amylase and lipase activities were elevated to about six times the normal upper limit. Abdominal ultrasonography showed moderate dilatation of common bile duct. The patient underwent endoscopic retrograde cholangiopancreatography (ERCP) revealing the papilla of Vater was suggestive of adenoma i.e., was markedly enlarged and covered with smooth surfaced mucosa. Sphincterotomy was performed and biopsies were taken from the inner part of the ampulla. The histopathological diagnosis from biopsy specimens was carcinoid tumor, staining positive for chromogranin A and synaptophysin. The patient underwent surgical transduodenal excision of the ampulla. The postoperative histopathological examination showed a composite tumor i.e. mixed neuroendocrine carcinoma (NE)-adenocarcinoma neoplasm (Figure 1A and B). Immunocytochemical studies were used to delineate the NE component (chromogranin, synaptophysin, EMA, cytokeratin). The NE-cell component represented about 80% of the tumor's area. NE tumor cells were well-differentiated, lacked significant atypia and showed low grade malignancy (Ki-67 labelling index was 5%). The glandular component presented well-differentiated tubules (grade I). No necrosis was found in either the NE or glandular areas. The tumor focally involved lymphatic vessels and nerves and infiltrated the muscle of the ampulla. The margins of the excised tissue specimen were free of neoplasm and no neoplastic cells were found in periduodenal lymphatic nodes (T1N0M0). The patient

has been put under endoscopic, serological (CA 19-9, CEA, chromogranin A) and ultrasound surveillance. Six months after surgery she is doing well.

DISCUSSION

Gastroenteropancreatic Neuroendocrine Tumors (GEP/NETs) are rare neoplasms originating from the diffuse neuroendocrine system, involving 15 types of highly differentiated ectodermal cells located in the gastrointestinal tract and pancreas. The most common location of GEP/NETs is in the appendix (62% of cases), but they may also be found in small intestine (27%), lung (15%), undefined primary locations with hepatic metastases (12%) and other organs (3%)^[1]. GEP/NETs secrete peptides and neuroamines that cause distinct clinical syndromes, including carcinoid syndrome. Many tumors are, however, clinically silent until late presentation associated with mass effects. The current WHO classification categorizes GEP/NETs into: (1) well-differentiated neuroendocrine tumor; (2) low-grade malignant carcinoma; (3) high-grade malignant carcinoma and (4) mixed tumor: adenocarcinoma/neuroendocrine carcinoma^[2]. At present 14 terms are used to define tumors with mixed exocrine-endocrine features. Volante *et al*^[3] proposed classification based on the extension of each component and structural features of the NE component. Three separate patterns can be distinguished i.e. (1) NE tumors with focal non-NE component occupying less than 30% of the tumor, (2) mixed exocrine-endocrine carcinomas (NE or non-NE cells > 30%) and (3) non-NE carcinoma with NE component (< 30%). The type of tumor influences the prognosis, which improves with increasing contribution of NE component^[3]. About 50% of GEP/NETs are carcinoids found by chance during laparotomy or as hepatic metastases.

Carcinoids involving the papilla of Vater are rare lesions, accounting for 0.35% of all gastrointestinal carcinoids. So far, only 110 cases have been reported in the literature, mostly as individual case reports^[4]. These tumors are predominantly found on ERCP in patients with obstructive jaundice or acute biliary pancreatitis. In the presented case the tumor histologically was a mixed carcinoid-adenocarcinoma neoplasm (type IV). The concurrence of carcinoid with adenocarcinoma is an unusual phenomenon in the gastrointestinal tract, that has been reported in the oesophagus, stomach, small intestine, appendix, colon and rectum^[5-7]. To our knowledge, this is the seventh report of this rare neoplastic association in the duodenal periampullary region.

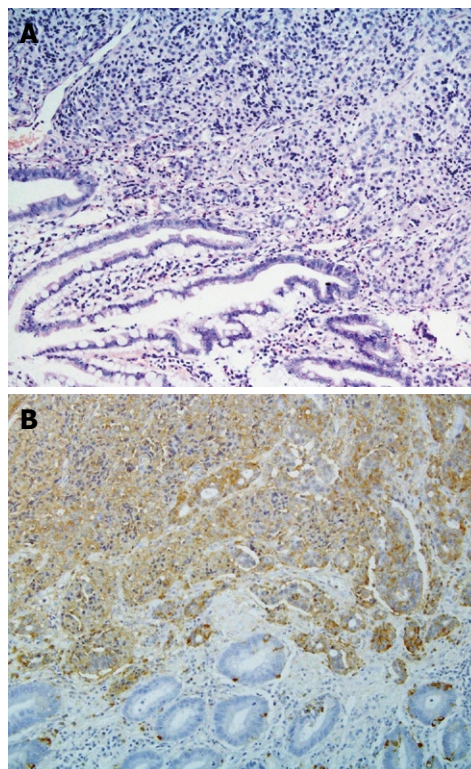


Figure 1 Histopathological examination. A: Mixed carcinoid-adenocarcinoma neoplasm ($\times 200$); B: Chromogranin A positive stain of tumour cells ($\times 200$).

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S- Editor Tian L L- Editor Lalor PF E- Editor Zheng XM



Placement of percutaneous transhepatic biliary stent using a silicone drain with channels

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Received: February 25, 2009 Revised: August 5, 2009

Accepted: August 12, 2009

Published online: September 7, 2009

Key words: Biliary tract; Drainage; Hepaticojejunostomy; Stenosis; Stents

Peer reviewer: Taku Aoki, MD, Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

Yoshida H, Mamada Y, Taniai N, Mineta S, Mizuguchi Y, Kawano Y, Sasaki J, Nakamura Y, Aimoto T, Tajiri T. Placement of percutaneous transhepatic biliary stent using a silicone drain with channels. *World J Gastroenterol* 2009; 15(33): 4201-4203 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4201.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4201>

Abstract

This report describes a method for percutaneous transhepatic biliary stenting with a BLAKE Silicone Drain, and discusses the usefulness of placement of the drain connected to a J-VAC Suction Reservoir for the treatment of stenotic hepaticojejunostomy. Percutaneous transhepatic biliary drainage was performed under ultrasonographic guidance in a patient with stenotic hepaticojejunostomy after hepatectomy for hepatic hilum malignancy. The technique used was as follows. After dilatation of the drainage root, an 11-Fr tube with several side holes was passed through the stenosis of the hepaticojejunostomy. A 10-Fr BLAKE Silicone Drain is flexible, which precludes one-step insertion. One week after insertion of the 11-Fr tube, a 0.035-inch guidewire was inserted into the tube. After removal of the 11-Fr tube, the guidewire was put into the channel of a 10-Fr BLAKE Silicone Drain. The drain was inserted into the jejunal limb through the intrahepatic bile duct and was connected to a J-VAC Suction Reservoir. Low-pressure continued suction was applied. Patients can be discharged after insertion of the 10-Fr BLAKE Silicone Drain connected to the J-VAC Suction Reservoir. Placement of a percutaneous transhepatic biliary stent using a 10-Fr BLAKE Silicone Drain connected to a J-VAC Suction Reservoir is useful for the treatment of stenotic hepaticojejunostomy.

INTRODUCTION

In most operations for hepatic hilum malignancy, the intrahepatic bile duct at the hilum is divided from the hepatic artery, portal vein, and surrounding tissues. Biliary complications, such as leakage or stenosis, are caused occasionally by the presence of several small bilioenteric anastomoses^[1]. Stenosis of a bilioenteric anastomosis increases the risk of cholangitis and requires the long-term placement of a stent.

The BLAKE Silicone Drain (Ethicon, NJ, USA) is made of silicone; the entire drain is flexible, and channels along the sides, instead of holes, facilitate drainage. It is always used with a continuous suction device (J-VAC Suction Reservoir; Ethicon), which creates a closed drainage system. Drainage is very efficient: a larger area is in contact with tissue as compared with perforated drains, and fluid is efficiently removed by capillary pressure.

We have reported previously the effectiveness of a BLAKE Silicone Drain connected to a J-VAC Suction Reservoir for the management of pancreatic fistula^[2]. We also reported that a 10-Fr BLAKE Silicone Drain has been used as an external stent for a bilioenteric anastomosis during surgery for hepatic hilar malignancy^[3].

This report describes a method for percutaneous transhepatic biliary stenting with a BLAKE Silicone Drain, and discusses the usefulness of placement of the drain connected to a J-VAC Suction Reservoir for the treatment of stenotic hepaticojejunostomy.



Figure 1 Percutaneous drainage catheters were inserted into each abscess, and pus was discharged. Abscessography demonstrated communications between the dilated intrahepatic bile duct of the anterior branch and each abscess.



Figure 3 One week after initial insertion of the 11-Fr tube, cholangiography was performed via the tube. After insertion of a 0.035-inch guide wire, the 11-Fr tube was switched to a 10-Fr BLAKE Silicone Drain.

CASE REPORT

A 68-year-old man with intrahepatic cholangiocarcinoma invading the hepatic hilum underwent extended left hepatectomy combined with *en bloc* resection of the extrahepatic bile duct and extended lymph node dissection.

Bilioenteric continuity was reestablished by two hepaticojejunostomies of the anterior and posterior branches of the hepatic ducts, performed using a Roux-en-Y jejunal limb. Silicone drains with an internal lumen and side holes, made by cutting a 5-Fr radiopaque tube for pancreatic drainage (Sumitomo Bakelite, Tokyo, Japan), were used as an internal stent for each bilioenteric anastomosis. Two external drainage catheters (19-Fr BLAKE Silicone Drain) were positioned at the cut surface of the liver and connected to the J-VAC Suction Reservoir. Fixation of the greater omentum was performed to avoid delayed gastric emptying^[4]. After operation, major bile leakage occurred, but resolved gradually. The drainage catheter was removed on postoperative day 12, and the patient was discharged on postoperative day 18.

Forty days after discharge, the patient was readmitted because of severe pyrexia. Upon admission, computed tomography demonstrated two low-density areas associated with dilatation of the intrahepatic bile ducts in the anterior segment. Hepatic abscesses caused by stenosis of the hepaticojejunostomy were diagnosed.



Figure 2 The catheter (11-Fr PTCS tube, Sumitomo Bakelite, Tokyo, Japan) with several side holes was placed in the jejunal limb through the intrahepatic bile duct and passed through the stenosis of the hepaticojejunostomy.

Percutaneous drainage catheters were inserted into each abscess, and pus was discharged. Abscessography demonstrated communications between the dilated intrahepatic bile ducts of the anterior branch and each of the abscesses (Figure 1). Pyrexia improved, and discharge of clear bile from one of the drainage catheters continued. One week after insertion of the drainage catheters, abscessography was repeated. It was difficult to insert the catheter from the abscess into the intrahepatic bile duct. Another percutaneous transhepatic biliary drainage catheter was inserted directly into the intrahepatic bile duct under ultrasonographic guidance. An 11-Fr tube (11 Fr PTCS tube; Sumitomo Bakelite) with several side holes was placed in the jejunal limb through the intrahepatic bile duct, and passed through the stenosis of the hepaticojejunostomy (Figure 2). Bile juice was discharged from the 11-Fr tube, and discharge from the drainage catheters of the abscess decreased. One of the catheters was removed from the abscess because pus discharge stopped. One week after initial insertion of the 11-Fr tube, cholangiography was performed *via* the tube. A 10-Fr BLAKE Silicone Drain is flexible, which made one-step insertion difficult. After insertion of a 0.035-inch guide wire, the 11-Fr tube was switched to a 10-Fr BLAKE Silicone Drain (Figure 3). The drain was connected to a J-VAC Suction Reservoir, and continued low-pressure suction was applied. The remaining catheter was removed from the abscess. The patient was discharged 5 d after switching the 11-Fr tube to a 10-Fr BLAKE Silicone Drain connected to the J-VAC Suction Reservoir. After 4 mo, the patient was readmitted because of liver failure. The portal vein was obstructed by recurrent carcinoma. Three weeks after readmission, the patient died. The 10-Fr Blake Silicone Drain was not occluded.

DISCUSSION

Diagnostic techniques for hepatobiliary disease have improved recently, but advanced hepatic hilum malignancy is still encountered frequently^[5-7]. Palliative treatment with a biliary stent is carried out in patients with inoperable malignancy in order to relieve symptoms related to obstructive jaundice^[8]. In patients undergoing hepatectomy for advanced hepatic hilum malignancy, aggressive dissection may compromise the ductal blood supply. Peripheral branches of blood vessels,

with small diameters and a poor blood supply, may be damaged along with the bile duct, which increases the risk of biliary leakage^[1]. The use of stents has been recommended to decompress the bile duct, reduce the risk of bile leakage, and decrease fibrotic narrowing of the anastomosis during early healing^[9-12]. However, the risk of biliary complications such as leakage or stenosis is increased by the presence of small several bilioenteric anastomoses^[1].

We performed placement of a percutaneous transhepatic biliary stent using a 10-Fr BLAKE Silicone Drain connected to a J-VAC Suction Reservoir for the treatment of stenotic hepaticojejunostomy. BLAKE Silicone Drains promote efficient drainage: a larger area is in contact with tissue as compared with perforated drains, which facilitates the effective removal of fluid by capillaries. These drains contain no plasticizing agents, which are considered to have deleterious effects in humans. The channels do not interfere with removal, and the drains can be removed easily, safely and securely. These drains also provide a high flow rate when connected to a closed suction device (J-VAC Suction Reservoir). The suction pressure of the J-VAC Suction Reservoir is lower than that with other portable low-pressure continuous suction devices. As a result of low suction pressure, the J-VAC Suction Reservoir does not damage tissue; therefore, we used this drainage system in the lumen of the bile duct and jejunum.

We have evaluated previously the efficacy of a BLAKE Silicone Drain connected to a J-VAC Suction Reservoir for the management of pancreatic fistula. In basic experiments, no collections of fluid were detected around the BLAKE Silicone Drain. When leakage occurred, it did not cause an abdominal abscess, and a “drain canal” linking the anastomosis with the extracorporeal orifice was formed all along the drainage route^[2].

In the patient described in this report, 5-Fr silicon drains were used as a stent for the bilioenteric anastomosis. Stenosis of the anastomosis occurred after leakage. We used a 10-Fr BLAKE Silicone Drain as an external stent for bilioenteric anastomosis after major hepatectomy with pancreatoduodenectomy. The drain is placed within the hepaticojejunostomy *via* the stump of the jejunal limb. It is then connected to the J-VAC Suction Reservoir. Bile and pancreatic juice can be drained by a single drain. Even if bilioenteric anastomosis leaks, a 10-Fr intraluminal diameter of the anastomosis is maintained^[3].

The treatment of stenotic bilioenteric anastomosis requires the long-term placement of a stent in the anastomosis. The BLAKE Silicone Drain is flexible and contains no plasticizing agents. It has four continuous channels instead of holes along the sides, and prevents obstruction of small biliary branches. The BLAKE Silicone Drain is therefore suited for long-term placement. The input port of the J-VAC Suction Reservoir has an anti-reflux valve, which reduces the risks of the reverse flow of fluid into the body and of retrograde infection. This valve enables the patient to use the reservoir without having to worry about the level of the drainage bag during position changes and

ambulation. The J-VAC Suction Reservoir is portable and convenient in size, which enables the patient to carry it around in a specially designed pochette on the shoulder. A BLAKE Silicone Drain connected to a J-VAC Suction Reservoir usually remains free of occlusion for about 6 mo.

In conclusion, placement of a percutaneous transhepatic biliary stent using a 10-Fr BLAKE Silicone Drain connected to a J-VAC Suction Reservoir was useful for the treatment of stenotic hepaticojejunostomy.

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CASE REPORT

Sclerosing epithelioid fibrosarcoma of the liver infiltrating the inferior vena cava

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Received: April 17, 2009 Revised: August 3, 2009

Accepted: August 10, 2009

Published online: September 7, 2009

to those of the primary tumor. To our knowledge, this is the first report of SEF of the liver with tumor invasion of the IVC.

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Key words: Sclerosing epithelioid fibrosarcoma; Liver tumor; Surgery; Inferior vena cava invasion; Vimentin

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INTRODUCTION

Sclerosing epithelioid fibrosarcoma (SEF) is a rare and poorly defined variant of fibrosarcoma, first described by Meis-Kindblom *et al*^[1] in 1995^[2-4]. It is a mesenchymal neoplasm characterized histopathologically by a predominant population of epithelioid cells arranged in strands, nests, and sheets, embedded in a fibrotic and extensively hyalinized stroma. Although the SEF belongs to the low-grade sarcoma group of neoplasms, approximately 50% of patients with the tumor develop local recurrence and/or metastases^[3-5].

Several investigators have reported cases of SEF arising from various sites such as upper extremities, lower extremities, limb girdles, trunk, and the head and neck area, but there have been no reports of SEF of the liver^[1-10]. Recently, we experienced a patient with SEF of the liver with invasion of the inferior vena cava (IVC). Although the SEF in the patient was surgically resected, it recurred after surgery. The recurrent SEF was also surgically resected. In addition to the rarity of the SEF of the liver, the SEF displayed a rare extension pattern

Abstract

Sclerosing epithelioid fibrosarcoma (SEF) is a rare and distinct variant of fibrosarcoma, composed of epithelioid tumor cells arranged in strands, nests, cords, or sheets embedded within a sclerotic collagenous matrix. We report a 39-year-old man with SEF of the liver, which infiltrated the inferior vena cava (IVC). The SEF of the liver was successfully resected, and the infiltrated IVC was also removed together with the liver tumor. Histopathological examination of the tumor showed typical histopathology of SEF. Immunohistochemically, the tumor was positive for vimentin. Recurrence was noted 7 mo after surgery. After chemotherapy, the recurrent tumor was resected surgically, and histopathological examination showed similar findings

of infiltration in the IVC. To our knowledge, there have been no previous reports of such kinds of tumor extension in SEF. In this article, we report the first case of SEF of the liver with invasion of the IVC.

CASE REPORT

A 39-year-old man presented with the chief complaint of general fatigue and abdominal uncomfotableness. Laboratory findings included no thrombocytopenia and liver enzymes above the normal range (alanine transaminase: 44 U/L, lactate dehydrogenase: 267 U/L, alkaline phosphatase: 412 U/L, and γ -glutamyltranspeptidase: 187 U/L). Hepatitis virus markers and autoantibodies were negative. Tumor markers including protein induced by vitamin K absence or antagonist II, α -fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen were all within the normal ranges. Serum soluble interleukin-2 receptor level was not elevated. The patient did not consume alcohol and had no history of exposure to radiation. Computed tomography (CT) and magnetic resonance imaging (MRI) demonstrated a huge liver tumor measuring about 70 mm in diameter located in segment 1 based on Couinaud's classification, with invasion of the IVC adjacent to the site adjacent to the right atrium^[11] (Figure 1A and B). In addition, another liver tumor measuring 18 mm was found in segment 8 on the CT and MRI. Fluorodeoxyglucose-positron emission tomography/CT showed abnormal accumulations in both tumors.

With a preoperative diagnosis of primary malignant liver tumor of unknown origin with invasion of the IVC and possibly intrahepatic metastasis, laparotomy was performed. The huge tumor, which was located mainly in the liver, adhered to the diaphragm, right lower lobe of the lung, and pericardium. The tumor also adhered to the suprahepatic IVC. By using the total hepatic vascular exclusion technique (THVE), extended left hepatectomy with resection of the caudate lobe of the liver was performed. The diaphragm, the right lower lobe of the lung, and the pericardium were resected. Next, after clamping the suprahepatic IVC below the right atrium and the retrohepatic IVC above the renal veins, the adhered IVC wall was removed together with the liver. The resected IVC wall was replaced with an expanded polytetrafluoroethylene graft. Since the tumor in segment 8 was suspicious of intrahepatic metastasis, partial hepatectomy of segment 8 was also performed. No other liver tumors were found during surgery.

A macroscopic view of the resected tumor was shown in Figure 2. The tumor, measuring 68 mm \times 54 mm, was mainly located in segment 1 of the liver. Microscopically, the tumor mainly existed in the liver with invasion into neighboring extrahepatic soft tissue including the wall of the IVC and diaphragm. Moreover, tumor thrombi were identified in the left portal vein and its branches. The tumor consisted of uniformly round or polygonal epithelioid cells with a faintly eosinophilic cytoplasm (Figure 3A and B). The tumor cells were arranged in strands, nests, cords or sheets and embedded in a heavily hyalinized matrix. The microscopic findings of the

tumor in segment 8 were similar to those of the primary tumor, and that tumor was diagnosed as an intrahepatic metastasis. Immunohistochemical staining of the main tumor was positive for vimentin (Dakopatts, Glostrup, Denmark), bcl-2 (Dakopatts), and CD99 (Dakopatts), and was negative for AE1/AE3 (Dakopatts), CAM5.2 (Becton Dickinson, San Jose, CA), desmin (Dakopatts), epithelial membrane antigen (EMA) (Dakopatts), CD34 (Nichirei, Tokyo, Japan), S100, α -smooth muscle actin (α -SMA) (Dakopatts), neuron-specific enolase (Dakopatts), CD56 (Dakopatts), leukocyte common antigen (LCA) (Dakopatts), CD30 (Dakopatts), HMB45 (Dakopatts), AFP (Dakopatts), and CA19-9 (Zymed Laboratories Inc., San Francisco, CA) (Figure 3C). The MIB-1 labeling index (Dakopatts) was 30%. Moreover, cytogenetic analysis did not show t (X; 18) chromosomal translocation, which is frequently observed in synovial sarcoma^[12,13]. Based on the results of histopathological examination, cytogenetic analysis, and immunohistochemical patterns, the tumor was finally diagnosed as SEF. Although the tumor was associated with invasion to the wall of the IVC and diaphragm, the most part of the tumor existed in segment 1 of the liver. Moreover, tumor thrombi were identified in the right portal vein and its peripheral branches, and intrahepatic metastases, which were frequently seen in the primary liver malignant tumor. Therefore, the SEF probably originated from the liver. The resection margins were free of tumor. The noncancerous area of the liver was histologically normal. The patient had an uneventful postoperative course and was discharged from the hospital 24 d after surgery.

Seven months after surgery, the patient complained of back pain. CT examination showed recurrent SEF in the extrahepatic retroperitoneal tissue (Figure 1C and D). Systemic chemotherapy consisting of adriamycin and ifosfamide was applied. Close follow-up showed arrest of tumor growth and no new metastatic lesions within the first 3 mo. Subsequently, laparotomy was performed for the excision of the recurrent tumor 12 mo after the initial surgery. The recurrent tumor, located in the retroperitoneal tissue and adherent to the remnant liver, also infiltrated the graft IVC. Using the THVE approach, the tumor was resected with the adherent liver tissue and the infiltrated IVC graft. The resected IVC was again replaced with an expanded polytetrafluoroethylene graft. The microscopic findings of the resected tumor were similar to those of the primary SEF. The immunohistochemical staining patterns of the primary tumor and the recurrent tumor were also similar. The patient had an uneventful postoperative course and was discharged from the hospital 30 d after the surgery.

At the last follow-up examination 6 mo after the second surgery, he remains in good condition, with no evidence of recurrence.

DISCUSSION

SEF is a rare tumor characterized histopathologically by a predominant population of epithelioid cells arranged in strands, nests, cords, or sheets, which are

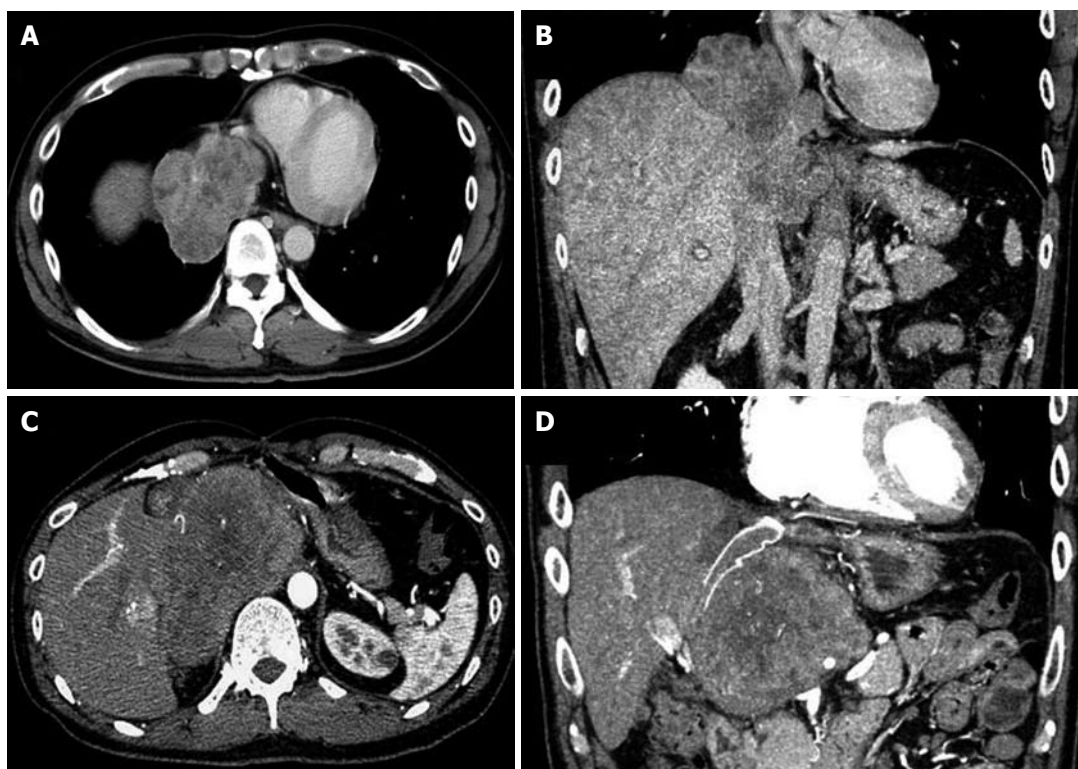


Figure 1 CT of the patient, showing the liver tumor infiltrating the suprahepatic inferior vena cava (A: Axial scan; B: Coronal scan), and showing the recurrent tumor (C: Axial scan; D: Coronal scan).

embedded within a sclerotic collagenous matrix^[1-4]. Immunohistochemically, staining for vimentin is positive in all SEF whereas staining for CD34, leukocyte markers, HMB45, CD68, desmin, glial fibrillary acidic protein, and TP53 is negative. Focal and weak immunostaining for EMA, S100 and more rarely for cytokeratins may be seen in a minority of cases.

The histopathological differential diagnosis of SEF generally includes a wide variety of tumors with sclerotic or epithelioid features, and thus immunohistochemical analysis is essential for precise diagnosis of SEF. In the present case, differential diagnosis was challenging for the following reasons. Undifferentiated hepatocellular carcinoma and infiltrating adenocarcinoma should be differentiated from SEF, but immunohistochemical staining for AFP, CAM5.2, and AE1/AE3 was negative in the present case. Synovial sarcoma should also be differentiated from SEF. Cytogenetic identification of t (X: 18), which is found in synovial sarcoma, could differentiate synovial sarcoma from SEF^[12,13]. No chromosomal translocation was observed in the present case. Furthermore, smooth muscle neoplasms, such as hyalinized leiomyoma or leiomyosarcoma, frequently resemble SEF histopathologically, but they are characterized by immunohistochemical positivity for α -SMA and desmin. Clear cell sarcoma and malignant peripheral nerve sheath tumors may need to be distinguished from SEF. However, these tumors are positive for S100 immunostaining. Moreover, sclerosing lymphoma and malignant melanoma of the soft part are also on the list of differential diagnoses,

but these tumors are usually positive for LCA and HMB45, respectively, and were negative in our case. Epithelioid hemangioendothelioma could be ruled out based on immunonegativity for CD34, and alveolar rhabdomyosarcoma could be excluded by the negative result of desmin and absence of rhabdomyoblasts. Moreover, absence of osteoid formation excluded traosseous osteosarcoma in the present case. Thus, taking into consideration not only the histopathological findings but also the results of cytogenetic analysis and immunohistochemical examinations, the tumor was finally diagnosed as SEF.

In reviewing previous reports, SEFs usually arise in the deep soft tissue and are frequently associated with the adjacent fascia or periosteum. Most SEFs are located in the lower extremities and limb girdles, followed by the trunk, upper extremities, and the head and neck area^[1-10].

In this case, although the tumor was associated with invasion to the wall of IVC and diaphragm, the most part of the tumor existed in the liver. Moreover, in this case, tumor thrombi and intrahepatic metastases were concurrently identified. These features might suggest that the tumor had originated in the liver. To our knowledge, there have been no reports of SEF originating from the liver.

The etiology of most malignant soft tissue tumors, including SEF, is generally unknown. A recent report reviewed 90 patients with SEF and found no significant gender difference with a mean patient age of 47 years (range, 14-87 years)^[5]. The average tumor size at diagnosis is about 8 cm. Several investigators reported previously

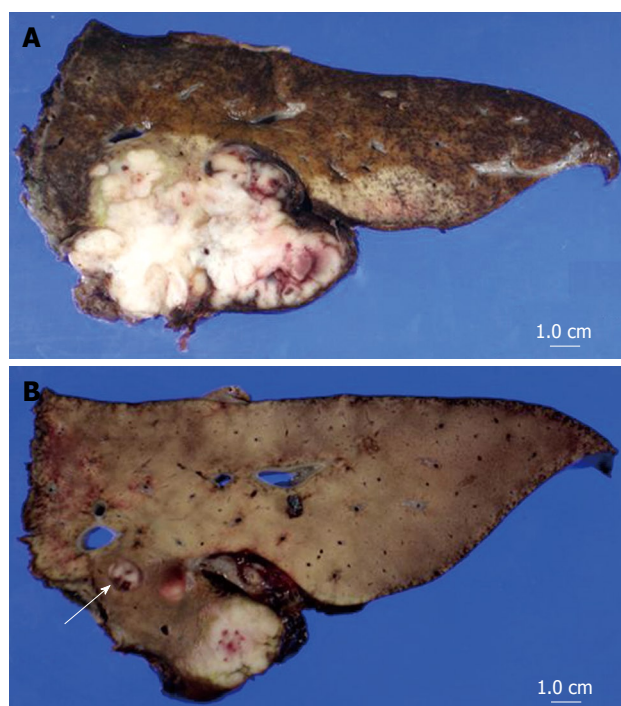


Figure 2 Macroscopic findings of the resected tumor. A: The tumor was directly contiguous to the normal liver parenchyma; B: Tumor thrombus was identified in the left portal vein (arrow).

the association between radiation and occurrence of fibrosarcoma, though our patient had no history of radiation^[9,14].

The established treatment for SEF is complete resection. There is no evidence to support the effect of chemotherapy and/or radiotherapy, though these therapies were used in some previous reports^[3,10]. As for prognosis, Chow *et al*^[4] reported 57 patients with SEF with a local recurrence rate of 48%, metastasis rate of 60%, and mortality rate of 35%. Moreover, in a study of 16 patients with SEF by Antonescu *et al*^[3], the local recurrence rate, metastasis rate, and mortality rate were 50%, 86% and 57%, respectively^[5]. Distant metastasis is reported to be most common in the lung, followed by bone, soft tissue, brain, and lymph nodes^[1-5]. These previous reports of poor prognosis suggest that SEF is a clinicopathologically distinct soft tissue tumor with malignant potential although it is categorized as a low-grade neoplasm in the sarcoma group.

In our case, since no distant metastasis was found preoperatively and since complete resection was thought possible, we selected surgical resection although the tumor had extended at the time of surgery into the IVC, in addition to local intrahepatic metastasis. Indeed, we could completely resect the tumor macroscopically, but the tumor recurred postoperatively. It is quite possible that malignant cells remained at the tumor bed after the resection despite our careful endeavor. Alternatively, considering the high recurrence rate of SEF reported previously, it is also possible that malignant cells might have already exfoliated from the tumor extending to IVC and presumably reached other organs before the resection. Thus, when surgical resection is selected for

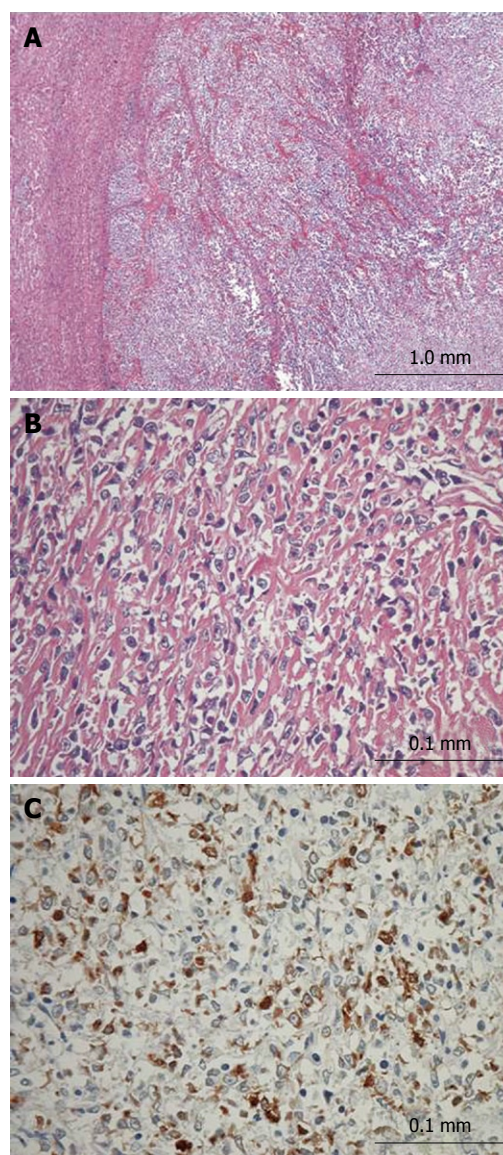


Figure 3 Histopathological findings of the resected liver tumor. A, B: Hematoxylin-eosin staining; C: Immunohistochemical staining for vimentin. A: The tumor (right side) was close to the normal liver parenchymal tissue (left side) ($\times 4$); B: The tumor consisted of uniformly round or polygonal epithelioid cells, which were arranged in strands, nests, cords or sheets and embedded in a heavily hyalinized matrix ($\times 40$); C: Positive immunohistochemical staining for vimentin ($\times 40$).

SEF, perioperative chemotherapy and/or radiotherapy, in addition to macroscopic complete resection, may be necessary to prevent recurrence, though there is no established effective and standardized regimen of chemotherapy and/or radiotherapy. To clarify the effect of perioperative chemoradiotherapy, in addition to surgical resection, further study with a larger number of SEF cases treated perioperatively is needed.

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S- Editor Tian L L- Editor O'Neill M E- Editor Zheng XM

Priapism secondary to penile metastasis of rectal cancer

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Received: May 19, 2009 Revised: August 4, 2009

Accepted: August 11, 2009

Published online: September 7, 2009

Abstract

Metastatic penile carcinoma is rare and usually originates from genitourinary tumors. The presenting symptoms or signs have been described as nonspecific except for priapism. Rectal adenocarcinoma is a very unusual source of metastatic penile carcinoma. We report a case of metastatic penile carcinoma that originated from the rectum. Symptomatic improvement occurred with palliative radiotherapy.

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Key words: Penile neoplasms; Neoplasm metastasis; Priapism; Rectal cancer

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Park JC, Lee WH, Kang MK, Park SY. Priapism secondary to penile metastasis of rectal cancer. *World J Gastroenterol* 2009; 15(33): 4209-4211 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4209.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4209>

INTRODUCTION

Metastatic tumors of the penis are rare despite an abundant blood supply to the penis. Approximately 300 cases have been reported in the clinical literature^[1]. Primary sites of origin are the bladder (33%), prostate (30%), colon (17%)

and kidney (7%)^[2]. Extra-pelvic sites including the lung, pancreas, stomach, esophagus, melanoma and testis have been noted^[3-6]. Fewer than 60 cases of penile metastasis from colon cancer have been reported. Penile metastasis has been a part of more widespread disease in about 90% of reported cases^[7]. We report a case of a painful penile metastasis as a manifestation of rectal cancer dissemination that was improved by the use of palliative radiotherapy.

CASE REPORT

A 43-year-old man was admitted to our clinic with a penile erection and pain. Two years earlier, the patient had undergone abdominoperineal resection for adenocarcinoma of the rectum, followed by adjuvant chemoradiotherapy with capecitabine. Postoperative pathology revealed a moderately differentiated adenocarcinoma and pathological T3 tumor with metastases in 15 out of 37 regional lymph nodes. Thirteen months earlier, recurrence of rectal cancer was detected in the abdominal para-aortic nodes. Various palliative chemotherapeutic agents were administered for 11 mo and the patient received no further treatment. One month earlier, penile erection and pain developed.

Physical examination showed no visible skin lesions, but multiple palpable hard nodules were present over the penile shaft. Tenderness developed following palpation of the penis.

The penile lesions had multifocal, irregular-shaped, low-density areas as depicted on pelvic computed tomography (CT) images, and these lesions had increased in size and number as compared with previous CT images obtained 3 mo previously (Figure 1). Positron emission tomography (PET) showed low 18F- fluorodeoxyglucose uptake in the lesions (Figure 2). Penile magnetic resonance imaging (MRI) showed low to iso-intensity signals as compared with the surrounding corpus cavernosum on an axial T1-weighted image, low to intermediate signal intensity on an axial T2-weighted image, and the presence of non-enhanced lesions on a gadolinium-enhanced image (Figure 3). Other sites of metastasis were noted in several para-aortic lymph nodes, lung and vertebral body of the L2 spine.

Fine needle aspiration of the nodules was performed and histological examination showed nests of acinar-like cells with cytological atypia, consistent with metastatic adenocarcinoma from primary rectal cancer (Figure 4).

Intravenous morphine infusion and spinal nerve block were given for control of penile pain, but were ineffective. Other treatment options were required but

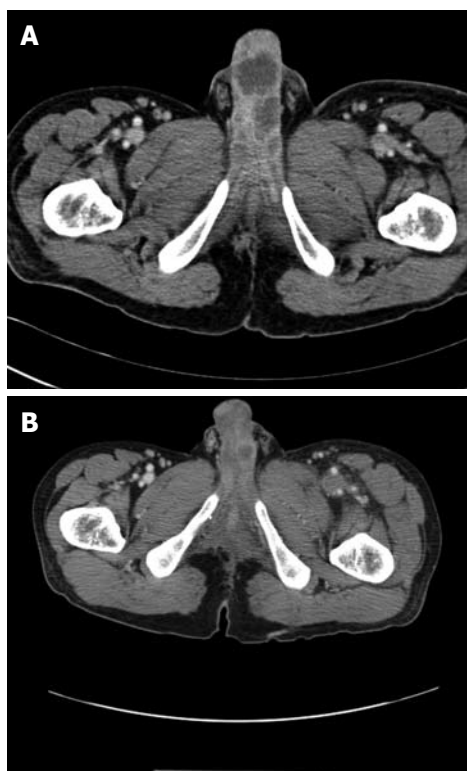


Figure 1 CT image. A: Multifocal irregular shaped low-density areas along the penis; B: Multifocal irregular shaped low density areas are also seen in an CT image obtained 3 mo earlier.

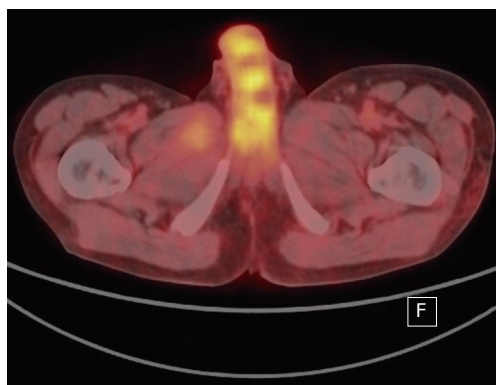


Figure 2 PET image showed low 18F-fluorodeoxyglucose uptake in the lesions.

the patient was in a chemotherapy refractory and systemic disseminated state. As a result of poor performance status, the patient was not allowed to undergo palliative partial or total penectomy. We offered the patient the option of undergoing palliative external beam radiation (2600 cGy, 13 fractions), which was preformed successfully. Penile pain was relieved and the patient was discharged from the hospital.

DISCUSSION

The reason why the penis is a rare site for metastasis despite rich vascularization is not clear. Various mechanisms for penile metastasis have been suggested. These include retrograde venous spread, retrograde lymphatic spread,



Figure 3 Penile MRI. A: Low to iso signal intensity as compared with the surrounding corpus cavernosum on an axial T1-weighted image; B: Low to intermediate signal intensity on an axial T2-weighted image; C: The presence of non-enhanced lesions on a gadolinium-enhanced image.

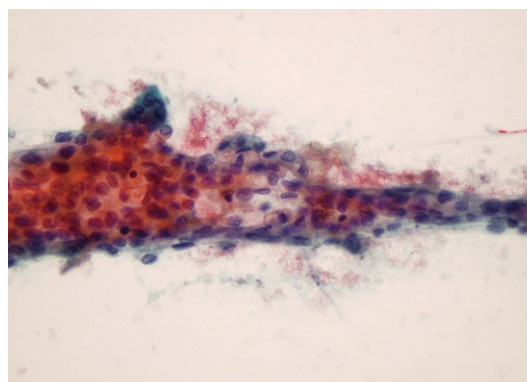


Figure 4 Fine-needle aspiration of penile mass showed nests of acinar-like cells with cytological atypia, consistent with metastatic adenocarcinoma (Papanicolaou stain, $\times 400$).

arterial embolism and local direct extension^[3]. Currently,

a route that involves retrograde venous spread from the pudendal into the dorsal venous system of the penis is considered as the most likely mechanism.

Presenting symptoms and signs in order of frequency are malignant priapism (40%), urinary retention, penile nodules, ulceration, perineal pain, edema, generalized swelling, broad infiltrative enlargement, dysuria and hematuria^[1]. Penile metastasis must be differentiated from primary penile cancer, chancre, chancroid, non-tumorous priapism, Peyronie's disease, tuberculosis and other inflammatory and suppurative diseases^[8]. The corpus cavernosum is usually the site of involvement of metastatic penile carcinoma. The glans penis and corpus spongiosum are involved rarely.

Penile lesions require an excisional or fine-needle aspiration biopsy for pathological confirmation. However, radiological evaluations such as CT, MRI and PET are useful noninvasive methods for the evaluation of lesions^[3,9].

Penile metastases typically manifest as multiple discrete masses in the corpus cavernosum. Noninvasive modalities such as CT, MRI and PET are being used increasingly. CT has been used in the evaluation of penile lesions. Due to only single-plane imaging, its use is limited and it may not detect penile metastasis^[9]. MRI is a most reliable alternative for diagnosis and assessing the extent of penile metastasis, with its multi-plane imaging and superior soft tissue contrast. These masses have low signal intensity relative to normal corporal tissues in T1-weighted images, and low or high signal intensity in T2-weighted images^[9-11]. PET is also useful for detection of clinically silent metastatic sites in hypermetabolic cancer^[5,11].

The treatment plan depends on the performance status and primary cancer state of the patient. Treatment modalities include local excision, penectomy, chemotherapy and radiotherapy. Most patients with penile metastasis already have widely disseminated disease, and > 80% of the patients will die within 6 mo, irrespective of the primary tumor and the treatment method, making palliative noninvasive treatment advisable.

In conclusion, we report a case of painful priapism secondary to penile metastasis of rectal cancer, which was improved by palliative radiotherapy. Most patients with metastatic penile carcinoma have developed systemic dissemination and early detection, precise diagnosis and noninvasive treatment are required for improvement of quality of life.

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S- Editor Tian L L- Editor Kerr C E- Editor Zheng XM

CASE REPORT

Retroperitoneal desmoplastic small round cell tumor: Pediatric patient treated with multimodal therapy

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Received: May 26, 2009 Revised: July 29, 2009

Accepted: August 5, 2009

Published online: September 7, 2009

Abstract

A desmoplastic small round cell tumor (DSRCT) is a rare, aggressive mesenchymal neoplasm. Although a DSRCT can develop at various sites, the intra-abdominal site is the most common location. These tumors are found most commonly among young adolescents and the prognosis is extremely poor. Multimodal treatment with surgery, chemotherapy and radiotherapy is very important for these rare cases, and this treatment can improve patient survival. In this report, we describe the case of an 8-year-old boy diagnosed with DSRCT located in the retroperitoneal space. The patient has undergone surgical resection and adjuvant chemoradiation therapy, and is currently alive without disease recurrence.

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Key words: Retroperitoneum; Desmoplastic small round cell tumor; Multimodal therapy

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Kim YS, Cha SJ, Choi YS, Kim BG, Park SJ, Chang IT. Retroperitoneal desmoplastic small round cell tumor: Pediatric patient treated with multimodal therapy. *World J Gastroenterol* 2009; 15(33): 4212-4214 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4212.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4212>

INTRODUCTION

A desmoplastic small round cell tumor (DSRCT) is a rare, aggressive mesenchymal neoplasm with an extremely poor prognosis. It was described initially as a distinct clinicopathological entity by Geral and Rossi in 1989^[1]. This rare, highly malignant tumor belongs to the primitive tumor family of small round cell tumors (lymphoma, neuroblastoma, alveolar rhabdomyosarcoma, Ewing's sarcoma, neuroectodermal tumor, and DSRCT)^[2,3]. Young adolescent males are most commonly affected^[4]. Its primary location is in intra-abdominal sites, but it can also be found at other sites such as the kidney and ovary, as well as in the retroperitoneal space^[5-7]. In addition, these tumors are associated with a specific reciprocal translocation t (11;22) (p13;q12) that leads to the fusion of the WT1 (Wilms' tumor gene) and EWS (Ewing's sarcoma gene). Patients with this tumor have a very poor prognosis, but multimodal treatment with surgery, chemotherapy and radiotherapy can improve survival^[8,9].

We describe here a case of retroperitoneal DSRCT in a boy, who was treated with surgery and adjuvant chemoradiotherapy.

CASE REPORT

An 8-year-old boy with no previous health problems was referred to our hospital with a 2-wk history of a palpable abdominal mass. The family history was non-contributory and there were no complaints except for abdominal pain and indigestion. The physical examination showed an approximately 20-cm palpable abdominal mass predominantly on the right side of the abdomen, with mild tenderness. The laboratory tests showed no significant abnormalities. Computed tomography (CT) showed a septated, very large (21 cm × 17 cm), predominantly cystic, lobulated mass in the right upper quadrant of the abdomen between the liver and the right kidney (Figure 1). The vena cava and the duodenum were deviated to the left side of the abdomen because of the mass effect of the lesion. Upon magnetic resonance imaging (MRI), the lesion had a T1-attenuated high signal suspicious for a high protein content, a predominantly cystic portion with a large solid portion on the medial aspect, and no definite direct invasion into the major vessels. The radiologist suspected an undifferentiated embryonal sarcoma that originated from the liver, or a

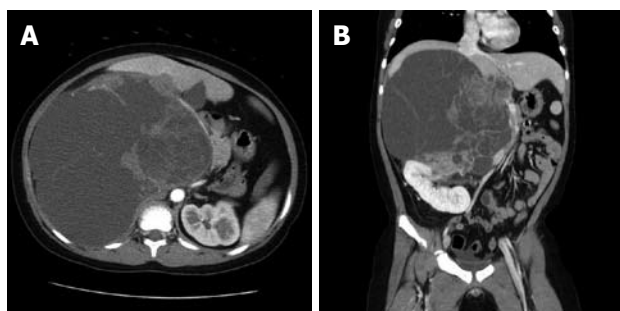


Figure 1 CT (A and B) shows a septated, very large (about 21.8 cm × 16.7 cm), predominantly cystic, lobulated mass in the right upper quadrant between the liver and the right kidney.

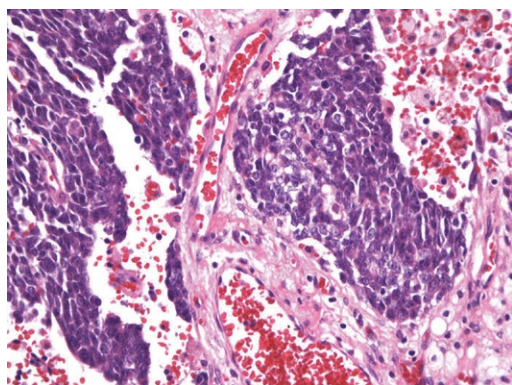


Figure 3 Histopathological findings. Tumor cells consist of small round, hyperchromatic nucleus and scanty cytoplasm (Hematoxylin and eosin stain, × 400).

mesenchymal hamartoma. Surgery was planned for a definitive diagnosis and a proper management.

The operative findings revealed a mass of about 20 cm, irregular in shape, with cystic and solid components. As it was located in the retroperitoneal space, the liver, inferior vena cava, duodenum, right kidney, and intestine were deviated to the left and downward. To obtain a good operative field, we aspirated about 500 mL of brown-colored cystic fluid from the cystic portion of the tumor, and a small portion was excised for frozen sections. Examination of frozen sections revealed that the tumor was highly suggestive of malignancy, and we excised the entire lesion. Since there were severe adhesions to the surrounding tissue and important abdominal vessels such as the inferior vena cava, right renal vessels and celiac trunk, the dissection required a long time and there was a large amount of bleeding. Grossly, there was no evidence of peritoneal seeding or hepatic metastasis. The mass was totally removed without significant problems (Figure 2). The operative time was 500 min, blood loss was about 2 L and eight units of packed red blood cells were transfused. The microscopic examination confirmed a retroperitoneal DSRCT, with the typical appearance of well-defined nests or clusters of small undifferentiated round cells, surrounded by a prominent desmoplastic stroma (Figure 3). Upon immunohistochemical staining, there were positive responses to cytokeratin, vimentin, CD99, and desmin (Figure 4). In addition, there were focal weak positive responses to S-100 and neuron-specific enolase (NSE).

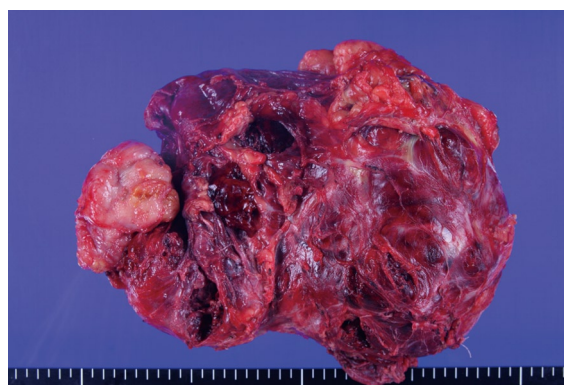


Figure 2 Gross findings after resection show a hemorrhagic and necrotic solid mass with a focal fibrotic, granular cut surface.

After confirmation of the diagnosis, postoperatively, the patient received adjuvant chemotherapy (vincristine, ifosfamide, doxorubicin, etoposide and cyclophosphamide) in combination with consolidating radiotherapy (25 times 180 cGy/d, a total dose of 45 Gy). The patient is free of disease recurrence 24 mo after resection and adjuvant chemoradiotherapy.

DISCUSSION

DSRCT is an aggressive malignant tumor with a poor prognosis. Abdominal pain or discomfort is the most common presentation for this rare neoplasm. Its primary location is the intra-abdominal peritoneal cavity, but many other sites have been described^[10]. The most common sites of peritoneal involvement have been reported to be the pelvic space, omentum, small bowel mesentery, and the retroperitoneal space. This tumor is typically found in young adolescent men (male:female ratio = 5:1). Usually, it is accompanied by extensive peritoneal seeding at the time of diagnosis^[9,10].

Although the findings are nonspecific, CT is the most widely used diagnostic modality for identifying this tumor. It can detect a bulky, lobulated and heterogeneous mass with or without calcification. Sometimes, ascites, adenopathy or liver metastases are found. Upon MRI, T2-weighted imaging shows a heterogeneous hyperintense signal with low or isodense T1-weighted signals. No specific tumor markers have been identified^[11].

Histopathologically, the characteristic findings are well-defined nests or clusters of undifferentiated small round cells, surrounded by a prominent desmoplastic stroma. The tumor cells are characterized by small hyperchromic nuclei with scanty cytoplasm. Immunohistochemistry can be positive for AE1/AE3 (88%), desmin (dot pattern, 81%), CD99 (23%), or NSE (84%)^[11]. This tumor is associated with a specific reciprocal translocation t (11:22) (p13;q12), which leads to fusion of the WT1 and EWS genes; this rearrangement is detected in almost all cases. The resulting chimeric protein is thought to be a transcriptional activator that fails to suppress tumor cell growth^[3].

Although the prognosis is very poor, aggressive surgical tumor resection is the major determinant of patient survival. Lal *et al*^[9] recommended surgical resection

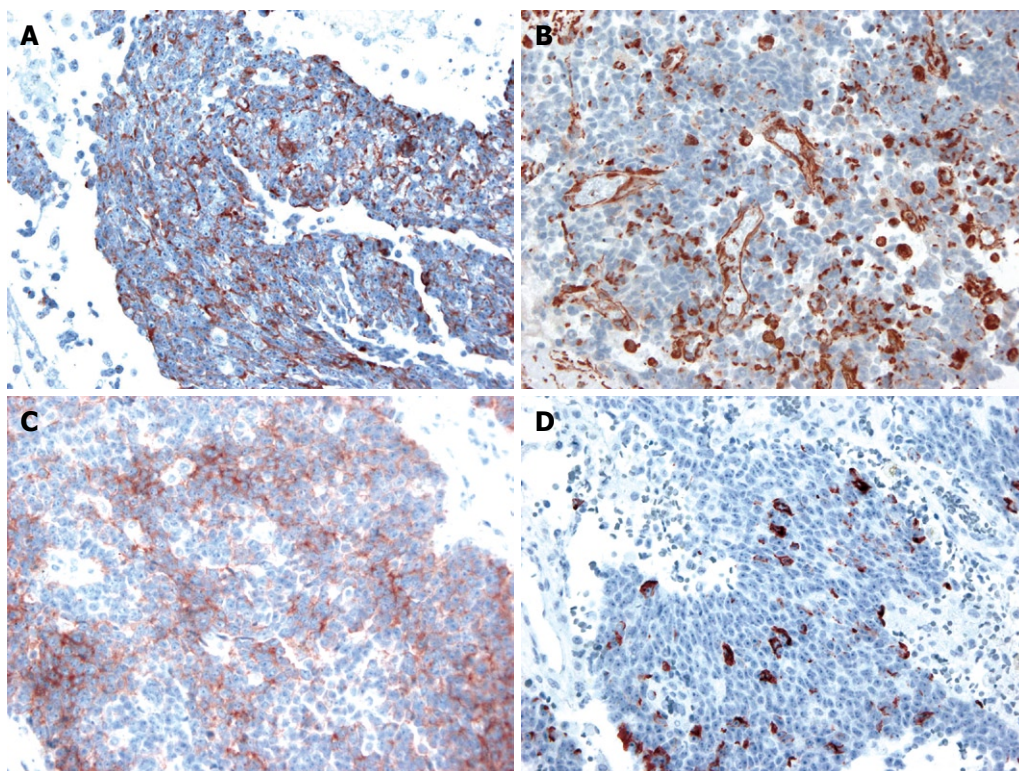


Figure 4 Immunohistochemical staining was positive for cytokeratin (A), vimentin (B), CD99 (C) and desmin (D) ($\times 400$).

of $> 90\%$ of the tumor burden. Attempts were not made to achieve microscopically negative resection margins. They used systemic chemotherapy, with the P6 protocol including cyclophosphamide, doxorubicin, vincristine, ifosfamide and etoposide. They reported a good response with this regimen. Adjuvant radiotherapy to the whole abdominopelvic area was also delivered. With this multimodal therapy, including the P6 protocol, $> 90\%$ resection, and adjuvant radiotherapy, there was a statistically significant prolongation of patient survival^[9]. The presence of nodal invasion and metastasis does not appear to correlate with overall patient survival. The expected 3-year survival was only 27% when multimodal treatment was not used^[9]. We used a similar strategy for the treatment of our patient; the patient is free of disease recurrence 2 years after surgery and adjuvant chemoradiotherapy. The platelet-derived growth factor receptor pathway inhibitor SU101 (leflunomide) is being studied in a pediatric phase I trial. The possibility of improved future treatments might help to prolong the survival of patients with DSRCT^[12].

In summary, we present a patient with retroperitoneal DSRCT, and expect a longer survival as a result of our aggressive surgical resection and adjuvant chemoradiotherapy.

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Enterovesical fistula caused by a bladder squamous cell carcinoma

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Received: June 1, 2009

Revised: July 15, 2009

Accepted: July 22, 2009

Published online: September 7, 2009

Abstract

Enterovesical fistulas are not uncommon in patients with inflammatory or malignant colonic disease, however, fistulas secondary to primary bladder carcinomas are extremely rare. We herein reported a patient presenting with intractable urinary tract infection due to enterovesical fistula formation caused by a squamous cell carcinoma of the urinary bladder. This patient underwent *en bloc* resection of the bladder dome and involved ileum, and recovered uneventfully without urinary complaint. To the best of our knowledge, this is the first case reported in the literature.

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Key words: Enterovesical fistula; Squamous cell carcinoma; Urinary bladder; Malignant fistula

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Ou Yang CH, Liu KH, Chen TC, Chang PL, Yeh TS. Enterovesical fistula caused by a bladder squamous cell carcinoma. *World J Gastroenterol* 2009; 15(33): 4215-4217 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4215.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4215>

INTRODUCTION

Enterovesical fistulas are not uncommon in patients with inflammatory or malignant colonic disease, however, fistulas secondary to primary bladder carcinomas are extremely rare^[1-3]. We herein report a patient presenting with intractable urinary tract infection due to enterovesical fistula formation caused by a squamous cell carcinoma of the urinary bladder. To the best of our knowledge, this is the first case reported in the literature.

CASE REPORT

An 83 year-old male presented with a 4-mo history of recurrent urinary tract infection. He had a history of benign prostate hyperplasia (American Urology Association score 24) that was under medical treatment. In order to correct urine retention and prevent urinary tract infection, photosensitive vaporization of the prostate had been performed. One month after the operation, the patient suffered from urinary tract infection again. The urine culture contained multiple flora of the gastro-intestinal tract including: *Escherichia coli*, *Viridans streptococcus*, *Klebsiella*, *Pneumoniae* and *Enterococcus faecium*. Retrograde urethrography showed leakage of the contrast and cystoscopy disclosed a fistula in the right posterior wall of the bladder with edematous tissue surrounding the fistula (Figure 1). Abdominal and pelvic computed tomography showed a very large neoplasm in the pelvic cavity with suspicion of vesicoileal fistula formation. Under the impression of malignant enterovesical fistula, the patient underwent surgical intervention. At laparotomy, a 10 cm-diameter whitish stony hard tumor was located in the pelvis, involving the distal ileum and urinary bladder, with a frank fistula formation. Resection of the urinary bladder dome and the involved ileum was performed in *en bloc* fashion (Figure 2). The pathology report yielded a moderately differentiated squamous cell carcinoma of the urinary bladder with direct invasion of the terminal ileum (Figure 3). This patient recovered uneventfully and was discharged without urinary tract infection on post-operative day 7.

DISCUSSION

Enterovesical fistula is a rare disease, with an estimated two to three patients per 10 000 hospital admissions, with annular incidence of 0.5 per 100 000^[1]. In a literature

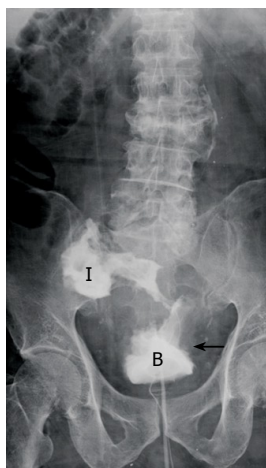


Figure 1 Cystography showed an enterovesical fistula (arrow). B: Urinary bladder; I: Ileum.

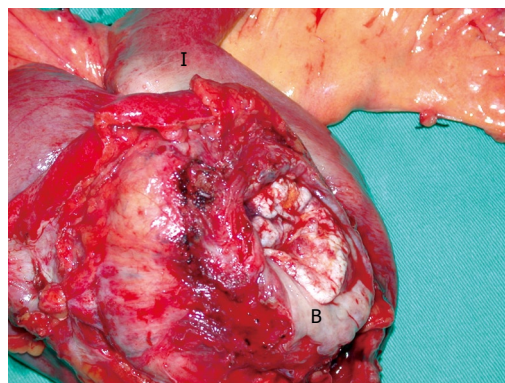


Figure 2 Surgical specimen of urinary bladder dome and ileum resected *en bloc*. B: Urinary bladder; I: Ileum.

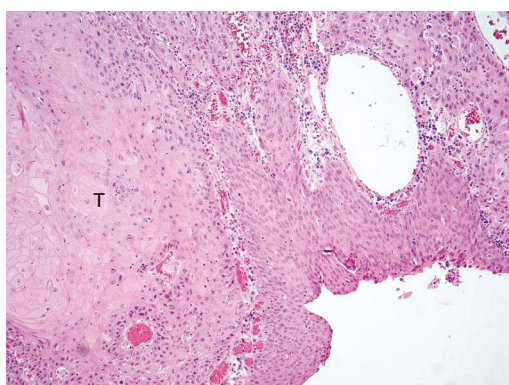


Figure 3 Microscopic examination yielded the diagnosis of squamous cell carcinoma of the urinary bladder. HE staining, original magnification $\times 40$. T: Tumor burden.

review, 20% of vesicoenteral fistulas were rectovesical fistulas and 4%-5% were appendicovesical fistulas. Carson *et al*^[2] reported 100 cases of enterovesical fistulas with 51% colonic diverticulitis, 16% colorectal cancer, 5% urinary bladder carcinoma and 16% due to other causes, including secondary to radiation necrosis, cervical cancer, tuberculoma and iatrogenic perforation.

The most common histologic type of bladder cancer leading to enterovesical fistula is transitional cell carcinoma. Primary squamous cell carcinoma of the bladder is a relatively rare tumor. The prevalence of squamous cell carcinoma varies depending on geographic location. It accounts for only 3%-7% of bladder cancers in the United States and 1% in England, but up to 75% in Egypt, where schistosomiasis is endemic^[4]. Squamous cell carcinoma are usually related to chronic infection, bladder stones, chronic indwelling catheters or bladder diverticula. To our knowledge, this is the first report of vesicoenteral fistula as a result of bladder squamous cell carcinoma. Only one case of vesicorectal fistula from invasive rectal squamous cell carcinoma cancer had been reported^[5]. Almost all squamous cell carcinomas are already advanced and muscle-infiltrative at the time of diagnosis^[6]. Squamous cell carcinomas of the bladder have an unfavorable prognosis due to a local advanced stage at the time of presentation.

The most common clinical presentations of entero-

vesical fistulas are urinary tract infection (100%), pneumaturia (66%), fecaluria (50%), and hematuria (22.6%)^[3].

Diagnostic tests include cystoscopy examination, retrograde cystography, and computed tomography. The diagnostic rate using cystoscopy examination is 77%-79%^[2,7], while retrograde cystogram and computed tomography showed the fistulous tract in 66.6% and 83.3%, respectively^[8,9]. Barium enema or small bowel series have a 20%-35% diagnostic rate to indicate the fistula^[4]. Under cystoscopy examination, the fistula may be seen within a hyperemic area at an early stage of the disease, and with cystic mucosal hyperplasia or localized granulation tissue at the late stage^[10,11]. Computed tomography has the advantage of showing the fistula tract or gas distinctly in the bladder. In addition, it is useful in delineating the extent of disease involvement, visualizing the anatomic relationship of the adjacent organs and detecting distant metastasis for malignant diseases, which accordingly allows tailoring of the management strategy^[7,12,13].

Optimal work-up of a malignant of enterovesical fistula includes detection of the fistula and anatomic extension using the aforementioned diagnostic tests and definitive pathological confirmation, if possible. Except for elderly people in generally poor condition, diverting enterostomy and indwelling urethral catheters are not recommended as permanent treatments for enterovesical fistulae^[3].

Whitely and Grabtald proposed that a one-stage *en bloc* resection of the colonic malignancy and involved bladder portion is a reasonable and safe procedure^[7,11], avoiding a total cystectomy. Patients must be well-prepared, non-obstructive and without systemic infection before the surgery^[1,11,12,14,15]. However, if resection of the bladder is too wide, resulting in a total cystectomy, reconstruction to restore intestinal continuity and provide adequate bladder capacity and continence is pertinent. On the other hand, if the tumor is deemed unresectable and there is a short life expectancy, a palliative surgical procedure with enterostomy, with or without urinary diversion, is suggested^[12,16].

The 5-year survival rate for colon cancer with enterovesical fistula is 81% in nodal negative patients (T4N0), compared with 27% in nodal positive patients (T4N1)^[17].

Looser *et al*^[16] also suggested that colonic cancer with a perforation into the bladder could be treated by resection for a curative purpose, obtaining long-term survival in 50% of the patients^[7,16]. Nevertheless, Vidal Sans *et al*^[3] reported that surgical morbidity and mortality is relatively high, especially in fistula resulting from malignancy. Despite various methods of treatment for enterovesical fistula, the prognosis for patients with an advanced stage of malignant fistulae is dismal.

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LETTERS TO THE EDITOR

Early aggressive therapy for severe extensive ulcerative colitis

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Received: June 28, 2009 Revised: July 23, 2009

Accepted: July 30, 2009

Published online: September 7, 2009

Abstract

The current ulcerative colitis (UC) treatment algorithm involves a step-up therapeutic strategy, mainly aiming at inducing and maintaining its clinical remission. Although this therapeutic strategy may seem to be cost-efficient and reduce the risk of side effects, recent trials and case reports have shown that top-down therapy using infliximab induces a rapid clinical response, enhances patient quality of life, promotes mucosal healing, reduces surgeries and indirect cost of treatment for patients with severe UC. Moreover, since long-term treatment with infliximab is safe and well tolerated, early aggressive top-down therapeutic strategy may be a more effective approach, at least in a subgroup of severe extensive UC patients.

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Key words: Infliximab; Ulcerative colitis; Top-down therapy

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Cui DJ. Early aggressive therapy for severe extensive ulcerative colitis. *World J Gastroenterol* 2009; 15(33): 4218-4219 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4218.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4218>

INTRODUCTION

Since ulcerative colitis (UC) cannot be cured by

medication, therapeutic strategy is very important for patients with UC. The UC treatment algorithm includes a early aggressive or top-down therapeutic strategy and a sequential or step-up regimen. Recently, the emergency of effective biological therapy in the treatment of UC has led to a clinical debate about “top-down *vs* step-up strategy” which describes two opposite approaches. Since one approach is not suitable for all patients with UC, therapeutic strategy ought to be tailored to the individual patient so as to maximize efficacy while balancing risk and cost. In this letter the benefits, safety and costs of alternative strategies will be critically appraised.

TO THE EDITOR

I read with great interest the case report recently published in the *World Journal of Gastroenterology* by Cury *et al*^[1]. The authors reported a case in which infliximab was safely and effectively administered to a patient with severe and extensive UC. Upon reading this interesting case report, two questions arose in my mind.

First, the white blood cell (WBC) count of 161 000/mm³ or $161 \times 10^9/L$ reported in the case, which is markedly elevated because the normal WBC concentration is 4000-10 000/mm³, remains to be elucidated. Although a high WBC count can occur in infections, toxins, acute hemolysis, trauma and malignancies, the leukocytosis described in the case report might be due to leukemoid reaction, leukemia and other myeloproliferative disorders since WBC concentration is over 30 000/mm³. However, the leukocytosis reported in the case report was not permanent and progressive, and infectious precipitants were ruled out^[1]. Moreover, since no endoscopic evidence is available to support toxic megacolon, there might be an error in the WBC count or in a leukemoid reaction in the case report, the cause for which is not clear.

Second, whether top-down therapeutic strategy should be implemented in patients with severe and extensive UC which extends beyond the splenic flexure but not to the cecum. In a recently published consensus, a sequential or step-up therapy, mainly aiming at inducing and maintaining its clinical remission, has been advocated for patients with severe extensive UC which is best defined by True-love and Witts criteria^[2]. The step-up therapeutic strategy may seem to be cost-efficient for the vast majority of UC patients and reduce the risk of side effects. However, this sequential strategy did not induce mucosal healing

and could not achieve the best attainable quality of life until infliximab was administered to the reported patient. In addition, early aggressive therapy with infliximab and azathioprine may reduce the indirect cost of treatment for patients. More recent studies have shown that top-down therapy using infliximab induces a rapid clinical response, has a steroid-sparing effect, enhances patient quality of life, promotes mucosal healing, and reduces hospital stay time and surgeries^[3-6]. The reasons why the step-up strategy is advantageous over the top-down are concerned with its side effects and costs of biological agents. However, it was reported that long-term treatment with infliximab is safe and well tolerated and not associated with excess mortality or malignancies^[5,7]. Moreover, an 8-wk maintenance treatment schedule with infliximab appears to be a cost-effective treatment option for adult patients suffering from moderate to severe UC^[8]. Therefore, the top-down approach is appealing and can result in a modification in the natural course of UC, at least in a subgroup of patients with severe and extensive UC.

Since the top down approach is not suitable for all patients with UC, the future challenge is to identify a subgroup of patients who will develop complicated diseases or are therapy refractory at a later time point and for whom infliximab treatment in the early phase may change the natural history of UC.

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S- Editor Tian L L- Editor Wang XL E- Editor Zheng XM

ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Meetings

Events Calendar 2009

January 12-15, 2009
 Hyatt Regency San Francisco, San Francisco, CA
 Mouse Models of Cancer

January 21-24, 2009
 Westin San Diego Hotel, San Diego, CA
 Advances in Prostate Cancer Research

February 3-6, 2009
 Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
 Second AACR Conference
 The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
 Hyatt Regency Boston, Boston, MA
 Translation of the Cancer Genome

February 8-11, 2009
 Westin New Orleans Canal Place, New Orleans, LA
 Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
 Hong Kong Convention and Exhibition Centre, Hong Kong, China
 19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
 Orlando, Florida
 AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
 Vienna, Austria
 EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
 Phoenix, Arizona
 AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
 Marriott Wardman Park Hotel
 Washington, DC
 13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
 Glasgow, Scotland
 British Society of Gastroenterology (BSG) Annual Meeting
 Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
 Silver Spring, Maryland
 2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
 Colorado Convention Center, Denver, CO
 AACR 100th Annual Meeting 2009

April 22-26, 2009
 Copenhagen, Denmark
 the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
<http://www.easl.ch/>

May 17-20, 2009
 Denver, Colorado, USA
 Digestive Disease Week 2009

May 29-June 2, 2009
 Orange County Convention Center
 Orlando, Florida
 45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
 Chicago, Illinois
 Endpoints Workshop: NASH

May 30-June 4, 2009
 McCormick Place, Chicago, IL
 DDW 2009
<http://www.ddw.org>

June 17-19, 2009
 North Bethesda, MD
 Accelerating Anticancer Agent Development

June 20-26, 2009
 Flims, Switzerland
 Methods in Clinical Cancer Research (Europe)

June 24-27 2009
 Barcelona, Spain
 ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
 Beijing International Convention Center (BICC), Beijing, China
 World Conference on Interventional Oncology
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009
 Snowmass, CO, United States
 Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
 Aspen, CO, United States
 Molecular Biology in Clinical Oncology

August 1-7, 2009
 Vail Marriott Mountain Resort, Vail, CO, United States
 Methods in Clinical Cancer Research

August 14-16, 2009
 Bell Harbor Conference Center, Seattle, Washington, United States
 Practical Solutions for Successful Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
 Beijing International Convention Center (BICC), Beijing, China
 19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
 Taipei, China
 Asian Pacific Digestive Week
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009
 Boston Park Plaza Hotel and Towers, Boston, MA, United States
 Frontiers in Basic Cancer Research

October 13-16, 2009
 Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
 Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
 Versailles, France
 Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
 Boston, MA, United States
 The Liver Meeting

November 15-19, 2009
 John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
 AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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Supportive foundations: The complete name and number of supportive foundations should be provided, e.g., Supported by National Natural Science Foundation of China, No. 30224801

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S,** Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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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/eid.htm>

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- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Volume 15 Number 34
September 14, 2009

World J Gastroenterol

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World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 34
September 14, 2009



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The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No.62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
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Beijing Kexin Printing House

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Beijing Bureau for Distribution of Newspapers and Journals (Code No. 82-261)
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PUBLICATION DATE

September 14, 2009

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CSSN

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Overview of immunosuppression in liver transplantation

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Received: July 1, 2009 Revised: July 30, 2009

Accepted: August 6, 2009

Published online: September 14, 2009

com/1007-9327/15/4225.asp DOI: <http://dx.doi.org/10.3748/wjg.15.4225>

Abstract

Continued advances in surgical techniques and immunosuppressive therapy have allowed liver transplantation to become an extremely successful treatment option for patients with end-stage liver disease. Beginning with the revolutionary discovery of cyclosporine in the 1970s, immunosuppressive regimens have evolved greatly and current statistics confirm one-year graft survival rates in excess of 80%. Immunosuppressive regimens include calcineurin inhibitors, anti-metabolites, mTOR inhibitors, steroids and antibody-based therapies. These agents target different sites in the T cell activation cascade, usually by inhibiting T cell activation or *via* T cell depletion. They are used as induction therapy in the immediate peri- and post-operative period, as long-term maintenance medications to preserve graft function and as salvage therapy for acute rejection in liver transplant recipients. This review will focus on existing immunosuppressive agents for liver transplantation and consider newer medications on the horizon.

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Key words: Immunosuppression; Liver transplantation; Induction therapy; Rejection

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Pillai AA, Levitsky J. Overview of immunosuppression in liver transplantation. *World J Gastroenterol* 2009; 15(34): 4225-4233 Available from: URL: <http://www.wjgnet.com>

INTRODUCTION

Due to advances in immunosuppression and improvements in surgical techniques, liver transplantation has become an extremely successful treatment option for patients with end-stage liver disease, with one-year graft survival rates exceeding 80%^[1]. Currently, there are eight patients worldwide who have survived more than three decades after liver transplantation^[2].

Organ transplantation initially came to light with the first successful kidney transplantation in 1954 on monozygotic twins; however, immunosuppression was limited to total body irradiation which was largely fatal^[3,4]. With the invention of 6-mercaptopurine (6-MP) and azathioprine (AZA) in the 1950s along with the introduction of corticosteroids as combination therapy by Starzl in the 1960s, there was noticeable improvement in kidney allograft survival, although one-year survival still did not exceed 50%^[4]. Multiple interventions including splenectomy, thymectomy and thoracic duct drainage were employed with minimal success.

The first successful human liver transplant was performed by Thomas Starzl in Denver in 1967 on an 18-month-old child with unresectable hepatoblastoma^[2]. The immunosuppressive regimen included anti-lymphocyte globulin (ALG), AZA and prednisolone and the child survived for more than a year.

However, the next significant breakthrough in immunosuppression did not occur until the discovery of cyclosporine (CYA) in 1972 from the soil fungus *Tolypocladium inflatum*. Borel *et al*^[5] first described its remarkable immunosuppressive properties in 1976 and by the 1980s there was international affirmation of its effectiveness. CYA quickly became the standard of care for maintenance immunosuppression in solid organ transplant recipients. This paved the way for the current era of liver transplantation, which has since continued to evolve with the discovery of multiple novel immunosuppressive agents.

IMMUNOSUPPRESSION

Effective immunosuppression in transplantation relies on preventing the immune system from rejecting the allograft while preserving immunologic control of infection and

Table 1 Drugs that increase CNI and sirolimus levels

Drugs that increase CNI levels
Macrolides: clarithromycin, erythromycin, azithromycin
Antifungals: fluconazole, itraconazole, ketoconazole, voriconazole, clotrimazole
Calcium channel blockers: verapamil, diltiazem, nifedipine
Others: cisapride, metaclopramide, amiodarone, cimetidine, protease inhibitors
Drugs that decrease CNI and sirolimus levels
Antibiotics: rifabutin, rifampin
Anticonvulsants: carbamazepine, phenobarbital, phenytoin, fosphenytoin
Others: St. John's Wort

CNI: Calcineurin inhibitor.

neoplasia. Although the mechanism is not completely understood, transplanted livers rarely reject compared to other organs, do not require an HLA-matched donor, and may offer a protective effect for other simultaneously transplanted organs^[6,7]. Both micro- and macrochimerism models have been used to explain this phenomenon, as well as that of hepatic dissolution of donor specific antibodies. Ideally, the long-term objective is to achieve immune tolerance or the ability to alter the recipient's immune system in order to promote long-term graft function without immunosuppressive therapy, while maintaining immunity to infectious agents^[8]. Unfortunately, except for a small minority of patients (approximately 20%) who have been successfully weaned off immunosuppressive medications, most experience immunologic rejection with the discontinuation of these drugs and have to be maintained on at least low doses of these medications^[9-13].

Immunosuppressive regimens include calcineurin inhibitors, anti-metabolites, mTOR inhibitors, steroids and antibody-based therapies. These agents target different sites in the T cell activation cascade, usually by inhibiting T cell activation or proliferation or *via* T cell depletion. The selection of agents is based on an individual's medical history as well as on institution experience and preference. Most immunosuppressive regimens combine drugs with different sites of action of T cell response, allowing for dosage adjustments to minimize side effects and toxicities. Currently, the mainstay of maintenance immunosuppressive regimens are calcineurin inhibitors (CNIs), used in greater than 95% of transplant centers upon discharge, although there is a known increased risk of renal impairment^[14,15], metabolic derangements, neurotoxicity and *de novo* malignancies^[16] with the long-term use of these medications.

CALCINEURIN INHIBITORS

CYA and tacrolimus are the two CNIs approved for use in organ transplantation and are the principal immunosuppressives used for maintenance therapy. The routine use of these medications in liver transplant recipients has dramatically decreased the incidence of rejection and graft loss. The primary mode of action is inhibition of T cell activation. CYA binds to

Table 2 Common side effects of immunosuppressive agents

Drug	Adverse effects
Tacrolimus	Nephrotoxicity, neurotoxicity ¹ , diabetes ¹ , hyperkalemia, metabolic acidosis, hypertension, hyperlipidemia
Cyclosporine	Nephrotoxicity, neurotoxicity, diabetes, hyperlipidemia ¹ , hypertension ¹ , hyperkalemia, metabolic acidosis, gingival hyperplasia, hypertrichosis
MMF	Myelosuppression, gastrointestinal side effects, viral infections (CMV, HSV), spontaneous abortions in pregnant women
Sirolimus	Hyperlipidemia, myelosuppression, proteinuria, poor wound healing, pneumonitis, skin rash
Corticosteroids	Diabetes, hypertension, obesity, osteoporosis, avascular necrosis, growth retardation, Cushingoid features, psychosis, poor wound healing, adrenal suppression, cataracts

¹More common in respective agent. MMF: Mycophenolate mofetil; CMV: Cytomegalovirus; HSV: Herpes simplex virus.

cyclophilin which results in inhibition of the calcium/calmodulin-dependent phosphatase, calcineurin. The binding to cyclophilin interferes with calcineurin's de-phosphorylation of nuclear factor of activated T cells (NFAT), preventing translocation of NFAT into the nucleus and up-regulation of pro-inflammatory cytokines. The end result is the inhibition of IL-2 gene transcription and T cell activation and proliferation^[4,8]. Tacrolimus also inhibits calcineurin but binds specifically to FK506-binding protein (FKBP-12).

The immunosuppressive effects of the CNIs are related to total drug exposure which can be estimated by measuring blood 12-h troughs. The potency of tacrolimus is estimated to be 100 times greater on a molar level^[8] when compared to CYA. Although several earlier studies showed tacrolimus to be superior to CYA in the prevention of cellular rejection^[17-19], another more recent multi-center trial showed no significant differences between the two medications with regard to acute rejection episodes, death or graft loss^[20]. Both CNIs are metabolized principally by the cytochrome P450 system and therefore have significant interactions with multiple medications requiring careful monitoring of drug levels (Table 1).

CNIs have a wide range of toxicities, many of which are dose-dependent (Table 2). Nephrotoxicity is a well-recognized side effect and it has been documented that nearly 20% of liver transplant recipients experience chronic renal failure within 5 years^[15]. This can be best managed by either discontinuation or reduction of the medication. Neurotoxicity is another common problem; one which is more predominant with tacrolimus. The clinical presentation varies from headaches and tremors to agitation, confusion, hallucinations or overt psychosis. Hypertension, hyperlipidemia, hyperkalemia, metabolic acidosis and diabetes are also frequent side effects. Diabetes is more common with tacrolimus use, whereas hypertension and hyperlipidemia tend to be more prominent with CYA use. Gingival hyperplasia and hypertrichosis are specific side effects of CYA only.

Another important feature of CNIs is their interaction with transforming growth factor- β (TGF- β), a cytokine that augments fibrosis development and promotes tumor cell invasiveness^[21]. TGF- β transcription is increased with CNI use, which is of concern given the possibility of hepatocellular carcinoma (HCC) recurrence or the emergence of post-transplant lymphoproliferative disorder (PTLD).

ANTIMETABOLITES

Both mycophenolate mofetil (MMF) and mycophenolate sodium (MPS) undergo immediate first-pass metabolism in the liver into the active compound mycophenolic acid (MPA), which was first discovered in 1893^[22]. However, the immunosuppressive properties of MPA were not recognized until the 1990s. MPA inhibits inosine-5'-monophosphate dehydrogenase (IMPDH)^[23], the rate-limiting enzyme in the *de novo* synthesis of guanosine nucleotides. Inhibition of the IMPDH pathway results in selective blockade of lymphocyte proliferation^[24].

The major advantage in using the MPAs is their lack of renal toxicity. In patients with pre-existing renal disease, they have been used in conjunction with low-dose CNIs as part of a renal-sparing protocol with promising results^[25,26]. Ideally, these medications should be initiated when renal dysfunction is first noted, although emerging data suggests the benefits of MPAs in reversing long-standing renal disease due to its association with decreased TGF- β levels^[27-29]. MPAs are rarely used as monotherapy in transplant recipients given their higher rates of rejection compared to the CNIs^[30,31], although more recent data demonstrate the safety of this approach when carried out carefully^[32,33]. However, in patients previously on CNIs or mTOR inhibitors with evidence of acute rejection, MPAs are often added as supplemental immunosuppressive therapy.

The predominant side effects of MPAs are related to gastrointestinal disorders and bone marrow suppression (Table 2). Diarrhea is the most common dose-limiting adverse effect, although abdominal pain, nausea and vomiting can frequently occur^[34]. Studies have also shown increased incidences of cytomegalovirus (CMV)^[35-37], herpes simplex virus (HSV)^[38,39], *Candida* infections, and, rarely, progressive multifocal leukoencephalopathy (PML)^[40] with the use of MPAs. In pregnant patients, increased risks of spontaneous abortions during the first trimester and serious congenital malformations have also been reported (www.fda.gov). Routine monitoring of MPA levels is not generally employed in clinical practice.

Azathioprine is another antimetabolite which was predominantly used for the prevention of rejection in the 1960s but has since been largely replaced by the MPAs. It is selectively used in a few centers in combination with other immunosuppressive medications, primarily CNIs and steroids.

mTOR INHIBITORS

The two mTOR inhibitors approved for organ trans-

plantation are sirolimus (SRL) and everolimus (EVL), although neither has been approved for use in liver transplantation to date. They bind intracellularly to FK506 binding protein (FKBP12) but unlike tacrolimus, they do not inhibit calcineurin activity. Rather, the complex is a highly specific inhibitor of mammalian target of rapamycin complex 1 (mTORC1)^[41] which has a direct effect on the cell signaling pathway required for cell cycle progression. This subsequently inhibits IL-2 signaling to T cells, thus preventing T cell proliferation. Similar to the CNIs, sirolimus is metabolized by the cytochrome P450 system and requires therapeutic drug monitoring (Table 1).

The first reported study illustrating the effectiveness of sirolimus monotherapy for maintenance of immunosuppression in liver transplantation was in 1999 by Watson *et al*^[42]. However, two subsequent large studies examining sirolimus *de novo* therapy with tacrolimus and corticosteroids were terminated early due to excess hepatic artery thrombosis (HAT). As a result, sirolimus carries a black box label warning which cautions against the possible development of early post-transplant HAT. Subsequent studies have since disputed this finding^[43-45], however, mTOR inhibitors are rarely used as *de novo* therapy.

Importantly, in patients with CNI-induced nephrotoxicity, conversion to sirolimus therapy has proved to be effective with ensuing improvements in renal function^[46-48]. Again, sirolimus conversion should be initiated early since late conversion rarely improves chronic renal dysfunction^[49]. In fact, several studies have shown that in patients with pre-existing renal disease, sirolimus can worsen nephrotoxicity and promote proteinuria^[50-52].

Recent studies have also shown potential anti-tumor properties of sirolimus^[53-56] which might be of importance in patients undergoing liver transplantation for HCC. Zimmerman *et al*^[57] examined the role of sirolimus-based maintenance therapy in post-transplant recipients with a history of HCC and found that overall survival was increased in the sirolimus arm compared to the CNI arm. Clinical trials examining the anti-cancer effects of mTOR inhibitors in liver transplant recipients with HCC have been encouraging^[44,58] and new trials are ongoing.

Metabolic side effects of mTOR inhibitors include proteinuria and increases in serum cholesterol and triglycerides (Table 2). Bone marrow suppression, interstitial pneumonitis, peripheral edema, dermatological effects (acne, mouth ulcers) and delayed wound healing are all well-documented. Inhibition of fibroblast growth factor by sirolimus impairs tissue repair and plays a role in delayed wound healing^[59]. Interstitial pneumonitis is rarely life-threatening, is dose-dependent and resolves on withdrawal of the drug^[60].

CORTICOSTEROIDS

Corticosteroids are well-known for their anti-inflammatory properties such as suppression of prostaglandin synthesis,

stabilization of lysosomal membranes and reduction of histamine and bradykinin release^[30,31]. They also exhibit various immunomodulatory effects including effects on antigen presentation by dendritic cells and induction of a decrease in the number of circulating CD4+ T cells, IL-1 transcription and IL-1-dependent lymphocyte activation^[4,8].

High-dose corticosteroids were used judiciously in the 1960s in post-transplant recipients, with resulting increased morbidity due to their well-known deleterious side effects. This led to several studies in the 1980s on renal transplant recipients which confirmed that graft and patient survivals, as well as rejection episodes, were similar in the high- and low-dose steroid groups as long as AZA was also used^[61-63]. Currently, intravenous corticosteroids are predominantly used as first-line therapy for the treatment of acute cellular rejection. Regarding maintenance therapy, they are often successfully withdrawn within 3-6 mo post-transplantation in patients without evidence of rejection or liver disease attributed to autoimmune disorders^[64]. The primary concern with corticosteroid use is exacerbation of hepatitis C virus (HCV) recurrence and liver fibrosis with high-dose pulsed therapy^[65]; however, this has not been evident with low, gradually tapered doses^[66,67].

Well-documented side effects of corticosteroids include diabetes, hypertension, central obesity, Cushingoid features, osteoporosis, avascular necrosis, psychosis, poor wound healing, adrenal suppression and cataracts (Table 2).

ANTIBODY-BASED THERAPIES

Polyclonal antibodies

Polyclonal antibodies, including anti-thymocyte (ATG) and anti-lymphocyte globulins (ALG), have been used since the early days of liver transplantation and are prepared by inoculating rabbits or horses with human lymphocytes or thymocytes^[4]. Their mechanism of action is rapid lymphocyte depletion due to complement-mediated cell lysis and uptake by the reticulo-endothelial system (RES) of opsonized T cells^[68]. In addition, they may also cause partial T cell activation and blockade of T cell proliferation^[69]. Polyclonal antibodies were routinely used as induction therapy in liver transplantation along with corticosteroids and AZA before the discovery of CYA.

Lymphocyte depletion is believed to play a role in preparing the recipient's immune system to adapt and recognize the transplanted organ as self and prevent destruction of the allograft. Accordingly, studies have shown that ATG administration results in regulatory T cell (Treg) expansion *in vitro* and *in vivo*^[70-72]. Tregs or suppressor T cells are responsible for preventing activation of the immune system and maintaining tolerance to self-antigens.

Currently, approximately 20% of transplant centers use these agents for induction purposes^[73] and recent data support the administration of thymoglobulin induction to delay CNi use and avoid renal toxicity without increasing the risk of rejection or HCV recurrence^[74-76]. A few

studies have also successfully shown the benefit of using these medications as induction therapy to avoid post-transplant corticosteroid use^[77,78] without an increased incidence of acute rejection. This is especially important in HCV recipients where high-dose pulsed corticosteroid therapy can significantly accelerate liver fibrosis. At present, anti-lymphocyte antibodies are used extensively to treat steroid-resistant acute rejection and are successful in 70%-96% of patients^[79-81].

The main side effect of these medications, affecting 80% of patients, is a "first-dose reaction" and febrile episode which can often be ameliorated by pre-medication with antipyretics, antihistamines and intravenous steroids. This effect is likely due to pyrogen release from the massive destruction of lymphocytes^[69,82]. Other adverse effects include thrombocytopenia, anemia, CMV infection, PTLN, pruritic skin rashes, serum sickness and anaphylaxis^[83-85].

Monoclonal antibodies

Monoclonal antibodies include the anti-IL-2 receptor (CD25) antibodies, anti-CD52 antibody and muromonab-CD3 (OKT3). The two anti-IL-2 receptor antibodies approved for clinical use are basiliximab (Simulect), a chimeric protein, and daclizumab (Zenapax), a humanized protein. Both antibodies are specific for the α chain of the IL-2 receptor, CD25, which is only expressed on activated T cells^[8]. These antibodies remain in the circulatory system for weeks after initiation of therapy and have been used successfully with low-dose CNIs in preventing acute rejection in the early post-transplant period^[86-88]. They also have fewer side effects compared to the anti-lymphocyte globulins, rarely cause the typical first-dose infusion reactions and are associated with less risk of opportunistic infections and PTLN.

Muromonab-CD3 (OKT3) targets the CD3 molecule on T cells and causes depletion of lymphocytes by massive T cell lysis^[89] and cytokine release^[90]. This profound cytokine release can lead to pulmonary edema and acute respiratory distress and rarely, intra-graft thrombosis and aseptic meningitis^[91,92]. As a result, antihistamines and intravenous steroids are routinely used as pre-medication to reduce this "cytokine release syndrome". Several days after OKT3 administration, T lymphocytes no longer express CD3 and are considered to be immunologically incompetent^[93]. OKT3 is primarily used in liver transplantation for steroid-resistant acute rejection^[94,95] and has a success rate of complete recovery in 50% of patients. OKT3 use should be limited in the HCV population as several studies have confirmed exacerbation of disease recurrence with this agent^[96,97].

The humanized anti-CD52 antibody, alemtuzumab (Campath-1) targets lymphocytes, monocytes, macrophages, natural killer cells and thymocytes but spares plasma cells and memory lymphocytes^[8,98]. It is unique in that it depletes lymphocytes from the circulation as well as peripheral lymph nodes. Several studies in renal transplant patients have shown its efficacy in preventing rejection when used in

combination with low-dose CNIs or sirolimus^[99-101]. Tzakis *et al.*^[102] compared the use of alemtuzumab induction therapy combined with low-dose tacrolimus in liver transplant recipients receiving standard doses of tacrolimus and corticosteroids. Although patients who received alemtuzumab had less renal dysfunction and acute rejection in the first two months post-transplant, the overall incidence of rejection was not significantly different between the two groups. Similarly, Marcos *et al.*^[103] proposed that alemtuzumab, in conjunction with minimal CNI use, is a successful treatment strategy in liver transplant recipients, improving overall graft and patient survival, especially in HCV-infected subjects.

FUTURE DIRECTION OF IMMUNOSUPPRESSION

Costimulation blockade (Belatacept)

Belatacept is a soluble cytotoxic T-lymphocyte antigen-4 (CTLA-4) agent which binds CD80 and CD86 and inhibits T cell activation^[4,8]. Belatacept competes with the CD28 receptor on T cells which normally binds CD80 and CD86 on the antigen presenting cell (APC) as a co-stimulatory signal required for T cell activation. Belatacept is administered intravenously once a month and does not carry the renal toxicity of CNIs. Clinical trials in liver transplant patients are currently ongoing with this agent.

Efalizumab

Efalizumab is a humanized leukocyte function-associated antigen-1 (LFA-1; CD11a) specific monoclonal antibody that inhibits T cell-APC stabilization and blocks lymphocyte adhesion to endothelial cells^[104,105]. This agent was approved for the treatment of psoriasis in 2003 and has not yet been used in liver transplantation, although a few clinical trials have been carried out in renal transplant patients with mixed results^[106]. Although the results regarding immunosuppression were promising, an increased risk for PTLT was shown when efalizumab was used in combination with high-dose CYA.

Other newer agents on the horizon undergoing phase II/III trials include Janus Kinase (JAK) 3 inhibitors, AEB071 (a protein kinase C (PKC) isoforms inhibitor), and Alefacept (a LFA3-IgG1 fusion receptor protein). JAKs are intermediaries between cytokine receptors and signal transducers and activators of transcription (STATs) which lead to immune cell activation^[107,108]. JAK-3, a cytoplasmic tyrosine kinase, is primarily found on hematopoietic cells and its stimulation is specific for the IL-2 family of cytokines which makes it a very attractive target for immunosuppression. Clinical trials are underway in renal transplant patients using these agents. AEB071 (PKC inhibitor) is an oral agent that blocks early T cell activation and IL-2 production^[109]. Three phase II renal transplant trials using AEB were started, two of which had to be stopped due to increased episodes of acute rejection; the third trial is ongoing

in Europe^[110]. Alefacept, a LFA3-IgG1 fusion receptor protein initially approved for the treatment of psoriasis, interferes with T-cell activation and produces a dose-dependent reduction in T-effector memory cells^[111]. A multi-center clinical trial in renal transplant recipients is currently underway.

CONCLUSION

The current era of immunosuppressive therapy continues to evolve with the discovery of novel agents, targeting different sites of the immune cascade. Important objectives when using these medications include decreasing the incidence of renal toxicity from CNIs while preserving graft function as well as optimizing immunosuppression without creating an environment for increased infections, aggressive recurrence of hepatitis C or triggering PTLT and other malignancies.

At our institution, high-dose intravenous corticosteroids are used in the immediate peri- and post-operative period and then tapered accordingly. In patients without renal dysfunction post-transplantation, CNIs are the mainstay of therapy with the long-term goal of low levels of immunosuppression and minimization of medication. In patients with renal insufficiency, we have had success with a combination of low-dose CNI therapy and MPAs or a switch to mTOR inhibitors to preserve graft function and prevent further renal deterioration. We typically avoid the switch to mTOR inhibitors within the first 3-6 mo post-transplantation given the risk of hepatic artery thrombosis, rejection, and wound healing. Patients are weaned off corticosteroids within 6 mo to 1 year, providing they do not have evidence of autoimmune disease or recurrent episodes of rejection.

As evidenced by prior studies, the recommended approach to the patient with HCV infection is gradual, cautious weaning of corticosteroids within the first 3-6 mo while continuing low levels of maintenance immunosuppression, typically with CNIs. While HCV recurrence is universal after liver transplantation, avoiding excessive and erratic changes in the immunosuppressive regimen should prevent clinically aggressive disease.

The ultimate goal remains the ability to induce tolerance in transplant recipients. While this is not a current available practice, data from selected patients demonstrate that it may become a viable option with advances in future research and improved understanding of the genetic make-up and predisposition of this population. Until then, finding the balance between preserving graft function and optimizing immunosuppression while minimizing toxicities remains a challenge.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH



EDITORIAL

Prevalence, predictors, and clinical consequences of medical adherence in IBD: How to improve it?

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Received: July 2, 2009 Revised: July 15, 2009
Accepted: July 22, 2009
Published online: September 14, 2009

Peer reviewer: Dr. Limas Kupcinskas, Professor, Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

Lakatos PL. Prevalence, predictors, and clinical consequences of medical adherence in IBD: How to improve it? *World J Gastroenterol* 2009; 15(34): 4234-4239 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4234.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4234>

Abstract

Inflammatory bowel diseases (IBD) are chronic diseases with a relapsing-remitting disease course necessitating lifelong treatment. However, non-adherence has been reported in over 40% of patients, especially those in remission taking maintenance therapies for IBD. The economical impact of non-adherence to medical therapy including absenteeism, hospitalization risk, and the health care costs in chronic conditions, is enormous. The causes of medication non-adherence are complex, where the patient-doctor relationship, treatment regimen, and other disease-related factors play key roles. Moreover, subjective assessment might underestimate adherence. Poor adherence may result in more frequent relapses, a disabling disease course, in ulcerative colitis, and an increased risk for colorectal cancer. Improving medication adherence in patients is an important challenge for physicians. Understanding the different patient types, the reasons given by patients for non-adherence, simpler and more convenient dosage regimens, dynamic communication within the health care team, a self-management package incorporating enhanced patient education and physician-patient interaction, and identifying the predictors of non-adherence will help devise suitable plans to optimize patient adherence. This editorial summarizes the available literature on frequency, predictors, clinical consequences, and strategies for improving medical adherence in patients with IBD.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Therapy; Adherence; Compliance; 5-aminosalicylate; Mesalazine; Azathioprine

INTRODUCTION

Inflammatory bowel disease (IBD) is a multifactorial entity with both genetic and environmental factors contributing to disease pathogenesis^[1]. Worldwide, the incidence rates for IBD vary from 0.5 to 24.5 per 100 000 person-years^[2], with the majority of patients being disabled during various parts of their lives. This characteristic may also suggest poor adherence (i.e. a percentage of the prescribed doses is not taken) outside the clinical trial settings^[3-5].

Treatment of IBD can involve several medications with varying regimens, dietary modifications, and potentially, surgery, depending on symptoms, severity of illness, and response to treatment. Adherence to the pharmacological treatment is a complex process, where the doctor-patient relationship, treatment regimen and other disease-related factors play key roles. The undesirable side effects of some medications (e.g. weight gain, cushingoid appearance, and immune suppression) and the complex treatment regimens for IBD patients (e.g. varying dosing schedules and pill quantities for each medication) are likely to disrupt adherence and the effective management of this condition. These data are consistent with the hypothesis that many patients engage in an implicit cost-benefit analysis in which beliefs about the necessity of their medication are weighed against concerns about the potential adverse effects of taking it, and that these beliefs are related to medication adherence, as in other chronic conditions^[6]. This scenario has been also proven in patients with IBD in a very recent paper^[7]. In contrast, the impact of medication non-adherence on the hospitalization risk and health care costs in chronic conditions (e.g. diabetes, hypertension, and congestive heart failure) is enormous^[8]. It has been estimated to cost as much as \$100 billion in the US annually, and accounts for 10% of all hospital admissions.

However, there are no studies that directly assess the costs associated with non-adherence in patients with IBD. Recently, it has been estimated in the UK that relapse was associated with a two- to threefold increase in the costs for those who did not require hospital care and a 20-fold increase for those who were hospitalized^[9].

Research on adherence in IBD is limited. Studies in adults have revealed medication non-adherence rates ranging from 35% to 45%^[3,5,10]. Unfortunately, most of these studies used different, unimodal indirect assessment methods including non-standardized self-report questionnaires, non-standardized patient/parent interviews, pill counts, pharmacy records, and measurements of health outcomes. A drawback of this method is that it can overestimate adherence, and its accuracy depends on the patient's cognitive abilities, the honesty of replies, as well as the interviewer's correct interpretation of responses. The patient may forget doses taken or missed. Prescription refills are also considered questionable for assessing dosing compliance because they provide no information on timing or quantity of pills ingested. In addition, pill counts are often erroneous because patients do not always return bottles with leftover pills. Thus, until now, the methods for assessing compliance have varied in terms of prospective *versus* retrospective or objective *versus* subjective measurement, target behaviour assessed (e.g. consumption of medication and refill of prescription), medication assessed, and method of assessment. This variability resulted in different estimates of the prevalence of non-adherence and diminished generalizability and the validity of data. Furthermore, these studies were limited by potential response bias in the self-report measures or behavioural manipulation, such as discarding pills to influence pill count data^[11].

In general, direct methods for measuring medication adherence include drug concentration monitoring through blood and urine assays. This strategy is expensive and inconvenient for patients, and, moreover, only a limited number of drugs can be monitored in this manner. The bioavailability and completeness of absorption of various drugs, as well as the rate of metabolism and excretion, are factors that make it difficult to correlate drug concentrations in blood or urine with adherence. The ability of direct methods to identify non-adherence also depends on the accuracy of the test and the degree to which the patient was non-adherent before the urine or blood sample was taken. Drug concentration monitoring can also be misleading because most drugs are rapidly absorbed following administration. Thus, even if numerous doses were omitted, yet a few doses were taken immediately prior to the blood test, the results would show the presence of a moderate amount of drug, or *vice versa*.

In IBD, bioassays measuring 6-thioguanine nucleotide (6-TGN) and 6-methylmercaptopurine nucleotide (6-MMPN) levels have been suggested as potentially useful objective adherence markers for 6-mercaptopurine (6-MP)/azathioprine (AZA)^[11,12]. However, they have not been validated against traditional measures of adherence. Moreover, like other bioassays, they are subject to pharmacokinetic variation in absorption, metabolism,

and excretion. Despite their limitations, bioassays provide key adherence data in that they can confirm ingestion. Non-therapeutic metabolite levels can suggest either non-adherence or pharmacokinetic influence, or both; cases where both 6-TGN and 6-MMPN levels are subtherapeutic/unquantifiable are likely to indicate non-adherence. Thus, although there is no gold standard in adherence assessment, and limitations exist with any measure of adherence, both behavioural and biological measures offer unique data that could be used to better understand non-adherence. Moreover, determining the most advantageous approach to assessing adherence is critical to the clinical care of these patients. This editorial summarizes the available literature on frequency, predictors, clinical consequences, and strategies for improving medical adherence in patients with IBD.

PREVALENCE OF NON-ADHERENCE IN IBD

In normal clinical practice, adult studies have revealed medication non-adherence prevalence rates ranging from 35% to 72%^[3,5,10,13,14]. For example a cross-sectional study of US outpatients with quiescent ulcerative colitis (UC) found that only 40% were adherent to maintenance mesalazine (mesalamine) therapy^[3]. In the UK, approximately 15% of patients fail to even redeem prescriptions at the pharmacy^[15]. Moreover, treatment non-adherence rates might vary considerably between countries. In Europe, a survey of 203 IBD patients revealed self-reported non-adherence rates ranging from 13% in France, to 26% in Italy, 33% in the UK and 46% in Germany. The overall non-adherence rate was 29% across Europe^[4], where non-adherence was defined as taking < 80% of prescribed medication. Similarly high rates of non-adherence were reported from Eastern Europe. Overall intentional non-adherence was reported by 38.9% of patients, and 18.6% of the patients at least once discontinued the treatment^[5]. In a Canadian study, UC diagnosis was associated with higher risk of non-adherence (OR: 4.42)^[16].

Significant differences may exist in children and adolescents, given the complex developmental challenges unique to childhood and adolescence, including the maturation of cognitive and behavioral patterns (e.g. health beliefs) that affect self-management. However, only a few studies have examined adherence rates in pediatric IBD, with the results indicating the prevalence of non-adherence ranging from 50% to 66%^[17,18]. Moreover, special attention should be paid to the method of assessment, because significant differences may be present in objective methods *versus* subjective self-report methods. In a recent paper, Hommel *et al*^[11] reported an objective non-adherence frequency of 38% for 6-MP/AZA and 49% for 5-ASA medications, while the subjective non-adherence frequency was reported to be as low as 6% for 6-MP/AZA and 3% for 5-ASA. In contrast, in a prospective, single-center study from Germany^[19] both objective (9.2%) and self-reported (7.1%) non-adherence rates were low in 65 adult Crohn's disease (CD) patients.

PREDICTIVE FACTORS FOR NON ADHERENCE AND CLINICAL CONSEQUENCES

Gender

Conflicting data are available on the role of gender in predicting non-adherence to medical therapy. Kane *et al*^[3] and Mantzaris *et al*^[20] related poor adherence with the male gender. In the study by Kane *et al*^[3] non-adherent patients were statistically more likely to be males (67% *vs* 52% in adherent patients). Gender interactions also proved relevant in a recent population-based study, in which young females proved to be less adherent than males^[17], while other studies could not find a significant difference^[5]. In addition there may be different factors affecting medication adherence in men and women. In the study by Ediger *et al*^[16] a diagnosis of ulcerative colitis (*vs* Crohn's disease) having high scores on the Obstacles to Medication Use Scale and a low level of the personality trait of agreeableness, were important predictors of low adherence in males. For women, important predictors of low adherence included an age younger than 30 years, having high scores on the Obstacles to Medication Use Scale, and having a low level of the personality trait of agreeableness. Immunosuppressant use was associated with high adherence in women.

Similarly, data are conflicting with regards to marital status, type of education, employment status, or type of disease. A higher education level and full time employment was also associated with a non-adherent patient behavior in some^[5,16,21], but not all, studies^[14].

Age and disease duration

Age seems to be an important factor, as younger patients tend to be less adherent than older patients^[10,18]. In a recent Italian study^[22] non-adherence was 43% in patients < 40 years old compared to 34% in those older than 40 years ($P = 0.041$, OR: 1.5, CI: 1.01-2.13). Recently, diagnosis and disease duration shorter than 5 years was also associated with significantly worse adherence (24% of the patients) than a longer-standing disease (15% of the patients; $P = 0.001$, OR: 2.1, CI: 1.30-3.39) in the same study. Moreover, non-adherence increased to 75% when both age (< 40 years) and disease duration (< 5 years) were considered. This may have to do with the fact that IBD primarily affects young individuals with greater personal and social goals, being busy at work, and having some degree of rebelliousness, but it may also be that a younger age is associated with a more recent diagnosis, with less experience with the burden of relapse or surgery. This was, however, not a universal finding^[5].

Phenotype, disease activity and surgery

In UC, Kane *et al*^[3] reported by means of univariate analysis, that male gender, being without a relationship partner, left-sided disease, and a history of more than four concomitant medications, were negatively associated with adherence. Conversely, being married, having a recent colonoscopy, and a greater extent of disease supported

adherence. A UK-based cross-sectional study, using data extracted from general practitioner (GP) clinical records, examined the usage of long-term aminosalicylate therapy in patients with UC^[13]. It was found that 38% of the patients with extensive colitis, 37% of the patients with left-sided colitis and 46% of those with proctitis did not take medications for maintenance therapy. This was not, however, confirmed in all studies^[5].

An association between medical adherence and complicated disease course in CD was reported by Spanish authors^[14]. Better adherence was significantly associated with a more complicated disease course (steroid dependency, steroid refractoriness, need for infliximab treatment, hospitalization, or surgery) in patients with short disease duration. Similarly, in a recent Hungarian study^[23], a higher number of previous surgeries was associated with improved self-reported adherence in patients with CD.

Active disease was associated with higher adherence, even if steroids were included in the treatment regimen in both CD and UC^[10]. In contrast, other studies reported low adherence rates after long-term remission^[3,22]. Very high non-adherence rates (74.3%) were reported for azathioprine in CD patients who were in long-term (> 48 mo) clinical remission^[20].

Moreover, a direct association between adherence and risk of relapse was reported in UC. Kane *et al*^[24] prospectively studied the risk factors associated with relapse among 99 patients who were in remission for more than six months and prescribed 5-ASA maintenance therapy. The clinical recurrence of UC was defined as four or more bowel movements per day. At a 12-mo follow-up, 19 of 86 patients had recurrent disease, 13 (68%) of whom were non-adherent. Patients who were non-adherent with medication had a greater risk of recurrence than adherent patients (OR: 5.5, 95% CI: 2.3-13). A Kaplan-Meier curve constructed to compare outcomes stratified by adherence status for 24 mo also showed that UC patients adherent to their 5-ASA therapy had a significantly greater chance of remaining in remission than those who were non-adherent (89% *vs* 39%; $P = 0.001$).

Drug type and dosing regimes

Non-adherence to therapy might also be due to the drug formulation causing discomfort (difficulty in swallowing tablets or using enemas) or side effects (pain, abdominal distension, or difficulty in retaining enemas). Most studies are consistent in finding that topical therapy with enemas, suppositories or foams is more likely to be associated with non-adherence than oral therapy. In an Italian study^[22], topical therapy with enemas was associated with significantly more non-adherence (68% of users) than oral therapy (40% of users; $P = 0.001$, OR: 0.25, CI: 0.11-0.60). Similarly, analyzing a national prescription-based database also showed that overall adherence to mesalazine was unexpectedly low and the rectal formulation was among the factors influencing non-adherence^[25]. Enemas were judged difficult to use, painful or to cause bloating, and were difficult to manage during working hours.

The association between the type of oral medications and non-adherence is more controversial. The undesirable

side effects of some medications (e.g. weight gain, cushingoid appearance, or immune suppression) and the complex treatment regimens for IBD patients (e.g. varying dosing schedules and pill quantities for each medication) are likely to disrupt adherence and effective management of this condition. Interestingly, some studies did not report a direct association. For example, in the study by Cervený *et al*^[5], the non-adherence rate at any time point was 40% on aminosalicylates, 29% in patients on systemic steroids, and 31% in patients on immunosuppressants in CD. Similar data were reported in UC, supporting the notion that adherence is influenced by multiple parallel factors, including gender, age, disease activity, and so on. Interestingly, the same study, using a factor analysis, reported a strong influence of adverse drug effects on adherence. Intentional non-adherent behavior due to adverse drug effects was the second most common cause reported during a patient interview. In addition, adverse drug effects were independently proven by factor analysis to affect a patient's confidence in treatment.

Reasons for non-adherence to oral therapy include multiple daily doses and a high number of concomitant medications. In the study by Kane *et al*^[3], besides being males, single, and having left-sided disease, non-adherent patients were statistically more likely to be taking four or more concomitant medications (60% *vs* 40%). Similarly, in the study of Shale and Riley^[21], in addition to being young, having education beyond the age of 16 years and being in full-time employment, being prescribed a 3-times-a-day regimen was identified as predictor for non-adherence. The need to take medicine during working hours ($P = 0.001$, OR: 3.5, 95% CI: 2.27-5.26), and multiple daily doses ($P = 0.045$, OR: 2.8, 95% CI: 0.99-7.70) were significantly associated with non-adherence in adults^[22], which was also confirmed by other studies^[20,21]. Similarly, adolescents whose regimen involved more than one daily medication administration had more adherence barriers^[26]. In addition, lack of time and medication side effects were also commonly reported barriers. Other adolescent-reported barriers included missing medication due to feeling well or discontinuing medication based on the belief that the medication was not working. In contrast, a recent retrospective cohort study suggests that adherence in UC patients is independent of drug formulation^[27]. Magowan *et al*^[27] used records from multiple US health plans to compare the refill prescription profiles of 1680 UC patients who had initiated 5-ASA therapy with one of four formulations: delayed-release mesalamine (Asacol), controlled-release mesalamine (Pentasa), sulfasalazine (Azulfidine), or balsalazide (Colazal). Upon initiation of treatment, the median daily dose and respective tablet/capsule load were 2.4 g (6 tablets) for delayed-release mesalamine, 4.0 g (16 capsules) for controlled-release mesalamine, 2.0 g (4 tablets) for sulfasalazine, and 6.75 g (9 capsules) for balsalazide. Comparison of the refill profiles over 12 mo, however, indicated that adherence in these patients was not affected by formulation type and/or dose regimen.

The use of once-daily treatment for improving medical compliance is further supported by a recent randomized, multicentre, investigator-blinded study of 362

patients who were randomised to receive mesalazine granules (Pentasa®) 2 g once daily or 1 g twice daily. It showed an 11.9% greater remission rate at one year (73.8% *vs* 63.6%, respectively) in the single daily dose group^[28]. Patient questionnaires showed significantly greater self-reported compliance ($P < 0.05$) and acceptability ($P < 0.001$) in the once-daily group. High compliance rates were reported for the once-daily MMX mesalazine and Salofalk® granules^[29,30]; therefore the effect is likely to be generic rather than compound-specific. Thus, new mesalazine formulations offer a simplified dose regime, resulting in presumably improved long-term compliance that can be considered an important advantage in the management of UC patients.

Patient-doctor relationship

The partnership between patient and the treating physician is of utmost importance in determining medical adherence, where effective patient-physician dialog is central to promoting patient adherence^[22]. Studies have also shown that the interaction between the patient and the physician has a huge impact on health outcomes and costs. Both the quality and quantity of the visits are important. Sewitch *et al*^[10] found an increased risk of intentional non-adherence to be associated with being treated by the same physician for more than one year, not scheduling another appointment, and greater total discordance between the patient and the physician. Similarly, a higher degree of intentional non-adherence in the study by López San Román *et al*^[31] was associated with greater patient depression and patient-physician discord. Patients trusted their physician less, and considered themselves to be less informed about their treatment.

A direct association between the total number of health care visits and medical adherence was proven in children with CD^[18]. In addition, patients under specialist care were significantly more likely to be taking an aminosalicylate than those definitely discharged to general practitioner's care in adults with UC^[13]. In contrast, however, a European cohort showed no correlation between the number of times an IBD patient had seen the physician and self-reported medication adherence rates^[4].

STRATEGIES TO IMPROVE ADHERENCE

A large body of evidence supports the key role of the physician-patient relationship in achieving higher patient medication-adherence rates. Psychology literature points out to using COPE principles as a way for physicians to improve their relationship with patients and optimize patient adherence to their medication. The COPE principles encompass the following: communicate with patients; obtain patient's commitment to therapeutic objectives; promote emotional/psychological/physical support as necessary; educate the patient and their family. In addition, trust in the physician and continuity of care by the same doctor are also important to patients.

In the everyday practice, the physician's willingness to allow patients to contribute input and become involved in their illness during the medical visits was suggested to

facilitate treatment decisions that are meaningful to both parties^[32]. The consultation style adopted by the physician is also an important factor in building the physician-patient relationship. Indeed, when physicians adopted a mutual, co-operative relationship, and exhibited less control dominance, a reported increase in patient adherence and satisfaction was observed.

During consultations, all factors affecting adherence need to be explored, including the patient's level of knowledge, belief systems and support environment, for example their network of family and friends.

Written and oral education (on the disease, management algorithm and medications) has been shown to increase adherence by approximately 6%-25%^[33]. Written information is more effective when verbally reinforced. In addition, a study of 69 patients with IBD demonstrated improved knowledge, patient satisfaction, and a positive trend towards greater adherence in patients who had undertaken the IBD education program (consisting of pamphlets and ad hoc physician education), which is the standard of care in many referral centers, compared with patients who received standard care^[34].

Guided self-management involving the provision of a shared set of guidelines containing action plans for the prevention of disease activity and/or symptom relief, have been used in the management of many chronic illnesses. A randomized, controlled study evaluating guided self-management programs in patients with IBD has demonstrated, a reduction in hospital visits without an increase in morbidity and greater confidence in the patient's ability to cope with IBD^[35]. Further studies are needed in order to assess whether such interventions will improve adherence to medication and clinical outcomes in patients with IBD. Furthermore, a special form of patient education could be successfully implemented using an internet-based patient education platform, as suggested by Elkjaer *et al*^[36].

Another approach that could be used to optimize patient adherence involves individualized therapy, where physicians review the patient's disease and therapeutic history, and identify which treatment(s) were effective/ineffective in the past to avoid prescribing the same unsuccessful medication. Simplification of treatment (e.g. reduced dosing frequency and the use of long-acting agents) and avoidance of unnecessary multiple concomitant medications is preferable, where feasible, and are associated with better adherence and improved clinical outcome^[3,21,24]. Furthermore, this patient review process could also provide predictive information on medication non-adherence behavior, and thus help identify those patients at high risk who might require longer consultation slots than those at low risk. Patients could also be prompted to take their medications via simple pill-taking cues, such as placing pills close to something they use daily, for example the toothpaste, breakfast table, glasses/contact lenses case, and so on. In addition, telephone support, postal reminders, and setting alarms on watches/mobile phones have been suggested. Nevertheless, combining education and behavioral interventions has been suggested to be the most effective approach to improving adherence.

CONCLUSION

Non-adherence is common in IBD and has been reported in 40%-60% of patients, especially those in remission and taking maintenance therapies for IBD. The economical impact of medication non-adherence, including absenteeism, hospitalization risk, and health care costs in chronic conditions, is enormous. The causes of medication non-adherence are multi-factorial, including forgetfulness, gender, new diagnosis, disease phenotype, patient-physician relationship, complicated dosing regimens, side-effect profile of the drugs, and treatment delivery methods. The associated factors may vary in each country because of the difference in the healthcare systems and the population. Moreover, a gold standard method to estimate the prevalence of non-adherence does not exist. Subjective assessment may underestimate adherence, while recent episodes of non-adherence may result in high non-adherence rates if measured by direct methods (e.g. drug concentration monitoring using blood and urine assays). Moreover, this latter strategy is expensive and inconvenient for patients, and only a limited number of drugs can be monitored in this way. Poor adherence may result in more frequent relapses, disabling disease course, and in ulcerative colitis, in increased risk for colorectal cancer. Improving medication adherence in patients is an important challenge for physicians. Understanding the different patient types, the reasons given by patients for non-adherence, simpler and more convenient dose regimens, dynamic communication within the healthcare team, self-management package incorporating enhanced patient education and physician-patient interaction and identifying the predictors of non-adherence, will help devise suitable plans to optimize patient adherence.

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S- Editor Tian L **L- Editor** Stewart GJ **E- Editor** Lin YP

REVIEW

Advances in diagnosis, treatment and palliation of cholangiocarcinoma: 1990-2009

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Author contributions: Aljiffry M and Molinari M contributed equally to this work by performing the literature search and by writing the paper; Walsh MJ reviewed and edited the manuscript.

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Received: August 5, 2008 Revised: July 15, 2009

Accepted: July 22, 2009

Published online: September 14, 2009

Key words: Cholangiocarcinoma; Epidemiology; Diagnosis; Therapy; Palliation; Systematic review

Peer reviewer: Simon D Taylor-Robinson, MD, Department of Medicine A, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0HS, United Kingdom

Aljiffry M, Walsh MJ, Molinari M. Advances in diagnosis, treatment and palliation of cholangiocarcinoma: 1990-2009. *World J Gastroenterol* 2009; 15(34): 4240-4262 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4240.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4240>

Abstract

Several advances in diagnosis, treatment and palliation of cholangiocarcinoma (CC) have occurred in the last decades. A multidisciplinary approach to this disease is therefore recommended. CC is a relatively rare tumor and the main risk factors are: chronic inflammation, genetic predisposition and congenital abnormalities of the biliary tree. While the incidence of intra-hepatic CC is increasing, the incidence of extra-hepatic CC is trending down. The only curative treatment for CC is surgical resection with negative margins. Liver transplantation has been proposed only for selected patients with hilar CC that cannot be resected who have no metastatic disease after a period of neoadjuvant chemo-radiation therapy. Magnetic resonance imaging/magnetic resonance cholangiopancreatography, positron emission tomography scan, endoscopic ultrasound and computed tomography scans are the most frequently used modalities for diagnosis and tumor staging. Adjuvant therapy, palliative chemotherapy and radiotherapy have been relatively ineffective for inoperable CC. For most of these patients biliary stenting provides effective palliation. Photodynamic therapy is an emerging palliative treatment that seems to provide pain relief, improve biliary patency and increase survival. The clinical utility of other emerging therapies such as transarterial chemoembolization, hepatic arterial chemoinfusion and high intensity intraductal ultrasound needs further study.

INTRODUCTION

Cholangiocarcinomas (CC) are malignant tumors originating from epithelial cells lining the biliary tree and gallbladder^[1]. Intrahepatic CCs (ICC) arise within the liver and extra-hepatic CCs (ECC) originate in the bile duct along the hepato-duodenal ligament. ICCs usually present as masses in the liver while jaundice is the most common presentation of ECCs. CCs are relatively rare tumors although their incidence is rising worldwide^[2,3]. Several advances in the diagnosis, therapy and palliation of patients affected by CC have occurred during the last decades. The aim of this article is to review the most recent high quality literature on this topic.

EVIDENCE ACQUISITION

We sought studies that reported at least one of the following aspects of CC: epidemiology, diagnosis, therapy (e.g. surgery, radiotherapy, chemotherapy, phototherapy), and palliation. Preference was given to randomized controlled trials (RCT) and prospective observational studies. For each of these topics we searched MEDLINE, Ovid MEDLINE In-Process, Cochrane Database of Systematic Reviews, Database of Systematic Reviews, Database of Abstracts of Reviews of Effects, EMBASE, PubMed, National Library of Medicine Gateway by established systematic review methods (Jadad Scale for RCT controlled studies, Downs and Black checklist for observational studies^[4-6]). We further reviewed reference lists and articles from the authors' libraries. We limited our search to English-language articles published from January 1990 to June 2009. We then developed a comprehensive

Table 1 Summary of the terms used singly or in combination for evidence acquisition

Primary MeSH terms	Secondary MeSH terms (Epidemiology, diagnosis)	Secondary MeSH terms (treatment, palliation)
Cholangiocarcinoma(s)	Epidemiology	Hepatectomy
Adenocarcinoma(s)	Classification	Resection
Carcinoma(s)	Diagnosis	Therapeutic(s)
Bile duct neoplasm(s)	Differential diagnosis	Treatment outcome(s)
Biliary tract neoplasm(s)	Early diagnosis	Surgery
Common bile duct neoplasm(s)	Risk factor(s)	Transplantation
Liver neoplasm(s)	Diagnostic imaging	Biliary tract
Bile duct(s)	Magnetic resonance imaging	Surgical procedures
Common bile duct	Endosonography	Liver transplantation
Intrahepatic bile duct(s)	Ultrasonography	Organ transplantation
Extrahepatic bile duct(s)	Emission computed tomography	Clinical trial
Biliary tract disease(s)	Radionuclide imaging	Controlled clinical trial(s)
Bile duct disease(s)	Positron emission tomography	Randomized controlled trial(s)
	X-ray	Clinical trial (phase I)
	Computed tomography	Clinical trial (phase II)
	Biopsy (needle)	Clinical trial (phase III)
	Biopsy (fine needle)	Clinical trial (phase IV)
	Cytology	Drug therapy
	Cytodiagnosis	Chemotherapy
	Tumor markers (biological) antigen(s)	Adjuvant
	Carcinoembryonic antigen	Antineoplastic agent(s)
	Ca 19-9 antigen	Combined modality therapy
	Ca 125 antigen	Antineoplastic
	Endoscopic retrograde cholangiopancreatography	Combined chemotherapy protocols
	Cholangiography	Neoadjuvant therapy
	<i>In situ</i> hybridization	Radiotherapy
	Fluorescence <i>in situ</i> hybridization	Adjuvant embolization
	Nucleic acid hybridization	Portal vein embolization
	Computed assisted image processing	Drainage
		Cholestasis
		Obstructive jaundice

and current database to catalog the medical literature on CC. The evidence database for the catalog was assembled only for CC arising in the intra- and extra-hepatic bile ducts. Our review did not include the management of gallbladder cancer, as several other comprehensive articles had already covered this topic^[7-10]. To identify all potential papers, we searched medical subject headings reported in Table 1. Two authors (Aljiffry M and Molinari M) independently performed the selection of the articles based on the content of titles and abstracts. When in doubt, each article was reviewed entirely. The decision to include articles in this review was reached by consensus. For conciseness, a full list of search strategies, search results, and quality assessment for each included study are available on request from the corresponding author.

EPIDEMIOLOGY

The incidence of CC is rising in most countries and it is the second most common primary malignancy of the liver after hepatocellular carcinoma^[1]. In the USA, approximately 5000 new cases are diagnosed every year^[11] accounting for almost 3% of all tumors of the gastrointestinal tract^[12]. While the incidence of ICC is rising, the occurrence of ECC is trending down^[13,14] suggesting that different risk factors may be involved^[15]. Caution should be used when interpreting these results as misclassification bias may have influenced these observations^[2,16]. In fact, analysis of the Surveillance Epidemiology and End Results database from 1975 until

1999 has shown that most hilar tumors (more than 90%) were classified as ICC^[2,16] while ECC were often combined with gallbladder cancers^[2,13]. Nevertheless, evidence that ICC and ECC may be dissimilar tumors is supported by the recent discovery that, *in vitro*, they express diverse cellular proteins and have different cellular shape, doubling time, chromosome karyotype and chemosensitivity^[17]. Similarly, researchers from France showed that hilar CC (HCC) express higher levels of MUC5AC (60% *vs* 22%), Akt2 (64% *vs* 36%), CK8 (98% *vs* 82%), annexin (56% *vs* 44%) and less vascular epithelial growth factor (22% *vs* 78%) in comparison to ICC^[18]. These findings support the hypothesis that the heterogeneous protein and receptor expression of ECC and ICC may be due to different carcinogenic pathways^[17,18].

ICC

The estimated age-adjusted incidence rates of ICC in the USA has increased by 165% over the last thirty years (from 0.32 per 100 000 in 1975-1979 to 0.85 per 100 000 in 1995-1999^[2,19] accounting for 10% to 15% of all primary hepatic cancers^[20]. The average age at presentation is the seventh decade of life^[2] with a male to female ratio of 1.5^[20]. The mortality rate and incidence of ICC have parallel trends^[13] as age-adjusted mortality rate increased from 0.07 per 100 000 in 1973 to 0.69 per 100 000 in 1997^[21].

ECC

In the USA, the age-adjusted incidence of ECC has

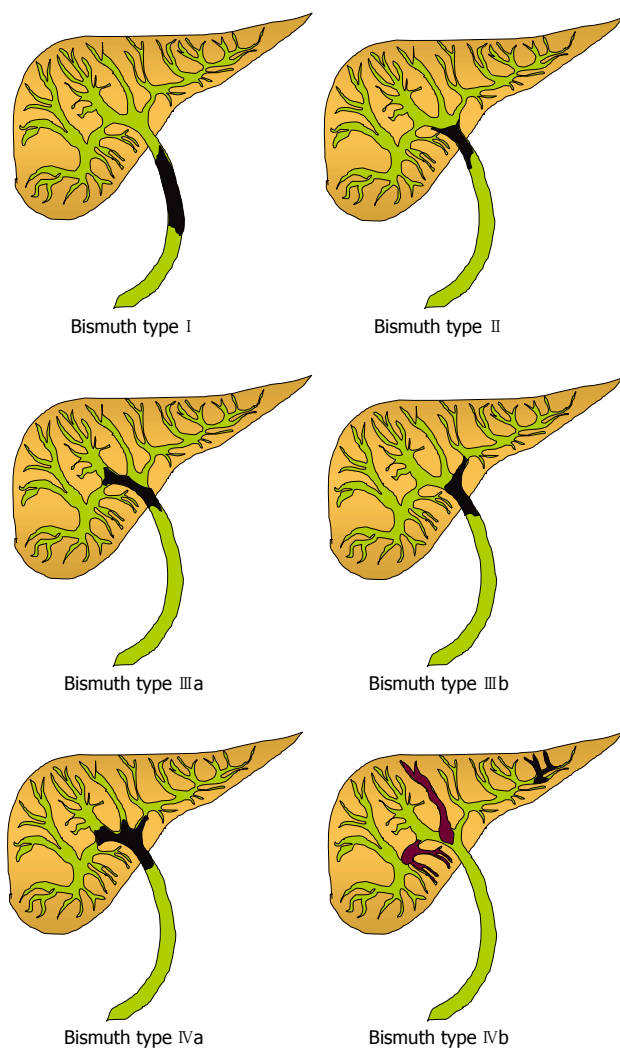


Figure 1 Bismuth's classification of cholangiocarcinomas. Type I: Cholangiocarcinoma is confined to the common hepatic duct; Type II: Cholangiocarcinoma involves the common hepatic duct bifurcation; Type IIIa: Cholangiocarcinoma affects the hepatic duct bifurcation and the right hepatic duct; Type IIIb: Cholangiocarcinoma affects the hepatic duct bifurcation and the left hepatic duct; Type IV: Cholangiocarcinoma is either located at the biliary confluence with both the right and left hepatic ducts involvement or has multifocal distribution.

decreased by 14% compared to data from two decades earlier. Currently it is 1.2 per 100 000 in men and 0.8 per 100 000 in women^[2,22]. Similarly to ICC, 65% of ECC present in the seventh decade of life^[22]. The mortality rate of ECC parallels its incidence and in the USA, the age-adjusted mortality rates for ECC declined from 0.6 per 100 000 in 1979 to 0.3 per 100 000 in 1998^[14,21].

CLASSIFICATION

Anatomical classification

ICCs arise within the liver parenchyma while ECCs involve the biliary tree within the hepatoduodenal ligament and gallbladder. ECCs are further divided into hilar or distal tumors. HCC, also called Klatskin tumors, are located within 2 cm from the bifurcation of the common duct and were described for the first time by Klatskin in 1965^[22]. Ten years later, Bismuth and Corlette proposed

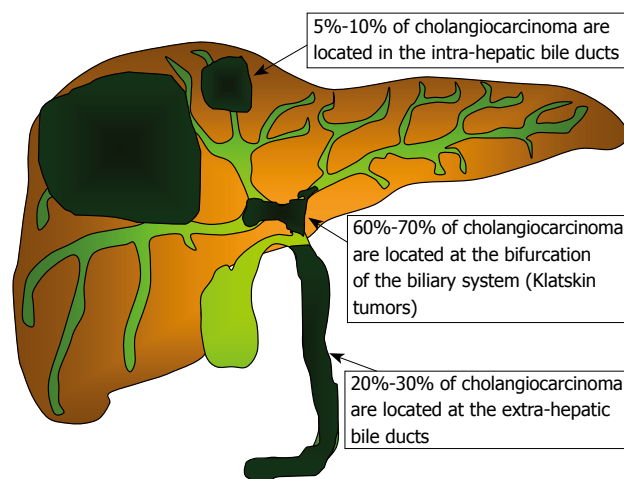


Figure 2 Anatomical presentation of cholangiocarcinomas. The majority of cholangiocarcinomas (60%-70%) present in the area of the biliary duct bifurcation and are called Klatskin tumors. The extra-hepatic bile duct is involved in 20%-30% of cases while intrahepatic cholangiocarcinomas represent 5%-10% of the tumors originating from the biliary system.

a clinical classification that stratifies these tumors by anatomical location (Figure 1)^[23]. Approximately 60% to 70% of CC are located in the hylum, 20% to 30% are ECC, and 5% to 10% are ICC (Figure 2)^[24,25].

Pathological classification

More than 90% of CC are well- to moderately-differentiated adenocarcinomas^[26,27] with tendency to develop desmoplastic reaction and early perineural invasion. Macroscopically, ICC may develop in solid masses, infiltrate periductal tissues, grow intraductally or have mixed characteristics. On the other hand, ECC develop nodular lesions, sclerosing strictures, or papillary growth patterns. Sclerosing CC are the most common^[28] while papillary adenocarcinomas are rare and associated with more favorable prognosis^[22].

RISK FACTORS FOR CHOLANGIOCARCINOMA

Only a minority of patients presenting with CC have known risk factors such as chronic biliary inflammation, cholestasis or congenital abnormalities (Table 2)^[29].

Primary sclerosing cholangitis (PSC)

In Western countries, PSC is the most important predisposing factor for CC^[30,31]. The cumulative annual risk of CC in patients with PSC is 1.5% per year after the development of jaundice^[32] and the prevalence of CC in patients with PSC ranges between 8% and 40%^[30,33,34]. A recent epidemiological study from the Netherlands has shown that the risk of CC for patients with PSC is 9% after 10 years from the time of the diagnosis^[35]. In patients with concomitant inflammatory bowel disease, the 10-year and 20-year risks for CC are 14% and 31% respectively, which are significantly higher than patients without inflammatory bowel disease (2% and 2%

Table 2 Known risk factors for cholangiocarcinomas

General risk factors
Old age (older than 65 years)
Smoking
Obesity
Diabetes
Post surgical
Biliary-enteric anastomosis
Chronic inflammatory diseases
Primary sclerosing cholangitis (PSC)
Hepatolithiasis (Oriental Cholangiohepatitis)
Hepatitis C
Hepatitis B
Human Immunodeficiency Virus (HIV)
Liver cirrhosis
Parasitic infections
<i>Opisthorchis viverrini</i>
<i>Clonorchis sinensis</i>
Congenital
Choledochal cysts
Caroli's disease
Congenital hepatic fibrosis
Chemical agents
Thorotrast
Dioxin
Nitrosamines
Asbestos
Medications
Oral Contraceptive Pills
Isoniazid

respectively; $P = 0.008$)^[35]. Individuals with PSC frequently develop CC at younger age (30-50 years) compared to the general population (60-70 years)^[50,32]. The diagnosis of CC in this group is challenging because clinical presentation and radiological findings of CC and PSC are similar. As a result, most cases of CC complicating PSC are detected at advanced stages and have poor prognosis^[36]. Predictive factors of CC in PSC patients are: sudden progressive jaundice, unintentional weight loss, marked dilation of bile ducts proximal to biliary strictures, serum level of Ca 19-9 tumor marker above 100 U/mL, and presence of cellular dysplasia on cytological specimens obtained by brushing of the biliary ducts^[37].

Parasitic infections

Infestation with liver flukes (*Opisthorchis viverrini* and *Clonorchis sinensis*) has been strongly associated with an increased risk of CC in South-East Asia^[3,38,39]. In areas where *Opisthorchis viverrini* is endemic, the adjusted prevalence for CC by age and gender is as high as 14%^[40]. The pathophysiology causing CC in these patients is not completely understood, however it is hypothesized that parasites colonize the biliary system causing chronic inflammation and predisposing to malignant transformation.

Intrahepatic biliary stones (Hepatolithiasis)

Oriental cholangiohepatitis (also known as recurrent pyogenic cholangiohepatitis) has a prevalence of 20% in South-East Asia^[41] and almost 10% of affected patients develop ICC^[42-45]. Recurrent episodes of cholangitis aggravate the chronic inflammatory process that persists

between flare ups. Risk factors associated with CC in these patients are: age over 40 years, long history of hepatolithiasis, unintentional weight loss, increasing serum alkaline phosphatase, decreasing serum albumin, and serum CEA tumor marker above 4.2 ng/mL^[46].

Congenital biliary cystic diseases

Patients with choledochal cysts have low risk for CC if the cyst is excised early in their life^[46]. On the other hand, the incidence of malignant degeneration is between 10% and 20% if the cyst is not excised by the age of 20 years^[28,47]. The mechanism of malignant transformation is not totally understood although biliary stasis and reflux of pancreatic secretions are suspected of causing neoplasia through chronic inflammation^[48]. CC can occur years after resection of the cyst suggesting that there might be a genetic defect predisposing to tumors of the biliary system^[49].

Liver cirrhosis and viral infections

The risk of developing CC in cirrhotic patients is ten-fold higher than the general population (0.7% *vs* 10.7%)^[2,50]. Among patients with CC in the USA, the prevalence of hepatitis C viral infection (HCV) was found to be four times higher than the general population (0.8% *vs* 0.2%)^[20]. These results have been confirmed in Italy^[51], in Taiwan^[52] and in Japan where HCV and viral hepatitis B (HBV) infection were detected in 23% and 11.5% of CC compared to 6% and 5.5% of controls, respectively, with cumulative rates 1000-times greater than the general population^[53]. Similar results were recently confirmed in a case-control study performed in China, where researchers found that at multivariate analysis, significant risk factors for the development of ICC were hepatolithiasis (adjusted OR: 5.7; 95% CI: 1.9-16.8) and HBV infection (adjusted OR: 8.8; 95% CI: 5.9-13.1)^[15]. A large epidemiological study from the United States validated that HCV infection is a risk factor for ICC (hazard ratio 2.55; 95% CI: 1.3-4.9) but not for ECC (hazard ratio 1.5; 95% CI: 0.6-1.85)^[54]. Although human immunodeficiency virus (HIV) does not cause cirrhosis *per se*, 0.5% of patients infected with HIV have been found to have CC in comparison to 0.1% among controls, confirming previous observations which suggested that chronic viral infections might predispose to neoplastic transformation of some cell lines^[20].

Chemical agents

Several compounds have been suspected of causing CC. Thorotrast (thorium dioxide) needs a special mention because it was used as a radiological contrast in the period between 1920-1950 and was found to increase the risk of CC up to 300-times in comparison to the general population^[16,55]. Because of its long biological half-life (400 years), the latency period of thorotrast-induced CC ranges between 16 to 45 years^[56], with the highest incidence between 20 to 30 years after exposure^[57]. A few studies have shown an association between CC and other chemical agents such as asbestos^[58], vinyl chloride^[59] and nitrosamines^[60]. Medications such as isoniazide^[61] and first generation of oral contraceptives^[62] are also suspected of increasing the risk of CC.

Other risk factors

Tobacco smoking is weakly associated with CC in the general population^[20] while it appears to be a strong risk factor for PSC patients^[63]. Other predisposing factors for CC are diabetes, obesity, presence of bile duct adenomas and biliary papillomatosis^[64,65]. Although there is no evidence that endoscopic sphincterotomy increases the risk of CC, biliary-enteric bypasses may do so^[66].

DIAGNOSIS

Clinical presentation

CCs are usually clinically silent or associated with nonspecific symptoms in early stage (Table 3)^[67,68]. ICCs are often diagnosed by imaging tests, and rarely during physical exams, as asymptomatic hepatic masses^[26]. On the other hand, ECC usually present with painless jaundice^[69] and symptoms related to biliary obstruction such as itching, clay-colored stool and hyperpigmented urine^[69]. Only 10% of cases present with ascending cholangitis^[70]. Jaundice is usually persistent and progressive while intermittent biliary obstruction may be observed in patients with papillary lesions that cause a ball-valve effect^[71]. Physical examination of patients with CC may reveal hepatomegaly, palpable gallbladder (Courvoisier sign), or signs of portal hypertension due to portal vein thrombosis secondary to tumor invasion or compression^[33,69].

Laboratory investigations

Serum biochemical tests usually support the clinical suspicion of CC but they are rarely diagnostic. Jaundice occurs only if there is obstruction of the two main intra-hepatic biliary ducts or common bile duct. In these circumstances, elevation of the serum levels of bilirubin and markers of biliary epithelial injury, such as alkaline phosphatase (ALP) and gamma glutamyltransferase (GTT)^[33], are common^[72,73]. On the other hand, in the presence of unilateral intrahepatic biliary obstruction, elevation of ALP or GTT may be present without increase in the serum bilirubin level^[33]. Other abnormal laboratory findings include hypoalbuminemia and prolonged prothrombin time, which reflect the combination of diminished hepatic synthetic function, cachexia and malabsorption of vitamin K^[33].

Serum tumor markers

Several tumor markers may support the diagnosis of CC, although none of them is sensitive enough to be used for screening purposes. The most commonly used markers are carbohydrate antigen (Ca 19-9) and carcinoembryonic antigen (CEA)^[73]. These tumor markers are not very specific as they can be elevated in the presence of other malignancies (e.g. pancreas and stomach) and with benign conditions such as cholangitis and hepatolithiasis^[73-75]. In patients without PSC, serum Ca 19-9 values above 100 U/mL have a sensitivity of 53% and specificity of 75%-90% for the diagnosis of CC^[74]. In patients with PSC, serum Ca 19-9 levels above 100 U/mL have sensitivity of 75%-89% and specificity of 80%-86% for the diagnosis

Table 3 Presenting symptoms of patients affected by cholangiocarcinomas

Symptoms	Percentage (%)
Jaundice	84
Weight loss	35
Abdominal pain	30
Nausea and vomiting	20
Fever	10

of CC^[75-77]. In a recent study from the Mayo Clinic, the optimal cutoff value for serum Ca 19-9 in patients with PSC was 20 U/mL which provided a sensitivity of 78%, specificity of 67%, positive predictive values of 23% and negative predictive value of 96%^[78]. Serum Ca 19-9 combined with either ultrasonography, computed tomography, or magnetic resonance imaging provided a sensitivity of 91%, 100% and 96% respectively for CC diagnosis^[78]. The levels of Ca 19-9 seem to correlate with the stage of the disease. Patel *et al*^[72] reported that the sensitivity of Ca 19-9 above 100 U/mL for the diagnosis of CC in patients with resectable tumors was 33% compared to 72% in patients with unresectable tumors. Using more than one tumor marker for patients with PSC may improve the detection rate of CC. In one study, using Ca 19-9 levels above 180 U/mL in combination with CEA levels above 5.2 ng/mL had a sensitivity of 100% and a specificity of 78.4%^[79]. Several new markers are currently being investigated. The human mucin 5, subtypes A and C (MUC5AC) are the most promising for future clinical use with sensitivity and specificity of 71% and 90%, respectively^[80].

Imaging modalities

Imaging modalities are essential for the diagnosis and treatment planning of patients with CC^[73].

Abdominal ultrasound (US)

US is usually the initial imaging test performed to evaluate patients with biliary obstruction^[81]. The sensitivity and accuracy of US for ECC diagnosis are 89%^[82] and 80%-95%, respectively^[83,84]. On the other hand, ICC are difficult to distinguish from other solid intra-hepatic masses as they lack specific US features^[83,84]. The use of duplex US with color Doppler technology is helpful in assessing portal venous invasion and hepatic parenchymal involvement. In a small series of patients with HCC, duplex US detected portal vein invasion correctly in 86% of patients^[85]. In a larger study, duplex US was 93% sensitive and 99% specific for detecting portal vein involvement^[86]. As the sensitivity and specificity of US are operator-dependent, most patients with suspected CC undergo further imaging modalities to confirm and stage suspected tumors^[87]. The sensitivity of US improves significantly in the presence of elevated serum tumor marker Ca 19-9^[80]. Serum level of Ca 19-9 above 20 U/mL in patients with PSC has been shown to increase the diagnostic sensitivity of abdominal US up to 91%, with specificity of 62%, positive predictive value of 23%, and negative predictive value of 98%^[80].

Computed tomography (CT)

Triple-phase CT scan is widely used to diagnose and stage CC^[88] as it provides valuable information regarding local spread, vascular invasion, lymph node involvement and presence of distant metastases^[89,90]. On CT scans, ICC usually present as hypodense lesions with irregular margins on initial images and a variable degree of delayed venous phase enhancement^[86]. These characteristics have been shown to correlate with prognosis as hyperattenuating CC have a more aggressive behavior^[91]. Other CT findings of ICC include dilatation and thickening of the peripheral intra-hepatic bile ducts and liver capsular retraction^[92]. ECC may be seen as a focal thickening of the ductal wall with various enhancement patterns^[93]. However, in many cases of ECC, visualization of the neoplasms is not definitive because they are too small to be detected. More recent studies^[92,94] have shown that modern contrast-enhanced multidetector row computed tomography was 78.6%-92.3% accurate for the diagnosis of ECC, although there was a strong tendency to underestimate the longitudinal extension of the tumor (77.8%) in comparison with the pathological results of the excised specimens^[95,96]. Four-channel multidetector-row CT has been shown to correctly diagnose hepatic artery invasion with 100% sensitivity and 90% specificity and portal vein invasion with 92.3% sensitivity and 90.2% specificity^[96]. Regular enhanced CT can be extremely useful by showing indirect signs of ECC such as biliary ductal dilatation and hepatic lobar atrophy. Atrophy of one hepatic lobe could be associated with hypertrophy of the opposite lobe, a condition known as the atrophy-hypertrophy complex. This phenomenon is seen when CC obstruct the biliary outflow of a single lobe and invade the ipsilateral portal vein causing compensatory hypertrophy of the opposite hepatic lobe^[97]. The sensitivity of triple-phase helical CT in the detection of HCC is in the range of 90% to 100%^[92,98] and it is even more sensitive in detecting ICC greater than 1 cm in size^[90]. These results show a marked improvement in the diagnostic yield of CT compared to previous reports in which the tumor detection rate was only 60%^[99]. CT is also useful for assessing the vascular and lymph node status of patients affected by CC. In a series of 55 patients with HCC, CT accurately predicted portal vein invasion, arterial invasion, and lymph node involvement in 86%, 93%, and 84% of patients, respectively^[100]. The overall accuracy of CT for determining resectability of CC is in the range of 60% to 85%^[90,100,101]. Recently, CT cholangiography (CTC) has been shown to be a promising modality for delineating the biliary tree. In a large study, CTC was superior to conventional CT or US and equal to endoscopic retrograde cholangiopancreatography (ERCP) for the diagnosis of HCC^[102]. In another smaller study, the sensitivity and specificity of CTC for malignant biliary obstruction were both 94%^[103]. One of the limitations of CTC is that optimal imaging quality depends on the secretory function of the liver^[104]. For patients affected by PSC, the combination of tumor serology (serum level of Ca 19-9 above 20 U/mL) and

contrast-enhanced abdominal CT scan has been shown to improve the diagnostic sensitivity (100%), specificity (38%), positive predictive value (22%) and negative predictive value (100%) of the test^[80].

Magnetic resonance imaging (MRI) and Magnetic resonance cholangiopancreatography (MRCP)

MRI with concurrent MRCP can provide three-dimensional reconstruction of the biliary tree by using magnetic resonance technology^[105]. Multiple studies have demonstrated the utility of MRCP in evaluating patients with CC^[106,107]. MRCP has diagnostic accuracy comparable to invasive cholangiographic techniques such as ERCP or percutaneous transhepatic cholangiography (PTC)^[108-111]. A further advantage of MRCP over invasive cholangiographies is that it does not require biliary instrumentation^[112]. Therefore, MRI along with MRCP is considered the radiological modality of choice for evaluating patients with suspected CC^[113]. MRCP/MRI allows definition of the anatomy and extent of CC within the hepatobiliary system^[108,110,114] vascular invasion, local lymphadenopathy and distant metastases^[108,113,115]. Ideally, MRCP should be performed before decompressing the biliary tree^[86]. ICC appear as a hypointense lesion on T1- and hyperintense on T2-weighted images with pooling of contrast within the tumor on delayed pictures as seen with CT^[116,117]. On MRCP, ECC may appear as extrahepatic lesions with similar signal intensity of ICC on both T1- and T2-weighted images, in addition to proximal biliary dilatation^[106,116]. A meta-analysis of 67 studies (4711 patients) evaluating MRCP performance in patients with suspected biliary diseases showed an overall sensitivity of 88% and specificity of 95%^[107]. In a series of 99 patients with HCC, MRCP accurately determined the longitudinal extension of the tumor in 88% of patients^[118]. In another smaller study, MRCP predicted the extent of biliary ductal involvement in 96% of cases with malignant hilar obstructions^[115]. Regarding surrounding structures, MRI has been shown to have 66% accuracy for detection of lymph node metastases^[119], 78% sensitivity and 91% specificity for portal vein invasion, 58%-73% sensitivity^[120] and 93% specificity for arterial invasion^[121]. In a comparative study the relationship of ICC to the vessels and surrounding organs was more easily evaluated on CT compared to MRI^[89]. For patients affected by PSC and CC, the diagnostic capacity of MRI is enhanced by the presence of serum tumor marker Ca 19-9 above 20 U/mL; as a recent study has shown that the sensitivity of the test in this case was 96%, specificity 37%, positive predictive value 24%, and negative predictive value 98%^[80].

Cholangiography

ERCP and PTC provide dynamic images but require invasive access to the biliary system. Both techniques can detect biliary abnormalities and determine the location and extent of ECC within the biliary tree. The choice between PTC and ERCP is generally dictated

by the availability of local expertise and the anatomical characteristics of the tumor^[69]. In patients with complete biliary obstruction, ERCP often cannot assess the proximal biliary tree while PTC cannot assess the distal extent of the tumor^[33,122].

The sensitivity and specificity of cholangiography range between 75%-85%, and 70%-75%, respectively^[110,116] with accuracy of 95%^[123]. Recent data have shown that in the presence of PSC, the association of an elevated level of serum Ca 19-9 increases the diagnostic utility of ERCP as its sensitivity was 91%, specificity 69%, positive predictive value 42%, and negative predictive value was 96%^[80]. A drawback of these invasive procedures is the risk of complications such as post-ERCP pancreatitis (4%-10%)^[123], bacteriobilia (30%-100%)^[73], bleeding, sepsis, vascular injury and death^[124]. On the other hand, ERCP and PTC have the advantage of providing brush cytology and bioptic specimens that can confirm the diagnosis of CC. The sensitivity of biopsy and brush cytology for diagnosing CC has been low due to the desmoplastic reaction associated with the tumor which is characterized by the presence of few malignant cholangiocytes within an extensive fibrous stroma^[11]. In a large prospective study, the sensitivity of routine cytology varied from 9%-24% and the specificity varied from 61%-100% with a high rate of inter-pathologist variation; the best diagnostic yield was obtained when the pathologists were aware of the patient's clinical condition^[125]. This was recently confirmed by a study from the Mayo Clinic which showed that in patients affected by PSC, the simultaneous presence of an elevated serum tumor marker level (Ca 19-9 above 20 U/mL) increased the sensitivity (50%), specificity (97%), positive predictive value (86%) and negative predictive value (88%) of cytological specimens^[80]. In another study, repeated brushing appeared to be a valuable strategy to improve the sensitivity of cytological analysis up to 44%^[126]. Endoscopic transpapillary forceps biopsies had a diagnostic sensitivity of 52% and a specificity of 100%^[127]. Advanced cytologic techniques, including digitized image analysis (DIA) and fluorescence *in situ* hybridization (FISH), have been recently used to increase the sensitivity of cytology^[128] especially in patients with PSC^[80]. The DIA technique quantitates nuclear DNA *via* special stains to assess the presence of aneuploidy, whereas FISH analysis detects chromosomal polysomy by using fluorescent probes. In a prospective study, DIA increased the sensitivity from 18% to 39% but decreased the specificity from 98% to 77%^[129]. In another comparative study, FISH increased the sensitivity from 15% to 34% compared to routine cytology, with similar specificities (91% for FISH and 98% for routine cytology)^[130]. For patients with PSC, the presence of elevated serum level of Ca 19-9 (above 20 U/mL) increases the diagnostic capacity of DIA and FISH; as a recent study measuring these parameters has shown that their sensitivity was 57% and 86%, specificity 94% and 83%, positive predictive value 89% and 80%, and negative predictive value was 74% and 88%, respectively^[80]. The use of peroral cholangioscopy

(POCS) or choledochoscopy has been shown to improve the diagnostic capacity of ERCP by directing tissue sampling. Fukuda *et al*^[131] reported a sensitivity of 100% and a specificity of 87% for diagnosing the etiology of bile duct strictures by adding POCS to ERCP. At this moment, the availability of POCS is limited to a few centers due to lack of expertise and the high costs of instrumentation. The introduction of new technologies such as SpyGlass[®], a single operator peroral cholangiopancreatography, has eliminated the need for two ERCP operators and has the potential of becoming an important tool to improve the diagnostic capacities of endoscopic techniques, and it is currently under investigation^[132-134].

Endoscopic ultrasound (EUS)

EUS is performed by using high frequency ultrasound probes placed on the endoscope. EUS has the advantage of interrogating tissues and organs in direct proximity to the stomach and duodenum, increasing the ability to detect abnormalities that would not be easily identified by percutaneous approach. In a prospective study of patients with suspected CC, EUS had a diagnostic sensitivity of 79% and specificity of 62%^[111]. This was confirmed in a recent meta-analysis where EUS had sensitivity and specificity of 78% and 84%, respectively^[135]. Two of the most attractive features of EUS are the ability to perform direct-guided fine needle aspirations (FNA) of the tumors in patients with negative cytology or the ability to sample enlarged lymph nodes for preoperative staging^[136-138]. However, caution should be applied in patients with potentially curative CC as this approach has some risk of peritoneal seeding^[65,130]. A recent prospective study evaluated the diagnostic yield of EUS-guided FNA of suspected HCC in potentially operable patients with negative brush cytology. The study showed sensitivity and specificity of 89% and 100%, respectively, and changed the preplanned surgical approach in 61% of patients^[136]. In another prospective study, EUS-guided FNA of suspected CC reported a diagnostic sensitivity of 86%, with a specificity of 100%. In the same study, EUS-guided FNA had a positive impact on the treatment management of 84% of patients^[139].

Intraductal ultrasound (IDUS)

IDUS is performed by using high frequency US probes placed into the common bile duct under ERCP guidance^[140]. Malignant biliary strictures often appear on IDUS as a hypoechoic infiltration of the ductal wall with irregular margins^[141,142]. In a prospective study of 62 patients with biliary strictures, IDUS had a diagnostic sensitivity of 90% and specificity of 93%^[143]. In another study by Stavropoulos *et al*^[144], IDUS increased the diagnostic accuracy of ERCP from 58% to 90% in a series of patients with biliary strictures and no mass detected on CT.

Positron emission tomography (PET)

PET is a non-invasive imaging modality that provides

functional images by detecting radiotracer 18F-fluorodeoxyglucose (FDG) uptake in neoplastic cells^[145]. Currently it is considered a standard modality for the staging of many malignancies^[146]. In the last decade, integrated PET and CT imaging systems (PET/CT) have combined the ability to obtain anatomical and functional images^[146,147]. PET and PET/CT are proven to be useful in the diagnosis and staging of CC. In a recent study, PET showed sensitivity and specificity of 90% and 78% respectively^[148]. In another study by Anderson *et al*^[149], PET had sensitivity of 85% for CC measuring at least 1 cm in size although its sensitivity was only 18% for infiltrating CC. These values were confirmed by Kluge *et al*^[150] who reported sensitivity of 92% and specificity of 93% for the detection of any type of CC by PET scan. A more recent study has shown that the sensitivity of PET/CT is correlated with the stage of CC as the sensitivity of the study was 25% for T2 tumors, 70% for T3 tumors and 66.7% for T4 tumors^[151]. The rate of detecting distant metastases by PET and PET/CT in patients with CC is in the range of 70% to 100%, while the detection of regional lymph node metastases is only about 12%^[152]. The sensitivity and specificity of PET/CT for detecting lymph node metastasis and distant metastasis were 41.7% and 80%, and 55.6% and 87.5%, respectively^[146]. Another study from the Memorial Sloan Kettering Cancer Center has shown that PET/CT had an overall sensitivity for identifying the primary tumor of 80% (78% for CC and 86% for gallbladder cancer) and changed management in nearly a quarter of all patients^[153]. PET has been shown to be useful in monitoring tumor response to treatment. In a small series by Chikamoto *et al*^[154], PET had a sensitivity of 80% for detecting local recurrence after resection in patients with HCC. One of the limitations of PET is that patients with biliary inflammatory conditions such as PSC or cholangitis may have false positive results^[152,155] while patients with mucinous CC may have falsely negative scans due to poor uptake of FDG^[155].

Optical coherence tomography (OCT)

OCT is a new technique that produces cross-sectional images using infrared light. Preliminary studies have demonstrated the ability of OCT to generate high resolution images of the biliary tree that correlate with histological findings^[156,157]. OCT has the potential to identify early CC^[104] but it is not widely available except in a few centers. Therefore the role of OCT in the diagnostic workup of CC is not yet established.

Non diagnostic work-up

Non-diagnostic cytology or biopsy results should not rule out CC in the presence of appropriate clinical and radiological findings^[73]. In the absence of other explainable causes of biliary strictures, patients should be considered to have CC and treated as such, accepting that 10% to 15% will have benign lesions on final pathology^[158,159]. For high risk patients, no surveillance or screening programs have been validated. Some authors

advocate annual follow up with non-invasive modalities (tumor markers and radiological tests), reserving invasive methods only when cytology and bioptic specimens or stenting are indicated^[160].

TUMOR SPREAD

Understanding the patterns of spread of CC is essential for staging and treatment planning. CC can spread along biliary ducts, invade perineural and vascular tissues, spread directly into adjacent structures, invade lymph nodes or develop distant metastasis. Longitudinal extension of CC consists of mucosal (superficial) or submucosal (invasive) infiltration depending on the tumor growth pattern. Mucosal extension is predominantly seen with papillary (intraductal) and nodular (mass-forming) tumors, while submucosal extension is mainly seen with sclerosing (infiltrating) tumors^[161]. The length of longitudinal extension is determined by the type of invasion, with a mean length of 6-10 mm for the submucosal spread and 10-20 mm for the mucosal spread^[162]. Therefore, a gross surgical margin of more than 1 cm in the infiltrating type and more than 2 cm in the papillary and nodular types is recommended to achieve negative microscopic resection margins. One of the special characteristics of CC is the presence of perineural invasion that is seen in about 75% of cases^[163,164]. Perineural invasion is a prognostic factor for poor survival^[164,165]. In a retrospective review by He *et al*^[164], the 5-year survival rate was 47% for patients without perineural invasion compared to 13% for those with perineural invasion. HCC can spread directly into the hepatic parenchyma and the hepatoduodenal ligament where the proper hepatic artery and the portal vein are in close proximity to the bile duct, while distal ECC may directly infiltrate into the pancreas or the duodenum^[166]. Up to 80% of HCC have extension into the liver parenchyma^[166,167] by direct infiltration or by longitudinal extension along the biliary ducts^[168]. The latter mechanism explains the caudate lobe involvement by HCC and tumors involving the left hepatic duct^[169]. Hence, the practice of partial hepatectomy with caudate lobectomy for the surgical treatment of patients with hilar tumors is associated with improved survival^[168]. Tumors at the biliary confluence involve the portal vein in 30% of cases and often result in hepatic lobar atrophy^[166,167]. The significance of portal vein involvement in patients' survival is controversial. Some studies have shown that tumor invasion of the portal vein has a negative impact^[170,171] while other investigators reported opposite findings^[166]. This is most likely due to the fact that patients with portal vein invasion may tolerate more extensive surgical resections as the contralateral lobe becomes hypertrophied, therefore decreasing the risk of perioperative mortality and enhancing the chances of negative resection margins. Lymph node metastases usually involve the regional hilar nodes and to a lesser extent the para-aortic lymphatic nodes^[172]. The prevalence of lymph node involvement is approximately 45% for all CC with distal ECC having the highest incidence of nodal metastases^[68,172]. Several studies have confirmed

Table 4 AJCC staging of ICC

Stage	Tumor	Node	Metastasis
I	T1	N0	M0
II	T2	N0	M0
III A	T3	N0	M0
III B	T4	N0	M0
III C	Any T	N1	M0
IV	Any T	Any N	M1

T1: Solitary tumor without vascular invasion; T2: Solitary tumor with vascular invasion or multiple tumors none > 5 cm; T3: Multiple tumors > 5 cm or tumor involving a major branch of the portal or hepatic vein(s); T4: Tumor(s) with direct invasion of adjacent organs other than the gallbladder or with perforation of visceral peritoneum; N0: No regional lymph node metastasis; N1: Regional lymph node metastasis; M0: No distant metastasis; M1: Distant metastasis. AJCC: American Joint Committee on Cancer; ICC: Intrahepatic cholangiocarcinoma.

that lymphatic tumor involvement is an important prognostic factor. Survival rates for patients undergoing surgical resection with positive lymphatic invasion at 5 years are 10% to 15% in comparison to 30% to 40% for patients without lymph node metastasis^[164,172]. Presence of distant metastases (e.g. lung, bone, peritoneal, distant lymph nodes) is observed in 30% of patients at the time of diagnosis and is associated with survival of only a few months^[166].

STAGING

ICC and ECC are staged differently.

ICC

ICC are classified as primary liver malignancies in the new American Joint Committee on Cancer (AJCC) staging system, also known as the TNM staging (Table 4)^[173]. The AJCC staging system for primary liver tumors was based on data provided by patients affected by hepatocellular carcinomas and therefore is not sufficiently accurate for ICC^[174]. A new staging system for ICC was proposed by Nathan *et al*^[174] based on the number of tumors, vascular invasion, lymph node status and presence of metastatic disease. The presence of multiple tumors may be indicative of satellite neoplastic deposits or intrahepatic metastatic disease from hematogenous or lymphatic spread; similarly to vascular and lymph node invasion, it is associated with poor survival.

ECC

Giving the proximity of ECCs to the portal vein and hepatic artery, the goal of staging is to determine the local extent of the disease as it predicts resectability and the extent of the resection. The AJCC staging system for ECC (Table 5)^[173] is based on pathological data useful in identifying the patients' prognosis but with little applicability for assessing the feasibility of surgical treatment^[174]. Bismuth-Corlette classification for HCC is useful for describing the tumor location and its spread within the biliary tree but it is not predictive of resectability. The Memorial Sloan-Kettering Cancer

Table 5 AJCC staging of ECC

Stage	Tumor	Node	Metastasis
0	Tis	N0	M0
I A	T1	N0	M0
I B	T2	N0	M0
II A	T3	N0	M0
II B	T1-T3	N1	M0
III	T4	Any N	M0
IV	Any T	Any N	M1

Tis: Carcinoma *in situ*; T1: Tumor confined to the bile duct histologically; T2: Tumor invades beyond the wall of the bile duct; T3: Tumor invades the liver, gallbladder, pancreas, and/or unilateral branches of the portal vein (right or left) or hepatic artery (right or left); T4: Tumor invades any of the following: main portal vein or its branches bilaterally, common hepatic artery, or other adjacent structures, such as the colon, stomach, duodenum, or abdominal wall; N0: No regional lymph node metastasis; N1: Regional lymph node metastasis; M0: No distant metastasis; M1: Distant metastasis.

Table 6 Proposed T-Stage criteria for hilar cholangiocarcinomas (MSKCC)

Stage	Criteria
T1	Tumor involving biliary confluence with or without unilateral extension to second-order biliary radicles
T2	Tumor involving biliary confluence with or without unilateral extension to second-order biliary radicles and ipsilateral portal vein involvement with or without ipsilateral hepatic lobar atrophy
T3	Tumor involving biliary confluence with bilateral extension to second-order biliary radicles; or unilateral extension to second-order biliary radicles with contralateral portal vein involvement; or unilateral extension to second-order biliary radicles with contralateral hepatic lobar atrophy; or main or bilateral portal vein involvement

Center (MSKCC) has proposed a staging system known as T-stage criteria^[174]. The MSKCC staging system is based on the location and extent of ductal involvement, presence or absence of portal vein invasion, and presence or absence of hepatic lobar atrophy irrespective of metastases or lymph node status (Table 6). The MSKCC staging system for HCC correlates with resectability and survival, as 59% of T1 lesions are resectable with median survival of 20 mo compared to 0% resectability for T3 lesions with a median survival of only 8 mo^[174].

THERAPY

Surgical resection

Tumor resection is the only potential cure for CC and the median survival of patients with unresectable disease is 6 to 12 mo^[26]. All patients with resectable ICC and HCC, and the majority of patients with ECC, require partial hepatectomy to increase the chances of negative resection margins. Preoperative patients' evaluation includes an extensive assessment of their fitness for major surgery, the absence of any metastatic disease and the possibility of resection margins free from cancer^[175]. If any of these conditions are not satisfied, surgical therapy is not indicated and palliative modalities should be recommended.

Table 7 Criteria for unresectability of HCC

Local tumor invasion
Bilateral hepatic duct involvement up to secondary biliary radicles
Encasement or occlusion of the main portal vein
Unilateral tumor extension to secondary biliary radicles with contralateral portal vein or hepatic artery encasement or occlusion
Hepatic lobar atrophy with contralateral portal vein or hepatic artery encasement or occlusion
Hepatic lobar atrophy with contralateral tumor extension to secondary biliary radicles
Metastatic disease
Lymph node metastases beyond the hepatoduodenal ligament (N2 lymph nodes) ¹
Distant metastasis (e.g. lung, liver, peritoneal)

¹Peripancreatic, periduodenal, periportal, celiac, or superior mesenteric lymph nodes.

Preoperative patient preparation

Many patients are not considered surgical candidates because of the presence of comorbidities or advanced age. A patient's performance status, nutritional conditions, and comorbidities need to be carefully evaluated before considering surgery^[176]. A retrospective review of patients with resected HCC showed that the presence of preoperative serum albumin less than 3 g/dL was a significant predictive factor for high postoperative mortality^[177]. In the same study, a preoperative serum total bilirubin above 10 mg/dL was associated with lower survival rates^[177]. The role of preoperative biliary drainage (PBD) in jaundiced patients remains controversial. A recent meta-analysis failed to demonstrate any benefit^[178]. Furthermore, PBD seems to increase the risk of perioperative infections and a longer postoperative hospital stay^[124,178].

Nevertheless, prolonged preoperative biliary obstruction is associated with increased postoperative morbidity and mortality after hepatic resection due to the presence of severe cholestatic liver dysfunction^[177,179]. Currently, preoperative PBD is not routinely recommended, but it has been shown to be beneficial in the presence of cholangitis, severe malnutrition, coagulation abnormalities^[180,181] and when patients require major hepatic resection^[175,182]. When preoperative drainage is performed, definitive surgery should be deferred for a few weeks to allow sufficient restoration of hepatic function^[168]. The use of liver volumetric and/or hepatic functional studies is warranted when anticipating an extended hepatic resection, to estimate the future liver remnant and minimize the risk of liver failure caused by insufficient function or small residual liver parenchyma.

Assessment of resectability

Meticulous interpretation of all the available clinical and radiological data is recommended to determine resectability and avoid unnecessary interventions. Despite the improvement of diagnostic modalities, about 16%-25% of patients are found to have more extensive disease preventing resection at the time of laparotomy^[68,183]. The major determinants of resectability

Table 8 Survival rates after resection of ICC

Author (yr)	Resections (n)	Overall 5-year survival (%)	R0 5-year survival (%)
DeOliveira <i>et al</i> ^[67] , 2007	34	40	63
Miwa <i>et al</i> ^[195] , 2006	41	29	36
Jan <i>et al</i> ^[196] , 2005	81	15	NR
Ohtsuka <i>et al</i> ^[197] , 2003	50	23	NR
Uenishi <i>et al</i> ^[198] , 2001	28	27	67
Inoue <i>et al</i> ^[199] , 2000	52	36	55
Yamamoto <i>et al</i> ^[200] , 1999	83	23	53
Madariaga <i>et al</i> ^[201] , 1998	34	35	41

NR: Not reported.

are the extent of tumor within the biliary tree, the amount of hepatic parenchyma involved, vascular invasion, hepatic lobar atrophy, and metastatic disease^[166,179]. A review of 294 cases of CC demonstrated that resectability rates are higher for more distal tumors^[69]. The determination of resectability is most challenging in patients with HCC. It is reported that about half of patients with HCC deemed to be resectable preoperatively have unresectable disease when explored^[174]. The radiological criteria defining unresectability in patients with HCC are listed in Table 7^[174,175]. With regard to distal ECC and ICC, AJCC stages III and IV are generally considered unresectable (Table 8).

Generally, invasion of the main portal vein or invasion of the vasculature supplying the planned hepatic remnant preclude resection. Nevertheless, recent reports have shown that en-bloc resection with vascular reconstruction may achieve negative margins and potential cure with only 10% perioperative mortality in very selected patients^[184,185]. The application of staging laparoscopy has been recently advocated as it can reduce the number of unnecessary laparotomies by identifying metastatic lesions in the liver and in the peritoneal cavity^[186]. The yield of laparoscopy for detecting unresectability in patients with potentially resectable CC on preoperative imaging modalities is about 25% with an overall accuracy of 50%^[187,188]. Moreover, laparoscopy offers the addition of intraoperative hepatic US, which can increase the diagnostic yield up to 42%^[189]. One of the limitations of laparoscopy is the inability to detect vascular or nodal involvement^[188,189]. Peritoneal washings to obtain cytology specimens have not been shown to predict occult metastasis in patients with CC^[190]. Ultimately, true resectability is determined after a complete abdominal exploration.

Operative procedures and survival

The goal of surgery is to obtain complete excision of the tumor with negative histological margins (R0 resection), as this is associated with marked survival advantages compared to margin positive resections (R1 or R2 resection)^[26,68,176,191]. To confirm histologically-negative margins, many authors advocate the use of intraoperative frozen section examinations of the bile ducts^[174]. A very important study from the MSKCC has recently evaluated the clinical significance of intraoperative frozen section for patients affected by HCC^[192]. The primary aim of

Table 9 Survival rates after resection of HCC

Author (yr)	Resections (n)	Liver resection (%)	R0 resection (%)	Overall 5-year survival (%)	R0 5-year survival (%)
Hasegawa <i>et al</i> ^[213] , 2007	49	92	78	40	50
DeOliveira <i>et al</i> ^[67] , 2007	173	20	19	10	30
Dinant <i>et al</i> ^[214] , 2006	99	38	31	27	33
Hemming <i>et al</i> ^[205] , 2005	53	98	80	35	45
Rea <i>et al</i> ^[208] , 2004	46	100	80	26	30
Kawasaki <i>et al</i> ^[182] , 2003 ¹	79	96	68	NR	40
Kawarada ^[215] , 2002	87	75	64	26	NR
Jarnagin <i>et al</i> ^[166] , 2001	80	78	78	37	NR
Tabata <i>et al</i> ^[216] , 2000	75	71	60	23	40
Kosuge <i>et al</i> ^[217] , 1999	65	88	52	35	52
Miyazaki <i>et al</i> ^[218] , 1998	76	86	71	26	40

¹Five-year survival for patients with R1 resection is 6%; NR: Not reported.

this study was to assess the importance of obtaining frozen sections of the bile duct margins for the planning of the extent of the surgical dissection. Frozen sections were obtained in 101 patients: among them 20 (19.8%) had positive and 81 (80.1%) had negative results. Among the patients who had negative frozen sections, 8 (9.8%) individuals were found to have positive margins at subsequent histopathology. In this study, intraoperative frozen section was shown to be 71.4% sensitive, 100% specific, and with a positive predictive value of 100% and negative predictive value of 80.2%^[192].

ICC

Surgical therapy for ICC is based on the same principles used for hepatic resections performed for hepatocellular carcinomas or secondary tumors. The operative approach should be aimed at ensuring R0 resection margins whenever it is possible. Lymph node dissection during resection of ICC is not recommended as it does not improve patients' survival^[193,194]. Current outcomes after surgical resection have improved in comparison to historical data with 5-year survival rates ranging from 20% to 40% (Table 9)^[195-201]. Predictors of poor outcomes include: positive resection margins, lymphatic and vascular invasion and periductal infiltrating disease^[202,203]. The most common site of recurrence after surgical resection is within the liver^[196].

HCC

Curative surgery of HCC usually requires the excision of the extrahepatic bile duct, regional lymphadenectomy, cholecystectomy and in most cases some sort of partial hepatectomy including the caudate lobe, especially for tumors mainly extending in the left hepatic duct^[174,207]. The rationale behind performing partial hepatectomies in HCC is to ensure histologically negative margins. Several studies have shown that this strategy increases R0 resections in up to 80% of patients^[174,182,205]. Extended lymphadenectomy is not recommended as there is no evidence showing survival advantage^[168,172].

Radical resection of HCC has 5%-10% perioperative mortality rate, especially when extended hepatectomy (5 or more segments) is required^[174,206-208]. This partly

Table 10 Survival rates after resection of distal ECC

Author (yr)	Resections (n)	Overall 5-year survival (%)	R0 5-year survival (%)
DeOliveira <i>et al</i> ^[67] , 2007	229	23	27
Cheng <i>et al</i> ^[219] , 2007	112	25	26
Murakami <i>et al</i> ^[224] , 2007	36	50	62
Yoshida <i>et al</i> ^[225] , 2002	26	37	44
Fong <i>et al</i> ^[222] , 1996	45	27	54 ¹

¹Patients had node negative tumors as well.

relates to the increased rate of postoperative liver failure with major hepatic resections. Portal vein embolization (PVE) is a valuable preoperative measure when anticipating extensive liver resections with subsequent small hepatic residual volume^[209]. A compensatory hypertrophy of the remnant hepatic parenchyma is induced by selectively occluding the main portal vein branch to the lobe that will be resected. This can increase the volume of the anticipated liver remnant by 12%-20%, thereby reducing the rate of postoperative liver dysfunction^[210,211]. PVE is useful when the anticipated liver remnant volume is less than 20%-25% of the total liver volume in patients with normal liver function, and when the anticipated liver remnant volume is 40% or less in patients with compromised liver function^[212].

The average 5-year survival rates post-resection for HCC are 25%-40% (Table 10)^[68,174,182,209,213-218]. Factors associated with favorable outcome include; R0 resection, no lymph node metastasis, absence of perineural invasion, and well-differentiated histological grade^[174,185].

ECC

The same principle of achieving a negative resection margin applies to ECC. The resectability rate has been reported as being up to 90% with distal extrahepatic tumors^[68,219]. Complete removal of distal ECC usually requires a pancreaticoduodenectomy (Whipple procedure)^[73,220,221]. Even in these circumstances, extended lymphadenectomy is not justified as it does not provide survival advantages and it is associated with increased perioperative morbidity^[222]. Segmental bile duct excision is rarely an option, except for CC located in the middle of the common duct in the absence of periductal invasion or spread to the surrounding structures. Only 10% of patients undergoing bile duct excision alone obtain curative resection margins on final pathology^[222,223]. Most commonly, when approaching patients with CC arising midway along the extrahepatic duct, surgeons should assess whether a pancreaticoduodenectomy or a partial hepatectomy is more appropriate with regard to the tumor extension. In these patients, curative resections are associated with a 25%-50% 5-year survival rate (Table 10)^[68,220,223-225]. The main determinants of poor outcomes are positive surgical margins and lymph node involvement^[220,223]. Other factors associated with unfavorable prognosis include pancreatic invasion, duodenal invasion, perineural invasion, and a poorly-differentiated histology^[68,220].

Liver transplantation

Transplantation is an emerging therapy for unresectable CC without evidence of metastatic disease. Candidates are individuals who would require a total hepatectomy to achieve clear margins and those with underlying liver failure precluding hepatic resection. The early experience of transplantation for CC reported early recurrence rates of more than 50% and a 5-year survival of 10%-20%^[226-228]. More recently, in highly selected patients undergoing neoadjuvant protocols, promising results have been reported. In 2002, Sudan *et al*^[229] reported a series of 11 patients transplanted for CC after neoadjuvant chemoradiation with 45% tumor free survival and median follow up of 7.5 years. Similar reports have been reported by Becker *et al*^[230] who observed a 45% 5-year survival for patients who were diagnosed as being affected by CC prior to undergoing transplantation, and a 33% 5-year survival was observed by Sotiropoulos *et al*^[231] in Germany. At the Mayo Clinic, Rosen *et al*^[232,233] have developed a liver transplantation protocol for HCC that provides a disease-free 5-year survival of 82%. This protocol is aimed at treating unresectable HCC or CC in PSC patients. To be eligible for this protocol, the diagnosis of CC is confirmed histologically, considered unresectable and with no evidence of metastatic disease. Eligible patients receive neoadjuvant chemoradiation therapy followed by staging laparotomy to rule out metastatic disease followed by living-related or cadaveric liver transplantation. Currently, the use of liver transplantation for the treatment of CC is reserved only for highly selected patients in specialized centers.

ADJUVANT THERAPY

The use of postoperative chemotherapy, radiotherapy or chemoradiation therapy have been evaluated as means of improving disease-free survival in patients with resected tumors since CC have high rates of local and distant recurrence.

Adjuvant chemotherapy

Postoperative chemotherapy has failed to show significant survival benefits^[234,235]. A recent multicenter RCT evaluated the effect of postoperative chemotherapy with mitomycin C and 5-fluorouracil (5FU) versus surgery alone for individuals affected by cancers of the pancreas and biliary system^[236]. Among 508 patients post-R0 resection, 139 individuals were affected by CC and for these individuals no survival benefit was seen after chemotherapy treatment^[237].

Adjuvant radiotherapy

The use of postoperative external beam radiation with or without intraoperative radiotherapy and intraluminal radiotherapy (brachytherapy) has been explored in the adjuvant setting without significant benefits after R0 resections^[125,237,238]. On the other hand, several studies showed that adjuvant radiotherapy may benefit patients with positive resection margins^[239-241]. Todoroki *et al*^[241] showed that the 5-year survival in patients with

R1 resections was 34% when adjuvant radiotherapy (intraoperative and external beam) was used compared to 14% with surgery alone.

Adjuvant chemoradiation therapy

The radiosensitizing effect of chemotherapeutic agents has been evaluated in the adjuvant setting with positive results only for distal ECC. In a retrospective cohort study of 94 individuals who underwent resection for CC, 34% received postoperative chemoradiation^[242]. Longer survival was seen in patients who received adjuvant therapy (median survival 41 mo *vs* 24 mo)^[243]. Other retrospective studies demonstrated similar results and showed that patients with distal ECC had a superior survival advantage in comparison to more proximal CC following adjuvant therapy^[243,244]. Recently, Hughes *et al*^[245] have confirmed a slight 5-year survival advantage with postoperative chemoradiation therapy in patients with distal ECC compared with surgical resection alone (35% *vs* 27%). In line with these findings, Figueras *et al*^[246] did not demonstrate a significant survival benefit with adjuvant chemoradiation therapy for HCC. These results need to be confirmed further with larger prospective trials. For ICC, evidence to support the use of adjuvant chemoradiation therapy is very limited. In a recent retrospective study of 3839 patients, Shinohara *et al*^[247] have shown that the overall survival rate was significantly different between groups receiving surgery alone and surgery plus adjuvant radiation therapy ($P = 0.014$) and between radiation therapy alone and no treatment ($P < 0.0001$). The combination of surgery and adjuvant radiation therapy conferred the greatest benefit on overall survival (HR: 0.40; 95% CI: 0.3-0.47), followed by surgery alone (HR: 0.49; 95% CI: 0.44-0.54) and radiation therapy alone (HR: 0.68; 95% CI: 0.59-0.77) compared with no treatment.

There is a lack of RCT evaluating the utility of adjuvant therapy following R0 resections of CC. Moreover, most of the current studies are small and retrospective in nature and incorporated CC with cancers of the gallbladder and pancreas. Therefore, no standard adjuvant modalities are universally embraced for the treatment of CC.

Neoadjuvant therapy

The role of preoperative chemoradiation therapy has been evaluated in a small series of patients with ECC^[248]. Among nine patients who underwent neoadjuvant therapy, McMasters *et al*^[248] observed pathological complete response in 3 individuals and negative resection margins were obtained in all subjects. More recently, neoadjuvant therapy has been used in the setting of liver transplantation for CC with promising results. Further trials are required to better assess its efficacy.

PALLIATION

Nearly half of the patients with CC are considered candidates only for palliative treatments due to the advanced stage of their disease at the time of diagnosis or the

presence of significant comorbidities that prevent surgical therapy^[68,174]. Therefore, palliation plays an important role in the management of these individuals. The primary aim of palliative interventions is to improve quality of life by relieving symptoms and prolonging survival by preventing cholestatic liver failure. In the presence of incurable CC, tissue diagnosis should be obtained whenever possible to direct palliative therapy planning.

Biliary drainage

Biliary obstruction is the major cause of morbidity and mortality in patients with CC. The goals of biliary decompression are to relieve jaundice, pain, pruritus, and to prevent cholangitis and cholestatic liver failure^[249]. Different modalities are currently available to drain the biliary system and these include: endoscopic, percutaneous and surgical bypass. The ideal palliative biliary decompression should be effective, provide durable results, and have low risks of morbidity and mortality.

Biliary endoprosthesis (stenting)

Biliary stenting can be achieved endoscopically or percutaneously. Endoscopic biliary stenting is the most widely used method and the percutaneous approach is usually performed when endoscopic drainage fails or cannot be performed. Percutaneous stents can be either internal, external or both. External stents have the disadvantage of draining bile without the ability of enteric recycling and are associated with more discomfort and reduced quality of life. Little is known as to whether percutaneous or endoscopic biliary drainage have different overall efficacy in palliating patients with unresectable disease. Generally, only patients with advanced tumors that are totally obstructed are candidates for percutaneous external biliary drainage. A recent multicenter retrospective study from South Korea has shown that the placement of percutaneous self-expanding metallic stents across HCCs is associated with a higher success rate and lower risk of procedure-induced cholangitis^[250].

Endoscopic stents can be either self-expanding metallic or plastic (polyethylene). Metal stents are more expensive than plastic stents but have larger diameters and provide better patency rates^[251]. Metal stents can be either uncovered or covered by sealing the metallic mesh with a membrane which prevents tumor growth through the stent, increasing patency rates. Plastic stents often need to be changed at 2 to 3-mo intervals, while metal stents can remain patent up to 9 mo^[252]. Several RCT have compared metal to plastic stents for the treatment of patients with inoperable malignant biliary obstruction^[253,254]. These studies concluded that metal stents are more cost-effective for patients who are expected to survive more than 5 mo as they need less interventions and shorter hospitalizations^[254,255]. Patency rates are generally higher for ECC^[255] and metal stents provide superior palliation for HCC as compared to plastic stents^[256,257]. Draining about 25% of the hepatic parenchyma is usually sufficient for adequate palliation in the absence of infection^[250]. A RCT comparing unilateral versus bilateral drainage in patients with malignant hilar obstruction found that

drainage of one functional hepatic lobe is sufficient to relieve obstruction with no difference in complication and survival rates^[258]. It is important to note that stents placed for hilar lesions will require re-intervention in about 30% of patients due to stent occlusion^[259,260]. A RCT comparing covered to uncovered stents in patients with unresectable distal biliary malignancies showed that the patency of covered stents was significantly higher than that of uncovered stents^[261]. However, multiple studies report an increased risk of cholecystitis (5%) with the covered stents due to cystic duct occlusion^[262].

Surgical biliary drainage

Biliary-enteric anastomosis can be performed by open or laparoscopic approach. Studies comparing surgical to non-surgical biliary drainage showed similar overall palliative effects but with higher perioperative morbidity and mortality^[263,264]. Surgical drainage has the advantage of superior patency rates and prevents the need for stent exchanges required when using endoscopic or percutaneous stents due to clogging^[265]. Currently, the main candidates for surgical drainage are patients found to have unresectable CC at the time of exploration, individuals who are not able to undergo repeat endoscopic or percutaneous stent exchanges, and those who have long expected survival and who are fit for surgery^[183,266].

Palliative radiotherapy

Palliative radiotherapy may benefit patients with locally advanced unresectable CC or those who have undergone palliative bypass in the absence of distant metastases. The use of palliative radiotherapy has beneficial effects on pain relief, biliary patency and overall patient survival^[267,268]. The two most commonly used radiotherapy modalities are external beam radiation with 30 to 50 Gy, intraluminal brachytherapy with 10 to 20 Gy or the combination of both. Intraluminal brachytherapy is delivered by using iridium-192 seeds mounted on a catheter that is deployed across the tumor by endoscopic or percutaneous approach^[269]. It appears that brachytherapy is able to deliver more effective doses of radiation without damaging the surrounding organs. Generally, the majority of studies that demonstrated benefit of radiotherapy used combinations of both modalities with median patient survival ranging between 9 and 14 mo^[270-273]. Palliative radiotherapy is associated with increased incidence of complications such as cholangitis, gastroduodenitis and longer hospital stay in comparison to best supportive care and therefore it is not routinely used in many centers^[274]. Moreover, higher doses of radiation (more than 55 Gy) may be required to obtain an improved survival, with increased toxicity rates^[275]. Controlled studies are required to better evaluate the effectiveness and safety of these palliative treatments. For ICC, brachytherapy can be delivered by radioembolization with yttrium-90 microspheres^[276]. This approach has been shown to provide partial response in 27% of patients and stable disease in 68% with limited side effects; therefore it is not surprising that it has become the leading modality for palliation of CC in centers where this technique is available^[277].

Palliative chemotherapy

There is no standard chemotherapy option for patients with CC. Patients with widespread disease considered for palliative chemotherapy undergo treatment in an attempt to control the disease and improve their overall survival. Owing to the lack of RCT and the retrospective nature of observational studies with heterogeneous patient populations currently available, the interpretation of the survival benefit of palliative chemotherapy is difficult. Various chemotherapeutic agents with different dosing regimens have been tested with overall poor survival improvement. Historically, 5FU was the first chemotherapeutic agent used for palliation of CC patients with only 10% response rates when used alone. Several subsequent studies have evaluated 5FU in combination with other agents such as leucovorin, interferon-alpha, cisplatin, and oxaliplatin, with an overall response rate of 25% to 55% and a median survival ranging between 6 and 12 mo^[278-281]. Multiple phase II trials have evaluated the use of oral 5FU prodrugs (uracil-tegafur and capecitabine) in patients with advanced CC^[282,283]. For example, the combination of capecitabine and cisplatin had mild toxicity and produced a response in 41% of patients with a median survival of 12 mo^[283]. Gemcitabine proved to have good efficacy as a single agent in biliary malignancies with response rates of 30%^[284]. Several gemcitabine-based combinations, including cisplatin, capecitabine, and oxaliplatin have reported response rates up to 36% and median survival of 10 to 15 mo^[285-287]. Finally, a recent analysis of all the published chemotherapy trials in individuals affected by advanced CC from 1985 to 2006 concluded that gemcitabine combined with platinum compounds (cisplatin or oxaliplatin) had the best patient response rates^[288,289]. Recently, the roles of transcatheter arterial chemoembolization (TACE) and transcatheter arterial chemoinfusion (TACI) have been assessed for patients affected by unresectable ICC^[290,291]. Although TACE and TACI with gemcitabine, cisplatin and doxorubicin in different combinations^[292] are well tolerated, survival benefits have not been proven in large studies and will require further evidence before becoming widely accepted in the scientific community.

Photodynamic therapy (PDT)

PDT is an emerging palliative strategy based on the intravenous administration of photosensitizing agents that preferentially accumulate in malignant cells. After the delivery of these photosensitizing agents, specific wavelengths of light are administered causing activation of the photosensitizer and thus tumor cell necrosis^[293]. The depth of tumor necrosis obtained by this technique is between 4 mm and 6 mm^[294]. This modality is currently used as a palliative measure in conjunction with biliary stenting for nonresectable CCs. Improvements in quality of life, biliary drainage, and survival in patients with advanced CCs post-PDT have been reported in several case series^[294]. Furthermore, a RCT compared PDT with endoscopic stenting to stenting alone in patients with unresectable CC^[295]. The

study was terminated prematurely because PDT proved to be markedly superior to simple stenting. The PDT group in that trial had higher median survival (493 d *vs* 98 d), improved biliary drainage and better quality of life than the stenting alone group^[296]. Recently, PDT was investigated as a neoadjuvant modality before surgical resection of advanced HCC in 7 patients^[296]. Tumor-free resection margins were achieved in all patients with a 1-year recurrence-free survival rate of 83%^[297]. A recent study confirmed that the use of PDT as a neoadjuvant therapy is safe and can downstage tumors from unresectable to resectable^[297]. The main side effects of PDT include photosensitivity caused by the administration of photosensitizer agents and cholangitis related to biliary instrumentation^[294,298].

Other palliative measures: Several other palliative modalities have shown some benefits in selected groups of patients. Radiofrequency ablation has been used for patients unfit for surgery who have small intrahepatic CC^[299]. In a single-centre cohort of patients with unresectable intrahepatic CC, TACE has shown some survival advantage in comparison to best supportive care (median survival: 23 mo)^[300]. Hepatic arterial chemoinfusion offers tumor-directed chemotherapy and it has been proven to be safe^[301] as has localized ablation of tumor cells by high intensity intraductal ultrasound^[302]. Another promising area is the use of molecular targeting agents for chemoprevention and adjuvant therapy of CC such as cyclooxygenase-2 and nitric oxide inhibitors^[303]. The clinical utility of these emerging therapies needs further investigation before gaining wide acceptance.

CONCLUSION

Over the last decades several advances have occurred in the fields of epidemiology, diagnostic modalities, medical and surgical treatment of CC as well as in palliation. The diagnosis, staging and further management of patients affected by this disease may be a complex issue and requires expertise in many fields. To optimize the outcome of patients with suspected or proven CC, a multidisciplinary approach is recommended.

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S- Editor Tian L L- Editor Logan S E- Editor Lin YP

Emerging treatments for complex perianal fistula in Crohn's disease

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Received: April 12, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: September 14, 2009

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Taxonera C, Schwartz DA, García-Olmo D. Emerging treatments for complex perianal fistula in Crohn's disease. *World J Gastroenterol* 2009; 15(34): 4263-4272 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4263.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4263>

INTRODUCTION

Crohn's disease is a chronic inflammatory disease of the intestine of unknown etiology. It is characterized by focal or segmental transmural inflammation which can occur in any part of the digestive tract with occasional granuloma formation. This transmural inflammation disrupts intestinal mucosal integrity, favoring the development of abscesses and fistulas. When fistulas form, they can track between intestinal segments or between an intestinal segment and other organs (bladder, vagina), adjacent tissue or the skin. Fistulas are classified as internal when they communicate with adjacent organs (e.g. entero-enteric and rectovaginal fistulas) and external when they communicate with the dermal surface (e.g. enterocutaneous, peristomal and perianal fistulas).

The cumulative incidence of perianal fistulas in Crohn's disease varies between 20% and 25% in population studies^[1-3]. Perianal disease is associated with high morbidity and, typically, with local pain and discharge; it therefore has a very negative impact on the quality of life of the affected patients. In Crohn's patients, perianal disease is more common when the colon is affected and particularly when the rectum is involved^[2].

The ideal therapeutic goal in perianal fistulizing Crohn's disease is complete and sustained closure of the fistulas without the development of abscesses, thereby avoiding the need for surgical interventions, and improving the patients' quality of life. In an appreciable number of patients, complete closure cannot be achieved despite intensive medical treatment (including infliximab) and surgery in accordance with normal practice. Currently the goal of therapy for these patients has shifted from complete fistula closure to reduction in drainage from the fistula tract in order to improve their

Abstract

Complex perianal fistulas have a negative impact on the quality of life of sufferers and should be treated. Correct diagnosis, characterization and classification of the fistulas are essential to optimize treatment. Nevertheless, in the case of patients whose fistulas are associated with Crohn's disease, complete closure is particularly difficult to achieve. Systemic medical treatments (antibiotics, thiopurines and other immunomodulatory agents, and, more recently, anti-tumor necrosis factor- α agents such as infliximab) have been tried with varying degrees of success. Combined medical (including infliximab) and less aggressive surgical therapy (drainage and seton placement) offer the best outcomes in complex Crohn's fistulas while more aggressive surgical procedures such as fistulotomy or fistulectomy may increase the risk of incontinence. This review will focus on emerging novel treatments for perianal disease in Crohn's patients. These include locally applied infliximab or tacrolimus, fistula plugs, instillation of fibrin glue and the use of adult expanded adipose-derived stem cell injection. More well-designed controlled studies are required to confirm the effectiveness of these emerging treatments.

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Key words: Crohn's disease; Perianal fistula; Drug therapy; Topical administration; Infliximab; Adalimumab; Adipose tissue; Stem cells

quality of life. In these patients with complex perianal fistulizing disease that persists despite intensive medical and surgical treatment, a therapeutic gap exists for new treatments which aim for complete and sustained closure of the perianal fistulas.

In this review, after a brief discussion of the diagnosis, characterization, classification, and current systemic treatments of perianal fistulas in patients with Crohn's disease, we shall discuss some of the newer local therapies and their potential applications.

DIAGNOSIS AND CHARACTERIZATION

The starting point for management of perianal fistulas is a complete and accurate diagnosis of the lesions, which requires careful exploration of the anal and perianal region. An inadequate examination which fails to detect occult lesions (abscesses or fistula branches) may result in perianal disease becoming persistent or recurrent. An endoscopic examination is needed to determine the presence of macroscopic inflammation in the rectum and/or rectal stenosis, as such findings are important for the prognosis and treatment of the disease. There is consensus among the American Gastroenterological Association (AGA)^[4,5] and European Crohn's & Colitis Organization (ECCO)^[6] working groups concerning the need to complement the study of perianal disease with other diagnostic tools such as examination under anesthesia (EUA), magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS).

In the hands of expert surgeons, EUA is considered the gold standard against which other techniques are compared. EUA has an accuracy of 90% for diagnosis and classification of fistulas and abscesses^[7]. With this technique, it is possible to perform concomitant surgery of the lesions: incision and drainage of abscesses with seton placement, and other procedures to treat fistulas. MRI has an accuracy of between 76% and 100% for diagnosis and classification of perianal fistulas^[7,8]. With MRI, the surgeon who performs EUA can obtain important additional information in 15%-21% of patients^[7,9]. In view of its harmless nature and the additional information it provides, MRI is the initial diagnostic technique of choice according to the ECCO consensus statement^[6]. EUS offers a diagnostic accuracy of between 56% and 100%, and the findings change the surgical approach in 10%-15% of cases^[7,10], helping to guide medical-surgical treatment of perianal fistulas in Crohn's disease, resulting in a high response rate^[11]. Sometimes, the pain caused by the lesions or stenosis makes EUS difficult.

The combination of either of these imaging techniques (MRI or EUS) with EUA yields a diagnostic accuracy of 100% for perianal disease^[7]. Imaging techniques are essential to provide surgeons with a virtual view which allows them to treat all lesions during the surgical procedure.

CLASSIFICATION OF PERIANAL FISTULAS

A number of classification systems have been proposed

in the past^[12,13], but perhaps the most anatomically accurate is the Parks classification^[14], which takes the external anal sphincter as the central reference point and describes 5 types of perianal fistula: superficial, inter-sphincteric, trans-sphincteric, supra-sphincteric and extra-sphincteric. The Parks classification is however limited in that it does not take into account the presence of abscesses and/or connections with other organs such as the vagina or bladder, even though such information is important for determining the medical and surgical management of the disease.

The AGA technical review proposed a more clinically useful classification system with just 2 categories: simple and complex fistulas^[4,5]. Simple fistulas are low (superficial, low inter-sphincteric or low intra-sphincteric), have a single external opening and are not associated with perianal abscess, connection to the vagina or bladder, rectal stenosis or macroscopic proctitis. In contrast, complex fistulas are high (high inter-sphincteric, high intra-sphincteric, supra-sphincteric or extra-sphincteric) and/or can have several external openings and may be associated with perianal abscess, connection to the vagina or bladder, rectal stenosis or macroscopic proctitis. This classification has greater clinical relevance: simple fistulas respond better to treatment whereas complex ones have lower cure rates with medical treatment and, with this type of fistula, an aggressive surgical procedure will often lead to incontinence.

MEASURES OF HEALING

The Crohn's Disease Activity Index (CDAI) is widely used in Crohn's disease as an outcome measure but it is not designed or able to assess perianal fistulous disease activity, thus the Perianal Disease Activity Index (PDAI) is often used as an equivalent of this index for measurement of morbidity associated with perianal disease^[15]. This index assesses 5 categories related to fistulas: discharge, pain, restriction of sexual activity, type of perianal disease and degree of induration. The advantage of the PDAI is that it assesses aspects of the quality of life that are most affected in patients with perianal disease and that it has been validated in recent clinical studies^[16].

The most widely used instrument for assessing treatment outcomes in clinical trials is the Fistula Drainage Assessment. This measure classifies fistulas as open (i.e. purulent material is expelled with gentle pressure) or closed^[17]. A fistula has to remain closed for 2 consecutive visits (at least 4 wk apart) to be considered in remission. The Fistula Drainage Assessment does not consider changes in anal pain, which is also an important marker of treatment response.

Consideration of complete re-epithelization of the external openings supported by MRI studies could represent a major improvement in the assessment of fistula closure. Long term maintenance of the healing is of great therapeutic relevance. Indeed, the drafted guideline of the European Medicines Agency (EMA)

on “The clinical development of new medicinal products for the treatment of Crohn's Disease (Doc. Ref. CPMP/EWP/2284/99 Rev. 1)” states that “the therapeutic goals of management of fistulizing Crohn's disease are to close fistulas and maintain their closure, to reduce the incidence of infections in persisting fistulas, and to limit the need for surgical interventions. Clinical studies in fistulizing Crohn's disease should reflect this. The primary endpoint of the clinical trials should then be complete closure of fistulas and maintenance of a closed fistula without development of new fistulas.”

An MRI-based activity score was developed to assess the anatomical evolution of perianal fistulas in Crohn's disease^[18]. MRI imaging demonstrates that despite closure of draining external orifices after infliximab treatment, inflammatory changes in the fistula track persist for a long time. It has been suggested that this residual activity may cause recurrent fistulas and pelvic abscesses but a *post hoc* analysis of the ACCENT II study showed that maintenance infliximab therapy does not result in increased abscess development in patients with fistulizing Crohn's disease^[19].

TREATMENT

Crohn's disease cannot be cured by medical or surgical treatment. The aim of therapy is to alleviate symptoms and treat complications of the disease in order to improve the patients' quality of life. The strong negative impact of symptomatic perianal disease on quality of life justifies aggressive treatment to facilitate healing. The spontaneous cure rate for perianal fistulas is very low, ranging from 6% to 13% in the placebo arm of 3 controlled studies^[17,20,21].

Medical treatments

Antibiotics: Bacteria may in theory play a role in the appearance and persistence of perianal fistulous disease. Thus antibiotics are sometimes used as first-line therapy for fistula healing. In other cases antibiotics, in view of their prophylactic effects against infections and abscesses, are used as adjuvant (or bridging) therapy. Most of the studies of perianal fistulizing disease treated with antibiotics are uncontrolled and the sample sizes are small. In these studies, both metronidazole^[22-24], and ciprofloxacin^[25], as well as a combination of the 2 drugs^[26], showed an initial beneficial effect on the perianal fistula. Response typically occurs after 6 to 8 wk of treatment and is usually manifest in the form of decreased drainage. Fistula closure is uncommon and symptoms tend to recur after suspending treatment^[24].

Recently, a small randomized, double-blinded, placebo-controlled study evaluated ciprofloxacin or metronidazole for the treatment of perianal fistulas in patients with Crohn's disease^[27]. Twenty-five patients were randomized to ciprofloxacin 500 mg (10 patients), metronidazole 500 mg (7 patients) or placebo (8 patients) twice daily for 10 wk. Response ($\geq 50\%$ reduction in the number of draining fistulas) at week 10 was seen in 4 patients (40%) treated with ciprofloxacin,

1 patient (14.3%) treated with metronidazole, and 1 patient (12.5%) treated with placebo ($P = 0.43$). One patient from both the ciprofloxacin and placebo arm and 5 (71.4%) treated with metronidazole dropped out of the study ($P < 0.02$). This study was probably too small to detect differences between treatment arms.

In two studies, antibiotics were used as an adjuvant or a bridge to other drugs. The use of metronidazole and/or ciprofloxacin induced a response ($\geq 50\%$ reduction in the number of draining fistulas) at week 8 with fistula closure occurring in 25% of cases^[28]. At week 20, those patients who received additional azathioprine therapy had a better medium-term response (48% *vs* 15%). It should be pointed out that most of the patients in that study had simple fistulas and that only 9 of the 52 cases were classed as complex fistulas. In a placebo-controlled study, all patients received infliximab (3 induction doses at weeks 6, 8 and 12) and were randomized to receive either 500 mg ciprofloxacin twice daily or a placebo for 12 wk^[16]. The response rate (defined as $\geq 50\%$ reduction from baseline in the number of draining fistulas) at week 18 showed a tendency in favor of ciprofloxacin in combination with infliximab compared to infliximab alone (OR: 2.37; 95% CI: 0.94-5.98)^[16].

Thiopurines: Azathioprine and 6-mercaptopurine have shown efficacy in the treatment of Crohn's perianal fistulas. In a meta-analysis of 5 controlled studies, a response (defined as complete closure or decreased drainage) was found in 54% of the patients treated with azathioprine or 6-mercaptopurine compared to 21% in the placebo group (OR: 4.44; 95% CI: 1.50-13.2)^[29]. This meta-analysis is limited in that fistula response was a secondary endpoint and not the primary one in all of the studies included. There have been no controlled trials in which the primary endpoint was assessment of the effect of thiopurines on the closure of fistulas in patients with Crohn's disease.

Anti-tumor necrosis factor (TNF)- α agents: The efficacy of the anti-TNF- α antibody infliximab in fistulizing perianal disease refractory to 3 mo of conventional treatment has been shown in a controlled clinical study^[19]. The most favorable outcomes were obtained at doses of 5 mg/kg body weight and 3 induction infusions at 0, 2 and 6 wk. This regimen achieved complete fistula closure (no drainage in 2 visits 4 wk apart) in 55% of the patients compared to only 13% in the placebo group. The mean time to response was 2 wk and the mean duration of response was 12 wk after the last infusion. The ACCENT II study later confirmed the response rate to induction (69% at week 14) in the open-label extension^[30]. Responders were randomized to infliximab 5 mg/kg body weight or placebo every 8 wk. At week 54, 36% of the patients on infliximab were able to maintain complete closure compared to 19% of those on placebo ($P = 0.009$). Similar results have also been reported in clinical practice in a large uncontrolled series^[31].

Infliximab maintenance treatment has been shown to decrease the use of hospital resources (fewer hospitalizations and less need for surgery) in patients with fistulizing Crohn's disease^[32]. Nevertheless, it has been reported that in perianal disease, early relapse was common after stopping infliximab treatment, with only 34% of patient maintaining remission at 1 year^[33].

Adalimumab, another anti-TNF- α antibody, may also prove effective in perianal Crohn's disease. In the CHARM study, 33% of the patients randomized to adalimumab achieved long term complete fistula closure versus 13% in the placebo group (secondary endpoint; placebo *vs* adalimumab group combined; $P = 0.043$)^[34]. Complete fistula healing was sustained for up to 2 years by most of the patients in the open extension trial ADHERE. In a prospective open-label study in patients with active perianal fistulous disease who stopped responding or developed intolerance to infliximab, adalimumab induction therapy (160 mg at week 0 and 80 mg at week 2) induced complete fistula closure at week 4 in 23% of the cases^[35].

Other immunomodulators: Randomized studies designed specifically to assess the efficacy of cyclosporine in the closure of fistulas in patients with fistulizing Crohn's disease have not been published. However, there are several uncontrolled case series which used continuous cyclosporine infusion in patients who had failed conventional therapy^[36]. Many patients showed an initial response and were switched to oral cyclosporine; however the response was rapidly lost on drug withdrawal.

Uncontrolled case series suggested that tacrolimus may be useful in the treatment of perianal disease^[37,38]. In a small controlled clinical trial, patients treated with tacrolimus (0.2 mg/kg per day) had a higher response rate (defined as closure of at least 50% of fistulas) at week 4 compared to placebo (43% *vs* 8%), but no differences were observed in terms of complete fistula closure (10% *vs* 8%)^[21].

In a retrospective study methotrexate was used in patients with fistulizing Crohn's disease; after 6 mo 44% of the patients had partial or complete fistula closure^[39]. An early case series suggested that the antimetabolite agent mycophenolate mofetil could be effective in Crohn's perianal disease^[40]. In a more recent uncontrolled study from the same group mycophenolate mofetil induced a partial response in 7 out of 8 patients with perianal fistulas, but the response was subsequently lost in 5 of these 7 patients for several reasons including side effects^[41].

In refractory Crohn's disease, small uncontrolled series showed that thalidomide may be effective in treating complex perianal fistulas^[42,43]. Severe side effects, including neuropathy, were common and limited the long term use of the drug. Lenalidomide, an analogue of thalidomide, with lower toxicity and powerful anti-TNF properties was not effective in active luminal Crohn's disease^[44], and has not yet been tested for perianal Crohn's disease.

Miscellaneous therapies: A pilot open-label study provided data suggesting granulocyte colony-stimulating factor (GM-CSF) is a safe and potentially effective agent for the treatment of active perianal Crohn's disease^[45]. GM-CSF has been used in a placebo-controlled study in patients with luminal Crohn's disease, some of whom had draining fistulas at study entry. At 6 mo, 4 out of 8 patients in the GM-CSF group and 2 out of 5 in the placebo group had complete fistula closure^[46].

Octreotide, a somatostatin analogue, may have a role in treating Crohn's enterocutaneous fistulas, but has not been used in perianal disease^[47]. The effect of elemental diet on perianal Crohn's disease has been studied in a small retrospective series. Fistulas improved in some patients but early relapse occurred in almost all the cases^[48]. In a review of 22 patients with active and refractory perianal Crohn's disease treated with hyperbaric oxygen, 73% achieved a response^[49]. In a randomized, placebo-controlled trial oral, spherical adsorptive carbon was effective for the control of perianal fistulas in patients with Crohn's disease (remission rates were 29.6% *vs* 6.7% for placebo)^[50]. There is not sufficient evidence for any of these agents to support their use in patients with Crohn's perianal fistulas outside of clinical trials.

Summary of medical treatment in current guidelines: Despite methodological limitations in the supporting studies, antibiotics and azathioprine or 6-mercaptopurine are considered first-line therapy in complex perianal disease in the ECCO consensus statement^[6], and infliximab is reserved as a second-line treatment in case of failure. In the AGA technical review^[4,5] infliximab is recommended for treatment of complex perianal disease along with azathioprine or 6-mercaptopurine and antibiotics for the induction phase. Maintenance is recommended with azathioprine or 6-mercaptopurine, in association with infliximab in some cases.

Surgical treatment

Surgical treatment of complex perianal fistulizing disease aims to control sepsis through abscess drainage and intervention in the fistula tracts, including placement of non-cutting setons^[51]. Fistulectomy or fistulotomy are rarely indicated in complex fistulas in view of the high rate of proctectomy because of nonhealing or incontinence associated with the procedure^[6,51,52]. In severe cases with high fistulas, endorectal flaps are useful^[51,53]. In patients with severe refractory disease, diversion with ostomy (loop ileostomy or end sigmoid colostomy) or even proctectomy might be necessary.

In an uncontrolled study carbon dioxide laser ablation has been used as an alternative treatment in patients with perianal Crohn's disease^[54].

Combined medical and surgical treatment

The ideal treatment goal for complex perianal fistulas associated with Crohn's disease is the closure of all the fistulas and the prevention of recurrence. The

best outcomes have been achieved in studies using a combination of medical and surgical therapy^[55,56].

Surgery may offer some advantages when combined with medical treatment, for example, infliximab. There is concern, however, that use of infliximab may cause abscesses by inducing rapid closure of the fistulas. This problem might be reduced by performing MRI or EUS-guided EUA to detect all fistula tracks and to insert draining setons. Regueiro *et al.*^[55] observed that patients who underwent an EUA before infliximab administration were significantly less likely to have fistula recurrence compared to those treated with infliximab alone (44% *vs* 79%).

Hyder *et al.*^[57] investigated such a strategy in patients with perianal Crohn's disease. After EUA, 12 out of 22 patients required abscess drainage and 17 out of 22 had at least one drainage seton inserted. The short-term efficacy of that strategy was as high as 85% as measured by the PDAI; although the authors noted that long term healing rates were low. Talbot *et al.*^[58] reported a similar strategy in patients with complex fistulas-setons were inserted and removed after the second infliximab infusion. Complete healing of the perianal fistula was obtained in 47% of the patients and all showed at least a partial response.

The presence of active proctitis has a negative impact on the outcome of the surgical treatment of Crohn's fistulas. A pilot study suggested that infliximab treatment has a beneficial additive effect in the multistep treatment to first improve the proctitis before performing surgery in complex perianal Crohn's disease with active proctitis^[59].

In one recent retrospective study, 21 patients with Crohn's perianal fistulas and symptomatic perianal disease were treated according to a treatment protocol of serial EUS examinations^[60]. Surgical and medical therapy was tailored to the results of the EUS findings with seton placement and incision and drainage procedures performed when appropriate. Follow-up EUS examination guided when to remove setons or when to stop infliximab or antibiotics. Median follow-up was 68 wk (35-101 wk). No abscesses developed in any patient. Eighteen out of 21 patients (86%) had complete drainage cessation initially, and 16 out of 21 (76%) had long term cessation of drainage. Eleven (52%) had no persistent fistula activity on EUS. In 7 of these, the fistula remained closed after stopping infliximab or antibiotics while the remaining 4 continued infliximab for mucosal disease. This study showed that EUS-guided combination surgical and medical treatment with infliximab had high short and long term fistula response rates^[60].

In a recent randomized prospective study, 10 patients with active Crohn's perianal fistulas were randomized to either EUS guidance or control^[61]. All patients underwent an initial EUS. Patients in the EUS cohort were evaluated by a colorectal surgeon who had access to the EUS findings. The surgeon was blinded to the results of the initial EUS for those in the control group. EUA with seton placement or incision and drainage

was done at the surgeon's discretion, and all patients received optimal medical therapy including antibiotics, azathioprine or 6-mercaptopurine and infliximab. Patients in the control group received further therapy at the surgeon's discretion without EUS guidance. Those in the EUS cohort had additional EUS evaluations at weeks 22 and 38 with further therapy based on EUS results. One of 5 (20%) in the control group and 4 of 5 (80%) in the EUS group had complete cessation of drainage. In this small study, EUS guidance for combination medical and surgical therapy in perianal Crohn's disease appeared to improve outcomes.

Local treatments

Fistula healing is not possible in a significant percentage of patients with complex fistulizing Crohn's disease managed according to the currently accepted treatment algorithms^[4-6]. In addition, systemic medical treatments may be subject to intolerance or loss of response and surgical treatments such as fistulotomy should be used with caution given the risk of incontinence. Thus there is a therapeutic gap in the management of perianal Crohn's disease, and a number of local therapies which aim to achieve complete closure are under development. Table 1 summarizes studies of these new local treatments. The following sections will discuss these new local treatments in more detail.

Topical tacrolimus: Topical tacrolimus has been used successfully in the treatment of skin diseases with an immune component such as atopic dermatitis^[62]. Casson *et al.*^[63] therefore decided to investigate whether an in-house-prepared topical formulation could be beneficial in a series of pediatric patients with different manifestations of Crohn's disease including one case of perianal fistula; the patient responded to treatment although details of fistula healing were not presented.

The efficacy of topical tacrolimus in perianal Crohn's disease was recently investigated in a randomized placebo-controlled study^[64]. In that study, 19 patients, 12 of whom had fistulizing perianal Crohn's disease, were randomized to topical tacrolimus (1 mg in 1 g ointment applied twice daily) or placebo for 12 wk. In the case of patients with fistulas, the primary outcome measure was improvement defined as $\geq 50\%$ decrease in actively draining fistulas on 2 consecutive visits. Treatment showed a beneficial effect on anal and perianal ulcerating disease but lacked efficacy in the treatment of fistulizing Crohn's disease.

Fibrin glue: Instilling fibrin glue into fistulas is a simple and safe procedure which does not preclude the use of other techniques or repeat procedures in the case of failure^[65]. Several studies have been published of series of patients treated with fibrin glue and success rates vary from 0% to 80% (Table 1). This variability can be attributed, among other things, to the different types of fistulas treated (simple or complex; cryptoglandular, Crohn's, or traumatic etiology), and the differences in the definition of healing.

Table 1 Summary of studies of local treatments

Intervention reference	Study design and patients	Main findings
Topical tacrolimus		
Casson <i>et al</i> ^[63] , 2000	Case study of series of pediatric Crohn's patients including 1 patient with perianal fistula	Response reported in patient with perianal fistula
Hart <i>et al</i> ^[64] , 2007	Randomized, placebo-controlled study in 19 patients (12 Crohn's)	Treatment found not to be beneficial in perianal fistulas
Fibrin glue		
Abel <i>et al</i> ^[79] , 1993	Uncontrolled study of use of fibrin glue in 10 patients (2 Crohn's)	0/2 patients with Crohn's disease achieved healing
Cintron <i>et al</i> ^[80] , 2000	79 patients (6 Crohn's) assigned to 3 types of fibrin glue treatment	2/6 Crohn's patients (33%) achieved healing (no drainage)
Lindsey <i>et al</i> ^[66] , 2002	Randomized trial comparing fibrin glue with conventional surgery (fistulotomy or loose seton placement) in 42 patients (6 Crohn's and complex perianal fistula)	Healing (no drainage) in 2/6 Crohn's patients (33%) who received fibrin glue. No Crohn's patients received conventional surgery
Sentovich ^[81] , 2003	Uncontrolled study: 48 patients (5 Crohn's) underwent seton placement followed by instillation of fibrin glue	Healing in 4/5 (80%) Crohn's patients
Zmora <i>et al</i> ^[82] , 2003	Retrospective review of 37 patients with perineal fistula (7 Crohn's) treated with fibrin glue	Healing in 3/7 Crohn's patients (43%) (2 patients also treated with endorectal advancement flap)
Loungnath <i>et al</i> ^[83] , 2004	Retrospective review of 42 patients with perianal fistula (13 Crohn's) treated with fibrin glue	Lasting fistula healing in 4/13 (31%)
Singer <i>et al</i> ^[84] , 2005	Randomized trial comparing fibrin glue + antibiotics, fibrin glue + surgery, and fibrin glue + antibiotic and surgery in 75 patients (3 Crohn's)	Treatment failed in all 3 Crohn's patients (fibrin glue + antibiotic in 1 patient and fibrin + antibiotic and surgery in 2 patients)
Intralesional infliximab		
Poggioli <i>et al</i> ^[68] , 2005	Uncontrolled study of 15 Crohn's patients with complex perianal fistulas	Healing in 10/15 patients after 3-12 infusions
Asteria <i>et al</i> ^[69] , 2006	Uncontrolled study of 11 Crohn's patients with complex perianal fistulas naïve to infliximab	8/11 patients responded ($\geq 50\%$ reduction in fistula drainage) to treatment
Adipose-derived stem cell (ASC) therapy with fibrin glue		
García-Olmo <i>et al</i> ^[72] , 2005	Uncontrolled proof-of-concept study in patients with fistulizing Crohn's disease, including 1 perineal fistula	Perineal fistula healed after 8 wk
García-Olmo <i>et al</i> ^[73] , 2009	Randomized controlled phase II study comparing fibrin glue + ASCs with fibrin glue in 49 patients with complex perianal fistula (14 Crohn's)	Healing in 5/7 Crohn's patients (71%) in fibrin glue + ASCs group compared to 1/7 (14%) in the control group
Fistula plugs		
O'Connor <i>et al</i> ^[75] , 2006	Uncontrolled study of fistula plug in 20 Crohn's patients with fistula tracts not amenable to fistulotomy	Success rate of 80%, lower in the case of complex fistulas
Schwandner <i>et al</i> ^[76] , 2008	Uncontrolled study of 19 patients (7 Crohn's) with trans-sphincteric anorectal fistulas	Treatment success in 6/7 patients with Crohn's disease (86%)
Ky <i>et al</i> ^[77] , 2008	Prospective analysis of 45 patients (20 with complex fistulas and 14 with Crohn's disease) receiving anal fistula plug	Healing in 4/14 Crohn's patients (29%) after a median follow-up of 6.5 mo

Only one controlled study with patients with Crohn's disease has compared fibrin glue with surgical treatment not involving fibrin glue. In that study, Lindsey *et al*^[66] randomized patients with simple and complex fistulas to treatment with fibrin glue or conventional treatment (fistulotomy or loose seton placement with or without subsequent flap advancement). For the purposes of the study, complex fistulas were defined as high fistulas, fistulas associated with Crohn's disease, and low fistulas with compromised sphincters. Both Crohn's patients with complex fistulas reported healing, in one case after a second procedure. Healing among Crohn's patients in the other arm was not reported.

Intralesional infliximab: Although systemic infliximab administration is considered one of the more efficacious therapeutic options available for complex perianal fistulas associated with Crohn's disease, several authors have investigated the efficacy of local application of this drug. The main rationale for this approach is to try and avoid the potential systemic toxicity associated with infliximab. The first study to employ this approach was

published by Lichtiger *et al*^[67] in 2001. Nine patients with perianal Crohn's disease refractory to antibiotics or 6-mercaptopurine were treated with a circumferential and intrafistulous injection of infliximab at 0, 4, and 7 wk. Remission or partial response was achieved in 83% of the patients.

Since then, a number of uncontrolled studies have been conducted to assess the feasibility of local infliximab therapy. Poggioli *et al*^[68] included 15 patients with complex perianal fistulas associated with Crohn's disease. In 9 of these, intravenous infusion of infliximab was felt to be contraindicated because of fibrostenotic disease. The patients were injected with 15-21 mg of infliximab at the internal and external openings and along the fistula tract. The injections were well tolerated and 10 of the 15 patients achieved healing after 3 to 12 injections. A similar study was reported by Asteria *et al*^[69], although patients were excluded if they had received prior treatment with infliximab. Up to 3 injections of 20 mg of infliximab were made along the fistula tract and at both openings. The efficacy endpoint was reduction in fistula drainage of 50% or more (response)

or complete cessation of fistula drainage for at least 4 wk (remission). Overall, 8 patients achieved a response (73%) and, of these, 3 achieved remission (27%). After a longer follow-up (mean 10.5 mo, range 7-18 mo), 6 patients were responders and 4 were in remission.

Adipose-derived stem cell therapy: Adult stem cell therapy has promising applications in a number of areas of medicine and has no ethical concerns. Given that liposuction is a relatively safe procedure, an appealing source of adult stem cells is lipoaspirate^[70]. The stromal cells obtained are subsequently cultured and expanded to produce autologous adipose-derived adult stem cells (ASCs). Trials of ASCs in the treatment of fistulizing Crohn's disease have delivered the expanded ASCs by injecting them around the fistula opening and directly into the fistula tract.

The first procedure of this type published in the literature was a case report of a 33-year-old woman with Crohn's disease and a rectovaginal fistula refractory to treatment^[71]. ASCs were injected into the rectal mucosa, close to the sutured internal opening. After resection of the posterior vaginal wall and construction of an advancement vaginal flap, the accessory perineal hole was sealed with 2 mL of fibrin glue. One week after the intervention, the wound had completely healed. In 3 mo of follow-up, no recurrence of the rectovaginal fistula was reported.

A subsequent phase I study assessed 9 ASC injection procedures in 4 patients with fistulizing Crohn's disease^[72]. The series included 3 rectovaginal fistulas and 1 perineal fistula. As before, cells were injected into the rectal mucosa, close to the sutured opening and fistula tracks were then filled with fibrin glue. Two of the 3 rectovaginal fistulas and the perineal fistula had healed after 8 wk.

One randomized clinical trial using ASCs has also been conducted. In a recently completed phase II study, 49 patients with perianal fistula-14 of whom had Crohn's disease-were randomized to receive ASC therapy and fibrin glue or to receive fibrin glue alone (control group)^[73]. The primary outcome measure was the proportion of patients with complete fistula closure. An investigator blinded to the treatment confirmed healing by examination of a digital photograph. Five of the 7 patients with Crohn's disease assigned to ASC therapy achieved healing compared to one of 7 patients in the control group. The difference was not statistically significant but the study was not powered to detect differences in small subgroups such as those with Crohn's disease. Healing in this trial was defined as the absence of drainage, as well as complete epithelization of external openings. No severe adverse events related to ASCs have been reported when utilized for fistulizing Crohn's disease.

Fistula plugs: Recently, the use of bioprosthetic plugs made from porcine intestinal submucosa has been tried in patients with perianal fistula. In a prospective study which excluded patients with Crohn's disease, Johnson

et al^[74] randomized patients with high transsphincteric or deeper fistulas to either fistula plug or fibrin glue therapy. Of the 10 patients who underwent fibrin glue treatment, 6 (60%) had persistence of one or more fistulas at 3 m compared to 2 out of 15 (13%) of those who underwent the procedure with the fistula plug ($P < 0.05$). In a subsequent prospective but uncontrolled study, 20 Crohn's patients with a total of 36 fistula tracts not amenable to fistulotomy were treated with fistula plugs^[75]. After irrigation with hydrogen peroxide, each primary opening was occluded with a fistula anal plug. The authors found an overall success rate of 80%, although they noted that patients with complex fistulas with multiple primary openings were less likely to achieve success. Success appeared to be independent of the presence of setons or the use of anti-TNF- α therapy.

Schwandner *et al*^[76] have reported their experience in a series of 19 patients with transsphincteric anorectal fistulas. Seven of these patients had Crohn's disease. The surgical procedure comprised irrigation of the fistula tract and placement and internal fixation of the anal fistula plug without flap advancement or excision of the fistula tract. Success was defined as closure of both the internal and external openings with no further interventions and absence of abscess formation. Six of the 7 patients with Crohn's disease achieved success (85.7%).

In another retrospective study, anal fistula plugs were more successful in the treatment of simple anorectal fistulas but were associated with a high failure rate in complex perianal fistulas and particularly in patients with Crohn's disease (closure rate of 26.6% of fistulas in this group)^[77].

In a recent retrospective review reported at the 2008 Digestive Diseases Week conference^[78], the use of anal fistula plugs was associated with a lower success rate than previously reported: only 2 of the 22 (15%) Crohn's disease-associated fistulas healed. In 87% of the procedures the reason for failure was sepsis. These controversial results in an uncontrolled series await confirmation by randomized trials.

CONCLUSION

Symptomatic perianal fistulas in patients with Crohn's disease can have a large negative impact on quality of life. Treatment of complex perianal fistulas remains a difficult problem. Use of anti-TNF- α antibody therapy is widespread and supported by randomized clinical trials. Nevertheless, some patients fail anti-TNF- α treatment and, given the risk of incontinence associated with aggressive surgical procedures, there remains an unmet therapeutic need. Some of the emerging local therapies have obtained promising results in patients with fistulizing Crohn's disease in uncontrolled studies and case series but, for the most part, this promise has still to be confirmed in randomized trials. Adipose-derived stem cell therapy has compared favorably with fibrin glue alone in a randomized phase II trial, and high

healing rates were observed. Once the efficacy of these new local therapies has been confirmed, further effort will be required to optimize their use in the management of fistulizing Crohn's disease, which is necessarily complex and multidisciplinary.

ACKNOWLEDGMENTS

The authors thank Greg Morley for his invaluable contribution in the writing of this article.

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Meta-analysis and systematic review of colorectal endoscopic mucosal resection

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Received: March 2, 2009 Revised: June 16, 2009

Accepted: June 23, 2009

Published online: September 14, 2009

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Key words: Meta-analysis; Systematic review; Polyps; Endoscopic mucosal resection; *En-bloc* resection

Peer reviewer: Zvi Fireman, MD, Associate Professor of Medicine, Head, Gastroenterology Department, Hillel Yaffe Med Ctr, PO Box 169, 38100, Hadera, Israel

Puli SR, Kakugawa Y, Gotoda T, Antillon D, Saito Y, Antillon MR. Meta-analysis and systematic review of colorectal endoscopic mucosal resection. *World J Gastroenterol* 2009; 15(34): 4273-4277 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4273.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4273>

Abstract

AIM: To evaluate the proportion of successful complete cure *en-bloc* resections of large colorectal polyps achieved by endoscopic mucosal resection (EMR).

METHODS: Studies using the EMR technique to resect large colorectal polyps were selected. Successful complete cure *en-bloc* resection was defined as one piece margin-free polyp resection. Articles were searched for in Medline, Pubmed, and the Cochrane Control Trial Registry, among other sources.

RESULTS: An initial search identified 2620 reference articles, from which 429 relevant articles were selected and reviewed. Data was extracted from 25 studies ($n = 5221$) which met the inclusion criteria. All the studies used snares to perform EMR. Pooled proportion of *en-bloc* resections using a random effect model was 62.85% (95% CI: 51.50-73.52). The pooled proportion for complete cure *en-bloc* resections using a random effect model was 58.66% (95% CI: 47.14-69.71). With higher patient load (> 200 patients), this complete cure *en-bloc* resection rate improves from 44.19% (95% CI: 24.31-65.09) to 69.17% (95% CI: 51.11-84.61).

CONCLUSION: EMR is an effective technique for the resection of large colorectal polyps and offers an alternative to surgery.

INTRODUCTION

The use of endoscopic mucosal resection (EMR), pioneered in Japan for the treatment of early gastric cancer, has expanded to include therapy of other early gastrointestinal malignancies and pre-cancerous lesions such as adenomas. At the same time, this technique has gained acceptance in Europe and in the US, especially for the treatment of Barrett's esophagus with high grade dysplasia^[1-3]. Several variations of the EMR technique have been devised such as inject-lift-cut, strip biopsy, suction cup (EMRC), and EMR with a ligating device.

Throughout the world, adenomas of the colorectum represent the single most important premalignant lesion of the GI tract. Large (> 2 cm) colorectal polyps have been found in 0.8%-5.2% of patients undergoing colonoscopies for different indications^[4].

Large sessile and flat polyps represent a major technical challenge to conventional snare resection. Additional procedures and therapies such as Argon plasma coagulation are frequently needed to destroy remnant tissue after resection^[5]. When these techniques are not used or possible, patients are frequently referred for surgical resection^[6].

EMR has been shown to be useful in the removal of large colorectal sessile and flat lesions^[7]. However, there are limits to the size of lesions which can be removed *en-bloc* with the various EMR techniques, with 1.5-2 cm generally being the upper limit^[8].

En-bloc removal of large polyps is desirable as it facilitates thorough histological evaluation related to the

completeness of resection, and is associated with a lower recurrence rate as compared to piecemeal removal^[19-14].

MATERIALS AND METHODS

Study selection criteria

Studies using EMR technique to resect large (> 2 cm) colorectal polyps were selected. Successful cure *en-bloc* resection was defined as one piece removal with tumor-free vertical and lateral margins.

Data collection and extraction

Articles were searched for in Medline, Pubmed, Ovid journals, Japanese language literature, Cumulative Index for Nursing & Allied Health Literature, ACP journal club, DARE, International Pharmaceutical Abstracts, old Medline, Medline non-indexed citations, OVID Healthstar, and the Cochrane Controlled Trials Registry. The search terms used were EMR, endoscopic mucosal resection, colon polyps, lateral spreading tumors, large polyps, nonpolypoid colon lesions, flat colon polyps, and flat adenomas. Two authors (SP and YK) independently searched and extracted the data for revising into an abstracted form. Any differences were resolved by mutual agreement.

Quality of studies

Clinical trials with a control arm can be assessed for the quality of the study. A number of criteria have been used to assess the quality of a study (e.g. randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome)^[15,16]. There is no consensus regarding how to assess studies without a control arm. Hence, these criteria do not apply to studies without a control arm^[16]. Therefore, for this meta-analysis and systematic review, studies were selected based on completeness of data and inclusion criteria.

Statistical methods

This meta-analysis was performed by calculating pooled proportions, i.e. pooled proportion of *en-bloc* resections and complete cure *en-bloc* resections. Firstly, the individual study proportions of successful resections were transformed into a quantity using Freeman-Tukey variant of the arcsine square root transformed proportion. The pooled proportion was calculated as the back-transform of the weighted mean of the transformed proportions, using inverse arcsine variance weights for the fixed effects model and DerSimonian-Laird weights for the random effects model^[17,18]. Forrest plots were drawn to show the point estimates in each study in relation to the summary pooled estimate. The width of the point estimates in the Forrest plots indicated the assigned weight to that study. The heterogeneity among studies was tested using Cochran's Q test based upon inverse variance weights^[19]. If P value was > 0.10, the null hypothesis was rejected that the studies were heterogeneous. The effects of publication and selection bias on the summary estimates were tested by Begg-Mazumdar bias indicator^[20]. Also, funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio^[21,22].

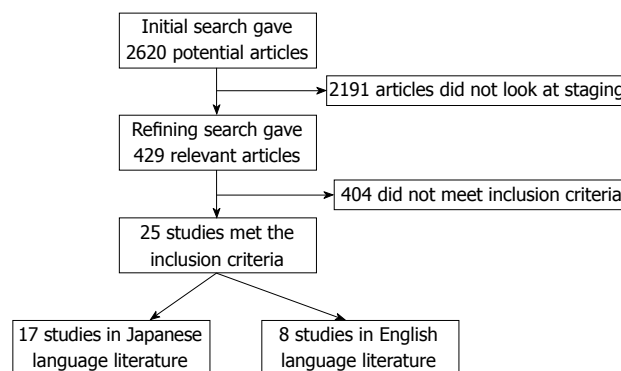


Figure 1 Search results.

RESULTS

An initial search identified 2620 reference articles from which 429 relevant articles were selected and reviewed. Data was extracted from 25 studies ($n = 5221$) which met the inclusion criteria^[23-46]. The search results are shown in Figure 1. All the studies used snare to perform EMR. Two studies used a strip biopsy technique^[42,43]. The mean size of the polyps was 22.48 ± 4.52 mm. There were 3755 successful *en-bloc* resections. The study characteristics are shown in Table 1.

The pooled proportion of *en-bloc* resections using a random effect model was 62.85% (95% CI: 51.50-73.52). Forest plot in Figure 2A depicts the individual study proportion of successful *en-bloc* resections in relation to the pooled estimate. The pooled proportion for complete cure *en-bloc* resections using a random effect model was 58.66% (95% CI: 47.14-69.71). Figure 2B shows Forrest plot depicting the individual study successful cure *en-bloc* resections in relation to the pooled estimate. The fixed effect model was not used because of the heterogeneity of studies.

Subgroup analysis was carried out by grouping studies according to the study population. This was done because the expertise needed to perform procedures might have affected the outcome. Studies were categorized into three groups: < 100 patients, 100-200 patients and > 200 patients. The proportions for successful *en-bloc* and successful cure *en-bloc* resections are shown in Table 2.

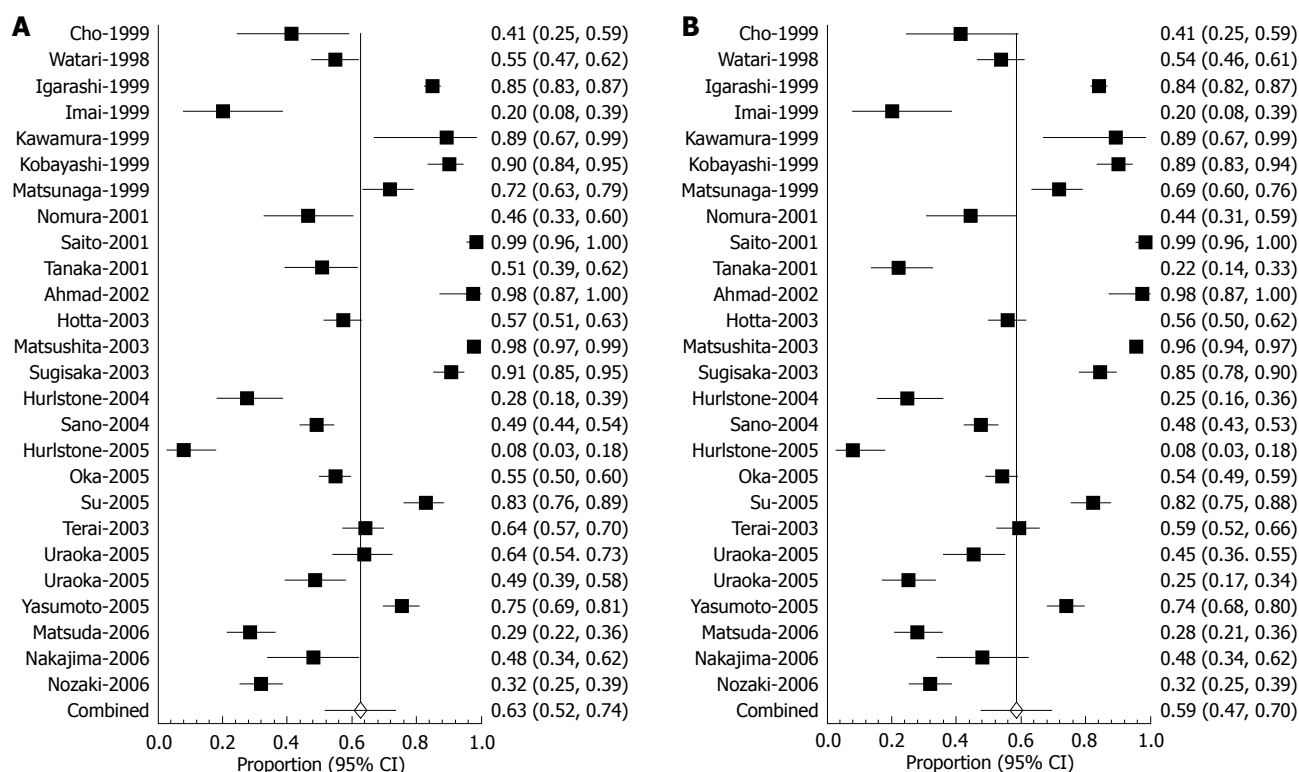
The publication bias calculated by Begg-Mazumdar bias indicator for successful cure *en-bloc* resections concluded that the Kendall's tau b value was -0.19 ($P = 0.17$). The funnel plot in Figure 3 shows that there was no publication bias for successful cure *en-bloc* resections.

DISCUSSION

Some colorectal cancers develop from adenomas. The risk of high grade dysplasia and cancer increases with the size of the lesion. Endoscopic removal of large (> 2 cm) sessile and flat polyps represents a difficult challenge for conventional snare resection and they are frequently managed by piecemeal resection or surgically^[6,47]. EMR was the definitive procedure in all the collated studies. The data for complications was not available for the majority of the studies, so this data was not collected. EMR is a technique that can be applied to sessile and flat

Table 1 Study characteristics

	Author, yr	Instrument used	n	Type of polyp	Technique
1	Matsushita <i>et al</i> ^[23] , 2003	Snare	935	No information	EMR
2	Imai <i>et al</i> ^[24] , 1999	Snare	30	No information	EMR
3	Igarashi <i>et al</i> ^[25] , 1999	Snare	884	No information	EMR
4	Oka <i>et al</i> ^[26] , 2005	Snare	410	Lateral spreading tumor	EMR
5	Sano <i>et al</i> ^[27] , 2004	Snare	392	Lateral spreading tumor	EMR
6	Hotta <i>et al</i> ^[28] , 2003	Snare	284	Protrusion 68, flat 213, depressed 3	EMR
7	Matsuda <i>et al</i> ^[29] , 2006	Snare	154	Is, Isp 33, LST-G 96, NG 25	EMR
8	Yasumoto <i>et al</i> ^[30] , 2005	Snare	240	LST-G 180, NG 60	EMR
9	Terai <i>et al</i> ^[31] , 2003	Snare	223	Lateral Spreading tumor	EMR
10	Nozaki <i>et al</i> ^[32] , 2006	Snare	198	Ip 3, Isp 34, Is 7, LST-G 85, NG 28	EMR
11	Watari <i>et al</i> ^[33] , 1998	Snare	186	Lateral spreading tumor	EMR
12	Sugisaka <i>et al</i> ^[34] , 2003	Snare	162	No information	EMR
13	Matsunaga <i>et al</i> ^[35] , 1999	Snare	134	No information	EMR
14	Nomura <i>et al</i> ^[36] , 2001	Snare	54	No information	EMR
15	Kobayashi <i>et al</i> ^[37] , 1999	Snare	131	No information	EMR
16	Nakajima <i>et al</i> ^[38] , 2006	Snare	52	No information	EMR
17	Cho <i>et al</i> ^[39] , 1999	Snare	34	No information	EMR
18	Saito <i>et al</i> ^[40] , 2001	Snare	170	Lateral spreading tumor	EMR
19	Tanaka <i>et al</i> ^[41] , 2001	Snare with needle spike	81	Lateral spreading tumor	EMR
20	Ahmad <i>et al</i> ^[42] , 2002	Snare with suction	41	Colon and rectum	EMR
21	Hurlstone <i>et al</i> ^[43] , 2004	Strip technique of Karita	80	Rectal villous adenoma	EMR
22	Hurlstone <i>et al</i> ^[43] , 2005	Strip technique of Karita	62	Rectal villous adenoma	EMR
23	Su <i>et al</i> ^[44] , 2005	Snare with needle spike	152	Colonic nonpolypoid lesions	EMR
24	Uraoka <i>et al</i> ^[45] , 2005	Snare	113	Lateral spreading tumor	EMR
25	Kawamura <i>et al</i> ^[46] , 1999	Snare	19	Submucosal invasive colorectal cancers	EMR

Figure 2 Forrest plot showing successful *en-bloc* (A) and cure *en-bloc* (B) resection.

lesions. Though initially used for the treatment of early gastric cancer in Japan, the technique has been expanded to the therapy of large colorectal neoplasms^[7].

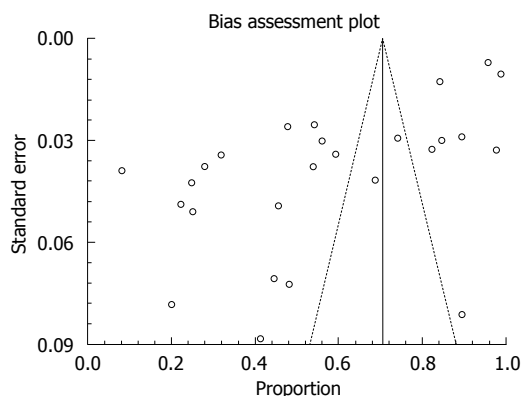
This meta-analysis revealed that *en-bloc* resection was achieved in 62.85% of lesions and tumor-free vertical and lateral margins were achieved in 58.6%. These results compare well to *en-bloc* resection rates achieved by conventional polypectomy snare, which have been reported

to be between 7% and 34% for large sessile polyps^[6,48].

Furthermore, our meta-analysis revealed that experience performing EMR plays an important role in achieving a better *en-bloc* resection and cure *en-bloc* tumor-free rate. Studies reporting more than 200 lesions removed reported a 71.39% *en-bloc* resection of lesions and tumor-free vertical and lateral margins in 69.17% of cases, while studies reporting less than a 100 lesions reported a

Table 2 Results based on study size

Study size	No. of studies	Successful <i>en-bloc</i> resection (95% CI)	Successful cure <i>en-bloc</i> resection (95% CI)
< 100 patients	9	48.07% (28.36-68.09)	44.19% (24.31-65.09)
100-200 patients	9	68.93% (50.39-84.76)	63.32% (43.50-81.04)
> 200 patients	7	71.39% (52.24-87.20)	69.17% (51.11-84.61)

Figure 3 Funnel plot showing publication bias for successful cure *en-bloc* resection.

48.07% *en-bloc* removal and tumor-free vertical and lateral margins in 44.19% of cases. This indicates that experience in the technique of EMR increase the cure *en-bloc* rate.

In the present meta-analysis we searched the world literature which included articles published in Japanese language literature. We believe that our results are a reasonable reflection of the status of EMR in the therapy of large colorectal polyps.

EMR is an effective technique for resection of large colorectal polyps. The technique offers an alternative to surgery. This meta-analysis shows that the success rate for *en-bloc* margin-free resection is not high but improves with experience. Improvements in techniques and equipment are needed to increase complete cure *en-bloc* resection rates.

COMMENTS

Background

Endoscopic mucosal resection (EMR) has emerged as an alternative to surgery for the resection of large colorectal polyps. Complete cure with tumor-free lateral and vertical margins would prevent further therapy. Published data regarding successful *en-bloc* resection with tumor-free margins by EMR has been varied.

Innovations and breakthroughs

EMR has been shown to be useful in the removal of large colorectal sessile and flat lesions. However, there are limits to the size of lesions which can be removed *en-bloc* with the various EMR techniques, with 1.5-2 cm generally being the upper limit. *En-bloc* removal of large polyps is desirable as it facilitates thorough histological evaluation related to the completeness of resection, and is associated with a lower recurrence rate as compared to piecemeal removal.

Applications

EMR is an effective technique for resection of large colorectal polyps and offers an alternative to surgery. This meta-analysis shows that the success rate for *en-bloc* margin-free resection is not high but improves with experience. Improvements in techniques and equipment are needed to increase complete cure *en-bloc* resection.

Peer review

The authors evaluated the proportion of successful complete cure *en-bloc*

resections of large colorectal polyps achieved by EMR. They found that EMR is an effective technique for resection of large colorectal polyps. This article is well written and easy to read.

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S- Editor Cheng JX L- Editor Logan S E- Editor Zheng XM



BRIEF ARTICLES

Sirolimus, bevacizumab, 5-Fluorouracil and irinotecan for advanced colorectal cancer: A pilot study

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Supported by A Grant From Association Pour la Recherche Contre le Cancer, Ligue Régionale De Bourgogne Contre le Cancer, INSERM, Fondation de France and GERTI (Groupe d'Etude en Thérapeutique Inovante) Study number GERTI-P001

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Received: June 1, 2009 Revised: July 16, 2009

Accepted: July 23, 2009

Published online: September 14, 2009

irinotecan, bevacizumab and sirolimus in advanced colorectal carcinoma after failure of classical treatment is feasible and promising. Further evaluation of this combination is required.

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Key words: Colorectal cancer; Bevacizumab; 5-Fluorouracil; Irinotecan; Sirolimus; Antineoplastic combined chemotherapy protocols; Angiogenesis

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Ghiringhelli F, Guiu B, Chauffert B, Ladoire S. Sirolimus, bevacizumab, 5-Fluorouracil and irinotecan for advanced colorectal cancer: A pilot study. *World J Gastroenterol* 2009; 15(34): 4278-4283 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4278.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4278>

Abstract

AIM: To evaluate the efficacy and the safety of combined 5-Fluorouracil, irinotecan, bevacizumab and sirolimus in refractory advanced colorectal carcinoma.

METHODS: We initiated a regimen with at day 1 an injection (*iv*) of bevacizumab at 5 mg/kg, followed by 180 mg/m² irinotecan, followed by Leucovorin 400 mg/m², followed by a 5-Fluorouracil bolus 400 mg/m² and a 46-h infusion 2400 mg/m². Sirolimus was given orally as continuous administration of 2 mg twice a day every days. This treatment was repeated every 14 d.

RESULTS: A total of 12 patients were enrolled. All patients presented with metastatic disease that had failed at least three lines of chemotherapy that contained oxaliplatin, irinotecan and bevacizumab. Cetuximab failure was also observed in all K-Ras wild-type patients. The median number of cycles was 8.5 (range 2-20) and clinical benefit was observed in eight patients. The median time to progression was 5 mo and the median survival was 8 mo. Grade 3 neutropenia developed in four patients, and grade 3 diarrhea and stomatitis in two.

CONCLUSION: The combination regimen of 5-Fluorouracil,

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide^[1]. Approximately 25% of patients with colorectal cancer present with overt metastatic disease, and metastatic disease develops in 40%-50% of newly diagnosed patients. Standard first-line treatments include fluorouracil (5-FU) with leucovorin and irinotecan^[2,3] or oxaliplatin^[4], alone or combined with bevacizumab^[5]. Cetuximab, a chimeric IgG1 monoclonal antibody against epidermal growth factor receptor (EGFR), has efficacy as monotherapy and in combination with irinotecan in irinotecan-resistant patients, if the tumor expresses wild-type K-RAS and B-RAF^[6,7]. However if these standard treatments fail, there are no accepted treatment options, and for such patients historical estimation of progression-free survival (PFS) and overall survival (OS) are about 2 and 4 mo, respectively^[8].

Recent results have suggested that inhibitors of mTor (mammalian target of rapamycin) signal are able to improve survival and induce tumor response in patients with poor-prognosis renal cell carcinoma^[9], and combination of mTor inhibitors and classical

chemotherapy is currently under clinical investigation. In colorectal carcinoma cell lines *in vitro*, rapamycin enhances the antitumor effect of irinotecan through Hypoxia-inducible factor-1 α inhibition (a key transcription factor that regulates angiogenesis) and by disruption of tumor vasculature^[10]. Therefore, we hypothesize that, in multi-treated colorectal patients, multimodal angiogenesis targeting using irinotecan, bevacizumab and the mTor inhibitor sirolimus can have a therapeutic effect.

The goal of this pilot study was to evaluate the safety and efficacy of sirolimus, bevacizumab, leucovorin, 5-FU, and irinotecan (FOLFIRI) in patients with advanced colorectal cancer that had progressed after treatment with 5-FU, irinotecan, oxaliplatin, bevacizumab and anti-EGFR therapy.

MATERIALS AND METHODS

Eligibility criteria

The eligibility and exclusion criteria, and pretreatment characteristics of the patients are presented in Table 1. Written informed consent was required before chemotherapy.

Treatment protocols and dose modification

On day 1, irinotecan (180 mg/m²) was administered as a 2-h *iv* infusion. Then leucovorin 400 mg/m² followed by a 5-Fluorouracil bolus 400 mg/m² was administered as a bolus *iv* injection. Then 5-Fluorouracil 2400 mg/m² was given as a 46-h *iv* infusion. Bevacizumab was given at 5 mg/kg as an *iv* infusion every two weeks over 60 min before the beginning of the chemotherapy. Sirolimus was given orally as continuous administration of 2 mg twice a day every days. The doses used for fluorouracil, irinotecan and bevacizumab were the classical recommended doses^[2-5]. For sirolimus, a dose of 4 mg/d was chosen because a phase I study has demonstrated that this is tolerable and sufficient to achieve mTor inhibition^[11]. Dose modifications of irinotecan or 5-FU were made for hematological or non-hematological toxicity, on the basis of the most severe grade of adverse effect that occurred during the previous cycle. Treatment was delayed until the absolute number of neutrophils was > 1000/ μ L, platelets were > 100 000/ μ L, and mucositis, diarrhea, or skin toxicity had recovered to grade 1 or less. The 5-FU dose was reduced after the occurrence of National Cancer Institute Common Toxicity Criteria (NCI-CTC) grade 3 diarrhea, stomitis or dermatitis. For toxicity of grade 3 or higher, a 20% dose reduction for irinotecan was prescribed by the protocol. Bevacizumab was retained for uncontrolled hypertension or proteinuria of > 3 g in 24 h. Bevacizumab was discontinued for grade 3 or 4 hemorrhage, thromboembolic events that required full dose anticoagulation, or any grade 4 toxicity. Sirolimus was discontinued for grade 3 or 4 diarrhea or stomatitis. Treatment was administered until the disease progressed, unacceptable toxic effects developed, or the patient refused further treatment.

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Histologically confirmed colorectal cancer (adenocarcinoma)	Thromboembolism that required therapeutic anticoagulation
Measurable disease	Central nervous system metastasis
No secondary malignancy	Major surgery within 6 wk
Age > 18 years	Nonhealing wounds
ECOG performance status 0-2	Uncontrolled hypertension
Adequate hematological, hepatic and renal function (including urinary excretion of no more than 500 mg protein/d)	Pregnant or lactating women
Failure of bevacizumab, oxaliplatin and irinotecan-based treatment	Bleeding diathesis, active or recent
For wild-type K-Ras: failure with cetuximab and irinotecan combination was required	Cardiovascular disease
Failure caused by significant intolerance to either drug was allowed	Cerebrovascular accident

Pretreatment and follow-up evaluation

Pretreatment evaluation included physical examination, complete blood cell counts, blood chemistry, tumor marker level (carcinoembryonic antigen, CEA), and computed tomography (CT) within 15 d of starting chemotherapy. Tumor responses were determined by RECIST and Choi criteria^[12,13]. Complete blood cell counts, serum chemistry, including liver and renal function, were performed at least every two weeks, and tumor assessment by CT was performed every three cycles.

Statistical analysis

Efficacy analysis was performed according to the intention-to-treat principle. Patients were considered assessable for response if they were eligible, had measurable disease, and had received at least one dose of study therapy. In the analysis of survival and subsequent treatment, all patients were followed until death, loss to follow-up, or termination of the study. PFS and OS were calculated using the Kaplan-Meier method. PFS was calculated from the date therapy started to the date of disease progression, and OS was calculated from the date therapy started to the date of death.

RESULTS

Patient characteristics

Between January 2008 and December 2008, a total of 12 patients were included in this pilot study at the Department of Medical Oncology, Georges-Francois Leclerc Cancer Center, Dijon, France. Demographic details of the patients included in the study are shown in Table 2. There were seven male and five female patients, median age 61 years (range 51-75). All patients had progressed after prior 5-FU, irinotecan and bevacizumab therapy, and 5-FU, oxaliplatin and bevacizumab therapy. Importantly, all patients had been treated previously with FOLFIRI bevacizumab chemotherapy and experienced progression according to the RECIST criteria following this treatment. Nine patients harbored wild-type K-Ras tumor and progressed on irinotecan plus cetuximab

Table 2 Patient characteristics

Characteristics	n
Median age (Range) (yr)	62 (51-75)
Sex	
Male	7
Female	5
ECOG performance status	
0-1	8
2	4
CEA level (range) (ng/mL)	882 (21-6327)
Primary site	
Colon	10
Rectum	2
Sites of metastasis	
Liver	9
Lung	7
Lymph nodes	6
Others	5
K-Ras status	
Wild-type	9
Mutated	3

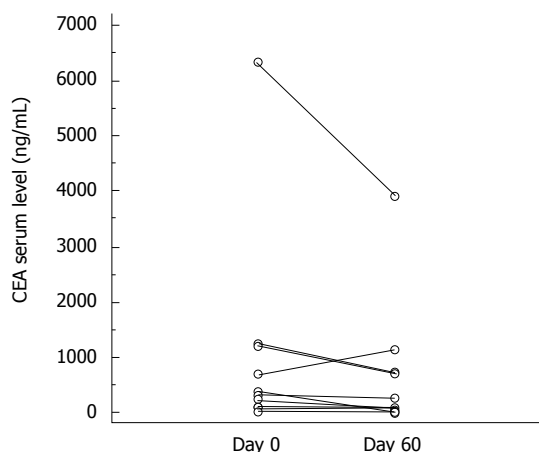


Figure 1 Carcinoembryonic antigen (CEA) serum level before and 2 mo after the beginning of the treatment.

therapy. All 12 patients were assessable for response, for toxicity and survival.

Objective tumor responses and survival

There were a median 8.5 cycles (range 2-20) of chemotherapy. Chemotherapy was stopped because of disease progression in 10 patients, and two discontinued because of toxicity (grade 3 diarrhea and stomatitis). Median follow-up duration was 9 mo. At 2 mo, CEA level decreased in all except one patient (mean level 882 ± 510 vs 579 ± 320 ; $P = 0.025$, Wilcoxon test; Figure 1). According to RECIST criteria, one patient had a partial response, seven had stable disease for > 3 mo, and four had progressive disease. According to the Choi criteria, three patients had a partial response, five had stable disease, and four had progressive disease (Figure 2). PFS was 5 mo (95% CI: 2.5-6), and median OS was 7 mo (95% CI: 4-NR). Figure 3 shows PFS and OS curves.

Toxicity

A total of 116 cycles of chemotherapy were administered.

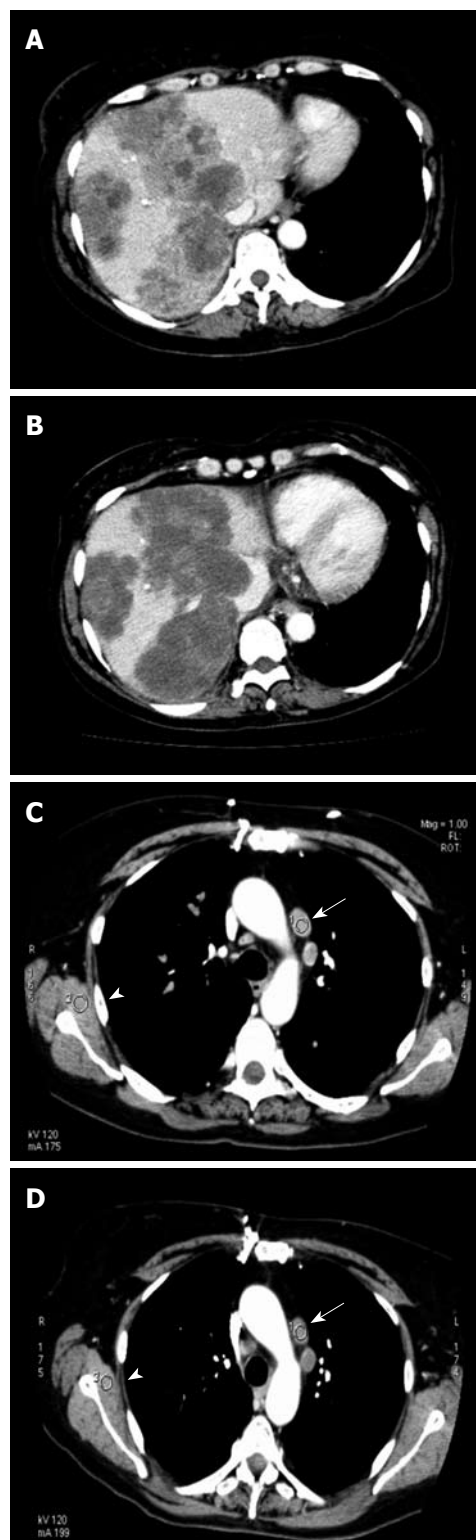


Figure 2 Representative CT scan of patients responding according to Choi criteria. A: (Patient 5) Contrast-enhanced CT-scan (arterial phase) showed three large liver metastases before inclusion; B: Two months later, a significant decrease in the enhancement of the metastases was observed by CT performed under the same conditions (arterial phase). Although lesions were classified as stable disease using RECIST criteria, they could be considered as a partial response using Choi criteria; C: (Patient 8) Contrast-enhanced CT-scan (arterial phase) showed two latero aortic lymph node metastases. Density was 82.8 UH (arrow). To assure comparability, density was also measured in muscle (72.8 UH) (arrow head); D: After 2 mo, although size of lymph node metastases was stable, there was a significant decrease in density (59 UH) (arrow), which led to a partial response using Choi criteria. In comparison, muscle exhibited no decrease in density (73 UH) (arrow head).

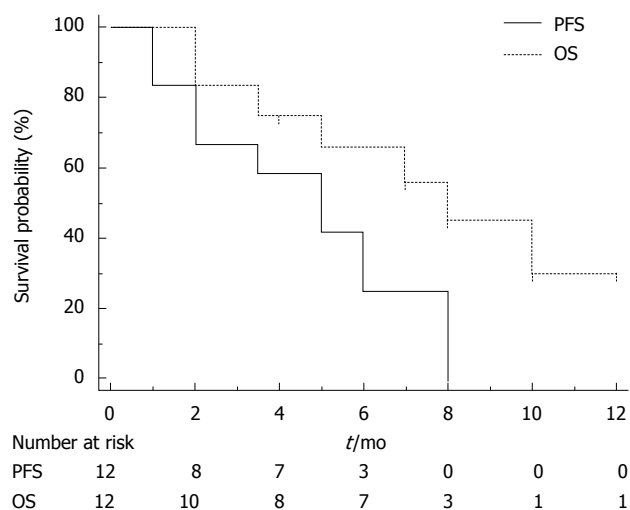


Figure 3 Kaplan-Meier curves of progression-free survival (PFS) and overall survival (OS).

All patients received at least 1 mo of the chemotherapy and sirolimus regimen. Dose modifications or interruptions were required in three patients. The incidence of hematological and non-hematological toxicity is summarized in Table 3. The major grade 3/4 hematological toxicity included neutropenia in three patients (25%) and thrombocytopenia in two (16%). No neutropenic fever was observed. Grade 1/2 nausea, vomiting and diarrhea developed in five patients; however, this toxicity was mild and manageable. In two patients, sirolimus-related grade 3 stomatitis led to treatment discontinuation. Only grade 2 nose bleeding were observed and did not require therapeutic modification. Hypertension occurred in three patients and was managed without drug modification. Proteinuria > 2 g/24 h occurred in one patient and was treated by angiotensin-converting enzyme inhibitors. All patients with stable disease had minor grade 1/2 manageable adverse effects. There was no treatment-related death. Eight deaths were caused by disease progression.

DISCUSSION

Recent trials with advanced colorectal carcinoma have shown that patients may survive for 24-30 mo^[14]. However, after failure of irinotecan, oxaliplatin, bevacizumab and anti-EGFR therapy, there are no accepted treatment options. Estimated PFS is about 2 mo and OS is 4 mo^[8].

Few nonrandomized trial have demonstrated that association of fluorouracil or capecitabine with mitomycin may give some response in heavily pretreated patients^[15-18]. Such treatments give an objective response rate of about 10% and PFS of 2-3 mo. Although, in these trials, patients did not benefit from new target therapies.

Recently, target therapy such as sorafenib and sunitinib have given some promising results in patients with end-stage colorectal cancer. Sunitinib was tested in a phase II trial in patients with previously treated colorectal metastases at a dose of 50 mg/d for 4 wk, followed by 2 wk off treatment^[19]. Median time to progression was 2.5 mo and median OS was 7 mo in bevacizumab-

Table 3 Observed toxicity according NCI-CTC grading (*n* = 12)

	NCI-CTC grade			
	1	2	3	4
Hematological				
Anemia	1	3		
Leucopenia		2	4	
Neutropenia		2	4	
Thrombocytopenia		1	2	
Non hematological				
Nausea/vomiting	2	5		
Mucositis	3	4	2	
Diarrhea		5	2	
Proteinuria		1		
Asthenia	3	5		
High blood pressure		3		

pretreated patients and 10 mo in those not pretreated with bevacizumab. Sorafenib has been tested in association with oxaliplatin in a phase I trial in patients with oxaliplatin-refractory colorectal cancer. The recommended dosage was 130 mg/m² oxaliplatin and continuous sorafenib 400 mg twice daily. This treatment showed promising efficacy^[20].

The phosphoinositide 3-kinase/mTOR axis is a pivotal pathway in cell growth and cell-cycle progression, in response to different stimuli, such as nutrients and growth receptors^[21]. The mTor pathway is activated aberrantly in around half of human tumors and plays a crucial role in angiogenesis. mTor inhibition is now considered as an important target for new anticancer drugs^[22]. In a phase I trial, mTor inhibitors showed minor efficacy in colorectal carcinoma^[23]. *In vitro* and preclinical studies have demonstrated the synergistic efficacy of combined rapamycin and irinotecan^[10]. In fact, irinotecan and rapamycin act on tumor growth by inhibiting angiogenesis by acting on Hypoxia inducible factor-1 α ^[24,25]. Recently, combination of everolimus and bevacizumab has demonstrated some activity in patients with refractory metastatic colorectal cancer who had progressed on a bevacizumab-based regimen, which suggests that bevacizumab and mTor inhibitors overcome resistance to bevacizumab^[26]. Therefore, we hypothesized that combination of sirolimus, bevacizumab and irinotecan may provide some synergistic antiangiogenic effects that can reverse resistance to bevacizumab- and irinotecan-based chemotherapy.

In our pilot study, combination of sirolimus, bevacizumab, 5-FU and irinotecan demonstrated that, for patients with advanced colorectal cancer, some clinical stabilization and prolonged stable disease can be obtained. CT monitoring suggested an antiangiogenic effect of the therapy, with tumor necrosis obtained in three patients. Median PFS of 5 mo and median OS of 8 mo suggest a potential clinical benefit of a such treatment. Despite its potent efficacy, this target therapy can have adverse effects, and we have to balance the efficacy, toxicity and cost of such therapy. Further studies will be needed to confirm our results.

COMMENTS

Background

Novel combinations of new chemotherapeutic agents and target therapies have demonstrated an improvement in tumor response and overall survival in patients with advanced colorectal cancer. Although, after failure of irinotecan, oxaliplatin, bevacizumab and cetuximab, no treatment demonstrates efficacy.

Research frontiers

The phosphoinositide 3-kinase/mTOR (mammalian target of rapamycin) axis is activated aberrantly in around half of human tumors and plays a crucial role in angiogenesis. In a phase I trial, mTor inhibitors showed minor efficacy in colorectal carcinoma. *In vitro* and preclinical studies have demonstrated the synergistic efficacy of combined rapamycin and irinotecan. Recently, combination of everolimus (an mTor inhibitor) and bevacizumab have demonstrated some activity in patients with refractory metastatic colorectal cancer who had progressed on a bevacizumab-based regimen, which suggests that bevacizumab and mTor inhibitors overcome resistance to bevacizumab. Therefore, the authors hypothesized that combination of sirolimus, bevacizumab and irinotecan provides some synergistic antiangiogenic effects that can reverse resistance to bevacizumab- and irinotecan-based chemotherapy.

Innovations and breakthroughs

This is believed to be the first study to investigate the capacity of an mTor inhibitor to reverse resistance to conventional therapies. The data suggest that both treatment regimens demonstrate efficacy and tolerable toxicity in this setting.

Applications

These data are in accordance with the preliminary findings from ASCO 2009, which demonstrate that sirolimus may reverse resistance of colorectal cancer to bevacizumab therapy. The data suggest that the therapy may have some clinical efficacy but substantial adverse effects.

Terminology

Sirolimus, also called rapamycin, is an mTor inhibitor. This drug is used as an immunosuppressant but demonstrates antitumor efficacy by inhibiting the mTor/AKT pathway. Irinotecan kills cells by inhibiting the enzyme topoisomerase I, which is also involved in DNA synthesis in proliferating cells. Bevacizumab is a monoclonal antibody that inhibits VEGF, which is expressed in some cases of colorectal cancer and aids the blood supply to tumors; bevacizumab inhibits this process and tumor cell growth.

Peer review

This is an interesting small pilot study that investigated fourth-line chemotherapy in patients with advanced metastatic colorectal cancer that had failed at least three previous chemotherapeutic regimens. A major finding was that clinical benefit was observed in 8/12 patients with severe adverse effects and relatively short overall survival.

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S- Editor Tian L L- Editor Kerr C E- Editor Ma WH



BRIEF ARTICLES

Effect of early propranolol administration on portal hypertensive gastropathy in cirrhotic rats

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Received: May 28, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: September 14, 2009

Abstract

AIM: To investigate any protective effect of early propranolol administration in the development of portal hypertensive gastropathy in cirrhotic rats.

METHODS: For the development of liver cirrhosis and portal hypertensive gastropathy, 60 rats underwent ligation of the left adrenal vein and complete devascularization of the left renal vein, followed by phenobarbital and carbon tetrachloride (CCl₄) administration. After two weeks of CCl₄ administration, the rats were randomly separated into two groups. In group A, propranolol was continuously administered intragastrically throughout the study, whereas in group B normal saline (placebo) was administered instead. Hemodynamic studies and vascular morphometric analysis of gastric sections were performed after complete induction of cirrhosis.

RESULTS: Vascular morphometric studies showed higher numbers of vessels in all mucosal layers in

the control group. Statistical analysis revealed a significantly higher total vascular surface in the control group compared to the propranolol group, but with no statistically significant difference between the mean vascular surfaces between the groups. Our study clearly shows that the increased mucosal blood flow is manifested by a marked increase of vessel count.

CONCLUSION: Early propranolol's administration in portal hypertensive cirrhotic rats seems to prevent intense gastric vascular congestion that characterizes portal hypertensive gastropathy.

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Key words: Portal hypertension; Portal hypertensive gastropathy; Hepatic cirrhosis; Carbon tetrachloride; Gastric mucosal lesion

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INTRODUCTION

Portal hypertension is a clinical syndrome characterized by elevation of portal pressure and accompanies most cases of hepatic cirrhosis. Most liver cirrhosis complications are attributable to concomitant portal hypertension and the consequent development of portosystemic collaterals and hyperdynamic circulation^[1-4]. Portal hypertensive

gastropathy (PHG) represents a clinical entity in portal hypertension and is endoscopically characterized by a mosaic-like or snake skin pattern of the gastric mucosa, mainly in the body and fundus of the stomach and more rarely in the gastric antrum^[5,6]. These gastric mucosal lesions represent another frequent cause of upper gastrointestinal bleeding, even though esophagogastric varices remain the major source of bleeding in patients with portal hypertension^[5,7].

Non-selective β -blockers have largely been used for primary prophylaxis of bleeding from gastroesophageal varices^[8-12]. Their effect though, as well as of many other agents, on the development of varices has yet to be clarified because there are conflicting results from several studies, both clinical and experimental^[13-17]. On the other hand, development of PHG seems to follow a different pathophysiological pathway and there are relatively few studies investigating drugs' effect on PHG. Propranolol, for example, has been shown to reduce bleeding related to PHG in small studies^[11,12] and these observations were confirmed in a randomized controlled trial of 56 patients with PHG^[18]. We therefore decided to investigate propranolol's effect on PHG and to clarify more precisely if early propranolol administration has any preventive effect on the development of PHG in rats with carbon-tetrachloride (CCl₄)-induced cirrhosis.

MATERIALS AND METHODS

Animals

Sixty four-month-old-male Wistar rats, weighting 280-350 g, were used. They were housed one per cage, kept on an artificial 12-h light-dark cycle and at stable room temperature of 20-22°C. They had free access to tap water and standard laboratory pulverized rat chow throughout the study.

For all animal experiments the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed. The study was approved by the Ethical Committee of the Aristotle University of Thessaloniki.

Experimental model

Liver cirrhosis, portal hypertension and esophagogastric varices were induced using a model, originally developed in our department, which has been proved to be very effective for the induction of cirrhotic portal hypertension as well as of esophageal and gastric varices^[19].

Briefly, all animals underwent ligation of the left adrenal vein and complete devascularization of the left renal vein. Two weeks later, induction of liver cirrhosis started according to the model of weekly intragastric administration of CCl₄ in the phenobarbitone-induced rat^[20,21].

Animal groups and drug administration

Two weeks after the beginning of carbon tetrachloride administration, the rats were randomly separated into two groups. In Group A, comprising 30 rats, propranolol was continuously administered throughout the study, whereas in Group B (30 rats), normal saline (placebo)

was continuously administered instead of propranolol. This early commencement of drug administration, before the full development of liver cirrhosis, aimed to simulate clinical practice, where any kind of preventive treatment should begin soon after initiation of the effect of a hepatotoxic agent. Propranolol (Inderal®, Wyeth Pharmaceuticals Inc., USA), dissolved in normal saline, was administered intragastrically, at a dose of 30 mg/kg per day.

Experimental period - animal sacrifice

CCl₄ was administered weekly until stable ascites developed (8-10 wk) as previously described^[16,17,19,22]. Ascites development was easily recognized by an abrupt increase in body weight and confirmed by the abdominal distention observed in the anesthetized rat in the prone position. Once stable ascites developed, CCl₄ administration was discontinued; one week later, rats were re-operated, portal pressure was measured and then the animals were sacrificed with an *iv* bolus administration of 0.5 mL of potassium chloride. The liver, stomach, and esophagus were carefully dissected and removed.

Portal pressure measurement

Portal pressure measurements were performed before animal sacrifice under light ether anesthesia; the rats were kept fasting for 12 h, with free access only to water. The peritoneal cavity of the animal was carefully accessed through the old midline incision, the presence of ascites was confirmed and ascitic fluid was carefully collected and measured. Portal pressure measurement was conducted by catheterization of a mesenteric vein with a PE-50 catheter, which was advanced until its tip reached the origin of the portal vein, while its other end was connected to a Space Labs, Inc. (Model 90308-11-14) pressure recorder. The external zero reference point was placed at the mid portion of the rat.

Histopathological study

The liver, stomach, and esophagus were fixed in 10% buffered formalin solution and embedded in paraffin soon after their removal.

Two sections of the stomach, the first at the cardioesophageal junction and the second at the body of the stomach, were stained with hematoxylin-eosin and initially examined on a light microscope (magnification $\times 4$ and $\times 10$). A liver section was also examined to confirm development of liver cirrhosis.

Morphometric analysis

Following light microscopy, all sections to be studied were scanned by a high resolution frame capture camera (JVC TK-F7300U), processed with computer software (Tema v1.00) and reproduced on a high-contrast, high-resolution PC monitor.

By use of the above mentioned software, delineating the outlines of vessels led to an easy calculation of the following parameters per optical field: (1) Total number of veins counted in gastric submucosa; (2) Total submucosal area occupied by vessels; (3) Mean

cross sectional vessel area (this variable was calculated by dividing total submucosal vessel area by the number of submucosal vessels); (4) Total number of superficial vessels in the gastric mucosa; (5) Total area of superficial vessels in the gastric mucosa; (6) Mean cross sectional vessel area of superficial gastric mucosal vessels; (7) Total number of deep gastric mucosal vessels; (8) Total area of deep gastric mucosal vessels; and (9) Mean cross sectional area of deep gastric mucosal vessels

All calculations were performed blindly by an experienced pathologist who was not informed as to the origin of the preparations.

Statistical analysis

Statistical version 6.0 (Stat Soft Inc.) was used for statistical analysis. First, the distribution of each parameter was determined according to its histograms and normal plots and was confirmed by application of the Shapiro-Wilk W test. Results were expressed as mean \pm SD for variables with normal distribution and as median - interquartile range for skewed distribution. Comparison between groups was performed using Student's t -test for unpaired data to evaluate differences in portal pressure and total submucosal area occupied by vessels; values of these variables followed a normal distribution. For all other variables, the non-parametric Mann Whitney U -test was applied. $P < 0.05$ were considered statistically significant.

RESULTS

Mortality

Forty-eight rats survived the study. There were no significant differences in body weight of rats among the two groups. There were seven deaths in group A and five in group B. As shown in Table 1, one of the propranolol group rats and two of the control group died from variceal bleeding before the end of the study (large amounts of blood were found in the stomach and upper jejunum). Two rats of group A and three rats of group B died from improper manipulation (administration of carbon tetrachloride into the tracheal-bronchial tree), while the deaths of four group A rats were attributed to CCl_4 toxicity.

Ascites

No significant difference in the amount of ascitic fluid was observed between the two groups ($P > 0.05$).

Portal pressure

Portal pressure values followed a normal distribution in both groups. Mean portal pressure was lower in the propranolol group (11.6 ± 1.36) compared to mean portal pressure of control group (14.61 ± 1.84) (Table 2). Comparison between groups revealed a portal pressure decrease of 21.5% in the propranolol group, which was proved to be statistically significant ($P < 0.05$).

Liver cirrhosis

All rats developed micronodular cirrhosis within 8-10 wk.

Table 1 Number of rats and causes of death

	Total number of deaths <i>n</i> (%)	Cause of death		
		Variceal bleeding	CCl_4 toxicity	Improper manipulation
Group A (<i>n</i> = 30)	7 (23.3)	1	4	2
Group B (<i>n</i> = 30)	5 (16.67)	2	-	3

Table 2 Portal pressure in group A and B

	Portal pressure (mmHg)			
	Mean	Minimum	Maximum	SD
Group A (<i>n</i> = 23)	11.60	9.20	14.3	1.36
Group B (<i>n</i> = 25)	14.61	11.3	18.2	1.84

Propranolol causes significant decrease ($P < 0.05$) in portal pressure.

Regenerating nodules surrounded by thickened septa of connective tissue with obvious architectural distortion were present on all hepatic sections. There was no obvious difference in the degree of hepatic fibrosis between the groups.

Gastric sections on light microscopy

Microscopic examination of the stomach revealed excessive mucosal and submucosal vascular congestion. Besides congestion, animals of group B (placebo treated groups) were found with more mucosal and submucosal vessels, while in some of them the development of smooth muscle cells in the mucosa was noticed.

Morphometric analysis of gastric mucosa and submucosa

Measurements and calculations were performed by image analysis in both groups. From the variables studied, only total and mean cross sectional area of superficial gastric mucosal vessels followed a normal distribution in both groups, while all other variables studied presented skewed distributions in either group or were non-continuous scale variables. Comparison between groups was performed using Student's t -test for unpaired data for the variables "total area of gastric superficial mucosal vessels" and "mean cross sectional area of gastric superficial mucosal vessels", and the non-parametric Mann Whitney U -test for all other variables. The summarized analysis and comparison of data are shown in Table 3. Statistically significant differences ($P < 0.05$) between groups were revealed for the variables "total area occupied by vessels" and "total number of counted veins" in the submucosa, the deep and superficial layers of gastric mucosa. On the other hand, the variable "mean cross sectional area" of gastric submucosal vessels as well as of deep and superficial gastric mucosal vessels did not differ significantly between the groups.

DISCUSSION

Esophageal varices have long been considered the major cause of upper gastrointestinal hemorrhage in patients

Table 3 Results of morphometric analysis and comparison between groups

	Group A	Group B	P
Total area of submucosal vessels (μm^2)	47 441.37 \pm 24 299.48	47 539.12 (33 295.84, 55 931.07)	0.0037
Mean cross sectional area of submucosal vessels (μm^2)	4682.70 (3571.92, 6350.68)	5911.17 \pm 1963.34	0.09
Number of submucosal vessels	5 (4, 7)	8 (7, 9)	0.01
Total area of superficial gastric mucosal vessels (μm^2)	7004.03 \pm 2438.37	10 994.49 \pm 3746.56	0.0001
Mean cross sectional area of superficial mucosal vessels (μm^2)	642.78 \pm 432.59	573.70 (475.08, 623.39)	0.25
Number of superficial gastric mucosal vessels	12 (9, 14)	20 (17, 24)	0.00001
Total area of deep gastric mucosal vessels (μm^2)	6916.76 (3694.98, 8016.40)	19 367.84 \pm 7034.08	0.0008
Mean cross sectional area of deep gastric mucosal vessels (μm^2)	834.88 (554.05, 953.04)	982.90 (697.11, 1249.35)	0.1
Number of deep gastric mucosal vessels	9 (7, 11)	20 (16, 22)	0.00001

Values are mean \pm SD or median (lower, upper quartiles).

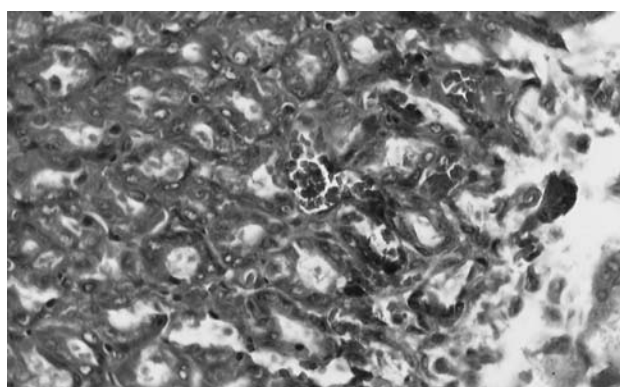


Figure 1 Vascular ectasia and congestion of gastric submucosa in group B (HE, \times 400).

with portal hypertension. However, gastric mucosal lesions have lately been considered as another frequent cause of upper gastrointestinal bleeding in these patients, accounting for 20% to 40% of all cases^[23,24]. Dilated precapillaries, capillaries and submucosal veins, extensive submucosal edema, thickening of the submucosal arteriolar walls and submucosal veins showing features of arterIALIZATION, are all observed in patients with portal hypertension^[5,25-28], while morphometric analyses have shown an increase of mean mucosal capillary cross-sectional area^[29-32]. Clinically significant bleeding is seen in association with severe portal hypertensive gastropathy (PHG) and non-selective beta-blockers, such as propranolol and nadolol, have been shown to reduce portal pressure and gastric mucosal blood flow. Previous experimental studies using propranolol^[13,14,33,34] and clonidine^[35] early in the process of portal hypertension induction have been proven effective in prevention of complications.

However, these studies, mainly based on hemodynamic measurements, are very sensitive and easily affected by a number of imponderable and in many cases unknown factors^[36-38]. This might explain why similar studies from various research centers often resulted in completely different conclusions^[39-43].

To avoid these problems, we decided to directly investigate the effects of early propranolol administration on gastric mucosal and submucosal pathology. The gastric mucosal and submucosal vein plexus (Figure 1) was meticulously studied and measurements of vessels'

and submucosa's areas were carefully performed with the aid of an image analysis system. Portal pressure was the only hemodynamic parameter studied. Measurements revealed a 21.5% decrease of portal pressure in propranolol treated rats; these results are fully compatible with literature data^[13,14,33,34]. On the other hand, careful analysis of morphometric data revealed that early propranolol administration significantly affects the total area of gastric submucosal and mucosal vessels as well as the number of gastric submucosal and mucosal vessels of cirrhotic rats, while the mean cross sectional area does not seem to be significantly affected.

In clinical practice, cirrhosis represents the major cause of portal hypertension. Induction of cirrhotic portal hypertension by carbon tetrachloride administration was therefore considered to be an appropriate experimental model for our study. In this model, portal hypertensive syndrome is fully developed after a reasonable time, permitting the study of chronic and early propranolol administration. All similar studies presented in the literature^[13,14,33-35], were carried out either in prehepatic portal hypertension or in cases of schistosomiasis, probably due to lack of reliable models capable of developing esophagogastric varices in cirrhotic rats. This is mainly due to the development of extended collaterals from the portal vein to the left renal vein via the left adrenal vein. These collaterals, which are non-functional in normal rats, prevent portosystemic shunt through the gastric and lower esophageal veins in case of portal hypertension. We overcame this problem by using a modification of the well-known model of carbon tetrachloride induced cirrhosis. This included the induction of cirrhosis in rats that had previously undergone ligation of the left adrenal vein and complete devascularization of the left renal vein. The effectiveness of this model has already been demonstrated in previous studies^[15-17,19].

Gastric submucosal vessels, as well as superficial and deep gastric mucosal vessels, were meticulously studied, and various measurements were carefully performed using an image analysis system, which permitted objective determination of numerous parameters. All gastric submucosal, as well as deep and superficial gastric mucosal vessels per optical field, were counted, and their borders were carefully delineated, to calculate the total and mean cross sectional area of submucosal gastric

veins, and deep and superficial gastric mucosal vessels. An accurate method of measuring vessel cross-sectional areas and comparing them would be to perfuse fix the vessels with a controlled perfusion pressure; however, the significant in vivo differences in portal pressure between the groups, and their effect on gastric vessels, would be masked. We also thought to perfuse fix vessels with pressures comparative to portal pressures; however, this was technically difficult. Thus, the final choice was to use no perfusion fixation and compare simple sections, by measuring several parameters, including vessel numbers. Several factors, besides portal pressure, affect vein development and gastric mucosal and submucosal congestion in cirrhotic animals. It is a general belief that portal pressure increase is the main causative factor for the development of portosystemic collaterals^[1-3], which are considered to be the result of widening, distension, and hypertrophy of pre-existing vessels. Additionally, active angiogenesis can also participate in their formation. Propranolol, by reducing the hepatic venous pressure gradient and azygos blood flow, seems to contribute to the reducing opening of pre-existing blood vessels. On the other hand, neoangiogenesis seems to be prevented by abolishing the norepinephrine inducing effect on vascular endothelial growth factor (VEGF) expression^[44-46]. Morphometric analysis in our study revealed a statistically significant difference ($P < 0.05$) between groups in the number of mucosal and submucosal vessels, as well as in the total area occupied by vessels, which was significantly greater ($P < 0.05$) in the placebo group compared to the propranolol treated group. On the other hand, there was no difference in mean cross sectional area of submucosal and mucosal vessels between the groups.

We can therefore claim, based on the results of this experimental study, that early propranolol administration in portal hypertensive cirrhotic rats could be useful in prevention of portal hypertensive gastropathy and its complications.

COMMENTS

Background

Patients with portal hypertension are at substantial risk of bleeding from small gastric mucosal lesions that have been largely described as portal hypertensive gastropathy.

Research frontiers

Propranolol is a well-known and extensively used beta-blocker that has been shown to reduce bleeding related to portal hypertensive gastropathy in small studies. In the area of prevention of portal hypertensive gastropathy, previous experimental studies have shown that early continuous administration of non-selective β -blockers, such as propranolol, could ameliorate portosystemic hemodynamics and therefore reduce complications. The sensitive and easily affected hemodynamic measurements used in previous studies have resulted in different conclusions and led to an effort to directly investigate the effects of early propranolol administration on gastric mucosal and submucosal pathology.

Innovations and breakthroughs

The concept that early continuous administration of drugs is capable of reducing portal pressure and could prevent the development of extended portosystemic collaterals and consequently esophagogastric varices or portal hypertensive gastropathy, has led to a series of experimental and clinical studies. Many agents with a known lowering effect on portal pressure (including propranolol, nadolol, clonidine, octreotide, isosorbite mononitrate and, more recently, endothelin

receptor antagonists) have already been tested for their effect on portosystemic shunting and development of esophageal varices, when administered early, that is before the full development of portal hypertension syndrome. Several studies have been published, both clinical and experimental, with controversial results on the protective role of non-selective beta-blockers. To avoid complicated and easily affected hemodynamic measurements the authors decided to directly investigate the effects of early propranolol administration on gastric mucosal and submucosal pathology. The gastric mucosal and submucosal vein plexus was meticulously studied and measurements of vessels' and submucosa's area were carefully performed with the aid of an image analysis system. Portal pressure was the only hemodynamic parameter studied in order to confirm the already known propranolol's effect on it.

Applications

The study results suggest that early propranolol administration in portal hypertensive cirrhotic rats could prevent intense gastric vascular congestion, which characterizes portal hypertensive gastropathy and could therefore be useful in preventing its complications.

Peer review

This is a well designed study to look at the effects of a non selective β blocker in the prevention of portal hypertensive gastropathy in a rat model for cirrhosis and portal hypertension.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Yin DH



BRIEF ARTICLES

Does *Helicobacter pylori* eradication therapy for peptic ulcer prevent gastric cancer?

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Received: June 29, 2009 Revised: July 29, 2009

Accepted: August 5, 2009

Published online: September 14, 2009

Abstract

AIM: To investigate the effects of *Helicobacter pylori* (*H. pylori*) eradication therapy for treatment of peptic ulcer on the incidence of gastric cancer.

METHODS: A multicenter prospective cohort study was conducted between November 2000 and December 2007 in Yamagata Prefecture, Japan. The study included patients with *H. pylori*-positive peptic ulcer who decided themselves whether to receive *H. pylori* eradication (eradication group) or conventional antacid therapy (non-eradication group). Incidence of gastric cancer in the two groups was determined based on the results of annual endoscopy and questionnaire surveys, as well as Yamagata Prefectural Cancer Registry data, and was compared between the two groups and by results of *H. pylori* therapy.

RESULTS: A total of 4133 patients aged between 13 and 91 years (mean 52.9 years) were registered, and 56 cases of gastric cancer were identified over a mean follow-up of 5.6 years. The sex- and age-adjusted incidence ratio of gastric cancer in the eradication group, as compared with the non-eradication group, was 0.58 (95% CI: 0.28-1.19) and ratios by follow-up period (< 1 year, 1-3 years, > 3 years) were 1.16 (0.27-5.00), 0.50 (0.17-1.49), and 0.34 (0.09-1.28), respectively. Longer follow-up tended to be associated with better prevention of gastric cancer, although not to a significant extent. No significant difference in incidence of gastric cancer was observed between patients with successful eradication therapy (32/2451 patients, 1.31%) and those with treatment failure (11/639 patients, 1.72%). Among patients with duodenal ulcer, which is known to be more prevalent in younger individuals, the incidence of gastric cancer was significantly less in those with successful eradication therapy (2/845 patients, 0.24%) than in those with treatment failure (3/216 patients, 1.39%).

CONCLUSION: *H. pylori* eradication therapy for peptic ulcer patients with a mean age of 52.9 years at registration did not significantly decrease the incidence of gastric cancer.

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Key words: *Helicobacter pylori*; Peptic ulcer; Gastric cancer; Eradication therapy; Cancer prevention

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Mabe K, Takahashi M, Oizumi H, Tsukuma H, Shibata A, Fukase K, Matsuda T, Takeda H, Kawata S. Does *Helicobacter pylori* eradication therapy for peptic ulcer prevent gastric cancer? *World J Gastroenterol* 2009; 15(34): 4290-4297 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4290.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4290>

INTRODUCTION

Yamagata Prefecture is an area in which gastric cancer

is particularly common. In Cancer Incidence in Five Continents, published by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO), it is reported that the incidence of gastric cancer for men in Yamagata Prefecture, was the second highest in the world, at 91.6/100 000 (ASR world) in 1993-1997^[1]. Yamagata Prefecture therefore has attempted aggressively to achieve secondary prevention of gastric cancer. In 1994, IARC/WHO concluded that *Helicobacter pylori* (*H. pylori*) is a definite carcinogen in humans^[2], and the results of a prospective cohort study^[3] and animal studies^[4-6] have demonstrated that *H. pylori* causes gastric cancer. The possible role of *H. pylori* eradication therapy in primary prevention of gastric cancer has thus attracted substantial interest in Yamagata Prefecture. In November 2000, when coverage of *H. pylori* eradication for patients with *H. pylori*-positive peptic ulcer by the National Health Insurance system in Japan began, the Yamagata *H. pylori* Clinical Study Group was established to design a multicenter prospective cohort study to investigate whether *H. pylori* eradication therapy for patients with peptic ulcer can decrease the incidence of gastric cancer.

Although animal studies have revealed that primary prevention of gastric cancer by *H. pylori* eradication is more effective as the duration between *H. pylori* infection and eradication is decreased^[7], the effects in humans of this type of prevention have not been determined sufficiently. Non-randomized prospective studies^[8,9] and retrospective studies^[10,11] in Japan have suggested that *H. pylori* eradication therapy prevents the development of gastric cancer, while a large-scale randomized controlled study in China did not support this conclusion. Although, a sub-population analysis of patients who did not have precancerous change at the time of eradication therapy has suggested gastric-cancer-preventive effects of *H. pylori* eradication therapy^[12]. No significant reduction in the incidence of gastric cancer by *H. pylori* eradication therapy was observed in a meta-analysis^[13]. In a multicenter, randomized controlled study in patients who underwent endoscopic resection of early gastric cancer and were thus at high risk of secondary gastric cancer, occurrence of secondary gastric cancer was prevented significantly by *H. pylori* eradication therapy^[14]. Evidence of prevention of gastric cancer by *H. pylori* eradication therapy thus needs to be obtained.

This report describes the results of a multicenter, prospective cohort study that investigated whether *H. pylori* eradication therapy in patients with peptic ulcer, living in an area where the incidence of gastric cancer is especially high, was effective in primary prevention of gastric cancer. The results of the present study, including endoscopy findings, were reconciled with those of the Yamagata Prefecture Cancer Registry to ensure accurate detection of gastric cancer.

MATERIALS AND METHODS

Study design

The present study was designed at Yamagata Prefectural Central Hospital, where the office of the Yamagata

H. pylori Clinical Study Group was located in May 2000. It included 82 participating institutions, 26 hospitals and 56 clinics, in Yamagata Prefecture. For ethical reasons, we selected performance of a non-randomized, multicenter, prospective cohort study in which patients decided themselves whether to receive *H. pylori* eradication therapy (eradication group) or conventional antacid therapy (non-eradication group) for the treatment of *H. pylori*-positive peptic ulcer. The sample size was calculated to detect a significant difference in incidence of gastric cancer in patients who received *H. pylori* eradication therapy or conventional antacid treatment over a 7-year period (a 2-year registration period and a 5-year follow-up period), with a power of 90% and an alpha error of 5% on the basis of the following assumptions: the incidence of gastric cancer in patients who received conventional antacid therapy was 0.5%; *H. pylori* eradication therapy decreased the incidence of gastric cancer by 50%-90%; the percentage of withdrawals was 20%; and patients were allocated to the non-eradication and eradication groups at a ratio of 1:5. We estimated that 560-2467 patients and 2797-12 333 patients were required for the non-eradication and eradication therapy groups, respectively. All tests and treatments performed were covered by the National Health Insurance (NHI) as determined by the Ministry of Health and Welfare of Japan (currently the Ministry of Health, Labor, and Welfare of Japan), and the study protocol was approved by the Ethics Committee of Yamagata Prefectural Central Hospital. All patients received a full explanation of the study using a standardized document, and provided written informed consent before registration in the study.

Patients with *H. pylori*-positive peptic ulcer were considered eligible. Patients with a history of gastric cancer and those in whom endoscopy or biopsy at the time of registration revealed gastric cancer were excluded from the study. Patients were registered between November 2000 and December 2003, and followed up until the end of December 2007.

Diagnosis of *H. pylori*-positive peptic ulcer and treatment

Prior to registration, all patients underwent upper gastrointestinal endoscopy and biopsy, if necessary, to diagnose peptic ulcer and exclude gastric cancer. During endoscopy, biopsy samples were collected from the greater curvature of the upper body and antrum of the stomach. The presence/absence of *H. pylori* infection was evaluated by rapid urease test.

H. pylori eradication therapy consisted of 30 mg lansoprazole or 20 mg omeprazole, plus 750 mg amoxicillin and 200 or 400 mg clarithromycin, all twice daily for 7 d. At least 1 mo after the completion of eradication therapy, patients underwent upper gastrointestinal endoscopy with the rapid urease test and urea breath test (with a cut-off value of 2.5‰; Ubit, Otsuka Pharmaceuticals, Tokyo, Japan). Successful *H. pylori* eradication was defined as negative results on the rapid urease and urea breath tests. When the results of the two tests were inconsistent, retesting was performed.

Conventional antacid therapy consisted of antacids such as proton-pump inhibitors and histamine- H_2 blockers.

Detection of gastric cancer

During the follow-up period up to December 2007, endoscopy was performed annually, in principle, to determine the presence/absence of gastric cancer. When follow-up endoscopy was performed, the investigators reported its results to the study office using a follow-up report form to provide information on the date of endoscopy, stage of ulcer, results of *H pylori* testing, and presence/absence of newly developed gastric cancer or other gastrointestinal diseases (such as reflux esophagitis, erosive gastritis/duodenitis, and esophageal adenocarcinoma).

To avoid overlooking gastric cancer due to the absence of annual endoscopy, a questionnaire survey was conducted and the data obtained in the present study were reconciled with those of the Yamagata Prefecture Cancer Registry. In October 2006, a questionnaire was mailed to all registered patients to determine the presence/absence of gastric cancer diagnosed after registration. The results of the questionnaire were compared with the data at registration to identify patients who might have developed gastric cancer after registration. Such cases were referred to the participating medical institutions to confirm the diagnosis of gastric cancer. In March 2008, record linkage between the cohort and Yamagata Prefectural Cancer Registry was conducted for identification of previous and new gastric cancer cases during the follow-up period up to the end of December 2007.

Statistical analysis

Person-years were calculated from the date of recruitment to the date of incidence of gastric cancer; end of follow-up in December 2007; date of change of residence to outside Yamagata Prefecture; death from causes other than gastric cancer; or the initiation of *H pylori* eradication therapy for patients in the antacid therapy group, whichever came first. For comparison between groups at baseline, Fisher's exact test was used for sex and type of peptic ulcer, and Student's *t* test or χ^2 test for histological type, location and stage of cancer, and treatment, with a level of significance of $P < 0.05$ (two-tailed).

Poisson regression was used to estimate the relative risk of gastric cancer in relation to eradication therapy for *H pylori*. Analyses were adjusted routinely for sex and age (< 60 , $60-70$, > 70 years), and stratified for duration of follow-up (< 1 , $1-3$, > 3 years) and location of ulcer (gastric, gastroduodenal or duodenal). We also examined the effects of adjustment for other risk factors including location of ulcer, intake of salt, and smoking. These statistical analyses were performed using Intercooled Stata 8.0 for Windows software (StataCorp LP, College Station, TX, USA).

The accumulated incidence of gastric cancer in patients with successful and unsuccessful *H pylori*

eradication was determined using data for patients for whom the results of *H pylori* eradication therapy were confirmed using the Kaplan-Meier method, and were tested for significance of difference between patients with and without successful eradication using the log-rank method. These analyses were performed using Dr. SPSS II for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Baseline characteristics of study subjects

A total of 4203 patients were registered, and 70 patients who had a history of gastric cancer or had gastric cancer at the time of registration were excluded. Intention-to-treat (ITT) analysis was performed for the data from the remaining 4133 patients (2964 male and 1169 female), who were aged between 13 and 91 years (mean 52.9 years).

H pylori eradication therapy was administered to 3781 patients (91.5%; eradication group) and conventional antacid therapy to 352 patients (8.5%; non-eradication group). Table 1 summarizes the characteristics of the 4133 patients included in the present study. There were no significant differences between the eradication and non-eradication groups in baseline characteristics such as distribution of sex or location of ulcers, while mean age was lower in the eradication than in the non-eradication group (52.4 years *vs* 58.1 years). No results of *H pylori* eradication therapy were reported for 691 (18.3%) of the 3781 patients who received *H pylori* eradication therapy. The eradication rate evaluated in the ITT analysis and the per-protocol (PP) analysis were 64.8% (2451/3781) and 79.3% (2451/3090), respectively. There were no significant differences in any factors, including age, between patients with and without successful *H pylori* eradication (mean ages of patients with and without successful *H pylori* eradication were 52.5 ± 12.3 years and 52.1 ± 13.5 , respectively; $P = 0.51$).

Development of gastric cancer

During a total of 22 900 person-years of follow-up (mean follow-up period: 5.6 years), gastric cancer was found in 56 patients, including 47/3781 patients (1.24%, 0.21%/year) who received *H pylori* eradication therapy and 9/352 patients (2.56%, 0.50%/year) who received conventional antacid therapy. There were no differences in sex distribution; location of ulcer lesions; histological type, location or stage of cancer; or type of treatment for gastric cancer between patients with and without *H pylori* eradication therapy (Table 2). Poisson regression analysis of factors that affected the incidence of gastric cancer revealed that sex, age group, and location of ulcers were independent factors that affected the differences in incidence rate ratio (IRR) of gastric cancer between patients receiving and not receiving *H pylori* eradication therapy (Table 3). The IRR of gastric cancer adjusted for sex and age group was 0.58 (95% CI: 0.28-1.19). *H pylori* eradication therapy decreased the incidence of gastric cancer by about 40%, although this change was not statistically significant. IRR was

Table 1 Baseline characteristics of 4133 patients

	Eradication group (n = 3781)	Non-eradication group (n = 352)	
Male/Female	2715/1066	249/103	$P = 0.67^1$
Male (%)	71.80	70.70	
Mean age	52.4 ± 12.7	58.1 ± 12.6	$P < 0.001^2$
Min-max	13.2-85.9	22.2-91.9	
Age (yr)			$P < 0.001^1$
< 60	2732 (72.3)	186 (52.8)	
60-70	701 (18.5)	102 (29.0)	
> 70	348 (9.2)	64 (18.2)	
Location of ulcer			$P = 0.41^1$
GU	2048 (54.2)	195 (55.4)	
GDU	418 (11.1)	45 (12.8)	
DU	1265 (33.5)	110 (31.3)	
Unknown	50 (1.3)	2 (0.6)	
Salt consumption			$P < 0.001^1$
Restricted	1377 (36.4)	173 (49.1)	
No interest in salt consumption	729 (19.3)	29 (8.2)	
Not restricted	1185 (31.3)	105 (29.8)	
Unknown	490 (13.0)	45 (12.8)	
Smoking history			$P < 0.001^1$
Non-smokers	1174 (31.0)	120 (34.1)	
Past smokers	565 (14.9)	48 (13.6)	
Current smokers	1931 (51.1)	159 (45.2)	
Unknown	111 (2.9)	25 (7.1)	
Mean duration of follow-up (yr)	5.6 ± 1.1	5.2 ± 1.8	$P < 0.001^2$
Min-max	0.09-7.96	0.11-8.41	

¹ χ^2 test; ²*t* test. Eradication group: patients who received *Helicobacter pylori* eradication therapy with or without successful eradication. Non-eradication group: Patients who received conventional antacid therapy. GU: Gastric ulcer; GDU: Gastroduodenal ulcer; DU: Duodenal ulcer.

by duration of follow-up 1.16 (0.27-5.00) for patients followed up for < 1 year, 0.50 (0.17-1.49) for 1-3 years, and 0.34 (0.09-1.28) for > 3 years. There were no significant differences in the incidence of gastric cancer between any subgroups of the eradication and non-eradication groups, although the difference in incidence between the groups tended to increase as the duration of follow-up was prolonged (Table 4). Gastric cancer was found in 6/1375 patients with duodenal ulcer (0.44%). All six patients were > 50 years of age at the time of registration. No cases of Barrett's adenocarcinoma, for which the possibility of increase in occurrence after *H. pylori* eradication therapy was a concern, were found in either of the two groups.

In a separate analysis, 3090 patients who received *H. pylori* eradication therapy, with known results, were compared for incidence of gastric cancer according to presence/absence of successful eradication. Gastric cancer was detected in 43 of the 3090 patients, including 32/2451 patients (1.31%) and 11/639 patients (1.72%) with and without successful eradication, respectively. No significant difference in incidence of gastric cancer was observed between patients with and without successful eradication. Analysis by type of peptic ulcer at the time of registration revealed that the incidence of gastric cancer did not differ between patients with and without successful eradication in subgroups of patients

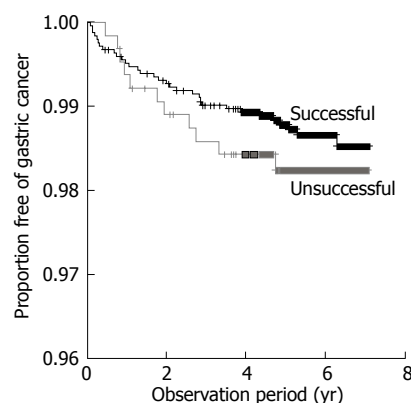


Figure 1 Proportion free of gastric cancer in the eradication group was compared according to results of eradication therapy, using Kaplan-Meier analysis. The incidence was 32/2451 (1.31%) in patients with successful eradication and 11/639 (1.72%) in patients with failure of eradication (log-rank test, $P = 0.43$).

with gastric or gastroduodenal ulcer, while successful eradication decreased the incidence of gastric cancer significantly in patients with duodenal ulcer (Figures 1 and 2).

DISCUSSION

This was a prospective, multicenter, cohort study in patients with *H. pylori*-positive peptic ulcer, which was designed to evaluate whether *H. pylori* eradication therapy decreased the incidence of gastric cancer in Yamagata Prefecture, a region in which gastric cancer is especially common. Since continuous follow-up is often difficult in clinical observation studies, the data from the present study were reconciled with those of the Yamagata Prefecture Cancer Registry to avoid overlooking gastric cancer. This was a unique feature of the present study.

During the follow-up of 4133 patients with peptic ulcer (mean age: 52.9 years) for a mean of 5.6 years, the incidence of gastric cancer in patients who received *H. pylori* eradication therapy was decreased by about 40% compared with that in patients who did not receive eradication therapy, although the difference between the groups was not statistically significant. Longer follow-up period tended to be associated with better prevention of gastric cancer, albeit not to a significant extent. Although there was no significant difference in incidence of gastric cancer according to the result of eradication therapy (success/failure) in those patients who received this treatment, successful eradication therapy did decrease significantly the incidence of gastric cancer in patients with duodenal ulcer.

There are four limitations to the interpretation of the results of the present study: (1) it was not a randomized controlled trial, and the number of patients not receiving eradication therapy was small; (2) the eradication rate was only 80%; (3) the follow-up period was not sufficiently long; and (4) the mean age of participants was high, at 53 years.

Factors (1) and (2) are limitations of the present study, in which randomization of patients was impossible

Table 2 Distribution of cases of gastric cancer

Sex	Male	Female			Total		
Eradication group	38	9			47	$P = 0.328^1$	
Non-eradication group	9	0			9		
Type of peptic ulcer	GU/GDU	DU					
Eradication group	43	4			47	$P = 0.244^1$	
Non-eradication group	7	2			9		
Histological type of cancer	Intestinal	Diffuse	Unknown				
Eradication group	35	10	2			47	$P = 0.304^2$
Non-eradication group	5	4	0			9	
Location of cancer	L	M	U	Unknown			
Eradication group	21	18	6	2	47	$P = 0.759^2$	
Non-eradication group	3	5	1	0	9		
Stage	Early	Advanced	Unknown				
Eradication group	34	11	2			47	$P = 0.198^2$
Non-eradication group	9	0	0			9	
Treatment	Endoscopy (EMR/ESD)	Surgery	Chemotherapy				
Eradication group	12	31	2			47	$P = 0.763^2$
Non-eradication group	2	7	0			9	

¹Fisher's direct test; ²Student's *t* test or χ^2 test. ESD: Endoscopic submucosal dissection; L: Lower third of the stomach; M: Middle third of the stomach; U: Upper third of the stomach.

Table 3 Results of poisson regression analysis

	IRR					
	Univariate	95% CI	<i>P</i>	Multivariate	95% CI	<i>P</i>
Eradication group	0.45	0.22-0.92	0.03	0.61	0.29-1.27	0.18
Non-eradication group	1.00			1.00		
Sex						
Male	1.00		0.05	1.00		0.03
Female	0.49	0.24-1.00		0.39	0.17-0.88	
Age (yr)	1.09	1.05-1.12	< 0.01			
< 60	1.00			1.00		
60-70	3.22	1.74-5.95	< 0.01	2.59	1.37-4.91	< 0.01
> 70	5.18	2.69-10.0	< 0.01	4.23	2.09-8.53	< 0.01
Location of ulcer	0.69	0.40-1.19	0.18			
Stomach/Stomach + duodenum	1.00			1.00		
Duodenum	0.28	0.13-0.63	< 0.01	0.37	0.16-0.83	0.02
Unknown	1.16	0.16-8.43	0.88	1.69	0.23-12.48	0.61
Salt consumption	0.94	0.84-1.06	0.27			
Restricted	1.00			1.00		
No interest in salt consumption	0.43	0.16-1.11	0.08	0.65	0.25-1.73	0.39
Not restricted	0.78	0.43-1.41	0.41	1.03	0.56-1.90	0.93
Unknown	0.58	0.24-1.39	0.22	0.58	0.22-1.60	0.29
Smoking history	1.04	0.89-1.22	0.63			
Non-smokers	1.00			1.00		
Past smokers	1.56	0.74-3.29	0.25	0.97	0.43-2.20	0.95
Current smokers	0.94	0.50-1.76	0.85	0.78	0.38-1.61	0.50
Unknown	1.71	0.50-5.88	0.39	1.65	0.41-6.65	0.48

IRR: Incidence rate ratio.

Table 4 IRRs of gastric cancer by follow-up period and location of ulcer

	Eradication group			Non-eradication group			IRR		IRR (sex- and age-adjusted)	
	per 1000 person-years	<i>n</i>	Incidence	per 1000 person-years	<i>n</i>	Incidence	95% CI		95% CI	
Overall	21.2	47	2.22	1.82	9	4.93	0.45	0.22-0.92	0.58	0.28-1.19
Follow-up period (yr)										
< 1	3.77	17	4.51	0.34	2	5.81	0.78	0.18-3.33	1.16	0.27-5.00
1-3	7.48	20	2.67	0.64	4	6.27	0.43	0.15-1.25	0.50	0.17-1.49
> 3	9.92	10	1.01	0.84	3	3.56	0.28	0.08-1.03	0.34	0.09-1.28
Gastric/Gastric + duodenal ulcers	13.8	41	2.97	1.21	7	5.76	0.52	0.23-1.15	0.62	0.27-1.39
Duodenal ulcers	7.12	5	0.70	0.60	2	3.35	0.21	0.04-1.08	0.31	0.06-1.61

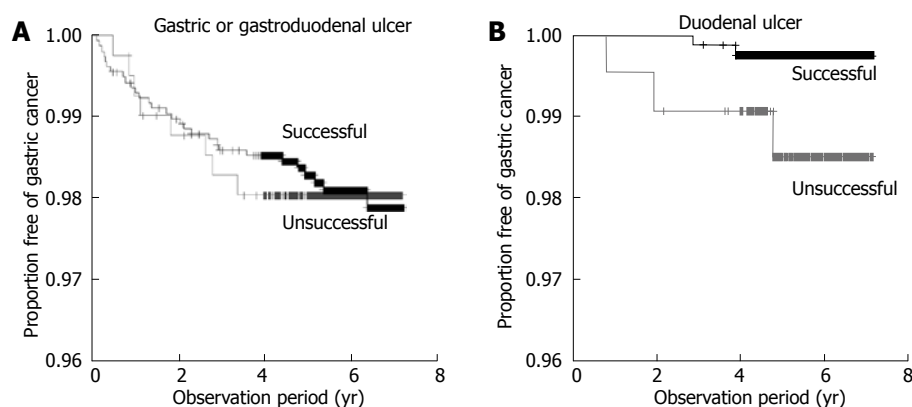


Figure 2 Proportion free of gastric cancer in patients with gastric or gastroduodenal ulcer (A) and duodenal ulcer (B) in the eradication group was compared according to results of eradication therapy, using Kaplan-Meier analysis. A: The incidence of gastric cancer was 29/1572 (1.85%) in patients with successful eradication and 8/413 (1.94%) in patients with failure of eradication (log-rank test, $P = 0.92$); B: The incidence of gastric cancer was 2/845 (0.24%) in patients with successful eradication and 3/216 (1.39%) in patients with failure of eradication (log-rank test, $P = 0.03$).

for ethical reasons, and secondary *H. pylori* eradication therapy was not covered by the NHI system. In addition to the four factors noted above, since the decrease in incidence of gastric cancer by *H. pylori* eradication was smaller than expected, and the numbers of patients did not reach those targeted, especially in the non-eradication group, the study did not have the statistical power required to detect a significant difference in the incidence of gastric cancer between patients who received eradication therapy and conventional antacid therapy.

The finding that the efficacy of *H. pylori* eradication in preventing gastric cancer tended to be better among patients with a longer follow-up period suggests that the length of follow-up in this study may have been insufficient. In a study of patients who underwent resection of gastric cancer, cancer in other locations was not detected upon preoperative evaluation^[15]. Although all patients evaluated in the present study underwent endoscopy and biopsy prior to registration, if required, the possibility cannot be ruled out that some patients had undetectable gastric cancer before registration. Gastric cancer lesions detected during the early phase after eradication therapy may in many cases have been present before therapy. More accurate determination of the efficacy of eradication therapy in preventing gastric cancer will require that patients be followed up for a long period of time. Since follow-up endoscopy cannot be continued for many years, it is important that our data be reconciled with those of the Yamagata Prefecture Cancer Registry to continue follow-up of the participants.

As pointed out by Wong *et al.*^[12], the precancerous state may represent the point of no return at which development of gastric cancer can no longer be prevented by *H. pylori* eradication. The participants in the present study were patients with peptic ulcer with a mean age of 53 years, and many patients with gastric ulcer also have atrophic gastritis or intestinal metaplasia. Among the registered patients, duodenal ulcer was more common in those < 50 years of age, while gastric ulcer

was common in patients > 50 years of age. The risk of gastric cancer was higher in patients with gastric or gastroduodenal ulcer than in those with duodenal ulcer, and a significant decrease in the incidence of gastric cancer according to successful *H. pylori* eradication was observed only in patients who underwent *H. pylori* eradication for the treatment of duodenal ulcer. Since *H. pylori* infection is usually established during childhood, it appears likely that antral gastritis and duodenal ulcer are common among young patients with a relatively short history of *H. pylori* infection, and that eradication of *H. pylori* may decrease the occurrence of gastric cancer. However, patients with a longer history of *H. pylori* infection often have corpus gastritis, and eradication therapy does not prevent gastric cancer to a significant extent. The results of an experiment in Mongolian gerbils has shown that eradication of *H. pylori* is more effective in preventing gastric cancer in animals with a shorter duration of *H. pylori* infection^[7]. These findings suggest the importance of the timing and target of *H. pylori* eradication therapy in preventing gastric cancer.

In a randomized clinical study on the effects of eradication of *H. pylori* after endoscopic mucosal resection of early gastric cancer, Fukase *et al.*^[14] have reported a significant decrease in the incidence of secondary gastric carcinoma during a 3-year follow-up period. The significant prevention of secondary gastric cancer by *H. pylori* eradication was considered to be caused by the following: (1) the risk of development of gastric cancer in patients following endoscopic mucosal resection for early gastric cancer is about 10-fold higher than in patients with *H. pylori*-positive peptic ulcer; (2) patients underwent accurate endoscopy several times before and after treatment for cancer; and (3) the number of cases of gastric cancer that were overlooked at the time of registration was therefore considered smaller than in other surveys.

Prior to initiation of the present study, there was concern regarding the possibility of a decrease in visits for endoscopy after symptomatic improvement, which could have resulted in an increase in advanced gastric

cancer. Although the investigators fully explained to subjects the risk of development of gastric cancer after *H pylori* eradication therapy and the importance of follow-up endoscopy, > 15% of them did not undergo examination to confirm the results of *H pylori* eradication therapy. The percentage of patients who received follow-up endoscopy as specified was significantly lower among those who received *H pylori* eradication therapy compared with conventional antacid therapy. Although this may have biased the rate of detection of gastric cancer, we attempted to decrease bias by reconciling our data with those of the Yamagata Prefecture Cancer Registry. Of the 56 cases of gastric cancer detected in the present study, 11 were detected as advanced gastric cancer. Cases of advanced gastric cancer mainly consisted of diffuse-type cancer and those with a mixture of intestinal and diffuse cancer cells. Careful observation is thus needed for the development of gastric carcinoma, especially diffuse-type gastric cancer, which may progress rapidly and is often difficult to detect.

Although it has been reported that gastric cancer does not develop in patients with duodenal ulcer^[3,8,9], gastric cancer developed in six patients with duodenal ulcer in the present study, including five aged ≥ 60 years and one 51-year-old patient. Since continuous infection with *H pylori* may result in the development of corpus gastritis in patients with antral gastritis associated with duodenal ulcer, middle-aged and elderly patients with duodenal ulcer often have gastritis in the corpus, and should thus be considered at high risk for development of gastric cancer, even after successful *H pylori* eradication therapy, especially in regions with a high incidence of gastric cancer.

In conclusion, *H pylori* eradication therapy for patients with peptic ulcer with a mean age of 52.9 years did not significantly decrease the incidence of gastric cancer during a mean follow-up period of 5.6 years. Although our results did not rule out a role for *H pylori* eradication therapy in preventing gastric cancer, the number of patients evaluated, duration of observation, and rate of eradication of *H pylori* were insufficient to obtain a significant difference in the incidence of gastric cancer between the groups with and without eradication therapy, and the mean age of patients was high. Further studies to clarify the efficacy of *H pylori* eradication in preventing gastric cancer will require eradication of *H pylori* as early as possible and careful and prolonged follow-up of patients.

ACKNOWLEDGMENTS

We acknowledge the cooperation with the study group of the 82 participating institutions and the registration of clinical data by 130 investigators.

COMMENTS

Background

Yamagata Prefecture in Japan has the second highest incidence of gastric

cancer in the world. *Helicobacter pylori* (*H pylori*) plays an important role in the development of gastric cancer. It is thus of crucial importance to determine whether *H pylori* eradication therapy in this geographical area is effective in primary prevention of gastric cancer.

Research frontiers

Although it has been demonstrated that *H pylori* is a definite carcinogen in humans, previous studies that have examined the efficacy of eradication therapy in preventing gastric cancer have yielded inconsistent findings. In the present study, the authors found that eradication therapy in patients with peptic ulcer with a high mean age of 53 years did not significantly decrease the incidence of gastric cancer, at least over the mean 5.6-year follow-up period.

Innovations and breakthroughs

Single-center prospective studies and retrospective studies have reported significant prevention of gastric cancer by eradication therapy, while one randomized clinical trial has revealed no overall effects, but significant prevention of gastric cancer in patients without precancerous lesions. The present multicenter, prospective cohort study, conducted in an area where the incidence of gastric cancer is especially high, revealed no overall efficacy of *H pylori* eradication for preventing gastric cancer in patients with peptic ulcer. In contrast, it demonstrated that eradication was associated with a significant decrease of gastric cancer in patients with duodenal ulcer, which is known to be more prevalent in younger individuals.

Applications

Given the overall lack of efficacy of eradication therapy for peptic ulcer in preventing gastric cancer, the findings highlight the importance of longer and careful follow-up after eradication therapy. Furthermore, the significant efficacy of treatment observed in younger patients suggests the need to eradicate *H pylori* as early as possible.

Terminology

Conventional antacid therapy: a conventional method of treatment, covered by the National Health Insurance (NHI) as determined by the Ministry of Health, Labor, and Welfare of Japan, which consists of treatment with antacids including proton-pump inhibitors and histamine- H_2 blockers given over 6-8 wk.

Peer review

The innovative content, as well as readability, reflects the advanced level of clinical research in gastroenterology both at home and abroad.

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S- Editor Tian L L- Editor Kerr C E- Editor Yin DH

BRIEF ARTICLES

Small sphincterotomy combined with endoscopic papillary large balloon dilation *versus* sphincterotomy

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Received: May 12, 2009 Revised: July 24, 2009

Accepted: July 31, 2009

Published online: September 14, 2009

CONCLUSION: SES + ELBD did not show significant benefits compared to conventional EST, especially for the removal of large (≥ 15 mm) bile duct stones.

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Key words: Sphincterotomy; Endoscopic; Balloon dilatation; Cholelithiasis; Lithotripsy

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Kim HG, Cheon YK, Cho YD, Moon JH, Park DH, Lee TH, Choi HJ, Park SH, Lee JS, Lee MS. Small sphincterotomy combined with endoscopic papillary large balloon dilation *versus* sphincterotomy. *World J Gastroenterol* 2009; 15(34): 4298-4304 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4298.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4298>

Abstract

AIM: To compare small sphincterotomy combined with endoscopic papillary large balloon dilation (SES + ELBD) and endoscopic sphincterotomy (EST) for large bile duct stones.

METHODS: We compared prospectively SES + ELBD (group A, $n = 27$) with conventional EST (group B, $n = 28$) for the treatment of large bile duct stones (≥ 15 mm). When the stone could not be removed with a normal basket, mechanical lithotripsy was performed. We compared the rates of complete stone removal with one session and application of mechanical lithotripsy.

RESULTS: No significant differences were observed in the mean largest stone size (A: 20.8 mm, B: 21.3 mm), bile duct diameter (A: 21.4 mm, B: 20.5 mm), number of stones (A: 2.2, B: 2.3), or procedure time (A: 18 min, B: 19 min) between the two groups. The rates of complete stone removal with one session was 85% in group A and 86% in group B ($P = 0.473$). Mechanical lithotripsy was required for stone removal in nine of 27 patients (33%) in group A and nine of 28 patients (32%, $P = 0.527$) in group B.

INTRODUCTION

The basic principle of common bile duct stone removal involves destruction or dilation of the bile duct orifice, which allows easy removal of the stone. Endoscopic sphincterotomy (EST) is accepted as the standard management for stone removal from the bile duct, but it is associated with serious complications such as hemorrhage, pancreatitis, perforation, and recurrent infection of the bile duct, which cause permanent functional loss of the sphincter of Oddi^[1-4]. Endoscopic papillary balloon dilation (EPBD) was introduced by Staritz *et al*^[5] and has been accepted widely as an alternative to EST^[6-10]. It has similar outcomes for common bile duct stone removal compared to EST, and has the advantages over EST of preserving papillary sphincter function and causing minimal complications such as hemorrhage and perforation^[11-19]. Despite these advantages, EPBD is associated with more severe and frequent occurrence of pancreatitis^[20-22]. In addition, EPBD has some technical difficulties for removing large stones because the biliary opening is not enlarged to the same degree as with EST^[23].

To overcome these limitations, Ersoz *et al*^[24]

introduced EBD with conventional EST for the removal of large (≥ 15 mm) bile duct stones that are difficult to remove by EBD alone. They have reported that EBD with conventional EST is more effective for the retrieval of large stones and shortens the procedure time. Recently, this technique has been modified slightly to endoscopic papillary large balloon dilatation (ELBD) with small incision EST, and many studies have reported on the outcome of stone removal and complication rate^[25-27]. However, these studies on the efficacy of ELBD with SES have concentrated on small stones, of which the majority are ≤ 1 cm^[25,26]. Therefore, the effectiveness of SES with ELBD for large stone removal (≥ 15 mm) has not been established.

We conducted a prospective randomized study to compare the efficacy and safety of SES + ELBD with conventional EST for the treatment of large (≥ 15 mm) common bile duct stones.

MATERIALS AND METHODS

Patients

From June 2006 to December 2008, 55 patients were enrolled, and all patients were diagnosed as having common bile duct stones by endoscopic retrograde cholangiography (ERCP) or magnetic resonance imaging (MRI). In all patients, the stone was at least 15 mm in maximum diameter. The exclusion criteria for this study were the following: (1) bleeding tendency with INR > 1.5 ; (2) platelet count $< 50\,000/\text{mL}$; (3) anticoagulation therapy within 72 h of the procedure; (4) bilio-colic fistula; (5) stone size > 50 mm; (6) acute cholecystitis; (7) acute pancreatitis; (8) cholangitis; (9) intrahepatic duct stones; (10) pancreatobiliary malignancy; and (11) surgical history involving the biliary tree (not including the gall bladder) or gastrointestinal tract, such as the stomach or small bowel, which can alter the papillary location. Patients chosen for our study protocol were divided randomly into two groups according to the order of the procedure. Twenty-seven patients underwent SES + ELBD (group A) and 28 patients underwent conventional EST (group B). This study was approved by the institutional review board of our hospital, and all patients provide written informed consent before entering the study.

Methods

Management such as pharyngeal anesthesia and premedication before the procedure was carried out in the same manner as for general endoscopy, and ERCP was performed with a side-viewing endoscope (TJF240; Olympus, Tokyo, Japan). After the bile duct stones were visualized following cholangiography, the stone was removed according to each protocol. In group A, we made an incision to the mid-portion of the papilla with a pull-type sphincterotome (Figure 1A) and then inserted a CRE balloon (15, 16.5, or 18 mm; Boston Scientific, Natick, MA, USA) over a guidewire. Balloon dilation was performed using wire-guided hydrostatic balloon catheters placed across the papilla. The balloon was inflated with dilute contrast media until the waistline was

obliterated under fluoroscopic monitoring (Figure 1B). Initially, we performed dilation with a 15-mm-diameter balloon, and if the balloon was not large enough to remove the stones, we repeated it with a larger balloon in the order 15 mm \rightarrow 16.5 mm \rightarrow 18 mm. When the papillary orifice was dilated after balloon dilation (Figure 1C), the stones were retrieved using a Dormia basket (WebTM extraction basket; Wilson-Cook Medical, Winston-Salem, NC, USA) (Figure 1D) or retrieval balloon catheter (double lumen retrieval balloon catheter; Boston Scientific). When the stones were not extracted from the biliary tract with initial basket trapping, mechanical lithotripsy (BML-4Q; Olympus) was performed to fragment the stones. In group B, EST was performed with a pull-type sphincterotome (KD-6Q; Olympus) as the standard method, which was accomplished by extending the incision up to the major horizontal fold of the papillary orifice. After EST, the stones were removed in the same way as in group A. If the stones could not be removed completely in one session, we performed another stone removal session in each group. Complete stone removal was documented with a final cholangiogram. The procedure time was measured as the time between selective cannulation and complete stone removal in the cases of successful stone removal in the first session. The maximum procedure time for the first session was limited to 40 min if the stone was difficult to remove in one session.

Measurements

Stone size and number and bile duct size were documented on the cholangiogram during ERCP. Stone size was assessed by comparing the largest diameter of the stone with the diameter of the TJF240 endoscope, as measured on the cholangiogram. The primary endpoint was the success rate for complete removal of stones within the initial ERCP session. The secondary outcomes included the time for the procedure of these initial-success cases, frequency of mechanical lithotripsy, and associated complications such as bleeding, pancreatitis, cholangitis, and perforation. To observe the complications, blood samples involving a complete blood count, liver function test, amylase, and lipase concentrations were taken before the procedure and 1 and 2 d after ERCP. Post-ERCP pancreatitis was defined as persistent abdominal pain of more than 24 h duration, associated with serum amylase more than three times the upper limit of normal. Bleeding complication was deemed a decrease in hemoglobin concentration of > 2 mg/dL or clinical signs of bleeding after the procedure, such as melena or hematemesis. Cholangitis was defined as a fever accompanied by leukocytosis and right upper quadrant pain after the procedure. All complications were classified and graded according to the consensus guidelines with some modification^[28]. After the stones were removed, ductal clearance was confirmed with a cholangiogram during the procedure.

Statistical analysis

Statistical analysis was performed using the statistical

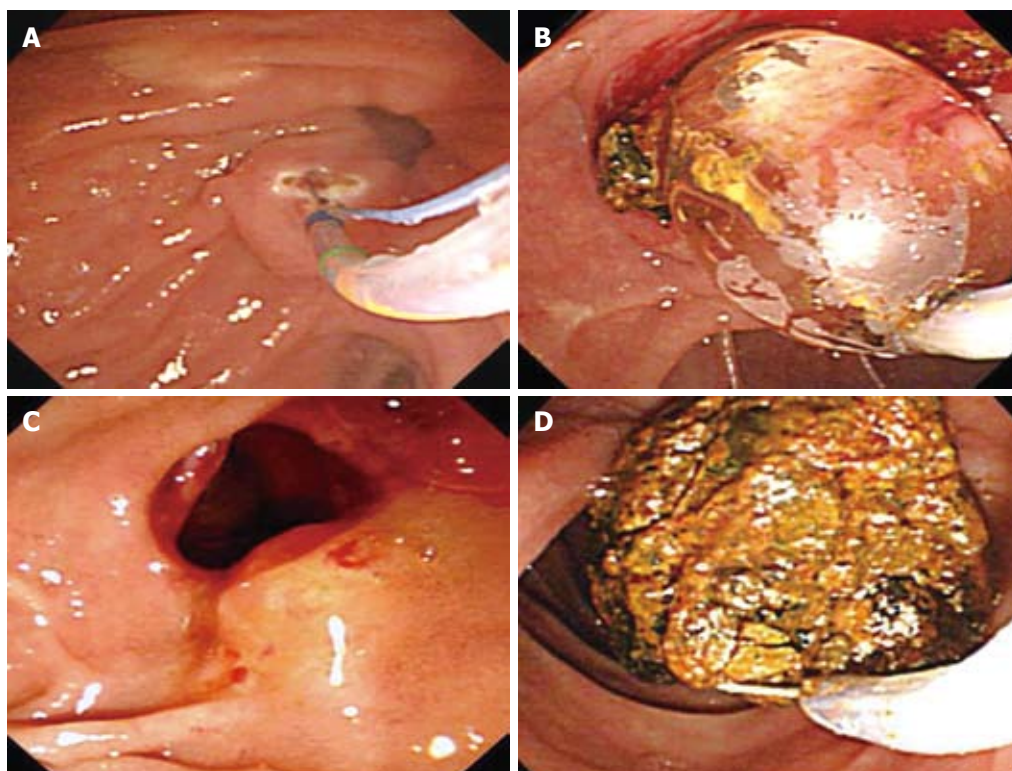


Figure 1 Endoscopic view. A: A small sphincterotomy using a pull-type sphincterotome; B: Endoscopic papillary balloon dilation with a large balloon after small endoscopic sphincterotomy; C: Dilated orifice after small EST + ELBD; D: Stone removal through the dilated orifice of the major papilla.

Table 1 Baseline characteristics of the patients

	Group A (n = 27)	Group B (n = 28)
Gender (M/F)	10/15	11/14
Mean age (yr) ¹	70.3 ± 8.7	69.8 ± 9.2
Mean diameter of stone (mm) ¹	20.8 ± 4.1	21.3 ± 5.2
Mean No. of stones ¹	2.2 ± 1.3	2.3 ± 1.2
Mean diameter of bile duct (mm) ¹	21.4 ± 6.3	20.5 ± 5.7
Periampullary diverticulum (%)	9 (33.3)	10 (35.7)
Previous cholecystectomy (%)	9 (33.3)	7 (25)
Distal CBD tapering (%)	11 (41)	10 (36)

¹mean ± SD; CBD: Common bile duct.

SPSS for Windows version 12.0 (Chicago, IL, USA). Data are presented as the mean ± SD or median with range. Categorical parameters were compared using the χ^2 or Fisher's exact test, and continuous variables were compared with Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

The gender ratio was similar in the two groups. The mean age was 70.3 years in group A and 69.8 in group B. The mean size of the stones was 20.8 mm (range 15-38.3 mm) in group A and 21.3 mm (range 15-48 mm) in group B. The mean number of stones was 2.2 in group A and 2.3 in group B. The maximal bile duct diameter did not differ significantly between the two groups. A peri-ampullary diverticulum was observed in nine patients in group A and 10 in group B. Sixteen

(29%) patients had a history of cholecystectomy. A tapered common bile duct was observed in 11 (41%) patients in group A and 10 (36%) in group B (Table 1).

Overall, complete removal of bile duct stones in the first session was achieved in 46 (84%) patients, while nine required additional sessions. The causes of failure in the first session were incomplete stone capture with the mechanical lithotripsy basket as a result of a large stone (two cases each in groups A and B), stone impaction (one case in group A), procedure-induced bleeding (one case in group B), and incomplete retrieval because of multiple stones (one case each in groups A and B). The stone clearance rate in the first session between the two groups did not differ significantly, and was 84% in both groups ($P = 0.473$). Mechanical lithotripsy was used for nine (33%) patients in group A and nine (32%) in group B ($P = 0.527$). The mean procedure time was compared in the cases involving successful removal of the stone in the initial session and did not differ statistically between the two groups. All stones were removed completely in all patients within three sessions (group A: 1.27 ± 0.53 sessions, group B: 1.31 ± 0.71 sessions, $P = 0.714$). The number of sessions of mechanical lithotripsy and mean procedure times did not differ significantly between the two groups (Table 2).

We also divided each group into subgroups according to the stone size (2 cm) and compared the stone removal rate and application of mechanical lithotripsy. The complete stone removal rate for each subgroup in the first session was similar in both groups: 85.7% (group A) and 86.6% (group B) in the subgroups with stones <

Table 2 Results of endoscopic stone removal after small EST + ELBD *vs* EST (stone size ≥ 15 mm)

	Group A (<i>n</i> = 27)	Group B (<i>n</i> = 28)	<i>P</i> value
Stone removal in the first session (%)	23 (85)	23 (86)	0.473
Mechanical lithotripsy (%)	9 (33)	9 (32)	0.527
Mean procedure time (min) ^{1,2}	18 \pm 12	19 \pm 13	0.917
Mean therapeutic session ¹	1.27 \pm 0.53	1.31 \pm 0.71	0.714

¹mean \pm SD; ²Calculated from initial success cases (*n* = 23 in both groups). Overall success rate of the first session: 84%.

Table 4 Comparison of stone removal in the first session and application of mechanical lithotripsy

	Group A (<i>n</i> = 11)	Group B (<i>n</i> = 10)	<i>P</i> value
Stone removal in the first session (%)	9 (81.8)	7 (70)	0.525
Mechanical lithotripsy (%)	6 (54.5)	6 (60)	0.801

2 cm in maximum diameter and 84.6% (group A) and 76.9% (group B) in the subgroups with stones ≥ 2 cm in maximum diameter. The rate of mechanical lithotripsy increased significantly with stone size irrespective of the group (*P* < 0.05 in each group, Table 3). Finally, we compared the stone removal rate in the first session and the need for mechanical lithotripsy in the cases with a tapered distal bile duct between the two groups. A tapered bile duct was deemed as one in which a portion of the distal common bile duct was narrowed with a steady curve on the cholangiogram. The stone removal rate was higher in group A (81.8%) than in group B (70%), but not significantly. In addition, the mechanical lithotripsy rate was similar between the two groups (Table 4).

Complications according to the consensus guidelines were not observed in either group, and we could not compare the complication rate between the two groups. Although mild amylase elevation less than three times the upper limit of normal was observed in four patients in group A (15%) and three in group B (11%), no instances of post-ERCP pancreatitis and cholangitis according to the consensus guidelines occurred in either group. We did not perform prophylactic pancreatic duct stenting during the procedure in any case. A small amount of bleeding was seen in four patients in group A (15%) and two in group B (7%). No procedure-related perforation was observed. Nine cases in which complete ductal clearance was not achieved in the first session underwent a second session on the next day or within a few days, and any additional protective procedure, such as biliary plastic stenting, was not performed until the next session.

DISCUSSION

EST is the most frequently used endoscopic technique for the clearance of stones from the bile duct. Its success rate exceeds 90%, and it has been accepted as the best nonsurgical treatment for common bile duct stones^[29-33]. However, EST is still associated with an 8%-12% rate

Table 3 Comparison of overall application of mechanical lithotripsy according to the size of the stone in each group

	Group A (<i>n</i> = 27)		Group B (<i>n</i> = 28)	
	< 2 cm (<i>n</i> = 14)	≥ 2 cm (<i>n</i> = 13)	< 2 cm (<i>n</i> = 15)	≥ 2 cm (<i>n</i> = 13)
Stone removal in the first session (%)	12 (85.7) ^a	11 (84.6) ^b	13 (86.6) ^c	10 (76.9) ^d
Mechanical lithotripsy (%)	2 (14.3) ^e	7 (53.8) ^f	2 (13.3) ^g	7 (53.8) ^h

Overall application of mechanical lithotripsy: 17/50 (34%). *P* value: a *vs* b, not significant; c *vs* d, not significant; e *vs* f, 0.018; g *vs* h, 0.008.

of acute complications, such as bleeding, perforation, cholangitis, and post-procedure pancreatitis^[11,25,34-37]. In addition, it permanently destroys the biliary sphincter, which can lead to chronic complications, such as duodenal biliary reflux, bacterial contamination, and chronic inflammation of the biliary system^[11].

EBD was introduced by Staritz *et al*^[5] in 1983 as an alternative method for the removal of bile duct stones. The main advantage of this technique is that it does not involve cutting the biliary sphincter, therefore preserving its function. However, major limitations of EBD exist, including difficulty in removing large stones and a high incidence of pancreatitis. Since balloon dilation does not enlarge the sphincter of Oddi to the same extent as EST, large stone removal with EBD is difficult, and mechanical lithotripsy is required more often than with EST^[11,21]. As a result, there is a need to modify the EBD technique to remove large bile duct stones and reduce the risk of pancreatitis. Similarly, EST is not a good method if the stones are too large to remove. Stone fragmentation procedures such as mechanical lithotripsy are required in this situation, regardless of the approach method. Ersoz *et al*^[24] first reported the use of EST followed by papillary balloon dilation. They reported an 83% success rate in the first session with a 7% rate of mechanical lithotripsy in 58 patients in whom endoscopic removal of bile duct stones using standard EST and balloon/basket extraction had failed. Recently, multiple published series have shown that the overall first session success rates of stone removal with EBD following EST ranged from 80% to 100%^[24,25,27,38], and these success rates were similar to those of EST. Although some recent studies have reported that the stone clearance rate for the initial session of EBD following EST is high, the outcome for large stone removal by ELBD following EST remains controversial. Since previous data from ELBD studies have included various sizes of stones, especially small stones < 1 cm, and comparison studies between SES + ELBD and conventional EST for large stone (≥ 15 mm) removal are not sufficient^[25,26,39]. Therefore, we could not clarify the effectiveness of ELBD following EST for large stone removal.

In our present study, we compared SES + ELBD to conventional EST in terms of usefulness and safety for the treatment of large stones. We also evaluated the number of applications of mechanical lithotripsy and compared this with previous studies, which reported

that EST + ELBD reduced the use of mechanical lithotripsy^[24,25,39]. The number of patients enrolled in our study was limited by the stone size and exclusion criteria. However, these criteria enabled us to compare the outcomes between the two groups more objectively.

Our findings showed that the initial success rate for the clearance of common bile duct stones was same in both groups and it was not significantly different. A previous series of EST + ELBD gave first session success rates of 70%-99% and mechanical lithotripsy rates of 1%-11%^[24-27,39]. In contrast, we had a 33% mechanical lithotripsy rate in group A and a 32% rate in group B. Compared to previous reports, the frequency of mechanical lithotripsy was markedly higher^[24-27,39], which might be attributable to the large stones (≥ 15 mm).

Previous studies likely reported lower rates of mechanical lithotripsy, because of smaller stones^[25,26,39], or a wider sphincterotomy^[24,40]. Of course, the frequency of mechanical lithotripsy might be related to various factors, such as the extent of EST, size of the stone and balloon, and shape of the stone and common bile duct. Removing large stones (≥ 15 mm) in patients with a tapered common bile duct without stone fragmentation might be difficult, despite orifice dilation using large balloon dilation. A retrospective pilot study of 50 patients revealed that patients that required mechanical lithotripsy were more often characterized by large stones combined with a tapered distal common bile duct rather than either of these features alone^[41].

Other recent studies have revealed that SES + ELBD reduced the frequency of mechanical lithotripsy and gave better results for the removal of stones^[25,27,39].

However, in our study, mechanical lithotripsy was not reduced with SES + ELBD, and no difference in the frequency of mechanical lithotripsy was observed between the two groups. We needed a stone fragmentation method such as mechanical lithotripsy, although we used a large balloon (maximum, 18 mm) to dilate the orifice; the larger stone size was associated with more frequent mechanical lithotripsy. The CRE balloon had a length of 8 cm, of which approximately half was positioned in the distal bile duct. Considering this point, we speculate that part of the terminal and distal bile duct could be dilated simultaneously using balloon dilation, and if the stone was small enough to pass through the dilated bile duct, it could be removed more easily. To remove large stones, however, some EST and large balloon dilation may help to dilate the sphincter of Oddi orifice, to allow the passage of large stones. Large balloon dilation alone cannot stretch the wall of the distal bile duct to the degree necessary for the effective removal of large stones. Hence, the configuration and wall lumen tension of the terminal bile duct may be more important factors for the removal of large stones than the size of the balloon and dilation of the bile duct. Therefore, if a stone is too large to remove *via* the dilated terminal bile duct and sphincter of Oddi, stone fragmentation using mechanical lithotripsy, for example, might be inevitable.

Complications according to the consensus guidelines did not occur in our study, which may be related to the

small number of patients enrolled. No procedure-related pancreatitis occurred. Only amylase elevation less than three times the upper limit of normal was observed in seven patients (four in group A and three in group B). Perforation did not occur in any patient.

A small amount of bleeding was observed in six patients in our study. Of these, stone removal was postponed to the next session for one patient in group B, but this case did not meet the criteria for bleeding complications according to the consensus guidelines^[28]. Other bleeding complications were easily controlled using argon-plasma coagulation, epinephrine spray, or compression by the balloon. Ersoz *et al*^[24] have reported a 9% bleeding rate in their EST + ELBD group, especially in patients with a tapered distal bile duct. With the larger balloon, the higher rate of bleeding could have been attributable to the moderate degree of EST. In addition, they performed major EST in their study. In the SES + ELBD group, the reported rate of bleeding ranged from 0% to 4.5%, and all of the cases were relatively mild^[25,39,40].

In conclusion, SES + ELBD did not show significant benefits compared to conventional EST and reducing the rate of mechanical lithotripsy, especially for the removal of large (≥ 15 mm) bile duct stones. Regarding the occurrence of complications, SES + ELBD showed a similar level of safety compared to conventional EST. Hence, SES + ELBD is a good alternative to conventional EST for the removal of large stones, especially for the unskilled endoscopist. However, a large-scale study of patients is required to clarify the difference in the efficacy of the two procedures.

COMMENTS

Background

Many recent studies on small sphincterotomy combined with endoscopic papillary large balloon dilation (SES + ELBD) have reported on the outcome of stone removal and the complication rate. As previous studies concentrated on the efficacy of small bile duct stone removal, the effectiveness of ELBD with SES for large stone removal (≥ 15 mm) has not been established.

Innovations and breakthroughs

Other recent studies have revealed that SES + ELBD reduced the frequency of mechanical lithotripsy and gave better results for the removal of stones. However, the present study found that SES + ELBD did not reduce the need for mechanical lithotripsy in removing large (≥ 15 mm) bile duct stones. Large balloon dilation alone cannot stretch the wall of the distal bile duct to the degree necessary for the effective removal of large stones. To remove large stones, the configuration and wall lumen tension of the terminal bile duct may be more important factors than the size of the balloon and dilation of the bile duct. Therefore, if a stone is too large to remove *via* the dilated terminal bile duct, stone fragmentation might be inevitable.

Applications

For the removal of large common bile duct stones, SES + ELBD is a good alternative to conventional endoscopic sphincterotomy (EST), especially for the unskilled endoscopist. However, a large-scale study is required to clarify differences in the efficacy of the two procedures.

Terminology

Endoscopic papillary balloon dilation (EPBD), an alternative method with similar outcomes compared to EST, is associated with frequent, severe pancreatitis. SES + ELBD is a modified EPBD technique.

Peer review

In this study, the numbers of the patients are too small to compare infrequent complications like bleeding or pancreatitis. However, it may be difficult to enroll many more patients with large bile duct stones.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Yin DH

Prognostic analysis of patients with pancreatic head adenocarcinoma less than 2 cm undergoing resection

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Received: June 20, 2009 Revised: August 5, 2009

Accepted: August 12, 2009

Published online: September 14, 2009

between the two groups. During a follow-up period ranging from 1.0 to 122.7 mo (median, 10.9 mo), S-PAC and L-PAC patients had a similar prognosis after resection ($P = 0.4805$). Among the S-PAC patients group, patients with higher albumin level (> 3.5 g/dL) had more favorable survival than those with lower albumin levels, which was the only favorable predictive prognostic factor. Meanwhile, early-staged (stage I, II) S-PAC patients tended to have a more favorable outcome than late-stage (stage III, IV) S-PAC patients, but this was not statistically significant.

CONCLUSION: S-PAC patients should not be regarded as early PAC. Only higher albumin level (> 3.5 g/dL) and early stage disease (stage I, II) were the favorable prognosis factors for S-PAC patients.

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Abstract

AIM: To investigate the differences in clinicopathological features between patients with pancreatic cancer greater or less than 2 cm situated over the pancreatic head and the prognostic factors for survival of patients with pancreatic cancer < 2 cm over the pancreatic head.

METHODS: From 1983 to 2006, 159 patients with histologically proven pancreatic adenocarcinoma (PAC) at the pancreatic head undergoing curative resection at the Department of Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan were reviewed, comprising 123 cases of large (L)-PAC (tumor > 2 cm) and 36 cases of small (S)-PAC (tumor ≤ 2 cm). We compared the clinicopathological characteristics and prognosis of L-PAC and S-PAC patients. The clinicopathological characteristics of S-PAC were investigated to clarify the prognosis predictive factors of S-PAC.

RESULTS: One hundred and fifty-nine PAC patients, aged 16-93 years (median, 59.0 years) with a tumor at the pancreatic head undergoing intentional curative resection were investigated. The S-PAC and L-PAC patients had similar demographic data, clinical features, and tumor markers (a similar positive rate of carcinoembryonic antigen and carbohydrate antigen 19-9). There were also similar rates of lymph node metastasis, portal vein invasion, stage distribution, tumor differentiation, positive resection margin, surgical morbidity and mortality observed

Key words: Prognostic factor; Pancreas; Pancreatic head area; Pancreatic cancer

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Chiang KC, Yeh CN, Lee WC, Jan YY, Hwang TL. Prognostic analysis of patients with pancreatic head adenocarcinoma less than 2 cm undergoing resection. *World J Gastroenterol* 2009; 15(34): 4305-4310 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4305.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4305>

INTRODUCTION

Pancreatic adenocarcinoma (PAC), one of the most lethal malignant cancers, ranks fifth in mortality related to cancer worldwide with the 5-year survival after resection ranging from 10% to 29%^[1-3]. Thirty-two thousand new PAC cases have been found in America each year^[4].

Surgery remains the only curative treatment of PAC and detection of PAC with small size (≤ 2 cm) (S-PAC) seems to be essential to improve the outcome, which is demonstrated in some reports^[5-7]. The 5-year survival rate varied from 19% to 41% for PAC patients undergoing pancreatectomy with the highest survival in patients with small tumors confined to the pancreas.

But the controversial question is “Dose PAC with tumor diameters of 2 cm or less necessarily indicate early-stage disease or good prognosis?” Recent data have shown the different opinions about this issue^[8,9], which warrants further studies addressing this topic.

To collect an adequate number of resected cases of S-PAC was difficult due to limitations of diagnostic equipment, explaining why most of the previous studies were multi-institutional^[7,10,11]. Recent advances in diagnosis have made it possible to detect PAC earlier and to increase the number of resected cases; consequently evaluation of S-PAC cases in one center becomes feasible, which could provide more accurate information.

We retrospectively reviewed 159 PAC cases with pancreatic head cancer undergoing pancreaticoduodenectomy from 1983 to 2006. Among them, 36 cases were diagnosed with S-PAC, and the remainder were PAC with tumor diameter > 2 cm, classified as large PAC (L-PAC). We compared the clinicopathological features and surgical outcomes between S-PAC and L-PAC to find the favorable prognostic factors.

MATERIALS AND METHODS

From 1983 to 2006, 159 patients with histopathologically proven PAC located at the pancreatic head undergoing intentional curative resection at the Department of Surgery, Chang-Gung Memorial Hospital, Taipei, Taiwan were reviewed. Curative resection was defined as a negative resection margin observed during histopathological examination. The 159 PAC patients comprised 96 men and 63 women with a median age of 64.0 years (range, 16-93 years). Among them, 36 patients (22.6%) had a tumor size \leq 2 cm classified as S-PAC and 123 PAC patients had tumor size > 2 cm (L-PAC). Tumor size was defined by histopathological examination. Surgical mortality was defined as death occurring within 1 mo after surgery. Laboratory tests were conducted on the day before surgery. Serum carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) were measured by radioimmunoassay. The tumors were preoperatively evaluated by abdominal ultrasonography, endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography, computed tomography, magnetic resonance image with cholangiopancreatography, and angiography, as appropriate. Tumor stage was defined according to the pathological tumor node metastasis (pTNM system) classification proposed by the UICC. Stages I and II were classified as early-stage, and stages III and IV as advanced stage PAC. Adjuvant chemotherapy was systemic administration of either 5-fluorouracil-based or gemcitabine-based regimen due to either the positive section margin or lymph node metastasis. Adjuvant radiotherapy was conducted by intra-operative radiotherapy, external beam radiotherapy and/or brachytherapy due to either a positive section margin or local recurrence.

Statistical analysis

All data are presented as percentage of patients or mean

with standard deviation. Numerical data were compared by independent two-sample *t*-tests. Nominal data were compared by Pearson chi-square test, or multiple forward stepwise logistic regression when appropriate. Survival was calculated and plots constructed according to the Kaplan-Meier method. Sixteen clinicopathological variables were selected for survival analysis, including demographic data, clinical features, laboratory data, operative findings, and pathological features. The log-rank test was performed for a univariate analysis for prognosis by using log-rank test and multivariate analysis was conducted with Cox's proportional hazard model. All statistical analyses were performed using the SPSS computer software package (Version 10.0, Chicago, IL, USA). A value of $P < 0.05$ was considered significant.

RESULTS

Clinicopathological features of L-PAC and S-PAC patients

The S-PAC group contained 22 men and 14 women, with a mean age of 61.9 ± 8.2 years. In the L-PAC group, there were 74 men and 49 women, with a mean age of 62.8 ± 10.5 years. Both groups had a similar age distribution, gender ratio, and laboratory data. In terms of symptoms and physical examination, both groups possessed a higher positive rate (> 80%) for non-specific symptoms and signs irrespective of the tumor size. Regarding tumor markers (CEA and CA 19-9), L-PAC and S-PAC groups had similar positive rates (35.9% and 75%, and 29.6% and 69%, respectively). In the light of lymph node metastasis and portal vein invasion, the two groups had similar positive rates. Even in the S-PAC groups, lymph node metastasis and portal vein invasion rates reached 63.9% and 11.1%, respectively. Both groups had almost the same curative resection rate. Portal vein invasion and retroperitoneal extension of the tumor explained the reasons for the positive margin. Both groups also had similar distributions of tumor differentiation and stage.

Morbidity and mortality rates between L-PAC and S-PAC groups

Surgical morbidity and mortality rates for the L-PAC group were 26.8% and 4.1%, respectively, similar to those of the S-PAC group which were 30.6% and 2.8%, respectively (Table 1). Almost the same percentage of patients received post-operative chemotherapy and radiation in the two groups, mainly for lymph node metastasis and positive margin.

Survival analysis between L-PAC and S-PAC

All of the 159 PAC patients undergoing resection were followed regularly until death with the duration of follow-up ranging from 1.1 to 213.5 mo (median, 16.4 mo). The 1-, 3- and 5-year survival rates of the 159 cases were 51.3%, 23.1% and 12.5%, respectively (Figure 1). The 3- and 5-year survival rates of the S-PAC and L-PAC patients were 26.4% and 6.6%, and 22.1% and 14.7%, respectively

Table 1 Clinicopathological features of 159 pancreatic head adenocarcinoma patients with tumor size smaller and larger than 2 cm *n* (%)

	S-PAC (<i>n</i> = 36)	L-PAC (<i>n</i> = 123)	<i>P</i>
Age (yr)	61.9 ± 8.2	62.8 ± 10.5	0.601
Sex (M/F)	22/14	74/49	0.918
Symptom (+/-)	35/1	123/0	0.226
Physical findings (+/-)	32/4	99/24	0.244
Albumin (g/dL)	3.74 ± 0.58	3.71 ± 0.61	0.827
AST (IU/L)	178.8 ± 258.5	138.1 ± 127.7	0.204
CEA (ng/mL) ≥ 5	8/27 (29.6)	33/92 (35.9)	0.549
CA 19-9 (IU/L) ≥ 37	20/29 (69.0)	72/96 (75.0)	0.518
Size (median)	1.5	3.5	0.0001
Operation time (min)	494.5 ± 141.5	469.2 ± 118.1	0.292
LN metastasis	23 (63.9)	72 (58.5)	0.565
PV invasion	4 (11.1)	12 (9.8)	0.760
Positive margin	10 (27.8)	35 (28.5)	0.244
Staging			0.459
I	12 (33.3)	39 (31.7)	
II	1 (2.8)	12 (9.8)	
III	22 (61.1)	71 (57.7)	
IV	1 (2.8)	1 (0.8)	
Tumor differentiation			0.723
W-D	11 (30.6)	38 (30.9)	
M-D	19 (52.8)	59 (48.0)	
P-D	6 (16.7)	26 (21.2)	
Morbidity	11 (30.6)	33 (26.8)	0.660
Postoperative CT	23 (63.9)	58 (47.2)	0.077
Postoperative RT	1 (2.8)	11 (8.9)	0.300
Mortality	1 (2.8)	5 (4.1)	0.760

AST: Aspartate aminotransferase; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; LN: Lymph node; PV: Portal vein; W-D: Well-differentiated; M-D: Moderate-differentiation; P-D: Poor-differentiation; CT: Chemotherapy; RT: Radiotherapy.

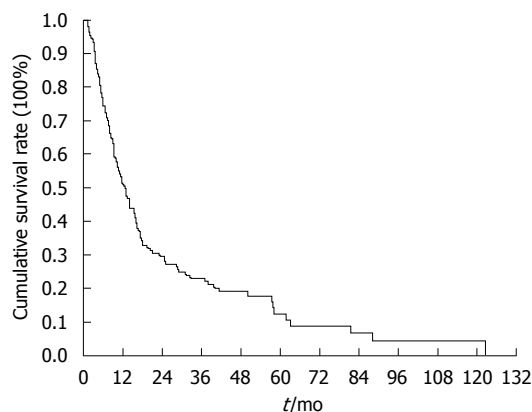
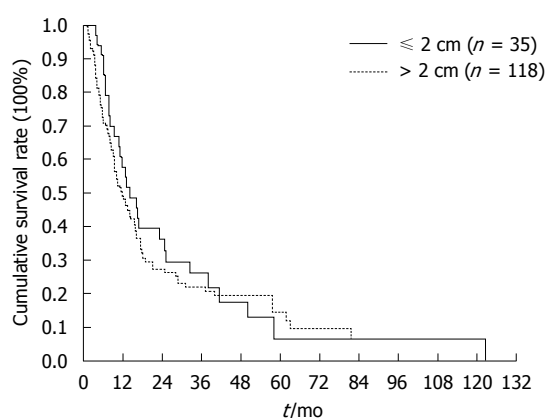
(Figure 2). The S-PAC patients had a similar overall survival rate to the L-PAC patients ($P = 0.48$) (Figure 2).

Prognosis predictive factors of S-PAC patients

S-PAC and L-PAC groups had the same prognosis in this study, which meant tumor size did not influence the outcome of this disease. So, we were interested in which factors could affect the prognosis of S-PAC patients. We chose 16 clinicopathological characteristics including demographic data, clinical biochemical laboratory values, tumor markers, operative procedure, tumor invasion, tumor differentiation, tumor stage, post-operative chemotherapy and radiation to determine which one could be the prognostic predictive factor (Table 2). Only the albumin level had a significant impact on survival of S-PAC patients ($P = 0.002$). The early stage S-PAC patients (Stage I, II) tended to have a favorable outcome compared with late stage S-PAC patients (Stage III, IV), but this difference did not reach statistical significance ($P = 0.0931$). Age, gender, operative procedures, biochemical data, tumor invasion, resection margin, and tumor differentiation did not associate with favorable prognosis.

DISCUSSION

PAC is one of most lethal human malignancies

**Figure 1** The overall survival rates of 159 pancreatic head adenocarcinoma patients.**Figure 2** The overall survival rates of S-PAC and L-PAC patients.

producing the fourth highest cancer-related mortality in the western world and the fifth highest cancer-related mortality rate worldwide^[4]. The overall 5-year survival rate of PAC has been reported to be around 1%-4%, which was attributed to its aggressive growth behavior, early local spread, early metastasis, and resistance to radiation and most systemic chemotherapies^[4]. Surgery remains the cornerstone of treatment. After resection, the 5-year survival rate can reach 10%-29%^[1-3].

For most malignancies, a smaller tumor size is usually deemed as a good indicator of early stage, and should be diagnosed and treated as soon as possible to improve the outcome. Some reports have demonstrated that tumor size was one of most important determinants of resectability and prognosis for pancreas cancer^[12-14]. Previously, Satake *et al*^[15] stated that PAC with tumor size < 40 mm represented a better prognosis. However, according to our report, even PAC with tumor size < 20 mm did not necessarily mean early stage disease and should not be treated by surgery alone^[7,9,16,17]. The reported 5-year survival for S-PAC patients ranges from 8% to 59% and is only 6.6% in our series^[3,7,9,16,18,19].

Several reasons could be found to explain this dismal outcome. S-PAC patients (tumor size < 2 cm) had similar clinicopathological features including positive clinical symptoms, tumor differentiation pattern and positive

Table 2 Univariate analysis of factors influencing the overall survival of the 35 small pancreatic head cancer patients undergoing resection

Factors	Survival time (mo)				P
	Median (mo)	Mean (mo)	3-yr (%)	5-yr (%)	
Gender					0.8417
Male (n = 21)	13.0	24.0	28.1	0	
Female (n = 14)	14.0	27.9	23.4	11.7	
Age (yr)					0.2931
≤ 60 (n = 13)	24.6	26.4	35.9	12.0	
> 60 (n = 22)	9.4	8.06-10.75	17.02	12.61	
Physical examination					0.9566
Positive (n = 31)	13.0	28.1	26.7	8.0	
Negative (n = 4)	16.1	22.5	25.0	0	
Bilirubin (mg/dL)					0.5049
≤ 2 (n = 8)	14.0	15.6	14.3	0	
> 2 (n = 27)	16.1	29.4	39.6	7.7	
Albumin (g/dL)					0.0002
≤ 3.5 (n = 13)	5.9	7.6	0	0	
> 3.5 (n = 22)	16.6	32.7	33.6	11.2	
Serum CEA (ng/mL)					0.6741
≤ 5 (n = 24)	16.6	22.4	23.6	7.9	
> 5 (n = 11)	9.1	19.8	25.0	15.42	
Serum CA 19-9 (IU/L)					0.7592
≤ 37 (n = 11)	16.6	19.4	11.1	11.1	
> 37 (n = 24)	14.0	23.5	33.6	0	
Operative procedure					0.6500
Whipple (n = 30)	16.1	27.9	28.8	7.2	
PPPD (n = 5)	14.0	15.5	0	0	
Portal vein invasion					0.1467
Positive (n = 2)	4.0	9.0	0	0	
Negative (n = 33)	16.1	28.0	28.1	7.0	
Resection margin					0.6704
Positive (n = 10)	10.9	19.5	30.0	0	
Negative (n = 25)	16.1	28.8	26.4	7.9	
TNM staging					0.0931
I + II (n = 13)	16.6	40.2	50.0	15.0	
III + IV (n = 22)	13.0	17.6	14.4	0	
Tumor differentiation					0.3202
W-D (10)	23.1	22.9	50.0	0	
M-D (19)	10.8	21.5	17.9	6.0	
P-D (6)	16.1	32.2	50.0	25.0	
Post-operative CT					0.7114
With (n = 23)	16.1	23.9	25.3	0	
Without (n = 12)	10.8	28.8	27.8	13.9	
Post-operative RT					0.9385
With (n = 1)	24.6	24.6	0	0	
Without (n = 24)	14.0	24.6	27.4	6.9	

IU: International unit; PPPD: Pylorus-preserving pancreaticoduodenectomy.

lymph node invasion rate to L-PAC patients (Table 1). Contrary to previous reports^[9,20], a well-differentiated type of adenocarcinoma is more frequently seen in PAC tumors with tumor size < 2 cm, but in our study, tumor size had no impact on tumor differentiation. S-PAC and L-PAC possessed the same distribution in terms of tumor differentiation. Jung *et al*^[20] also indicated that pancreas tumor size < 2 cm would tend to be symptomless, however, in our study, we could not find this difference. Both groups had a very low negative rate of symptoms. Contrary to Shimada's report^[8], we showed the incidence of lymph node metastasis was similar between S-PAC and L-PAC (as high as 63.9%), demonstrating that S-PAC with tumor size < 20 mm did not necessarily mean early stage disease. Such findings showed that PAC was attributed to its aggressive growth

behavior, early local spread and early metastasis no matter what its size.

CEA and CA 19-9 are widely used to screen malignancies in the general population. An 80% CA19-9 positive rate in pancreas cancer and high levels of CA19-9 associated with more advanced disease were reported^[21-23]. Jung *et al*^[20] reported that CA19-9 and CEA in patients with PAC tumor size > 6 cm would be higher than in smaller cancers. Steinberg revealed that CEA and CA 19-9 would not increase in patients with pancreas cancer < 2 cm^[24]. But in our study, we found the sensitivity of CEA and CA 19-9 for S-PAC and L-PAC patients were 29.6% and 69%, and 35.9% and 75%, respectively. Tumor marker values did increase in the S-PAC group and both groups had similar positive rates of CEA and CA 19-9.

Regarding prognostic analysis for S-PAC patients, only albumin level could be a favorable prognostic factor for S-PAC statistically ($P = 0.0002$). Early stage S-PAC (Stage I, II) tended to have a better prognosis than late stage S-PAC (Stage III, IV), but this was not statistically significant ($P = 0.0931$). In our study, most non-curative resection cases were due to portal vein invasion and retroperitoneum tumor extension. However, curative resection or not did not influence the final survival of S-PAC and tumor differentiation of S-PAC did not have any influence on survival either. Lymph node metastasis, poorly differentiated tumors, and positive margins were usually regarded as poor prognostic factors for pancreas cancer^[13,25,26]. In this study, we did not find these as unfavorable factors for prognosis. Limited case numbers should be the main cause. In terms of these issues, we need more time to collect more cases to answer these questions. Detection of PAC with small size (≤ 2 cm) (S-PAC) still warrants more efforts to improve the outcome.

In conclusion, S-PAC and L-PAC had similar clinicopathological characteristics. They expressed similar tumor biology, such as lymph node metastasis, portal vein invasion and tumor differentiation. So both groups had the same survival rate, explaining why S-PAC should not be deemed as an early stage disease and treated by surgery alone. For S-PAC groups, only albumin level could be a prognostic predictor. Early stage S-PAC tended to have a more favorable prognosis than late stage S-PAC, although this did not reach statistical significance.

COMMENTS

Background

Pancreatic cancer is a devastating cancer. Previously, cancer size has been deemed as an important prognostic factor for pancreatic cancer. However, growing evidence recently demonstrated the controversial conclusion against the previous viewpoint. Herein, the authors collected data from pancreatic cancer patients with cancer on the pancreatic head and they compared the clinicopathologic characteristics and survival between small pancreatic adenocarcinoma (S-PAC) and large (L)-PAC groups. In addition, they also tried to find out the prognostic factor for S-PAC patients.

Research frontiers

The tumor biology, including differentiation, lymph node metastasis, portal vein invasion and so on is similar between S-PAC and L-PAC patients, which would give rise to further studies of whether there are any different gene mutations in terms of the size of pancreatic cancer.

Innovations and breakthroughs

In this report, albumin level is the only prognostic factor for survival of S-PAC patients. Traditionally, lymph node metastasis and portal vein invasion are deemed as important prognostic factors for pancreatic cancer. Although such findings were not shown in their report, they considered these could be blamed partly on the limited number of cases.

Applications

Through the findings of their report, S-PAC should not be regarded as a early pancreatic cancer. Aggressive management such as post-operative chemotherapy and radiation are justified.

Peer review

This is an interesting clinical report. However, due to the poor prognosis of S-PAC patients and thus the small number of cases who survived for 5 years, we have to be careful in making extended conclusions from the data.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM



Baseline predictors of virological response for chronic hepatitis B patients

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Supported by "973" Program No. 2007CB512800; National Natural Science Foundation of China, No. 30771905; Mega-projects of Science Research, No. 008ZX10002-008; Beijing Municipal Science & Technology Commission, No. D08050700650803

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Received: July 7, 2009 Revised: July 29, 2009

Accepted: August 5, 2009

Published online: September 14, 2009

CONCLUSION: Baseline serum ALT, TSH, and TT4 levels, especially in combination, have high predictive values of virological response to Peginterferon α -2b in HBeAg-positive CHB patients.

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Key words: Chronic hepatitis B; Hepatitis B virus; Predictors; Virological response; Peginterferon

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Abstract

AIM: To determine which baseline factors of chronic hepatitis B patients are predictive of virological response to Peginterferon α -2b therapy.

METHODS: A total of 21 HBeAg-positive chronic hepatitis B (CHB) patients treated with Peginterferon α -2b were recruited. They were treated with Peginterferon α -2b (0.5-1.0 μ g/kg per week) for 24 wk and followed up for 24 wk. Clinical and laboratory data of the patients were determined at pretreatment and at week 12, at 24 during treatment, and at week 48 during follow up.

RESULTS: Ten patients achieved a virological response at the end of treatment. Their baseline serum alanine aminotransferase (ALT), thyroid-stimulating hormone (TSH), and total thyroxin (TT4) levels were significantly different from those who failed treatment. The positive predictive values (PPV) and negative predictive values (NPV) of ALT, TSH, and TT4 were 75% and 89 %, 75% and 89 %, and 75% and 75%, respectively. Moreover, combinations of the baseline ALT and TT4, ALT and TSH, TT4 and TSH levels had much higher PPV and NPV (86% and 88%, 89% and 100%, 83% and 100%, respectively).

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a worldwide health problem. More than 400 million people are chronically infected with HBV and are at risk of developing liver cirrhosis and hepatocellular carcinoma. Each year more than one million people die from HBV-related liver diseases^[1-4].

At present, the two main categories of antiviral drugs for chronic hepatitis B are interferon (including Peginterferon) and nucleoside/nucleotide analogs. Many studies have shown that an elevated serum ALT level is associated with virological response and HBeAg seroconversion in CHB patients^[5-8]. Besides higher serum ALT level, some studies also showed that higher aspartate aminotransferase (AST) level, increased histological activity in biopsy specimens, female sex, and lower serum HBV DNA levels are associated with a higher probability of HBeAg seroconversion in CHB patients treated with interferon-based therapies^[9-13]. It is also reported that the HBV genotype is an important predictor of response to interferon-based therapies^[14-17].

Recently, serum HBeAg levels have been used as

outcome predictors of sustained virological response to Peginterferon α -2a in HBeAg-positive CHB patients and showed high negative predictive values (NPVs) at week 24 of therapy^[18]. Another study showed that early serum HBsAg drops also had high predictive values of sustained virological response to Peginterferon α -2a in HBeAg-negative chronic hepatitis B patients both at week 12 and 24^[19].

However, the predictive values of other factors, especially the baseline factors for virological response to Peginterferon α -2b therapy are not clear. Therefore, in this study, we aimed to determine how well the baseline factors predicted the virological response to Peginterferon α -2b therapy in HBeAg-positive CHB patients.

MATERIALS AND METHODS

Ethics

The study was approved by the Investigation and Ethics Committee for Human Research at the Peking University First Hospital (Beijing, China). All patients provided informed written consent.

Patients and study design

Twenty-one consecutive HBeAg-positive chronic hepatitis B patients were evaluated. Patients were treated with Peginterferon α -2b at a dose of 0.5-1.0 μ g/kg per week for 24 wk. Clinical and laboratory data of the patients were determined before treatment, at week 12, and 24 during treatment. Thereafter they were scheduled for follow-up visits every 12 wk. End of treatment (EOT) response was defined as more than 2 log₁₀ IU/mL reduction in HBV DNA levels at the EOT. Non-response was defined as less than 2 log₁₀ IU/mL reduction in HBV DNA levels at the EOT.

Measurement of serologic markers of HBV

HBsAg, antibody to HBsAg, HBeAg, antibody to HBeAg and anti-HBc were measured using a microparticle enzyme immunoassay (Abbott Laboratories, North Chicago, IL). The HBV genotype was determined using the INNO LiPA HBV genotyping assay. Serum HBV DNA was measured using the TaqMan polymerase chain reaction assay [COBAS TaqMan, Roche Molecular System (lower limit of detection, 20 IU/mL)].

Measurement of biochemical markers

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a Hitachi Model 7600 Series Automatic Analyzer (Hitachi). Thyroid-stimulating hormone (TSH), total triiodothyronine (TT3), and total thyroxine (TT4) levels were measured using a Centaur Automated Chemiluminescence System (Bayer).

Statistical analysis

Quantitative variables were expressed as the median with interquartile ranges (IQR), and categorical variables

as frequencies. Comparisons between groups of quantitative and qualitative variables were performed using the Mann-Whitney *U* test and the Fisher's exact test, respectively. The accuracy of serum factors to predict virological response was assessed using the receiver operating characteristic curve. The cutoff value was chosen according to the receiver operating characteristic curve when the sensitivity and specificity were both relatively high for the selective baseline factor. All tests were two-sided and used a significance level of 0.05. Data handling and analysis were performed with SPSS software for windows, version 13.0 (SPSS Inc., Chicago, IL).

RESULTS

Baseline characteristics of patients

The baseline characteristics of the 21 HBeAg-positive CHB patients are shown in Table 1. The median age was 25 years (range, 20-39), and 81% of them were male (17/21). The median value of serum HBV DNA levels was 8.2 log₁₀ IU/mL (IQR, 7.5-8.7 log₁₀ IU/mL). The distribution of HBV genotype was: B, 24%; C, 76%. The median values of serum ALT, AST, TSH, TT3, and TT4 level were 147 IU/L (IQR, 123-201 IU/L), 65 IU/L (IQR, 51-97 IU/L), 2.06 mIU/L (IQR, 1.41-3.10 mIU/L), 2.22 nmol/L (IQR, 2.04-3.03 nmol/L), and 111.4 nmol/L (IQR, 96.8-140.6 nmol/L) respectively. The baseline TT3 and TT4 values of one patient were not assayed at pretreatment. Serological tests were negative for hepatitis C virus, hepatitis D virus, and human immunodeficiency virus in all patients.

Virological response

Of the 21 patients, ten (48%) showed an EOT response, and eleven (52%) were non-responders. Four patients (19%) obtained HBeAg seroconversion at the end of treatment (week 24). However, two of the four HBeAg seroconversion patients lost anti-Hbe, while another six patients achieved HBeAg seroconversion at week 48. The median value of serum HBV DNA levels were 2.7 log₁₀ IU/mL (IQR, 1.9-4.0 log₁₀ IU/mL) and 3.1 log₁₀ IU/mL (IQR, 1.8-6.6 log₁₀ IU/mL) in responders at week 24 and 48 respectively. In non-responders, The median value of serum HBV DNA levels were 7.4 log₁₀ IU/mL (IQR, 6.8-7.9 log₁₀ IU/mL) and 7.6 log₁₀ IU/mL (IQR, 7.1-8.7 log₁₀ IU/mL) at week 24 and 48 respectively. The baseline ALT and TT4 level were significantly higher in responders than in non-responders (both *P* < 0.05, Table 1). However, the baseline TSH level was significantly lower in responders than in non-responders (*P* < 0.05, Table 1). The baseline age was similar between responders and non-responders.

Predictability

To determine how well the baseline ALT, TSH and TT4 levels predicted virological response to Peginterferon α -2b therapy, we performed receiver operating characteristic curves for each parameter. The areas under the curves of

Table 1 Baseline characteristics of patients

Characteristic	All patients (n = 21)	Responders (n = 10)	Non-responders (n = 11)	P value
Median age, range (yr)	25 (20-39)	25 (20-38)	25 (20-39)	0.749
Gender, male (%)	81	70	91	0.311
HBV genotype (%B, C)	24, 76	10, 90	36, 64	0.311
Median HBV DNA levels, range [log (IU/mL)]	8.2 (7.5-8.7)	7.7 (7.2-8.4)	8.4 (8.1-8.8)	0.090
Median ALT level, range (IU/L)	147 (123-201)	184 (146-247)	124 (112-148)	0.011 ^a
Median AST level, range (IU/L)	65 (51-97)	90 (57-132)	64 (45-73)	0.072
Median TSH levels, range (mIU/L)	2.06 (1.41-3.10)	1.82 (1.14-2.08)	2.55 (1.68-4.11)	0.035 ^c
Median TT3 levels, range (nmol/L)	2.22 (2.04-3.03)	2.85 (2.02-3.85)	2.20 (2.02-2.54)	0.305
Median TT4 levels, range (nmol/L)	111.4 (96.8-140.6)	132.7 (109.0-168.5)	107.8 (88.4-117.3)	0.037 ^c

Data are expressed as the median (IQR) and as percentages. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TSH: Thyroid-stimulating hormone. ^a $P < 0.05$ differences of baseline serum ALT level between responders and non-responders; ^c $P < 0.05$ differences of baseline serum TSH level between responders and non-responders; ^c $P < 0.05$ differences of baseline serum TT4 level between responders and non-responders.

Table 2 Predictive value of single and combined baseline factors

Parameters	Responders	Non-responders	Predictive value (%)
ALT \geq 140	9	3	PPV = 75
ALT < 140	1	8	NPV = 89
TSH < 2.4	9	3	PPV = 75
TSH \geq 2.4	1	8	NPV = 89
TT4 \geq 120	6	2	PPV = 75
TT4 < 120	3	9	NPV = 75
ALT \geq 140, TT4 \geq 120	6	1	PPV = 86
ALT < 140, TT4 < 120	1	7	NPV = 88
ALT \geq 140, TSH < 2.4	8	1	PPV = 89
ALT < 140, TSH \geq 2.4	0	6	NPV = 100
TT4 \geq 120, TSH < 2.4	5	1	PPV = 83
TT4 < 120, TSH \geq 2.4	0	7	NPV = 100

Data are expressed as numbers of instances. PPV: Positive predictive values; NPV: Negative predictive values.

ALT, TSH, and TT4 were 0.827 ($P = 0.011$), 0.773 ($P = 0.035$), and 0.778 ($P = 0.037$), respectively. Accordingly, we chose cutoff values of 140 IU/L, 2.4 mIU/L, and 120 nmol/L for ALT, TSH, and TT4, respectively. Correspondingly, their positive predictive values (PPV) and negative predictive values (NPV) were 75% and 89 %, 75% and 89 %, and 75% and 75% (Table 2). We further performed the combination of the baseline ALT and TT4, ALT and TSH, and TT4 and TSH to predict the virological response. We found that their PPV and NPV were 86% and 88%, 89% and 100%, and 83% and 100%, respectively (Table 2).

DISCUSSION

Nowadays, more and more doctors are taking the initiative in individualized treatment for chronic hepatitis B patients. With the purpose of taking individualized treatment, it is important to evaluate the baseline status of each patient at the start of treatment and to then decide which antiviral drug is the best choice. For those patients who are not likely to benefit from Peginterferon α -2b therapy, an early switch to nucleoside/nucleotide analogs is essential.

Recently, a study showed that HBeAg levels had high negative predictive values (NPVs) at week 24 of

sustained virological response to Peginterferon α -2a in HBeAg-positive CHB patients^[18]. While in HBeAg-negative CHB patients, early serum HBsAg drops also had high predictive values of sustained virological response to Peginterferon α -2a at week 12 and 24^[19].

In our study, 21 HBeAg-positive CHB patients were treated with Peginterferon α -2b for 24 wk and followed up for 24 wk. We found that baseline serum ALT, TSH, and TT4 levels, and especially the combination of these factors, had high predictive values of virological response to Peginterferon α -2b therapy.

To identify the baseline predictors of virological response, we performed univariate analysis and receiver operating characteristic curves for baseline serum ALT, TSH, and TT4 levels, and found that the cutoff value of 140 IU/L of baseline serum ALT level had a relatively high predictive value of virological response. The cutoff values for TSH and TT4 were 2.4 (mIU/L) and 120 (nmol/L), respectively. Moreover, we found that combinations of these factors could further improve the PPV and NPV scores.

Some studies have shown that the rates of HBeAg loss and seroconversion were correlated with the baseline level of ALT. In patients with a higher baseline level of ALT, the rates of HBeAg loss and seroconversion during lamivudine therapy were also significantly higher at the end of year three^[7]. A previous study showed that CHB patients with normal ALT levels respond very poorly to interferon α -2a therapy. However, the response was significantly better in patients with elevated ALT levels^[13]. In HBeAg-negative CHB patients treated with Peginterferon α -2a, with or without lamivudine, a high baseline ALT level was identified as a significant predictor of virological response at weeks 24 post-treatment^[8].

Besides high baseline serum ALT level, we also found that higher TT4 level and lower baseline serum TSH level were associated with better outcome of Peginterferon α -2b therapy in HBeAg-positive CHB patients.

Although no study exploring the predictive value of virological response for baseline serum TT4 in chronic hepatitis B patients has been reported, several studies have demonstrated a reciprocal relationship between the endocrine and immune systems. Recently a study showed that triiodothyronine and thyroxine concentrations were

positively associated with markers of inflammation, natural killer-like T cells, activated monocytes derived interleukin-6 (IL-6), higher expression of IL-2 receptor on CD3+ T-lymphocytes, and percentage expression of memory T-lymphocytes, memory T-helper lymphocytes and memory T-cytotoxic lymphocytes within normal physiological ranges^[20]. This is supported by previous findings that thyroid hormone was involved in primary and secondary lymphopoiesis, and blastogenic responses to T and B cell mitogens were also enhanced following thyroxine administration^[21,22]. Other studies showed that thyroxine did not induce resting T lymphocyte proliferation but increased mitogen ConA-induced stimulation after three days of culture, in a dose-dependent manner. Thyroxine substitutive treatment restored the euthyroid status and reversed the impairment of T-cell activation induced by chronic stress in mice^[23,24]. Interestingly, the age-dependent immunological deterioration in old mice could be recovered by thyroxine treatment^[25]. These results indicated that thyroxine could enhance the immune response. Thus, this may be the reason why the responders who had higher baseline TT4 level achieved virological response more easily during Peginterferon α -2b therapy in our study.

Another major finding was the lower baseline TSH level of responders was also associated with higher virological response rate. This could be caused by the negative feedback mechanism due to their higher baseline serum TT4 level.

In conclusion, the identification and application of baseline factors to predict virological response of chronic hepatitis B patients before antiviral therapy is important. Using this method, we can identify patients who will most likely benefit from Peginterferon α -2b therapy before treatment. However, because of the small cohort of patients enrolled in our study, large-scale studies are needed to further confirm our results and to identify simpler and more appropriate factors that have high predictive values of virological response in chronic hepatitis B patients.

COMMENTS

Background

Early prediction of virological response for chronic hepatitis B patients treated with antiviral drugs is important. Some factors such as HBsAg and HBeAg reduction have been found to have high predictive values of sustained virological response in chronic hepatitis B patients treated with Peginterferon α -2a. However, the predictive values of other factors, especially the baseline factors for virological response to Peginterferon α -2b therapy, are not clear.

Research frontiers

Many studies have shown that an elevated serum alanine aminotransferase (ALT) level was associated with virological response and HBeAg seroconversion in CHB patients. Recent studies showed a reciprocal relationship between the endocrine and immune system. In this study, the authors showed that baseline serum ALT, thyroid-stimulating hormone (TSH), and total thyroxine (TT4) levels, and especially combinations of these factors, have high predictive values of virological response to Peginterferon α -2b in HBeAg-positive CHB patients.

Innovations and breakthroughs

The present study demonstrated that baseline serum ALT, TSH, and TT4 levels, and especially combinations of these factors, have high predictive values of virological response to Peginterferon α -2b in HBeAg-positive chronic hepatitis B (CHB) patients before treatment.

Applications

This study might represent a future strategy for identifying chronic hepatitis B patients who will most likely benefit from Peginterferon α -2b therapy before treatment.

Terminology

ALT is an enzyme that is normally present in liver and heart cells. ALT is released into blood when the liver or heart is damaged. TSH is a peptide hormone synthesized and secreted by thyrotrope cells in the anterior pituitary gland which regulates the endocrine function of the thyroid gland. Thyroxine (T4) is a form of thyroid hormone which is the major hormone secreted by the follicular cells of the thyroid gland.

Peer review

This study is of interest as it describes the relationship of virological response to Peginterferon α -2b therapy and serum parameters at pretreatment, although this was obtained in a very small cohort of patients.

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S- Editor Li LF **L- Editor** Stewart GJ **E- Editor** Yin DH

BRIEF ARTICLES

Expression of thymidylate synthase and glutathione-s-transferase π in patients with esophageal squamous cell carcinoma

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Supported by The Key Medical Talent Foundation of Jiangsu Province, China, No. RC2007036

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Received: May 27, 2009 Revised: August 1, 2009

Accepted: August 8, 2009

Published online: September 14, 2009

Abstract

AIM: To investigate the expression of thymidylate synthase (TS) and glutathione-s-transferase π (GST- π) in esophageal squamous cell carcinoma and their association with the clinicopathologic characteristics.

METHODS: Immunohistochemical methods were used to detect the expression of TS and GST- π in surgically resected formalin-fixed, paraffin-embedded esophageal squamous cell carcinoma (ESCC) tissue sections from 102 patients (median age, 58 years) and in 28 normal esophageal mucosa (NEM) samples. The relationship between TS and GST- π expression and clinicopathologic factors was examined.

RESULTS: The expression of TS and GST- π was not statistically significantly associated with age of the patients, tumor size, lymph node metastasis, depth of invasion or tumor stage. TS staining was positive in 17.86% of normal esophageal mucosa and in 42.16% of ESCC samples ($P < 0.05$). The expression level of TS

was not only significantly lower in well-differentiated (21.88%) than in poorly-differentiated carcinomas (51.43%, $P < 0.05$), but was also significantly higher in samples from male patients (46.51%) than from female patients (18.75%, $P < 0.05$). GST- π was positively stained in 78.57% of normal esophageal mucosa and in 53.92% of ESCC samples ($P < 0.05$). The expression level of GST- π was also significantly higher in well-differentiated carcinomas (65.63%) than in poorly-differentiated carcinomas (35.00%, $P < 0.05$).

CONCLUSION: The expression of TS and of GST- π may be used as molecular markers for the characterization of ESCC. Poorly-differentiated cells showed increased expression of TS and reduced expression of GST- π .

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Key words: Esophageal squamous cell carcinoma; Glutathione-s-transferase π ; Immunohistochemistry; Thymidylate synthase; Tumor markers

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Huang JX, Li FY, Xiao W, Song ZX, Qian RY, Chen P, Salminen E. Expression of thymidylate synthase and glutathione-s-transferase π in patients with esophageal squamous cell carcinoma. *World J Gastroenterol* 2009; 15(34): 4316-4321 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4316.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4316>

INTRODUCTION

Nearly 50% of patients with the diagnosis of esophageal cancer present with overt metastatic disease, and chemotherapy is the mainstay of palliation in this setting. With the increasing use of chemotherapy as an adjunct to surgical management, systemic chemotherapy will ultimately be used to treat the majority of patients with esophageal cancer. The combination of 5-fluorouracil

(5-FU) and cisplatin is widely used in the treatment of esophageal cancer. Alternatively, taxane or irinotecan is applied in combination with either 5-FU or cisplatin^[1]. The response to cisplatin and 5-FU has been low, ranging from 35% to 40%^[2]. Therefore, there is great interest in identifying additional biochemical markers that might be predictive of chemotherapy response and resistance. As Ilson *et al.*^[1] stated: “future strategies in the treatment of esophageal carcinoma will undoubtedly be based on advances in the understanding of the molecular biology of the disease”.

Thymidylate synthase (TS) is a key enzyme for DNA and RNA synthesis. The anticancer activity of 5-FU is based on this molecular target. Glutathione-S-transferase π (GST- π) actively binds to platinum and allows it to be removed from the cytosol^[3]. Several studies have suggested that the expression of TS and GST- π could be associated with chemotherapy resistance and prognosis in esophageal cancer and gastric cancer patients^[3-6]. To our knowledge, the significance of TS and GST- π expression in esophageal squamous cell carcinoma (SCC) has not been reported to date in a Chinese population.

The current study was conducted (1) to investigate the expression characteristics of TS and GST- π in esophageal SCC (ESCC) in a Chinese population, and (2) to study the association between TS, GST- π and the clinical characteristics of the patients.

Immunohistochemical analysis has been used to assess the expression of molecular markers in malignant tumors. TS or GST- π has been shown to predict chemotherapy response and resistance in several cancers^[7-12].

MATERIALS AND METHODS

Patients and specimens

In this study, we determined the expression of TS and GST- π in surgically resected formalin-fixed, paraffin-embedded ESCC tissue sections from 102 patients and in 28 normal esophageal mucosa samples using immunohistochemical methods. The patients (86 males and 16 females) with ESCC underwent surgical resection at the Department of Thoracic Surgery, People's Hospital of Taizhou (Taizhou Medical School, Yangzhou & Nantong University), between August 2005 and September 2007. All patients had undergone a subtotal or total esophagectomy and radical lymph node dissection.

Histopathological specimens were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. All specimens were obtained from patients who had not received chemo- or radiotherapy prior to surgical resection. All hematoxylin and eosin stained sections were reviewed and reexamined by pathologists. The grade of tumor differentiation was determined according to the classification of the World Health Organization^[13], and staged according to the TNM classification^[14].

The patients were 35-76 years of age with a median age of 58.0 years. The location of the tumors was as

follows: upper intra-thoracic esophagus in 11 cases (10.7%); middle intra-thoracic esophagus in 55 cases (53.9%); lower intra-thoracic esophagus in 36 cases (35.2%). Histological degree of differentiation was well-differentiated in 32 cases (31.4%), moderately-differentiated in 50 cases (49.1%) and poorly-differentiated in 20 cases (19.6%). Five cases were Stage I, 47 cases were Stage II, 33 cases were Stage III and 17 cases were Stage IV. NEM samples were taken from 28 patients from an area more than 5 cm from the cancerous tissue, as control non-tumor samples.

Antibodies

The following antibodies were used in this study: mouse monoclonal antibody, anti-human TS and GST- π , the PV-9000 test kit (Zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China) was also used.

Immunohistochemical staining

The specimens with adjacent non-cancerous esophageal mucosa were cut into 4-5- μ m thick sections and mounted onto slides, deparaffinized with xylene, and rehydrated with graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubating with 3% hydrogen peroxide (H₂O₂) in deionized water for 10 min. The slides were washed three times with TBS buffer (10 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.5) for 2 min. Before application of the TS primary antibody, an antigen retrieval technique was used (10 mmol/L sodium citrate solution, pH 6.0 in a rice cooker, at 640 W for 30 min). After three washes with TBS, an aliquot of 100 μ L of primary antibody was then applied to each section and incubated at 4°C overnight. It is not necessary to perform an antigen retrieval technique for GST- π . After washing 3 times with TBS and following the directions in the kit manual, agent one and then agent two (including the kit) were applied for 20 min at RT. Finally, the sections were washed 3 times with TBS, and the immunoreactions were visualized with 0.0067% diaminobenzidine as the substrate with 0.03% H₂O₂ in 100 mmol/L Tris-HCl buffer for 3 min. The sections were lightly counterstained in Harris hematoxylin solution for microscopic examination. Simultaneously, each section was incubated with TBS instead of the primary antibody as an internal negative control.

The immunostained specimens were analysed by two independent pathologists. Cytoplasm and/or nuclear staining (brown reaction product) was regarded as a positive result. Five fields in each tumor and non-tumor section were evaluated at medium power ($\times 200$) to determine the proportion of tumor cells and the staining intensity of the cytoplasm and/or nuclei in each section. The percentage of positive tumor cells was assigned to one of the following categories: 0 (0%-4%), 1 (5%-24%), 2 (25%-49%), 3 (50%-74%), or 4 (75%-100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), and 2+ (strong). Additionally, an immunoreactive score was calculated by multiplying the percentage of positive cells and the staining intensity. The

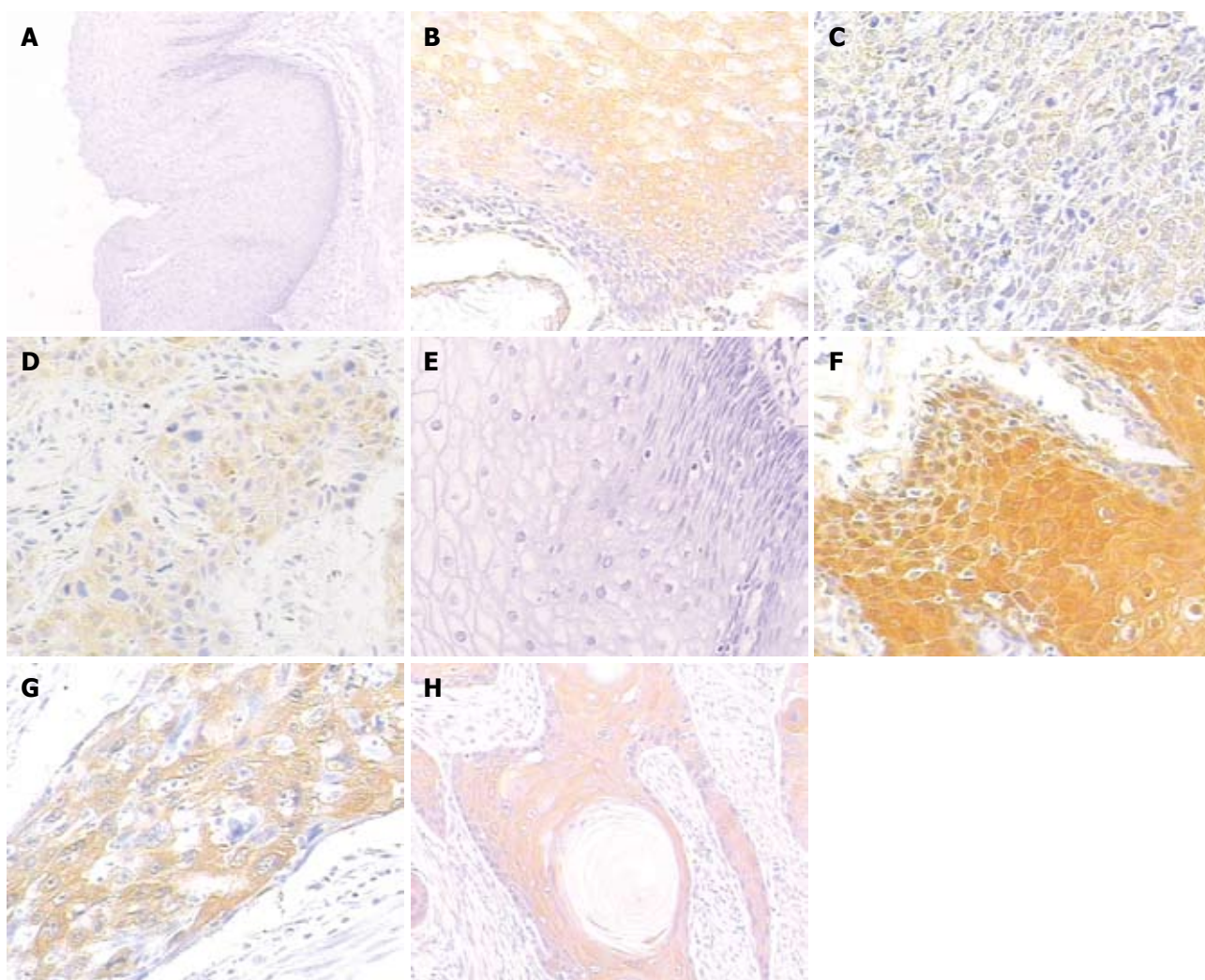


Figure 1 Expression of thymidylate synthase (TS) and glutathione-s-transferase π (GST- π) in normal esophageal mucosa and esophageal squamous cell carcinoma. A and E: Negative control for TS and GST- π in normal esophageal mucosa, positive staining can not be detected (using TBS as primary antibody) ($\times 100$); B and F: Positive staining was located in the cytoplasm in normal mucosa ($\times 200$); C and G: Poorly-differentiated SCC, note the diffuse strong TS immunostaining in esophageal SCC ($\times 200$), for GST- π , positive staining was shown in the cytoplasm ($\times 200$); D and H: Moderately-differentiated SCC, TS positive staining is in the cytoplasm ($\times 200$); Well-differentiated SCC, diffuse strong GST- π immunostaining can be seen in esophageal SCC ($\times 200$).

average score in each tumor and non-tumor section was calculated, and the expression was considered positive when the score was $> 2^{[15]}$. To confirm the reproducibility of the results, all sections were scored twice, the highest score between the 2 observers are thus reported.

Statistical analysis

The correlations between the expression of TS, GST- π and clinicopathological factors were determined using the χ^2 or Fisher test (SPSS 15.0 software package) at the 5% level.

RESULTS

Expression pattern of TS in normal esophageal mucosa and in ESCC

Without specific primary antibody to TS, no staining was observed in esophageal specimens (Figure 1A). The staining of TS was mainly concentrated in the cytoplasm of

cells, occasionally, the nuclei were also stained (Figure 1B). The expression rates of TS in normal esophageal mucosa and in ESCC were 17.86% (5/28) and 42.16% (43/102), respectively. This suggested that the expression level of TS in ESCC was significantly higher than that in normal esophageal mucosa ($\chi^2 = 5.50$, $P = 0.018$).

TS staining and clinicopathological factors

The correlations between the expression of TS and the clinicopathologic features of ESCC are summarized in Table 1. The expression of TS was not significantly associated with age of the patients, tumor size, lymph node metastasis, depth of invasion or tumor stage. The expression of TS was significantly higher in poorly- and moderately-differentiated ESCC (Figure 1C) than in well-differentiated ESCC (Figure 1D) ($\chi^2 = 7.866$, $P < 0.01$). Female patients had tumors with low TS expression (18.75%) more frequently than male patients (46.51%) ($\chi^2 = 4.264$, $P < 0.05$).

Table 1 Relationship between TS and clinicopathological characteristics of ESCC

Factor	<i>n</i>	(+)	(-)	Positive (%)	<i>P</i> value
Sex					
M	86	40	46	46.51	0.039
F	16	3	13	18.75	
Age (yr)					
< 60	58	27	31	46.55	0.302
≥ 60	44	16	28	36.36	
Histological grade					
Well	32	7	25	21.88	0.005
Moderate & poor	70	36	34	51.43	
Lymph node metastasis					
(-)	55	24	31	43.64	0.743
(+)	47	19	28	40.43	
Location ¹					
Upper	11	4	7	36.36	0.822
Middle	55	22	33	40.00	
Lower	36	17	19	47.22	
T Stage					
T ₀₋₁	7	4	3	57.14	0.405
T ₂₋₄	95	39	56	41.04	

¹Location: Upper, upper intra-thoracic esophagus; Middle, middle intra-thoracic esophagus; Lower, lower intra-thoracic esophagus. TS: Thymidylate synthase; ESCC: Esophageal squamous cell carcinoma.

Expression pattern of GST- π in normal esophageal mucosa and in ESCC

Without specific primary antibody to GST- π , no staining was observed in esophageal specimens (Figure 1E). While the staining of GST- π was mainly concentrated in the cytoplasm, occasionally, the nuclei of cells were also stained (Figure 1F). The positive expression rates of GST- π in normal esophageal mucosa and in ESCC were 78.57% (22/28) and 53.92% (55/102), respectively. This showed that the expression level of GST- π in normal esophageal mucosa was significantly higher than that in ESCC ($\chi^2 = 5.528$, $P < 0.05$).

GST- π staining and clinicopathological factors

The correlations between the positive expression rates of GST- π and the clinicopathologic features of ESCC are summarized in Table 2. The positive expression rates of GST- π were not significantly associated with the sex or age of the patients, tumor size, lymph node metastasis, depth of invasion or tumor stage. However, positive expression was significantly higher in well-differentiated ESCC (Figure 1H) than in poorly-differentiated ESCC (Figure 1G) ($\chi^2 = 4.645$, $P < 0.05$).

DISCUSSION

The present study was designed to evaluate the expression characteristics of TS and GST- π in ESCC, and to assess the relationship between TS, GST- π and clinical characteristics. Chemotherapy with cisplatin/5-FU is accepted as a standard treatment in squamous cell and adenocarcinoma of the esophagus. TS is the enzyme targeted by 5-FU, and this may be a potential marker of chemotherapy response, whereas an increase in expression of TS may indicate resistance to 5-FU^[16].

Table 2 Relationship between GST- π and clinicopathological characteristics of ESCC

Factor	<i>n</i>	(+)	(-)	Positive (%)	<i>P</i> value
Sex					
M	86	46	40	53.49	0.839
F	16	9	7	56.25	
Age (yr)					
< 60	58	33	25	56.90	0.489
≥ 60	44	22	22	50.00	
Histological grade					
Well	32	21	11	65.63	0.031
Moderate	50	27	23	54.00	
Poor	20	7	13	35.00	
Lymph node metastasis					
(-)	55	31	24	56.36	0.592
(+)	47	24	23	51.06	
Location ¹					
Upper	11	5	6	45.45	0.660
Middle	55	29	26	52.73	
Lower	36	21	15	58.33	
T Stage					
T ₀₋₁	7	3	4	2.86	0.543
T ₂₋₄	95	52	43	54.74	

¹Location: Upper, upper intra-thoracic esophagus; Middle, middle intra-thoracic esophagus; Lower, lower intra-thoracic esophagus. GST- π : Glutathione-s-transferase π .

Assessment of the probability of chemotherapy resistance using immunohistochemistry methods detecting TS and GST- π expression may allow for the selection of a more effective chemotherapeutic regimen in several cancer patients^[8-12].

TS plays an important role in folate metabolism. Using the methyltetrahydrofolic acid as a substrate, TS catalyses the methylation of deoxyuridylic acid, transferring it into deoxythymidylic acid, which is an important nucleotide in the synthesis and reparation of DNA^[17]. This study showed that the expression of TS in ESCC was higher than that in normal tissue, and that the expression in moderately- and poorly-differentiated ESCC was higher than that in well-differentiated ESCC. This study also revealed that there is a potential to select patients according to whether TS expression is correlated with chemosensitivity to 5-FU. Some studies have indicated that esophageal cancer in patients with low expression of TS is more sensitive to chemotherapy than those with high expression^[3,4].

In the current study, the expression level of TS was observed to be associated with gender. The expression level of TS was significantly higher in males than in females, contrary to the findings reported by Dong^[4]. More studies are needed to investigate the significance of this difference, if any, to the outcome of patients.

In our study, the proportion of male to female patients was 5.4 to 1, this was similar to that of Joshi (6.07 to 1)^[3]. If we can verify that the expression of TS is higher in male than female patients, then this result may help us to understand why esophageal squamous cell carcinoma is related to gender.

GST is a group of isozymes with the function of detoxification and combining proteins. In humans, GST

contains α , μ , π , σ and θ , 5 family constellations and 13 different enzymes. The constellations are encoded by GSTA, GSTM, GSTP, GSTS, GSTT, respectively, and the relationship between GST- π and tumors has attracted much attention^[18]. GST- π not only affects cisplatin by shifting it away from the cells, but it can also release oxygen free radicals, a mechanism which reduces radiation damage^[2]. The expression of GST- π was higher in normal esophageal mucosa than in ESCC, and was higher in well-differentiated tumors compared to poorly-differentiated tumors. These results suggest that the loss of GST- π expression in esophageal epithelium may be an early pre-cancerous sign. The expression of GST- π had no significant association with gender, age, location of the tumor, lymph node metastasis or T stage, consistent with a previous report^[19]. Recently, a study in gastric cancer indicated that the expression of GST- π may be associated with the efficacy of cisplatin^[6].

In conclusion, the present results indicate that expression of TS and GST- π in ESCC in a Chinese population may add to understanding tumor characteristics and to predict response to chemotherapy. It is possible to predict chemotherapy response and resistance by detecting these biological markers. Thus, we are planning to investigate the relationship between expression of TS, GST- π , the curative effect of chemotherapy, and survival rate in patients with ESCC in a future study.

COMMENTS

Background

Esophageal cancer is a serious threat to human health, and nearly 50% of patients with a diagnosis of esophageal cancer present with overt metastatic disease. Chemotherapy has played a crucial role in the treatment of esophageal cancer. The combination of 5-fluorouracil and cisplatin is widely used in these patients. The response to these two drugs has been low. Therefore, it is important to know which drugs may induce a response in these patients.

Research frontiers

Thymidylate synthase (TS) is a key enzyme for DNA and RNA synthesis. The anticancer activity of 5-FU is based on this molecular target. Glutathione-S-transferase π (GST- π) actively binds to platinum and allows it to be removed from the cytosol. In this study, we demonstrated that the expression of TS and GST- π was related to clinicopathological factors of esophageal cancer.

Innovations and breakthroughs

Recently, a number of studies have suggested that the expression of TS and GST- π is associated with chemotherapy resistance and prognosis in esophageal cancer and gastric cancer patients. However, the study of TS and GST- π in esophageal squamous cell carcinoma (ESCC) has not been reported in a Chinese population. Their study showed that the expression features of TS and GST- π were obviously different in normal esophageal mucosa and ESCC. Furthermore, the expression level of TS and GST- π is related to clinicopathological factors of ESCC in a Chinese population.

Applications

By understanding the anti-cancer molecular target of 5-FU and platinum and their relationships with TS and GST- π , this study indicated that it may be possible to predict response and resistance to chemotherapy by detecting TS and GST- π in patients with ESCC.

Terminology

The expression level of TS in ESCC was higher than in normal esophageal mucosa. In contrast, GST- π in normal esophageal mucosa was higher than that in ESCC. These enzymes can be used as diagnostic molecular markers for ESCC. The results demonstrated that tumor tissues were poorly differentiated when the expression of TS was increased and the expression of GST- π was reduced.

Peer review

This is well written article, describing the correlation between expression of TS and GST- π with clinicopathological features in patients with ESCC.

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S-Editor Li LF **L-Editor** Webster JR **E-Editor** Yin DH



BRIEF ARTICLES

Application of endoscopic hemoclips for nonvariceal bleeding in the upper gastrointestinal tract

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Received: May 8, 2009

Revised: August 7, 2009

Accepted: August 14, 2009

Published online: September 14, 2009

an effective and safe method for acute nonvariceal bleeding in the upper GI tract with satisfactory outcomes.

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Key words: Gastrointestinal hemorrhage; Endoscopy; Hemoclip; Hemostasis

Peer reviewer: Abdellah Essaid, Professor, Hospital Ibn Sina, Rabat 10100, Morocco

Guo SB, Gong AX, Leng J, Ma J, Ge LM. Application of endoscopic hemoclips for nonvariceal bleeding in the upper gastrointestinal tract. *World J Gastroenterol* 2009; 15(34): 4322-4326 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4322.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4322>

Abstract

AIM: To investigate acute nonvariceal bleeding in the upper gastrointestinal (GI) tract and evaluate the effects of endoscopic hemoclippping.

METHODS: Sixty-eight cases of acute nonvariceal bleeding in the upper GI tract were given endoscopic treatment with hemoclip application. Clinical data, endoscopic findings, and the effects of the therapy were evaluated.

RESULTS: The 68 cases (male:female = 42:26, age from 9 to 70 years, average 54.4) presented with hematemesis in 26 cases (38.2%), melena in nine cases (13.3%), and both in 33 cases (48.5%). The causes of the bleeding included gastric ulcer (29 cases), duodenal ulcer (11 cases), Dieulafoy's lesion (11 cases), Mallory-Weiss syndrome (six cases), post-operative (three cases), post-polypectomy bleeding (five cases), and post-sphincterotomy bleeding (three cases); 42 cases had active bleeding. The mean number of hemoclips applied was four. Permanent hemostasis was obtained by hemoclip application in 59 cases; 6 cases required emergent surgery (three cases had peptic ulcers, one had Dieulafoy's lesion, and two were caused by sphincterotomy); three patients died (two had Dieulafoy's lesion and one was caused by sphincterotomy); and one had recurrent bleeding with Dieulafoy's lesion 10 mo later, but in a different location.

CONCLUSION: Endoscopic hemoclip application was

INTRODUCTION

Bleeding in the upper gastrointestinal (GI) tract is very common. The majority of patients benefit from conservative treatments; however, for those who have active bleeding, or have a high risk of recurrence of bleeding, it is still a serious problem for both endoscopists and surgeons^[1]. At present, endoscopic therapy has been recommended as the first choice for the treatment of acute upper GI bleeding^[2]. Effective methods for the control of bleeding in the upper GI tract include local injection (epinephrine or ethanol), thermal coagulation (laser; heater probe), and mechanical methods (hemoclips; elastic bands)^[3,4]. Among these methods, hemoclips can achieve immediate hemostasis^[5] by obstructing the vessel and have the special advantage of lack of additional tissue damage^[6]. During January 2000 to January 2007, 68 patients were given endoscopic hemoclippping treatment for nonvariceal bleeding in the upper GI tract. In this retrospective study, clinical data and endoscopic findings are described, and the outcomes of the therapy are also evaluated.

MATERIALS AND METHODS

During January 2000 to January 2007, a total of 632 patients had emergent endoscopy for bleeding in the upper GI tract in our hospital, and 155 patients were given endoscopic therapy. Among them, 68 cases

with nonvariceal bleeding were given endoscopic hemoclip application. Written informed consent was obtained from all the patients or their relatives before the treatment. The 68 cases had ages ranging from 9 to 70 years (average 54.4, male:female = 42:26). The presenting manifestations were hematemesis in 26 cases (38.2%), melena in nine cases (13.3%), and both in 33 cases (48.5%). Some of the patients had basal disease, including cardiovascular disease (myocardial infarction, congestive heart failure, or significant cardiac arrhythmia) in eight cases (11.8%), liver cirrhosis in two cases (2.94%) and respiratory disease (chronic obstructive pulmonary disease) in six cases (8.82%). Twenty-eight cases were in a state of shock, and 44 cases were given blood transfusions of more than 400 mL; the systolic blood pressures of 12 cases were still less than 90 mmHg when they were given the endoscopic treatment. The electrocardiogram, blood pressure, and oxygen saturation were monitored for those who were in a severe condition.

The type of hemoclip applied was MD 850 (Olympus Corp.) with a rotatable clip application device (HX-5L, Olympus Corp.). After finding the bleeding point, we exposed the clip from the sheath, rotated it to a desired axis, and opened the clip to the maximum width. The clip was then pressed against the lesion and deployed. If needed, the procedure was repeated. The mean number of hemoclips applied was four. All of the patients were given physical care after endoscopic therapy, such as monitoring vital signs, fasting, intravenous fluid, intravenous administration of Histamine-2 receptor antagonists or proton pump inhibitors, hemostatic agents, and some were given blood transfusions.

RESULTS

The causes of the nonvariceal bleeding in the upper GI tract can be listed as followings: gastric ulcer in 29 cases, duodenal ulcer in 11 cases, Dieulafoy's lesion in 11 cases, Mallory-Weiss syndrome in six cases, post-operative in three cases, post-polypectomy bleeding in five cases, and post-sphincterotomy bleeding in three cases.

Hemostasis was defined as endoscopic cessation of bleeding for at least one minute after hemoclip application. Clinically, hemostasis was defined as no decrease in hemoglobin concentration, and correction of shock by blood transfusion and intravenous fluid. Hemostasis was obtained by hemoclip placement in 59 cases. Six patients underwent emergent surgery, in which three cases had peptic ulcers (two located in the posterior wall of the gastric body and one duodenal ulcer located in the posterior wall near the lesser curvature), one case had Dieulafoy's lesion, and two cases were caused by sphincterotomy. Three patients died due to cardiovascular failure and liver cirrhosis (two had Dieulafoy's lesion and one was caused by sphincterotomy). To evaluate the long-term outcomes of the treatment, the patients were followed-up for 30 d. All 59 cases achieved permanent hemostasis, and one of them had recurrent bleeding because of

Dieulafoy's lesion 10 mo later, but in a different location (initially in the proximal one third of the stomach and later in the duodena). The patient underwent endoscopic hemoclip application again, and also achieved a satisfactory result.

DISCUSSION

Despite the development of pharmacology and endoscopic therapy, nonvariceal bleeding in the upper GI tract remains a serious problem, especially for those who have active bleeding. It is associated with an approximately 20% rebleeding rate and its mortality ranges from 10% to 36%^[7-9]. The etiology of acute nonvariceal bleeding in the upper GI tract has changed little in the past 20 years, peptic ulcers (including gastric ulcer and duodenal ulcer) are still the most common causes of acute hemorrhage in the upper GI tract^[10]. In our group, it accounted for 58.8% of bleeding episodes. After endoscopic therapy, acid suppression is essential for those who have bleeding caused by peptic ulcer disease. In a low pH environment, platelets can lose their function, and blood clots might be dissolved by pepsin, resulting in further bleeding. Among the 40 bleeding peptic ulcers, 92.5% achieved permanent hemostasis, only three cases underwent emergent surgery.

Tears at the gastroesophageal junction (Mallory-Weiss syndrome) account for 5% to 15% of all cases of nonvariceal bleeding in the upper GI tract^[11]. These lesions are usually associated with repeated nausea and vomiting. For nonbleeding cases, conservative treatments are usually sufficient. In our group, six cases with active bleeding were given hemoclip application and all had excellent outcomes. Compared with other endoscopic treatments, such as sclerotherapy, epinephrine injection, and heater probe, the hemoclip is a safer choice, without adverse effects^[12].

Dieulafoy's lesion, an important cause of potentially life-threatening GI bleeding, was first described in 1896 and is a submucosal artery protruding from a minute defective mucosa surrounded by normal tissue^[13,14]. Its histopathologic description is "a caliber-persistent artery" in the submucosal tissue^[14]. It was regarded as a rare disease in the past because the caliber-persistent artery often retracts after bleeding^[9], but with the development of technology and familiarity with this disease, it is now estimated to represent about 5% the etiology of acute upper-GI bleeding^[9]. Endoscopic therapy is now considered the first-line method of achieving hemostasis, and hemoclip application has achieved satisfactory results with no reported ulcerative complications^[10]. It can cause occlusion of the bleeding vessel, which results of immediate local hemostasis and prevent delayed recanalization and recurrent bleeding^[5]. In most cases, the hemoclip can replace surgery as the first choice therapy for patients with Dieulafoy's lesion. However, because the lesions are often located in the proximal stomach, usually along the lesser curvature, it might be technically difficulty to apply a hemoclip. In our group, one case underwent emergent surgery and

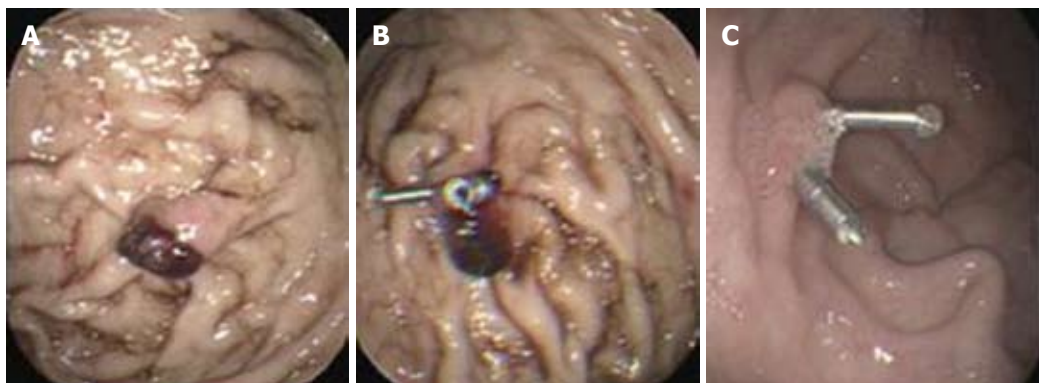


Figure 1 Endoscopic view of a Dieulafoy's lesion before and after endoscopic hemoclippping. A: Endoscopic view of a Dieulafoy's lesion with a protruding vessel in the gastric fundus; B: Endoscopic view showing complete closure of the mucosal defect with a protruding vessel by hemoclips; C: Endoscopic view of the same patient three months later.

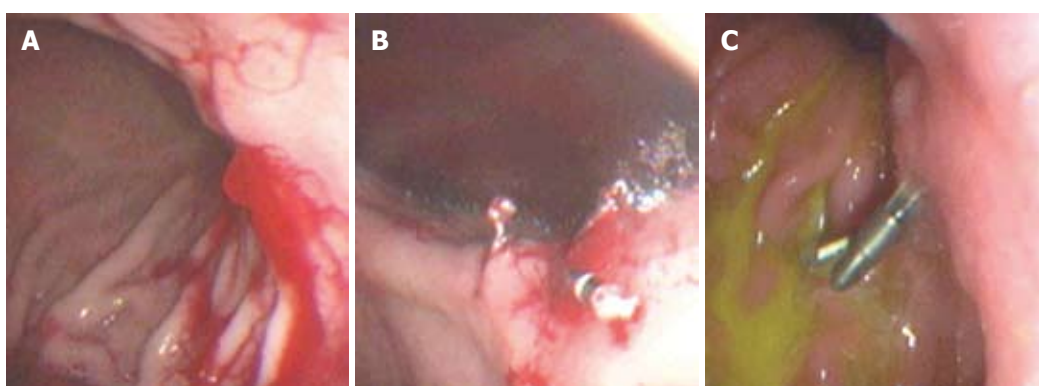


Figure 2 Endoscopic view of a Dieulafoy's lesion before and after endoscopic hemoclippping. A: Endoscopic view of a Dieulafoy's lesion with active bleeding at the posterior wall of the proximal one third of the stomach. B: View after hemoclips application to bleeding site; bleeding has stopped. C: Endoscopic view of the same patient three months later.

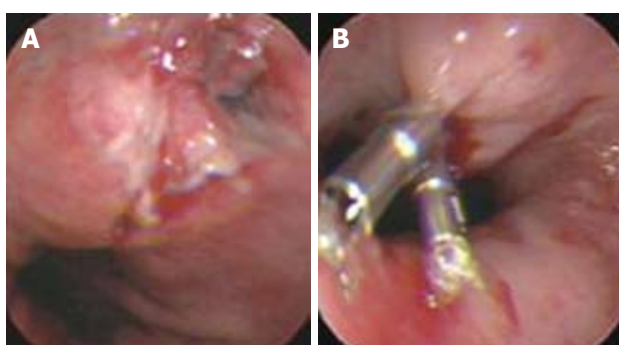


Figure 3 Endoscopic view of a Mallory-Weiss tear at the esophagogastric junction before and after endoscopic hemoclippping. A: Endoscopic view of a Mallory-Weiss tear at the esophagogastric junction with active bleeding; B: View after hemoclips application to bleeding vessel; bleeding has stopped.

two cases were dead due to cardiovascular failure.

Upper GI bleeding caused by endoscopic treatment, such as resection of polyps, mucosal resection, and sphincterotomy, is becoming more and more frequent in clinics due to the increased endoscopic treatments. In our group, five cases were caused by resection of polyps by endoscopy and three cases were caused by sphincterotomy by endoscopy. Among them, two cases underwent emergent surgery and one case died from GI bleeding. These three cases were all caused

by sphincterotomy. In most cases, hemoclippping can achieve satisfactory results; however, it is difficult to accomplish hemostasis through endoscopy in upper GI bleeding caused by sphincterotomy, even for experienced endoscopists, so if necessary, emergent surgery might be a better choice.

Patients who have active bleeding or have a high risk of recurrence of bleeding require effective hemostasis^[15]. At present, endoscopic therapy has been recommended as the first choice for the treatment of acute nonvariceal upper GI bleeding^[2]. Some endoscopic therapies, such as heat probe coagulation, injection of epinephrine, or sclerotic agency, have been proved to be effective for achieving hemostasis, but they might cause tissue injury at the same time, causing necrosis or even perforation^[16,17].

As a mechanical method of hemostasis, the hemoclip application was first introduced in 1975^[18,19]. Due to its simplicity, low cost, easy availability, repetition, minimal damage to the localized field, and reduced risk of adverse effects, hemoclips have been widely used for the treatment of nonvariceal bleeding in the upper GI tract, such as bleeding peptic ulcer^[20,21], Dieulafoy's lesion bleeding^[22,23] (Figures 1 and 2), Mallory-Weiss syndrome^[24,25] (Figure 3), post-polypectomy bleeding^[26], and post-sphincterotomy bleeding^[27]. It is suggested that

hemoclips are particularly helpful when active bleeding is encountered and/or there is a specific point of bleeding^[28]. In these cases, hemoclip application is fast and very effective in controlling bleeding. Theoretically^[6], clips can provide immediate hemostasis comparable with surgery by ligation of the bleeding vessel, with minimal injury to the adjacent tissue. DiMaio *et al*^[10] reported that Hemoclip application has excellent results for initial (97.6%) and permanent hemostasis (95.1%). An experimental study^[29] showed that only this mechanical method was effective for control of bleeding from vessels greater than 2 mm in diameter. Cipolletta *et al*^[30] also thought that clipping was superior to a standard therapy such as injection epinephrine, with significantly less further bleeding, fewer units of blood transfused, a shorter hospital stay, and limited damage to surrounding tissue. However, Gevers *et al*^[31] and Lin *et al*^[32] produced different results.

These inconsistent results in randomized controlled trials suggest that some other factors, such as age, the reasons and the locations of the lesions, shock, presence of multiple comorbidities, could all be associated with the failure of endoscopic hemoclips for bleeding^[33]. For example, some lesions are located in difficult-to-reach sites, which make it hard to apply the clips to the bleeding spot with a perpendicular angle^[34]. The tissue of an ulcer is very brittle, the clip can easily fall off if located on it, so the clip must be located on the normal tissue across the ulcer. If the ulcer is very large and beyond the width of the clip, we can not achieve hemostasis using a hemoclip. However, the experience of endoscopists appears to play a major role in successful clip application. In some cases, it is difficult to deploy hemoclips to the lesion with active bleeding, and only experienced endoscopists can accomplish this task. Thus, hemoclippping is more operator-dependent than other therapies, with some endoscopists achieving excellent results and others having less success. This could explain why the results have great variation in the hemoclip group. The devices themselves might also affect the final results. At present, a number of design improvements have been made to achieve better results. For example, the clip device can be rotated to a desired axis, which makes it easier to adjust the clip position before deployment. The installation of the clips is easier than before, which can save time, because in many cases, more than one clip is needed to achieve hemostasis. Of course, the results will be better if the device could deploy multiple clips at the same time and larger, stronger clips are designed to control the bleeding from large vessels. In summary, endoscopic hemoclip application is an effective and safe method for control nonvariceal bleeding in the upper GI tract with satisfactory outcomes, but the clip and the application device require further improvements.

COMMENTS

Background

Endoscopic hemoclippping has been proved to be effective for achieving hemostasis for nonvariceal gastrointestinal (GI) hemorrhage. However, the

efficacy for different causes of acute nonvariceal upper GI hemorrhage has been rarely reported. The aim of this study was to assess retrospectively the efficacy of endoscopic hemoclippping for different causes of acute nonvariceal upper GI hemorrhage, such as bleeding peptic ulcers, Dieulafoy's lesion bleeding, Mallory-Weiss syndrome, post-polypectomy bleeding, and post-sphincterotomy bleeding. The authors also wanted to determine the factors associated with failure of endoscopic hemoclippping to achieve hemostasis.

Research frontiers

The hemoclip has been widely used for the treatment of nonvariceal bleeding in the upper GI tract. The research hotspots is how to improve the success of hemostasis by endoscopic hemoclip application.

Innovations and breakthroughs

Many studies on the use of a hemoclip for the treatment of nonvariceal bleeding in the upper GI tract have been reported recently, but the results are inconsistent. In this article, The authors analyzed the common causes of acute nonvariceal bleeding in the upper GI tract, evaluated the efficiency of endoscopic hemoclippping for different causes of hemorrhage, and analyzed the factors associated with the failure of endoscopic hemoclippping to achieve hemostasis.

Applications

The study results suggest that endoscopic hemoclip application is an effective and safe method for controlling nonvariceal bleeding in the upper GI tract, with satisfactory outcomes and no adverse effects. Many factors, such as age, the causes and the locations of the lesions, shock, presence of multiple comorbidities, the devices and the experience of endoscopist might all be associated with the failure of endoscopic hemoclip to achieve hemostasis; and the clip and application device need to be further improved.

Peer review

It's a good idea to publish this clinical experience. This study is short (68 cases) but it's interesting to read it especially by endoscopists and it encourages other physicians to publish their experiences.

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Cytomegalovirus enteritis mimicking Crohn's disease in a lupus nephritis patient: A case report

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Received: June 18, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: September 14, 2009

Abstract

Cytomegalovirus (CMV) infection of the gastrointestinal (GI) tract has been reported in both immunocompetent and, more frequently, in immunocompromised patients. We describe a case of a 19-year-old male who developed CMV infection of the terminal ileum while receiving immunosuppression for lupus nephritis. This was a distinctly unusual site of infection which clinically mimicked Crohn's ileitis. We note that reports of terminal ileal CMV infection have been infrequent. Despite a complicated hospital course, ganciclovir therapy was effective in resolving his symptoms and normalizing his ileal mucosa. This report highlights the importance of accurate histological diagnosis and clinical follow-up of lupus patients with GI symptoms undergoing intense immunosuppression.

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Key words: Cytomegalovirus; Enteritis; Lupus nephritis; Terminal ileitis

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Khan FN, Prasad V, Klein MD. Cytomegalovirus enteritis mimicking Crohn's disease in a lupus nephritis patient: A case report. *World J Gastroenterol* 2009; 15(34): 4327-4330 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4327.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4327>

INTRODUCTION

Cytomegalovirus (CMV) infection of the gastrointestinal (GI) tract has been reported in both immunocompetent and, more frequently, in immunocompromised patients^[1,2]. Sometimes the presentation of this infection can mimic other illnesses, making accurate diagnosis difficult. As the therapy for this infection can be very toxic, and discontinuation of therapeutic immunosuppression can cause worsening of the underlying pathology, accurate diagnosis is essential. The case presented here illustrates the difficulty in making an accurate diagnosis, while highlighting the fascinating mimicry which CMV can display.

CASE REPORT

A 19-year-old white male presented with a 3-wk history of increasing malaise, weakness, fever, and arthralgia. He had no significant past medical history or family medical history and was on no medications. Outpatient workup revealed elevated acute serum Lyme and Ehrlichia titers for which he received oral doxycycline. Two weeks later, he presented with fever, arthralgia and a malar rash, along with mild diffuse abdominal pain and diarrhea. Physical examination demonstrated hypertension (blood pressure 150/90 mmHg), lower extremity edema, and mild diffuse abdominal tenderness. A complete blood count and comprehensive chemistry profile revealed pancytopenia (white blood cells 3000/mm³, hematocrit 25% and platelets 100 000/mm³), elevated transaminases (aspartate transaminase 250 IU/L and alanine transaminase 271 IU/L), albumin 2 g/dL, nephrotic range proteinuria (5 g/d) and a urinalysis with red blood cells and red cell casts, consistent with glomerular hematuria. His creatinine was 0.8 mg/dL with an estimated glomerular filtration rate of 130 mL/min. Serologic examination was significant for depressed complement components C3 and C4, a high titer antinuclear antibody 1:320, a positive anti-double stranded DNA antibody, erythrocyte sedimentation rate of 65 with negativity for c-ANCA (antineutrophil cytoplasmic antibody) and p-ANCA. A bone marrow biopsy was non diagnostic. Renal biopsy revealed features of World Health Organization class IV and class V lupus nephritis. He was treated with pulsed methylprednisolone 1 g daily for 3 d along with 1.2 g (0.7 g/m²) cyclophosphamide by intravenous infusion.



Figure 1 Abdominal gastrointestinal series X-ray revealing the typical string sign.

He continued on prednisone 80 mg/d orally. His immediate course was complicated by a transient psychotic reaction which required rapid tapering of the steroids. Subsequently his blood counts, liver function and clinical symptoms improved, and he was discharged home with close outpatient follow-up.

Approximately one month after receiving cyclophosphamide, he developed fever, severe debilitating watery diarrhea with abdominal pain, and presented to the emergency room in acute renal failure (creatinine 2.0 mg/dL). The renal dysfunction resolved completely after the administration of intravenous saline, but the GI symptoms persisted. Urinalysis demonstrated persistent hematuria and proteinuria. Routine stool studies, including cultures, occult blood and *Clostridium difficile* toxin were negative. The diarrhea was intractable with a large stool osmolar gap, hypoalbuminemia of 1.4 g/dL, and depressed cyanocobalamin levels, consistent with malabsorption and protein-losing enteropathy. Abdominal computed tomography (CT) demonstrated edema of the small intestinal wall, particularly in the ileum. A small bowel barium study clearly demonstrated a “string sign” with long narrowed segments of distal jejunum and ileum consistent with Crohn’s jejuno-ileitis (Figure 1). At the time, the differential diagnosis also included lupus vasculitis of the GI tract, ileal tuberculosis, actinomycosis, lymphoma, amoebiasis, or viral infection. Stool acid-fast bacilli and amoebic serologies were negative. An initial colonoscopy showed an inflammatory stricture with friable, erythematous and edematous mucosa at the terminal ileum, 2 cm proximal to the ileocecal junction through which the scope could not be passed.

Multiple biopsies were obtained but were not diagnostic. A CT angiogram, performed to rule out lupus vasculitis demonstrated a normal mesenteric vasculature. A subsequent colonoscopy one week later demonstrated persistently inflamed and denuded mucosa with a persistent terminal ileal stricture. Terminal ileal biopsies clearly revealed intranuclear basophilic inclusions consistent with CMV infection which was confirmed by immunocytochemical staining (Figure 2). No evidence of granulomas or vasculitis was seen on histology. Based on this finding alone, further immunosuppression was

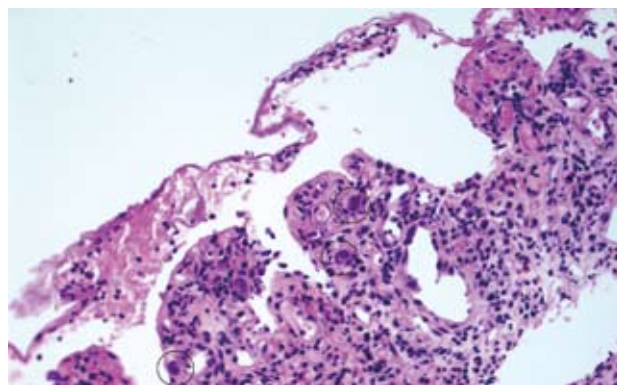


Figure 2 Hematoxylin and eosin staining of ileal tissue revealing cytomegalovirus inclusions, cytomegaly and intranuclear inclusions.

deferred, and the patient was started on intravenous ganciclovir 5 mg/kg twice daily. His subsequent hospital course was complicated by continued diarrhea, protein-losing enteropathy, severe malnutrition requiring total parenteral nutrition, fungal sepsis, and subclavian deep venous thrombosis. Ganciclovir was continued for 4 wk. Ultimately he recovered fully. His abdominal symptoms resolved, and he was able to tolerate an oral diet before discharge.

A repeat colonoscopy 4 mo later found resolution of the stricture, with no inclusion bodies seen on repeat biopsy. After this confirmation, cyclophosphamide and glucocorticoid therapies for lupus nephritis were resumed, with oral ganciclovir prophylaxis. There were no further infectious sequelae. He has had no further complications on follow-up and was switched to mycophenolate mofetil maintenance therapy. Currently he has normal renal function, no proteinuria or hematuria and continued quiescent lupus serology.

DISCUSSION

CMV is a double-stranded DNA virus and a member of the herpesviridae family.

During primary infection, T-cells are vital in controlling the viral replication, but do not eliminate the virus completely. This leads to a latent infection. Acute CMV infection in immunocompetent hosts can manifest with transient nonspecific symptoms, or as a systemic disease with significant organ involvement^[3]. It is estimated that 50%-80% of the adult population is seropositive for the virus^[4]. In immunocompromised hosts, re-activation or re-infection can lead to overt disease e.g., pneumonitis, hepatitis, pancreatitis, colitis, encephalitis, retinitis, or pericarditis leading to substantial morbidity and mortality. Transplant recipients and HIV infected patients with CMV enteritis had a mortality rate as high as 44% in one study^[5].

Immunosuppressive therapies for lupus have well documented infectious risks. Cyclophosphamide is a potent alkylating agent that impairs T-cell immunity at even low to moderate doses. In contrast to cancer chemotherapeutic doses, the doses given for lupus nephritis do not usually result in profound effects.

However, leukopenia and opportunistic infections can sometimes supervene. Data on the incidence of CMV disease with the cyclophosphamide induction protocol for lupus are scarce. The Euro-Lupus Nephritis Trial documented 3 cases out of 45 lupus nephritis patients on intravenous cyclophosphamide^[6].

The protean manifestations of lupus, together with the numerous possible complications of therapy, present unique problems for the treating physician. Lupus vasculitis has been reported to mimic Crohn's ileitis^[7]. The perplexing question in this case was whether the patient's symptoms represented this rare manifestation of GI lupus vasculitis, versus the coexistence of systemic lupus erythematosus (SLE) and Crohn's disease, an infectious complication of immunosuppression, or a fourth, less likely, possibility that the patient's CMV infection was the etiology of his abdominal pain at the time of his initial presentation with SLE, and thus predated his immunosuppression. The transient leukopenia and elevated transaminases on initial presentation, though not unusual for active systemic lupus, could have been the result of primary CMV infection in this spontaneously immunocompromised host.

The acute onset of CMV disease has been described in up to 46% of patients with connective tissue disease undergoing immunosuppressive therapy^[8]. It has also been noted that patients with connective tissue diseases treated with immunosuppression are at high risk for reactivation of latent CMV disease^[9]. Any part of the GI tract may be affected by CMV. Colitis is the most common manifestation of gastrointestinal CMV and can occur either alone or with other systemic involvement. CMV infection of the small bowel, though reported, is distinctly rare, especially in apparently immunocompetent hosts, and only involves 4.3% of CMV infections of the GI tract^[3,10]. CMV enteritis presents most commonly with fever, abdominal pain, diarrhea, or hemorrhage. The virus directly infects the bowel causing mucosal erosions or ulcerations. In severe cases, tissue necrosis and bowel wall perforation can occur^[1]. Histology of the affected mucosa shows a nonspecific inflammatory reaction and giant cells with ovoid nuclei containing basophilic "Cowdry" inclusion bodies. Mesenchymal cells are infected most frequently (97%) followed by endothelial cells (35%), smooth muscle cells (6%) and epithelial cells (3%). Mucosal ulcers are seen in more than half the cases^[10,11]. CMV ileitis in lupus patients is rare, but has been reported to cause ileal perforation^[12]. Though one case found CT evidence of bowel wall thickening^[7], we found no cases described in association with the radiographic "string sign". As mentioned, it is also possible that the initial presentation of abdominal pain and diarrhea in this case, prior to the diagnosis of lupus nephritis, may have actually been manifestations of CMV enteritis. Such infections have been reported to coincide with the immune dysregulation associated with SLE^[13,14].

Interestingly, there have even been reports suggesting that CMV disease itself may induce autoimmune abnormalities^[15]. There are a few case reports of acute CMV infection with elevated CMV antibody titers at

the time of diagnosis of SLE leading to speculation about a possible role in precipitating lupus activity^[16]. There have also been isolated case reports of SLE and Crohn's disease manifesting simultaneously in the same patient^[17], an association which could be attributed to the immunological basis of both diseases. CMV ileitis masquerading as Crohn's disease has also been reported, with documented mucosal and CT findings consistent with that diagnosis^[3]. However, the appearance of a radiographic "string sign" has never been described.

In this case, the persistently low complements and the ileal "string sign", in light of reports of lupus vasculitis of the GI tract mimicking Crohn's disease^[18], led to a therapeutic quandary. Specifically, it was uncertain whether the patient required intensification of his immunosuppression regimen for presumed vasculitis, or whether discontinuation of immunosuppression and concurrent antibiotic therapy was indicated. Given the well-documented infectious risks of therapies in both lupus and Crohn's disease, it was decided, despite decreasing complements and continued intractable diarrhea, to defer further immunosuppression pending definitive diagnosis of the underlying ileal pathology. This decision may have saved the patient from potentially catastrophic enhancement of his immunosuppression.

It is instructive to clinicians to be made aware of a rare complication of this common infection in an increasing number of potentially at-risk patients. A negative routine workup in a lupus nephritis patient with acute abdominal pain and diarrhea should provoke a high index of suspicion for occult CMV infection of the GI tract. Such symptoms may also result from mesenteric lupus vasculitis or from a manifestation of inflammatory bowel disease. Since morbidity increases with delay in initiation of effective therapy in all cases, early diagnosis and definitive treatment is vital for a favorable outcome. This case also raises the question in lupus patients as to whether CMV antigenemia and/or polymerase chain reaction for CMV DNA should be done routinely before initiating immunosuppression. The question whether empiric antiviral prophylaxis should be given to CMV seropositive patients remains unanswered.

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S- Editor Tian L L- Editor Cant MR E- Editor Yin DH



Rejection of Permacol® mesh used in abdominal wall repair: A case report

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Received: June 10, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: September 14, 2009

abdominal wall repair: A case report. *World J Gastroenterol* 2009; 15(34): 4331-4333 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4331.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4331>

Abstract

Permacol® mesh has shown promise when used in abdominal wall repair, especially in the presence of a contaminated surgical field. This biomaterial, derived from porcine dermis collagen, has proposed advantages over synthetic materials due to increased biocompatibility and reduced foreign body reaction within human tissues. However, we present a case report describing a patient who displayed rejection to a Permacol® mesh when used in the repair of abdominal wound dehiscence following an emergency laparotomy. Review of the English language literature using PubMed and Medline, showed only two previously published cases of explanation of Permacol® due to sepsis or wound breakdown. The authors believe this is the first case of severe foreign body reaction leading to rejection of Permacol®. Both animal and human studies show conflicting evidence of biocompatibility. There are several reports of successful use of Permacol® to repair complex incisional herniae or abdominal walls in the presence of significant contamination. It appears from the literature that Permacol® is a promising material, but as we have demonstrated, it has the potential to evoke a foreign body reaction and rejection in certain subjects.

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Key words: Abdominal wound closure; Permacol rejection; Foreign body reaction; Biocompatibility

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Wotton FT, Akoh JA. Rejection of Permacol® mesh used in

INTRODUCTION

Abdominal wall closure in the presence of overt sepsis is associated with a high failure rate. Biological prostheses are often used to reduce the risk of sepsis and ensure a trouble-free recovery. This is a case report of an experience involving the use of Permacol® mesh in abdominal wound dehiscence following an emergency laparotomy for caecal perforation. This patient later exhibited a severe foreign body reaction to the implant requiring its removal. Below, we outline an overview of the case followed by a review of the literature (using PubMed and Medline keywords: Permacol; porcine dermis collagen, abdominal wall repair; hernia repair), and resulting conclusions.

CASE REPORT

This case describes a 72-year-old man who was admitted as an emergency with acute abdominal pain and vomiting. On examination he had a distended rigid abdomen with reduced bowel sounds. He underwent an emergency laparotomy and right hemicolectomy for a perforated caecum, with localised abscess and generalised peritonitis. The wound was closed with “0” loop Polydioxanone (PDS) single layer with staples to the skin. Postoperatively he was admitted to the High Dependency Unit (HDU) but discharged to the ward the next day as he was making good progress. He developed a wound infection, manifested as discharge, on the 7th postoperative day. Some skin staples were removed to allow wound drainage and a vacuum assisted closure (VAC) dressing was applied on the 14th postoperative day. Two days after this, he was taken back to theatre to deal with a full thickness dehiscence of the abdominal wound. Following a thorough lavage, a Permacol® mesh was used to close the abdominal wound, partly as a bridge prosthesis as the fascial edges could not be approximated. This second operation was complicated by superficial wound dehiscence of the wound seven days later. A VAC dressing was reapplied

and the patient was discharged into the community. After sometime, the infection was controlled and there was pink granulation tissue formation in the wound. However, there was no demonstrable wound contraction or attempt at skin cover. He had application of silver nitrate to areas of over-granulation without significant response. Four months later, he underwent an elective exploration of the wound. At the time of surgery there was macroscopic evidence of rejection of the Permacol® mesh with nodular foreign body reaction and no attempt at wound healing at the level of the skin. The Permacol® mesh was excised and replaced with a Surgipro® mesh, with an uneventful postoperative period. Review of the wound 11 wk post-exchange of Permacol® with Surgipro® revealed evidence of wound healing in the superior section, although there remained an area (about 3 cm × 2 cm) of non-healing in the inferior section with the suspicion of a small piece of Permacol® remaining in the wound. An elective excision of abdominal wound sinus was carried out. Histology revealed features of acute and chronic inflammation superficially and granulomatous inflammation in the deep layer consistent with a “stitch granuloma”.

DISCUSSION

Permacol® (Tissue Science Laboratory, Covington, USA) is a biomaterial that has been used across a variety of surgical specialties since the 1980s, for urological, plastic, and gynaecological procedures^[1,2]. It was reported to have encouraging results when used in the form of a mesh for the repair of abdominal wall defects, and parastomal and inguinal hernias^[3]. Permacol® is derived from porcine skin and undergoes the removal of cellular components and genetic material before cross-linking the remaining extracellular matrix. The aim of this process is to produce a material that induces minimal foreign body reaction in tissues and is resistant to biodegradation by native collagenases. This is in contrast to Surgipro® (Cook Surgical, USA) which comprises monofilament fibres of polypropylene polymers to form a strong non-absorbable mesh, inducing a fibrous reaction which is the mainstay for the current repair of abdominal wall herniae and fascial defects^[3].

The proposed advantages of using a biomaterial over non-biomaterials are reduced infection; reduced risk of adhesion and fistula formation; and less rejection and erosion^[2,3]. Also, it is claimed that Permacol® is more suitable for use in contaminated surgical fields, where the risk of infection with a non-absorbable prosthesis is high^[2,4]. Permacol® initially takes on a structural role before becoming vascularized, followed by the incorporation of host cells, leading to remodeled tissue similar to that of the host. However, it has been proposed that there is a higher associated risk of hernia recurrence with biomaterials when compared to synthetic materials^[2,4].

This is an unusual case of extensive tissue reaction leading to rejection of a bioprosthesis (Permacol®). Animal studies (in a rat model) have demonstrated only a

minor chronic inflammatory response, limited evidence of collagen deposition or vascular ingrowth, and no foreign body reaction^[5,6]. However, Petter-Puchner *et al*^[7] who studied tissue responses to porcine cross-linked collagen implants in 10 rats at 17 d and three months showed extensive signs of foreign body inflammatory reaction, with three rats requiring euthanasia due to the migration of implants transcutaneously, and concluded that porcine dermal collagen shows suboptimal biocompatibility. Human studies revealed conflicting evidence of biocompatibility, lack of fibroblast penetration into the graft due to cross-linking of the porcine collagen matrix, absent acute polymorph cellular reaction, and occasional chronic foreign body reaction^[8-10]. Although the prosthesis had to be removed in this case, several studies have reported the successful use of Permacol® in abdominal wall or hernia repair^[2,11]. Hsu *et al*^[11] successfully used Permacol® in the reconstruction of incisional hernias or open abdomens in 28 patients with none requiring the prosthesis to be removed.

The decision to use Permacol® in this case is supported by others^[12,13] who described successful repair of complicated incisional herniae involving contaminated or uncontaminated surgical fields, with no post operative complications, wound infections or recurrence of herniae. Furthermore, Jehle *et al*^[14] described a case of complete wound dehiscence post elective panproctocolectomy where Permacol® was used to reconstruct the abdominal wall defect, and combined it with topical negative pressure dressing to achieve wound healing at five months. In another case where an emergency Hartmann's procedure for a sigmoid stercoral perforation was complicated by wound dehiscence and polyglactin absorbable mesh reconstruction of the abdominal wall resulted in an enterocutaneous fistula, resection and abdominal wall closure was achieved with Permacol® mesh.

Permacol® was used to repair complex abdominal wall defects in nine patients with incisional hernias following the removal of infected mesh, excision of abdominal wall tumour, wound infections and strangulated hernia repair. Despite the contaminated surgical field, five out of the nine patients had no complications due to infection. Two reported cases of explantation of Permacol® involved a patient who developed an abdominal wall abscess seven months after surgery^[15]. A paediatric renal transplant patient required Permacol® insertion as an adjunct to abdominal wall closure following transplantation, but suffered skin dehiscence 23 d postoperatively^[16].

In conclusion, our report provides the third reported case of Permacol® removal but for a very different reason-rejection. There was no sign of infection but the wound would not heal. Histology showed a mixture of acute and chronic inflammation, and foreign body inflammation. We believe this is the first documented case of Permacol® rejection in humans. Review of the literature has revealed the proposed biocompatibility of Permacol®, which is substantiated by the reported successes of its use in the repair of

incisional hernia and abdominal wall repair, including those with a contaminated surgical field. Most common complications include seromas, wound dehiscence or infection with only two reported cases in the literature where Permacol® was required to be removed. It would appear that Permacol® is a promising biomaterial but, as we have reported, it has the potential to induce severe foreign body reaction or rejection in certain subjects.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Yin DH



CASE REPORT

Resection of the uncinate process of the pancreas due to a ganglioneuroma

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Received: June 8, 2009 Revised: August 2, 2009

Accepted: August 9, 2009

Published online: September 14, 2009

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Key words: Ganglioneuroma; Uncinectomy; Retroperitoneal tumors; Resection of the uncinate process; Pancreatic tumors; Segmental pancreatic resection

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Poves I, Burdío F, Iglesias M, Martínez-Serrano M^A, Aguilar G, Grande L. Resection of the uncinate process of the pancreas due to a ganglioneuroma. *World J Gastroenterol* 2009; 15(34): 4334-4338 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4334.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4334>

Abstract

A 33-year-old woman who presented with epigastric discomfort and diarrhea underwent an abdominal ultrasound (US). This investigation and subsequent contrast-enhanced computed tomography, magnetic resonance imaging and endoscopic US with fine needle aspiration (FNA) revealed a 40 mm well-circumscribed mass in the uncinate process of the pancreas. Findings were suggestive of a mucinous or solid-cystic pseudopapillary tumor of the pancreas, although other lesions such as a non-functioning neuroendocrine tumor could not be ruled out. FNA samples were negative for malignant cells, but of limited value due to poor cellularity. It was decided to surgically remove the tumor because malignancy could not be discounted. Multiple intraoperative biopsies were suggestive of mesenchymal tumor and consequently a conservative resection (uncinectomy) was performed. The postoperative course was uneventful. The definitive diagnosis was ganglioneuroma. Immunocytochemistry showed positive staining with vimentin, S-100 protein, neurofilament and neuron-specific enolase. Ganglioneuroma is a rare benign tumor that can also present as a pancreatic tumor. Uncinectomy is feasible, safe and a good surgical technique for the treatment of non-malignant tumors located in the uncinate process of the pancreas.

INTRODUCTION

Ganglioneuroma is a rare benign soft tissue tumor that arises from sympathetic nerve fibers. It is most frequently discovered during childhood or in young adults. The two most common presentations are in the mediastinum and retroperitoneum^[1,2]. Although often difficult and not always possible, a preoperative diagnosis can be obtained by fine-needle aspiration (FNA)^[1-3]. Clinical features, FNA and histological findings have been accurately described^[3]. Adequate treatment consists of radical resection of the whole tumor and definitive diagnosis is obtained after surgical removal by morphologic examination of the complete specimen.

Most solid pancreatic masses are malignant and extended radical surgery is usually the standard of care. Conservative surgery of the pancreas has been advocated mostly for benign or premalignant lesions, most of which are cystic or neuroendocrine tumors (NETs)^[4,5]. Uncinectomy is a relatively novel surgical technique recently described for the treatment of non-malignant lesions^[6].

We present the case of a young woman who had an apparent solid-cystic pancreatic mass in the uncinate process of the head of the pancreas and who was treated by conservative resection (uncinectomy), with a definitive diagnosis of ganglioneuroma. Informed consent for writing this article has been obtained from the patient.



Figure 1 A hypodense and non-infiltrative tumor is occupying the uncinate process of the pancreas (1); It is in close contact with the superior mesenteric vein (2) and superior mesenteric artery (3).



Figure 2 Hypointense-T1 and heterogeneous tumor that does not present any modifications following IV contrast administration with Gadolinium.

CASE REPORT

A 33-year-old woman was diagnosed with a well-circumscribed pancreatic mass measuring 40 mm in diameter in the head of the pancreas. The mass was discovered during the course of an abdominal ultrasound performed while the patient was being assessed for epigastric pain and diarrhea. The physical examination did not reveal any relevant findings. Serum analysis showed a discrete hypochromic microcytic anemia only. On initial abdominal ultrasound (US), the lesion appeared to have a solid consistency. An abdominal contrast-enhanced computed tomography (CT), contrast-enhanced magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS) with FNA biopsy were all carried out to complete the patient's assessment. Tumoral (CA 19.9, CEA) and hormonal markers were normal. Following the guidelines of our centre, the study protocol for pancreatic nodules was followed.

CT (Figure 1)

This examination showed the presence of a hypodense, non-infiltrative tumor measuring 36 mm × 22 mm × 36 mm in both arterial and portal phases, with the appearance of having internal walls, located in the head of the pancreas and occupying the uncinate process in close contact with the superior mesenteric artery and vein, celiac trunk and hepatic artery. These findings were suggestive of a cystic or solid-cystic pseudopapillary tumor of the pancreas.

MRI (Figure 2)

This examination showed a hypointense-T1 lesion that did not present any modifications following IV contrast administration (Gadolinium) that would be suggestive of a mucinous pancreatic tumor.

EUS

This examination showed a 42 mm × 21 mm well-defined, non-infiltrative and heterogeneous mass in the uncinate process of the pancreas, in contact with the portal-mesenteric axis. There were no pathologic lymph nodes and the remaining pancreas looked normal. The

pancreatic duct was not dilated. Repeated direct FNA showed a tumor of extremely hard consistency, which made it difficult to obtain good quality samples. The ones obtained were negative for malignant cells, but of limited cellularity.

Although the diagnosis was not clear, it was decided to surgically remove the tumor because of a suspected solid-cystic pseudopapillary tumor or a non-functioning NET. Informed consent for conducting the protocol study and posterior operation was obtained from the patient. The operation was carried out through a right subcostal incision extended slightly to the left. During the exploration a well-delineated, non-infiltrative mass that occupied the uncinate process of the head of the pancreas was found. The mass was attached to the portal and superior mesenteric veins and to the superior mesenteric artery. Intraoperative multiple tumor biopsies (three tru-cut) showed fibrous tissue with some histiocytic cells suggestive of mesenchymal tumor. Intraoperative excisional biopsy of the intraaortic-cava, portal and hepatic lymph nodes showed no malignancy. Inferior pancreato-duodenal vessels were preserved. A complete resection of the uncinate process with preservation of the duodenum and the head of the pancreas was then performed (Figure 3A). Harmonic scalpel (Ethicon Endo-Surgery, Johnson & Johnson, Cincinnati, OH, USA) was used for cutting the pancreatic parenchyma. Cholecystectomy was carried out and a posterior cholangiography showed no leaking in the bile or in the pancreatic ducts. TachoSil® (Nycomed Pharma S.A.) sealant was applied, wrapping the head of the pancreas to prevent the formation of a pancreatic fistula (Figure 3B). An aspirative drain was established from the bed of the pancreatic resection site. The postoperative course was uneventful, and the patient was discharged on the sixth day.

The definitive diagnosis was a ganglioneuroma (Figures 4-6). The immunocytochemistry study showed positive staining with vimentin, S-100 protein, neurofilament and neuron-specific enolase (Figure 6).

DISCUSSION

Ganglioneuromas are very rare soft tissue tumors composed of gangliocytes and mature stroma^[1,3,7]. They usually

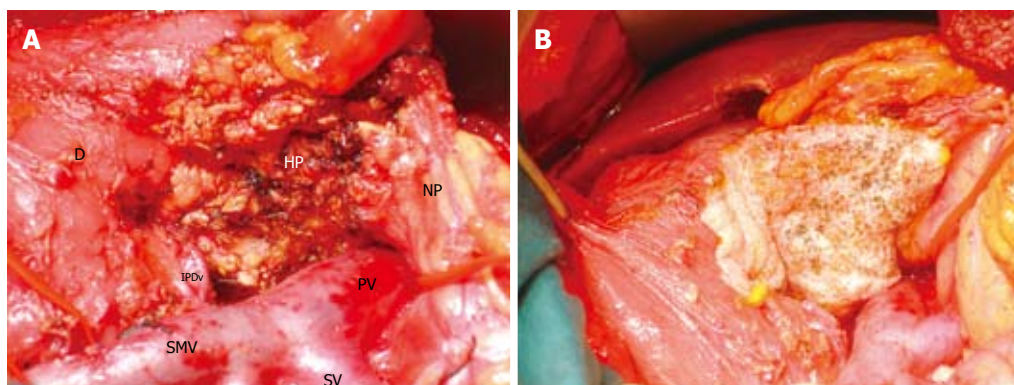


Figure 3 Appearance of surgical field with specimen resected. A: Uncinate process resected. Duodenum (D), neck of the pancreas (NP) and rest of the head of the pancreas (HP) are preserved. Superior mesenteric vein (SMV) is separated from uncinate process that is removed in the picture. Portal (PV) and splenic vein (SV) are also dissected. Inferior pancreaticoduodenal vessels (IPDv) are preserved; B: TachoSil® sealant is applied over the surface of the surgical bed for prevention of pancreatic fistula.

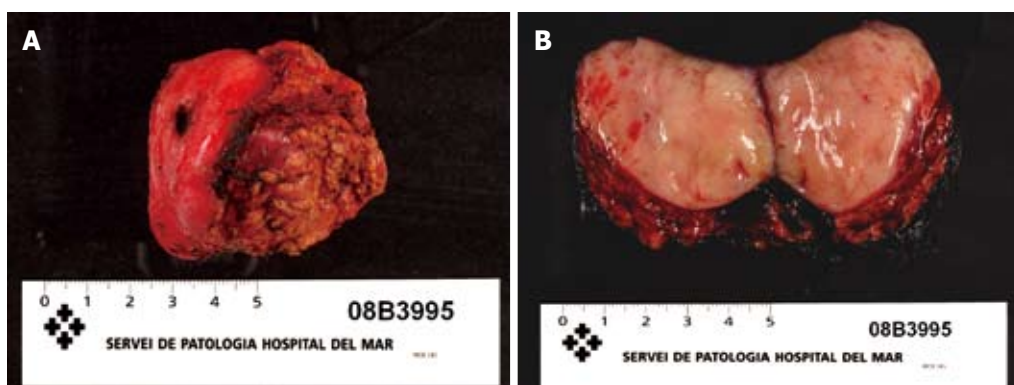


Figure 4 Macroscopic appearance of the surgical specimen. A: Nodular lesion with well-demarcated margins with normal residual pancreatic tissue adjoining; B: The tumor is well-circumscribed and firm. The inner surface is tan without evidence of necrosis or hemorrhage.

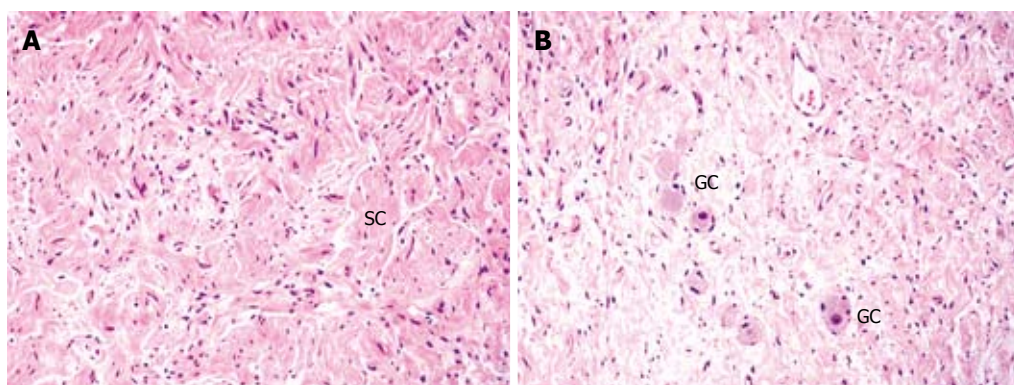


Figure 5 Microscopic images of the tumor (HE, × 10). A: The lesion is composed of a proliferation of spindle-shaped cells in a whorled or fascicular pattern [Schwann cells (SC) and nerve fibers]. The cells have elongated and wavy nuclei with eosinophilic cytoplasm; B: Scattered mature ganglion cells (GC) are another of the histopathologic components of the tumor.

consist of benign masses that produce symptoms caused by their growth and location. Ganglioneuromas are usually asymptomatic and are often discovered casually during the course of explorations when looking for other diseases. Although the most frequent presentations are in the mediastinum and in the retroperitoneum^[1-3], ganglioneuromas can also be found at any other location of the body such as in the neck or pelvis. In our review of the literature we have not been able to find any previous description of

a pancreatic localization. Diagnostic imaging is very difficult because ganglioneuromas present as a non-specific solid mass. MRI seems to be the most accurate diagnostic imaging method although not a definitive one^[7]. Unlike in other tumors, FNA is not always definitive, but its usefulness has been proven in some cases. A definitive diagnosis can only be reached after morphological examination of the removed specimen. Adequate treatment of ganglioneuromas is total surgical removal.

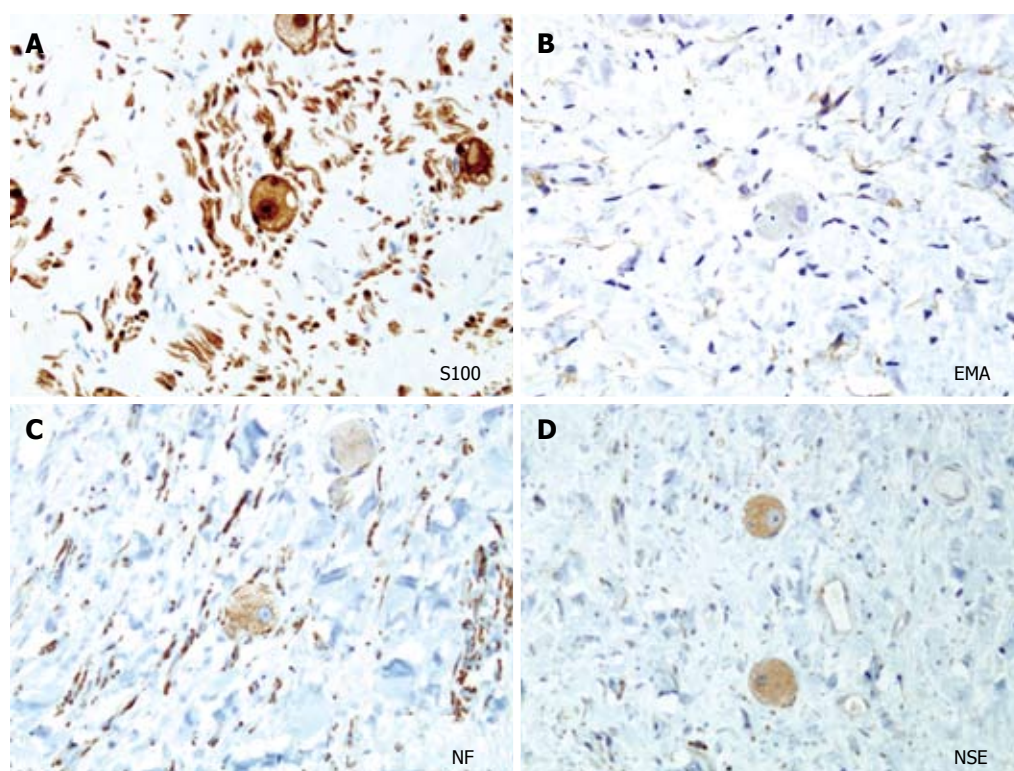


Figure 6 Representative immunocytochemistry samples of the typical ganglioneuroma ($\times 20$). A: S100 protein stain showing mostly Schwann cells and ganglion cells; B, C: Epithelial membrane antigen (EMA) and neurofilament (NF) stain showing the nerve fibers, highlighting the perineural cells and the axons of the nerve fibers; D: Ganglion cells are positive for neuronal-specific enolase (NSE).

Retroperitoneal ganglioneuromas usually present as sarcomatous soft tissue tumors. In the case we present here, the macroscopic appearance of the tumor was that of a pancreatic mass with limits that extended into the pancreas as confirmed by all the imaging studies performed. Although it can be hypothesized that these tumors usually originate from peripancreatic nerve fibers, they invade pancreatic tissue, as it is impossible to find a plane between the tumor and the pancreatic parenchyma. In fact, in this case the morphological macroscopic study showed that the mass was completely attached to the pancreas in a continuous manner (Figure 4A). The only way to obtain a definitive diagnosis and to provide total certainty of no malignancy was total excision of the tumor. A differential diagnosis, taking into account tumor characteristics and preoperative imaging results, was done to differentiate between mucinous cystadenoma, non-functioning NET and solid-cystic papillary tumor, all of which could have the potential to become malignant. NETs can also present as cystic lesions although this occurrence is rare^[8]. Somatostatin receptor scintigraphy with octreoscan has been recommended as the best imaging technique in NETs, being able to visualize more than 70% of these type of tumors^[9]. In our case, we decided not to perform this exploration because the decision for radical resection was proposed independently of this result. We preferred to carry out multiple tru-cut biopsies intraoperatively, prior to deciding what type of pancreatic resection to perform. Biopsy results were suggestive of a benign soft tissue tumor, although some malignant entities such as pseudoinflam-

matory tumor or primary malignant fibrous histiocytoma could not be completely ruled out before the tumor was removed in its entirety. Local resection without standard lymphadenectomy was performed.

Further knowledge gained over the last few years regarding the anatomy of the pancreas has helped to develop the concept of conservative surgery. Conservative surgery has gained popularity among surgeons and not only for lesions located in the left pancreas^[10]. Preserving the duodeno-pancreatic region is an issue for tumors that are also nested in the head of the pancreas. Resection of the uncinate process is an alternative to pancreato-duodenectomy (PD) for the treatment of non-malignant or pre-malignant lesions located in this area. Sharma *et al*^[6] described the technique used to perform an uncinectomy for the treatment of intraductal papillary mucinous neoplasms. Some other procedures such as resection of the head of the pancreas with preservation of the duodenum, median or central pancreatectomy, or enucleation, can be carried out depending on the type and precise location of the lesion. When these procedures have to be carried out, it is mandatory to obtain adequate preoperative or intraoperative confirmation of the benign nature of the samples. The postoperative course and long term sequelae are much better for conservative surgery than for PD.

As with enucleation, the main concern when performing an uncinectomy is the risk of leakage into the main pancreatic duct. To minimize the risk of this severe complication, direct intraoperative US exploration is highly recommended during resection. Nevertheless, in

many cases the pancreatic duct is narrow and is hard to identify. Intraoperative cholangiography and retrograde contrast back filling of the pancreatic duct can be an option for assessing the integrity of both the biliary and pancreatic ducts.

In conclusion, ganglioneuroma is a rare benign tumor that can also present as a pancreatic tumor. When a favorable location permits it, conservative resection of the pancreas is the treatment of choice. Uncinectomy is feasible, safe and a good surgical technique for the treatment of non-malignant tumors located in the uncinate process of the pancreas.

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S- Editor Li LF L- Editor Logan S E- Editor Ma WH



Novel *ABCB11* mutations in a Thai infant with progressive familial intrahepatic cholestasis

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Supported by "IKEM MZO 00023001"

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Received: June 4, 2009 Revised: July 29, 2009

Accepted: August 6, 2009

Published online: September 14, 2009

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INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) refers to a heterogeneous group of autosomal recessive liver disorders of childhood in which cholestasis of hepatocellular origin often presents in the neonatal period or first year of life, and leads to death from liver failure at ages ranging from infancy to adolescence^[1,2]. Three types of PFIC have been found; each is related to mutations in hepatocellular transport system genes involved in bile formation^[1-3]. PFIC type 1 (Byler disease) is caused by mutations in *ATP8B1* (chromosome 18q21-22), which encodes familial intrahepatic cholestasis 1 (FIC1). PFIC type 2 is caused by mutations in *ABCB11* (chromosome 2q24), which encodes bile salt export pump (BSEP). Mutations in *ABCB4* (chromosome 7q21), which encodes multidrug resistance protein 3 (MDR3), which is responsible for biliary secretion of phospholipids, cause PFIC type 3. In PFIC types 1 and 2, low or normal serum γ -glutamyltransferase (GGT) levels are found, whereas GGT levels are high in PFIC type 3^[1,2].

The diagnosis of PFIC can be difficult, especially where genetic testing is not readily available, as in Thailand. We report here a Thai infant diagnosed with PFIC type 2.

CASE REPORT

A 2-mo-old female infant presented with a 1-mo history of icteric sclerae associated with pale yellowish stools. She was a normal full-term infant (birth weight 2700 g). Hypothyroidism had been diagnosed at age 1 mo [free

Abstract

Progressive familial intrahepatic cholestasis (PFIC) type 2 is caused by mutations in *ABCB11*, which encodes bile salt export pump (BSEP). We report a Thai female infant who presented with progressive cholestatic jaundice since 1 mo of age, with normal serum γ -glutamyltransferase. Immunohistochemical staining of the liver did not demonstrate BSEP along the canaliculi, while multidrug resistance protein 3 was expressed adequately. Novel mutations in *ABCB11*, a four-nucleotide deletion in exon 3, c.90_93delGAAA, and a single-nucleotide insertion in exon 5, c.249_250insT, were identified, with confirmation in her parents. These mutations were predicted to lead to synthesis of truncated forms of BSEP. Immunostaining and mutation analysis thus established the diagnosis of PFIC type 2.

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Key words: *ABCB11*; Bile salt export pump; Progressive familial intrahepatic cholestasis

Table 1 Evolution of clinical biochemistry test results with body weight and height

Age (mo)	ALP (U/L) ¹	AST (U/L) (15-37)	ALT (U/L) (30-65)	GGT (U/L) ²	Alb (g/L) (34-50)	TB (mg/dL) (0-1.5)	DB (mg/dL) (0-0.5)	Chol (mg/dL) (114-203)	BW (kg)	Ht (cm)
2	417	354	264	47	37.4	5.7	3.9	155	4.95	56.0
6	298	375	344	37	49.8	7.7	6.4	229	7.20	64.0
12	267	472	354	66	33.8	5.3	4.5	191	8.20	68.0
18	332	311	237	59	38.1	6.2	5.2	151	10.4	71.4
24	269	239	171	59	44.1	6.1	4.5	121	11.4	78.0
30	373	340	240	60	41.2	7.9	6.8	102	11.5	80.0

ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Alb: Albumin; TB: Total bilirubin; DB: Direct bilirubin; Chol: Cholesterol; BW: Body weight; Ht: Height. Units and expected ranges for test-result values are given in parentheses. ¹Normal values for female children < 1 year, 185-555; 1-2 years, 185-520; 3 years, 185-425 U/L^[17]; ²Normal values for children 1-2 mo, 12-123; 2-4 mo, 8-90; 4 mo-10 years, 5-32 U/L^[18].

T4 0.94 ng/dL, thyroid stimulating hormone (TSH) 20.5 mIU/L], and thyroxine therapy begun. Growth and development were otherwise normal. No family history of liver disease was elicited. She had mildly icteric sclerae and hepatomegaly without splenomegaly or ascites. All other physical-examination findings were normal.

Conjugated-bilirubin and transaminase values were elevated but albumin, cholesterol, and GGT values were within expected ranges (Table 1). Prolonged coagulogram values were corrected by intravenous vitamin K administration. No IgM-class antibodies against cytomegalovirus were found, and VDRL testing was non-reactive. Plasma amino acid analysis found only mildly elevated methionine levels, interpreted as a nonspecific consequence of liver disease. Urine-reducing substances were absent. Other laboratory investigation results were normal, including free T4 and TSH, complete blood count, electrolytes, glucose, urea nitrogen, creatinine, ammonia and alpha-fetoprotein levels. Ultrasonography revealed a normal liver and bile duct. DISIDA scanning showed good hepatic function with demonstrable intraduodenal tracer at 3 h. Microscopy of a liver-biopsy specimen found changes interpreted as neonatal hepatitis. Ursodeoxycholic acid (UDCA) and fat-soluble vitamins were given.

Cholestasis persisted (Table 1), with development of severe pruritus and hepatosplenomegaly. On repeat liver biopsy aged 10 mo, hepatocellular swelling with multinucleation was found, as was canalicular and hepatocellular cholestasis (Figure 1A). Mild portal lymphocytic infiltration and fibrosis were also observed (Figure 1B). BSEP was not detected along the canaliculi on immunostaining (Figure 1C and D), while the homologous transport protein MDR3 was expressed adequately, which demonstrated that tissue fixation was adequate and permitted the inference that lack of BSEP expression was BSEP-specific. As these results were compatible with PFIC type 2, *ABCB11*, which encodes BSEP, was sequenced after parental consent was obtained.

Treatment with UDCA and fat-soluble vitamins was continued. At the time of writing, the patient is 30 mo old, with persistent jaundice and growth delay [Table 1; body weight 11.5 kg (P25) and height 80 cm (< P3)]. Serum alpha-fetoprotein concentrations are normal and sonographic monitoring has found no focal changes. She awaits liver transplantation.

Mutation analysis

ABCB11 was analyzed by direct sequencing of PCR products obtained from genomic DNA extracted from peripheral blood leukocytes. All exons, together with the adjacent parts of the intronic sequences, were amplified by PCR with intronic oligonucleotide primers as reported previously^[4]. The amplicons were gel-purified, extracted with QIAquick spin columns (Qiagen, Hilden, Germany), and used as templates for the sequencing reaction with Big Dye Terminator kit v3.1 (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The products were analyzed on a Genetic Analyzer 3130 (Applied Biosystems).

Two suspected sequence disturbances were predicted from the dual sequence readings at 20 bp from the potential mutation start site in both directions. The presence of both mutations was verified by molecular cloning into a plasmid vector pCR4.1-TOPO (Invitrogen, Carlsbad, CA), and sequencing the cloned wild-type and the mutated allele separately. In addition, the presence of suspected mutations was also examined by direct sequencing of appropriate amplicons obtained from the proband's parents. Two different heterozygous *ABCB11* mutations were found in the patient: a four-nucleotide deletion in exon 3 (protein coding exon 2), c.90_93delGAAA, inherited from the patient's mother, and a single-nucleotide insertion in exon 5 (protein coding exon 4), c.249_250insT, inherited from the patient's father (Figure 2). Both mutations are predicted to cause reading frame shift and premature termination of DNA translation, respectively p.Lys30AsnfsX31 and p.Gly84TrpfsX9, and therefore are considered to be pathogenic.

DISCUSSION

To the best of our knowledge, this is the first case report of PFIC type 2 in Thailand. The patient had a typical clinical presentation of PFIC type 2 with cholestasis of onset in early infancy, with development of hepatomegaly and severe pruritus^[1,2]. In PFIC type 2 serum bile acid concentrations are high, biliary bile-salt concentrations are very low, and GGT and cholesterol values are normal^[2,5]. While serum bile-acid and biliary bile-salt concentrations could not be determined, as such studies are unavailable in Thailand, her severe pruritus

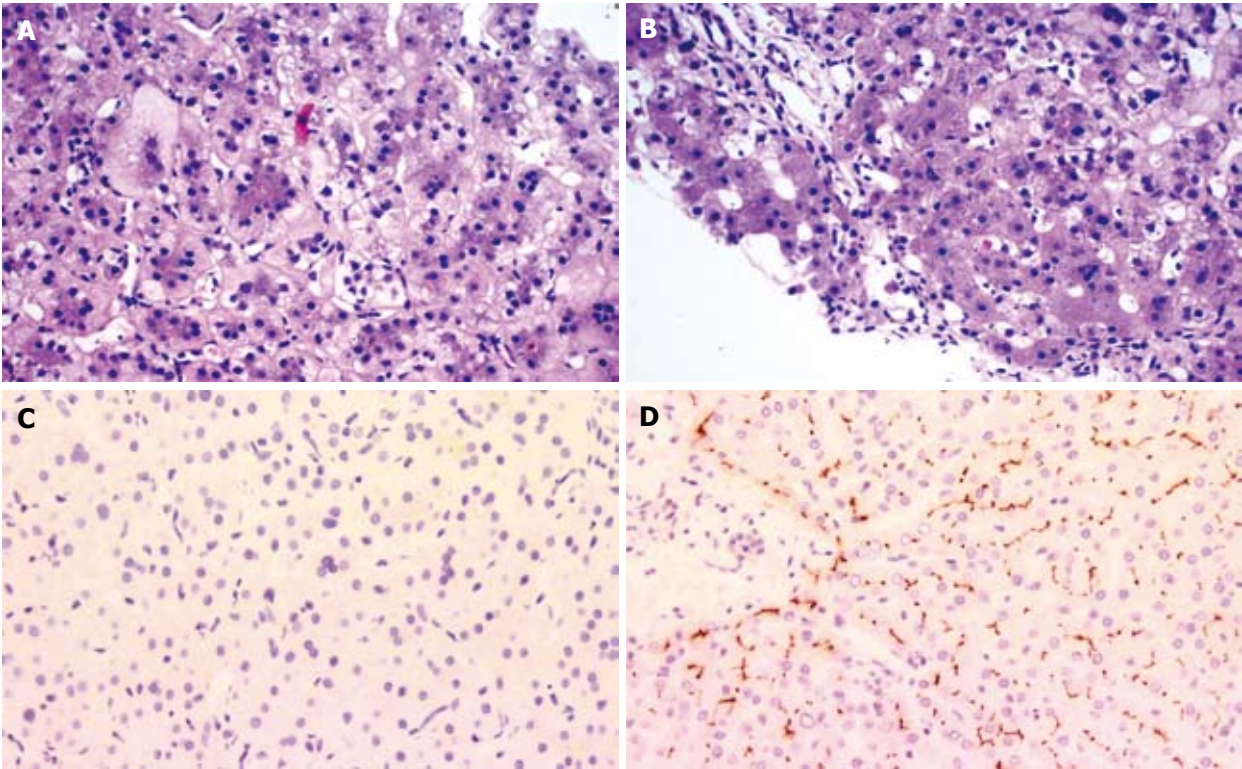


Figure 1 Liver, second biopsy (10 mo). Hepatocellular swelling and multinucleation, with intralobular cholestasis (A), accompanying mild portal-tract lymphocytic infiltration and fibrosis (B). On immunostaining, bile salt export pump (BSEP) expression was not detected (C); canaliculi in control liver stained normally for BSEP (D). [Hematoxylin/eosin, A and B; rabbit anti-BSEP polyclonal antibody (generous gift of Dr B Stieger)/hematoxylin, C and D; original magnification, all images, × 200].

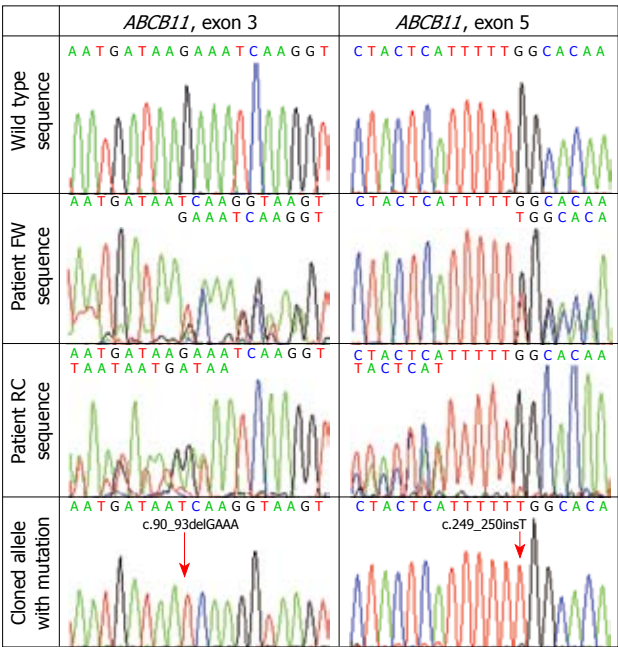


Figure 2 Mutations c.90_93delGAAA and c.249_250insT detected in exon 3 and 5 of *ABCB11*. FW: Forward reading (coding strand sequence); RC: Reversed and complemented reading (reversed and complemented sequence of the complementary strand).

indicated hypercholanemia. This feature and our patient’s GGT and cholesterol values were compatible with PFIC type 2. Although hypothyroidism in this patient might have caused cholestasis by delayed emptying of the biliary tract, and by changes in bile composition and

excretion rate^[6], normalization of free T4 and TSH levels after thyroxine therapy argued against this possibility. Since hypoglycemia was not present and initial growth was unremarkable, hypopituitarism was not pursued.

While PFIC type 1 has initial clinical and laboratory findings similar to those of PFIC type 2, histological features may differ. In PFIC type 1, bland canalicular cholestasis with variable fibrosis is found. In PFIC type 2, variable features include canalicular cholestasis and a neonatal hepatitis pattern, with hepatocellular swelling and giant cell transformation^[1,2]. Lobular and portal inflammation and fibrosis are more pronounced in PFIC type 2 than type 1^[2]. Immunostaining is a useful diagnostic tool for PFIC type 2 since most patients with *ABCB11* mutations and hepatobiliary disease of onset in infancy have no canalicular BSEP expression^[7]. At initial liver biopsy (2 mo) changes of neonatal hepatitis were found. At follow-up biopsy, these changes persisted, and BSEP was not detected along the canaliculi. The clinicopathological picture, felt to be compatible with PFIC type 2, thus prompted *ABCB11* analysis.

BSEP is an ATP-dependent transporter located on the canalicular membrane of hepatocytes^[8,9]. It is a major exporter of primary bile salts from hepatocyte cytoplasm into the bile-canalculus lumen, and works against an extreme concentration gradient^[2,9]. Mutations in *ABCB11* that lead to failure of BSEP expression, or to expression of functionally defective BSEP, in turn lead to accumulation of bile salts inside hepatocytes, with ongoing severe hepatocellular damage and diminished bile flow.

To date, more than 100 mutations in *ABCB11* have been identified^[7,10-14]; however, genotype-phenotype correlations are not wholly clear. Severe phenotypes are often associated with mutations that lead to premature protein truncation or failure of protein production. Insertion, deletion, nonsense, and splicing mutations result in damaging effects, and patients who have clinical PFIC associated with such mutations exhibit little or no BSEP expression in hepatocyte canaliculi^[2,7]. Missense mutations are also common. These can affect protein processing and trafficking or disrupt functional domains and protein structure^[2,7,15]. Detectable BSEP expression does not exclude functional BSEP deficiency^[2,7].

E297G and D482G are the two most common mutations in persons of European descent, and account for approximately 58% of BSEP mutations in European studies^[7]. In Asian patients, few reports of mutations in PFIC type 2 exist^[12-14]. Goto *et al*^[14] have reported four mutations in *ABCB11*, predicted to yield V330X, R487H, R575X and E636G, in two Japanese PFIC patients. Chen *et al*^[13] have reported seven BSEP mutations (M183V, V284L, R303K, R487H, W493X, G1004D and 1145delC) in four PFIC patients of Chinese descent; none of these mutations has been described in Caucasian patients. To the best of our knowledge, the mutations identified in our patient are novel. These mutations are predicted to lead to synthesis of truncated forms of BSEP.

Patients with PFIC type 2 are at risk for hepatobiliary malignancy^[7,16]. Hepatocellular carcinoma or cholangiocarcinoma developed in 19 of 128 patients (15%)^[7] and those who had two protein-truncating mutations were at particular risk^[7]. Close surveillance of BSEP-deficient patients who retain their native liver is therefore essential.

In conclusion, we report a Thai infant with clinical features of normal-GGT PFIC. Her liver did not express immunohistochemically demonstrable BSEP. Novel mutations in *ABCB11* were identified in the infant, with confirmation in her parents. These mutations were predicted to lead to synthesis of truncated forms of BSEP. Immunostaining and mutation analysis thus established the diagnosis of PFIC type 2.

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S- Editor Tian L L- Editor Kerr C E- Editor Yin DH

Primary squamous cell carcinoma of pancreas diagnosed by EUS-FNA: A case report

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Supported by Medical University of South Carolina, United States

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Received: June 16, 2009 Revised: July 15, 2009

Accepted: July 22, 2009

Published online: September 14, 2009

Abstract

Squamous cell carcinoma of the pancreas has been sparsely described since the 1940s, and generally has a poor prognosis. Herein, we present a case of primary squamous cell carcinoma of the pancreas with liver metastasis, both confirmed by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). To the best of our knowledge, this is the first case report in literature utilizing EUS-FNA for a cell-type specific diagnosis of primary pancreatic squamous cell carcinoma with a liver metastasis.

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Key words: Computed tomography; Cytology; Endoscopic ultrasound

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INTRODUCTION

Primary squamous cell carcinoma is rare among all pancreatic neoplasms, constituting less than 1% of cases. Herein, we present a case of primary squamous cell carcinoma of the pancreas with liver metastasis, both confirmed by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA).

CASE REPORT

A 76-year-old American African female was referred with a few weeks history of dull epigastric pain with radiation to the back, and weight loss of 10 pounds. Initial physical examination revealed only minimal tenderness over the epigastrium, and blood tests were all normal including serum lipase (29 IU/L, reference: 10-50 IU/L) and CA 19-9 (2 IU/mL, reference: 0-37 IU/mL). Upper endoscopy showed mild gastritis. Subsequent computed tomography (CT) of the thorax and abdomen revealed a 5 cm partial cystic mass in the tail of the pancreas and a 1 cm subtle hypodense non-enhanced lesion in the left lobe of the liver, with normal chest and mediastinum. CT-guided biopsy of the liver mass was performed, however, preliminary histology only revealed atypical cells. Endoscopic ultrasound (EUS) was performed for clarification of the pathology. A complex cystic mass with a large solid component was seen in the tail of the pancreas measuring up to 68 mm. Another 14 mm × 18 mm ill-defined, almost isoechoic lesion was also noted in the left lobe of the liver (Figure 1). Transgastric EUS-FNA, using a 25-gauge needle, of both the liver and tail of pancreas lesions was carried out, which revealed squamous cell carcinoma in both sites, although on-site interpretation was difficult due to the unusual cell type (Figure 2).

DISCUSSION

Squamous cell carcinoma of the pancreas has been

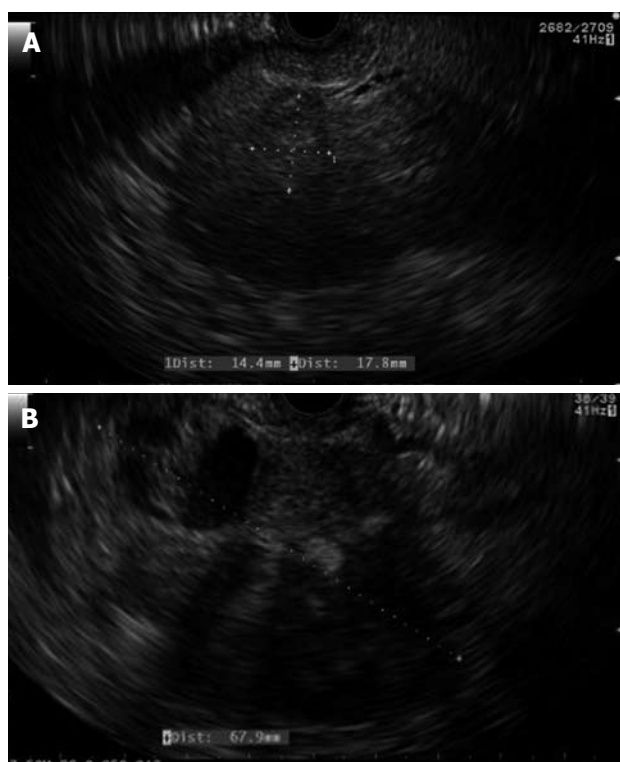


Figure 1 Endoscopic ultrasound image of the heterogenous, nearly isoechoic lesion in the left lobe of the liver (A) and the complex cystic mass in the tail of pancreas (B).

rarely described since the 1940s, and generally has a poor prognosis^[1]. It is hypothesized that squamous metaplasia of pancreatic ductal epithelium after chronic inflammation (e.g. chronic pancreatitis), could be one of the possible oncogenic mechanisms^[2]. A subset of pancreatic adenocarcinoma, adenosquamous carcinoma is occasionally found in surgical specimens after Whipple's operation. In one series, dual differentiation towards both adenocarcinoma and squamous cell carcinoma was seen in 25 pancreatic cancer patients^[3]. However, pure squamous cell carcinoma of the pancreas is extremely rare, and is often mistaken as either benign squamous cells from upper gastrointestinal contamination when it is well-differentiated, or metastasis from other sites (e.g. lung and upper aerodigestive tract) when it is obviously malignant. A MEDLINE search only identified 14 case reports in the English literature so far^[4-17], and all diagnoses were based on surgical specimens. Hypervascularity on contrast CT has been reported as a characteristic finding, but was not seen in this case^[15].

Since the introduction of EUS-FNA for investigating pancreatic cancer in the 1990s^[18], it has now become the standard procedure for pancreatic lesions. Cytopathological confirmation can be obtained with EUS-FNA, so as to avoid unnecessary pancreatic resection^[19]. From our knowledge, this is the first case report in the literature utilizing EUS-FNA for a cell-type specific diagnosis of primary pancreatic squamous cell carcinoma with a liver metastasis. The distinction of well-differentiated squamous cell carcinoma from benign disease such as lymphoepithelial cysts (LEC) of the

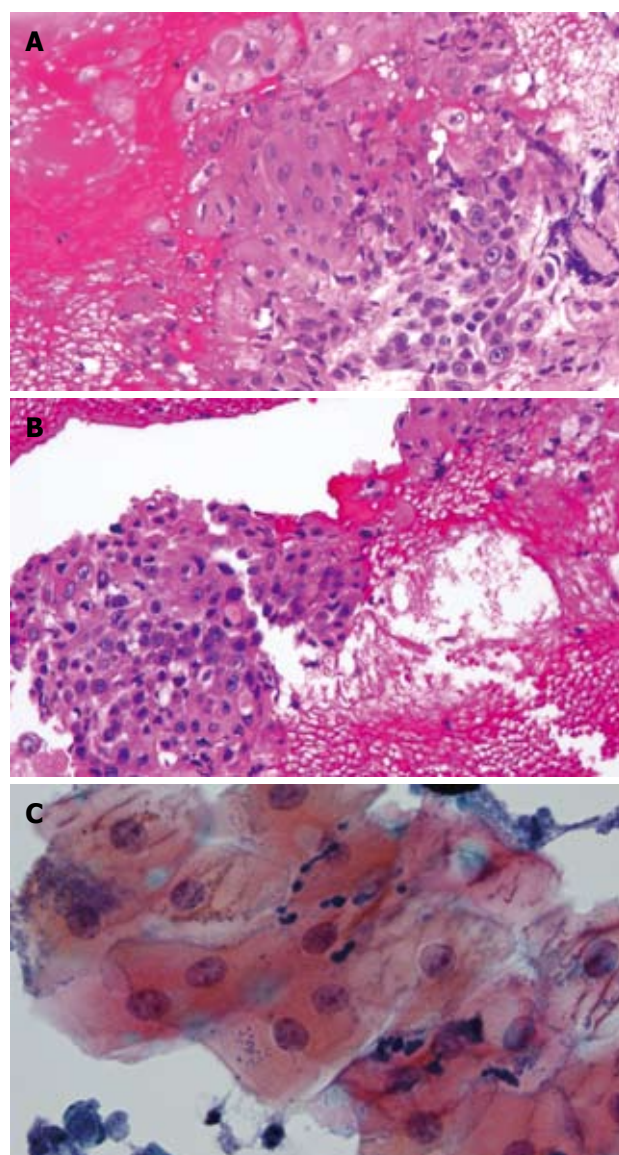


Figure 2 Fine-needle aspiration specimen. A: Liver mass (HE, × 100); B: Pancreatic mass (HE, × 100). The cell block shows atypical squamous cells consistent with keratinizing squamous cell carcinoma. C: Liver mass (ThinPrep, × 400). Squamous cell contamination from the esophagus as evidenced by the presence of bacteria and fungal organisms.

pancreas may be difficult. LEC is an infrequent benign condition and EUS-FNA typically shows squamous cells, lymphocytes, notched crystals and keratin debris^[20-22]. Because cystic degeneration can occur in pancreatic cancers, as in our case^[23], differentiation between benign and malignant squamous cell lesions could be difficult. If there is a solid component, it should be targeted during EUS-FNA rather than the cyst. In general, dense orangeophilic keratin debris, atypical parakeratosis, and nuclear atypia including hyperchromasia and nuclear membrane irregularities help us to differentiate cancer from reactive squamous metaplastic cells^[24]. A glandular component should also be sought when noting atypical squamous carcinoma cells, as adenosquamous carcinoma is more common than the pure squamous cell type^[3]. Squamous cell contaminants could also contribute to diagnostic uncertainty; although transgastric and

transduodenal routes for EUS-FNA should not produce many of these cells, however, they appear to have been noted in our case (Figure 2C)^[25]. In this patient, diagnosis of primary squamous cell carcinoma of the pancreas with liver metastasis was confirmed, as EUS-FNA obtained the same type of cancer cells from both pancreatic and liver lesions, and CT and upper endoscopy did not identify other possible primary squamous cell malignancy.

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S- Editor Tian L L- Editor Webster JR E- Editor Yin DH

LETTERS TO THE EDITOR

Biomarkers for noninvasive biochemical diagnosis of nonalcoholic steatohepatitis: Tools or decorations?

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Received: May 11, 2009 Revised: July 24, 2009

Accepted: July 31, 2009

Published online: September 14, 2009

Abstract

In light of the growing epidemics of nonalcoholic fatty liver disease (NAFLD), identification and validation of the novel biochemical surrogate markers for nonalcoholic steatohepatitis (NASH) are paramount to reduce the necessity for liver biopsy. The availability of such markers has tremendous potential to radically alter the management strategies of NAFLD patients and to monitor the disease activity. Although current biomarkers do not entirely fulfill the many requirements for the identification of patients with NASH, they should not discourage our quest, but remind us that we need to cognize the challenges ahead.

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Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Biomarkers; Liver biopsy

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Yilmaz Y, Dolar E. Biomarkers for noninvasive biochemical diagnosis of nonalcoholic steatohepatitis: Tools or decorations? *World J Gastroenterol* 2009; 15(34): 4346-4347 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4346.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4346>

TO THE EDITOR

In light of the dramatic increase in the prevalence of nonalcoholic fatty liver disease (NAFLD), noninvasive, simple, reproducible, and reliable biomarkers that can allow identifying patients with nonalcoholic steatohepatitis (NASH) among NAFLD patients are greatly needed^[1]. The availability of such biomarkers has tremendous potential to radically alter the diagnostic and monitoring strategies through the reduction in the need for liver biopsy^[2].

To be introduced in the clinical practice, the ideal biomarker for NASH must fulfill many requirements, such as ease of interpretation by clinicians, accurateness, reproducibility obtained in a standardized fashion, as well as high sensitivity and specificity. This latter point is both important and dependent on the design of study.

The recent report by Uslusoy *et al*^[3] published in the *World Journal of Gastroenterology* has provided evidence that certain noninvasive markers for liver injury, including aminotransferase levels and AST/ALT ratio, do not entirely reflect the histological aspects of liver biopsy in patients with NASH. Based on their results, the authors concluded that aminotransferase levels and AST/ALT ratio do not seem to be reliable predictors for NASH. Although numerous non-invasive biomarkers are available, all patients with fatty liver should undergo liver biopsy^[3]. It is feasible, however, that this radical conclusion may be too far to reach given the important caveats of this study. Firstly, the authors limited their analysis to aminotransferase levels. It has been previously shown, in this regard, that serum levels of caspase-cleaved cytokeratin 18 may be a potential biochemical marker for NASH in NAFLD patients with normal aminotransferase levels^[4]. Secondly, the statistical analysis of data, demonstrating the lack of an association of aminotransferase levels and AST/ALT ratio with NASH, is likely to be underpowered as the study enrolled too few participants to identify such differences. Underpowered studies are overly prone to making false-negative conclusions, or committing what epidemiologists call type II errors^[5]. Finally, appropriate use of biomarker results requires use of a Bayesian approach^[6], i.e. integrating pretest probabilities with biomarker test results (expressed as sensitivity and specificity) to estimate the posttest probability of disease.

Prerequisites for the clinical use of biomarkers for NASH include the elucidation of specific indications, the standardization of analytical methods, the characterization of analytical features, the assessment of performance characteristics, the incremental yield of different markers for given clinical indications, and the demonstration of cost-effectiveness. Although the development of NASH biomarkers fulfilling these features is challenging, it should not discourage our quest, but remind us that we need to cognize the challenges ahead. Technological advances will likely facilitate the use of multimarker profiling^[7] to identify patients with NASH in the near future.

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S- Editor Tian L **L- Editor** Wang XL **E- Editor** Lin YP

ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Meetings

Events Calendar 2009

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January 21-24, 2009
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February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

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Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

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EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
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Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

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AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
<http://www.easl.ch/>

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Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
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North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27, 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
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June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
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Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
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Molecular Biology in Clinical Oncology

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Bell Harbor Conference Center, Seattle, Washington, United States
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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

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- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G,** Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 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]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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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/eid.htm>

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- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 14, 2008



World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 35
September 21, 2009



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NAME OF JOURNAL

World Journal of Gastroenterology

RESPONSIBLE INSTITUTION

Department of Science and Technology of Shanxi Province

SPONSOR

Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHING

The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No.62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
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PRINTING

Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR

Beijing Bureau for Distribution of Newspapers and Journals (Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE

September 21, 2009

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Lian-Sheng Ma, *Beijing*

SUBSCRIPTION

RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN

ISSN 1007-9327
CN 14-1219/R

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5-ASA in ulcerative colitis: Improving treatment compliance

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Received: April 7, 2009 Revised: July 27, 2009

Accepted: August 3, 2009

Published online: September 21, 2009

Abstract

5-aminosalicylic acid (5-ASA) compounds are a highly effective treatment for ulcerative colitis (UC). While UC patient compliance in clinical studies is over 90%, only 40% of patients in every day life take their prescribed therapy. Adherence to medication has been emphasized recently by a Cochrane meta-analysis that has suggested that future trials of 5-ASA in UC should look at patient compliance rather than drug efficacy. Better compliance can be obtained by reducing the number of tablets and times of administration. Given that the 5-ASA formulations have different delivery systems that split the active moiety in various regions of the intestine, it is particularly important that an adequate dose of the drug arrives at the inflamed part of the colon. 5-ASA Multi matrix (MMx) is a novel, high strength (1.2 g), oral formulation designed for once-daily dosing. It releases the active moiety throughout the colon. Different studies with this compound have shown that it is as effective as 5-ASA enema in the treatment of mild-to-moderate, left-sided UC, and is comparable to a pH-dependent, delayed release 5-ASA (Asacol®), even if given once daily. Recently, the effectiveness in the acute phase of UC has been confirmed also in maintenance. In conclusion, at present, 5-ASA MMx seems theoretically the best agent for maintaining patient compliance, and consequently, treatment effectiveness.

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Key words: 5-aminosalicylic acid; Mesalamine; Multi matrix; Patient compliance; Ulcerative colitis

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Prantera C, Rizzi M. 5-ASA in ulcerative colitis: Improving treatment compliance. *World J Gastroenterol* 2009; 15(35): 4353-4355 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4353.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4353>

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease that involves the rectum and can extend to the colon in a caudal-cranial direction. The worldwide incidence is 0.5-24 new cases per 100 000 individuals, and prevalence is 100-200 cases per 100 000. From 60% to 80% of patients have distal and left-sided colitis^[1].

After the first introduction of sulfasalazine (Salazopyrin), composed of sulphapyridine (SP) and 5-ASA, for treating UC, the discovery that the active moiety is 5-ASA has prompted the industry to develop 5-ASA compounds with different delivery systems, because the SP molecule has been associated with important adverse effects^[2].

The delivery systems that have been designed for conveying 5-ASA to the colon include various pH-dependent polymers, microgranules encapsulated into ethyl cellulose, or azo-bound derivatives. None of these is as effective as the topical formulations for inducing remission in distal forms of UC^[3].

However, patients do not easily accept local therapy. The low compliance with topical administration is a determinant factor of late relapse^[4].

NEW SYSTEM FOR DELIVERING 5-ASA

5-ASA Multi matrix (MMx) 1.2-g tablets is a novel formulation of mesalamine that is characterized by a patented polymeric lipophilic and hydrophilic matrix enclosed within a gastro-resistant, pH-dependent coating^[5,6]. This coating permits the delay of 5-ASA release until the tablet is exposed to a pH of 7 (normally in the terminal ileum), while the interaction of lipophilic and hydrophilic excipients with intestinal fluids makes the release of 5-ASA slow and gradual throughout the entire colon^[7]. This system allows the delivery of a larger amount of 5-ASA to the colonic lumen than is possible with other mesalamine formulations. Moreover, another

advantage may be a longer residence of 5-ASA in the colon, as shown by a scintigraphic study that demonstrated that MMx was still detectable in the sigmoid colon 24 h after ingestion^[8].

Do these characteristics really confer a benefit over the other 5-ASA compounds? The first step was to discover whether MMx was comparable to 5-ASA enema, which is considered the gold standard therapy for treating left-sided UC.

5-ASA MMx IN ACTIVE UC

In an Italian study, 79 patients were randomized to receive oral MMx 3.6 g/d with a bedtime placebo enema, or oral placebo tablets with a bedtime enema of mesalamine 4 g/100 mL. At 8 wk, clinical remission rate was 60% in the MMx arm *vs* 50% in the enema arm; endoscopic and histological remission rates were 45% and 15% *vs* 37% and 8%, respectively. Compliance rate for the MMx group was 97% overall compared with 87.5% in the mesalamine enema group. The remission rate in the enema group declined after 4 wk because the patients in remission spontaneously reduced administration^[5].

A further explorative study was a phase II, multicenter, randomized, double-blind, dose-ranging small trial in patients with mild-to-moderate UC^[7]. Thirty-eight patients were randomized to once-daily dosing of MMx mesalamine at a dose of 1.2, 2.4 or 4.8 g, for 8 wk. Six patients achieved complete remission at 8 wk: 4 (30.8%) in the 2.4 g/d group, 2 (18.2%) in the 4.8 g/d group, and none in the 1.2 g/d group. The difference in remission rates between the groups was not statistically significant given the small sample size. In general, MMx was well-tolerated among the three groups. Within the limitation of the small sample size, the data suggest that MMx mesalamine at 2.4 g/d or 4.8 g/d is an effective once-daily treatment for mild-to-moderate UC.

The following two large phase III studies showed that MMx given once or twice daily at 2.4-4.8 g was effective for induction of remission, and endoscopic and symptomatic improvement in patients with mild-to-moderate UC at 8 wk^[9,10].

The first was a randomized, double-blind, placebo-controlled multicenter trial of 280 patients with mild-to-moderate UC^[9]. Patients received MMx 2.4 g/d (twice daily) or 4.8 g/d (once daily), or placebo for 8 wk. Clinical and endoscopic remission rates at week 8 were higher in the MMx 2.4 g twice daily (34.1%) and 4.8 g once daily (29.2%) than in the placebo (12.9%) group ($P < 0.001$). Clinical improvement rates were 55.7% and 59.6% *vs* 25.9%, respectively ($P < 0.001$). Endoscopic improvement rates were 61.4% and 69.7% *vs* 35.5%, respectively ($P < 0.01$).

In the second randomized, double-blind, double-dummy, placebo-controlled multicenter trial, 343 patients with mild-to-moderate UC received MMx 2.4 g once daily, MMx 4.8 g once daily, pH release mesalamine (Asacol[®]) 2.4 g (800 mg three times daily), or placebo for 8 wk^[10]. Clinical and endoscopic remissions at 8 wk were

higher in patients receiving MMx 2.4 g/d (40.5%, $P = 0.01$) or MMx 4.8 g/d (41.2%, $P = 0.007$) than placebo, while the differences between MMx and Asacol[®] (32.6%) and between Asacol[®] and placebo (22.1%) were not statistically significant.

STUDIES WITH 5-ASA MMx IN MAINTENANCE OF UC REMISSION

The 459 patients, who achieved remission in the previous two studies, were enrolled in a randomized, multicenter, open label trial^[11]. The aim was to evaluate the safety and efficacy of 2.4 g/d MMx once or twice daily as 12 mo maintenance therapy in patients with UC. One hundred and seventy-four of 459 patients (37.9%) experienced 384 adverse events, the majority of which were mild or moderate in intensity. Most serious adverse events were of a gastrointestinal nature. At month 12, 88.9% of patients in the once-daily treatment group and 93.2% in the twice-daily group maintained clinical remission.

The first placebo-controlled trial that reported the efficacy of MMx in maintenance therapy was a multicenter study^[12]. Three hundred and thirty-one European patients with UC were randomized to receive MMx 2.4 g/d once daily, or Asacol[®] 2.4 g/d twice daily, administered in a double-dummy fashion for 12 mo. All patients were in remission with at least one documented relapse in the previous year. The data from this study indicate that MMx 2.4 g/d once daily and Asacol[®] 2.4 g/d twice daily are similarly tolerated and effective in the maintenance of remission of left-sided UC. Overall, 68.0% of patients in the MMx group and 65.9% in the Asacol[®] group, were in clinical remission at 12 mo. Clinical and endoscopic remission was maintained in 60.9% and 61.7% in the MMx and Asacol[®] groups, respectively, at 12 mo. The proportion of patients in remission was not distributed equally: Polish and Ukrainian showed a higher proportion of patients in remission than did Italian centers (country effect, $P < 0.001$). This variation may reflect differences in practice between the national health services in each study country. Poland and Ukraine reported quite high remission rates in all study populations, with 77.8%-96.7% of patients maintaining remission at 12 mo.

In contrast, patients from the Italian centers showed consistently lower remission rates 56.2% (MMx group) and 54.6% (Asacol[®] group) at 12 mo, which is consistent with the currently available literature. A possible explanation for this discrepancy is that 66.5% of the Italian population were taking at baseline an adequate maintenance dose of 5-ASA (≥ 1.6 g/d). In contrast, only 49.0% and 36.1% of patients in Poland and Ukraine were using comparable maintenance therapy. It is therefore possible that investigators in Poland and Ukraine included patients with milder UC, who were in remission without adequate therapy.

Examining only the Italian population, including

the diary card data, a significant difference was detected between the MMx and Asacol[®] groups, in the intention-to-treat ($P = 0.026$) and per-protocol ($P = 0.010$) population. This study, however, was not able to test if the compliance with once-daily administration of MMx improved the efficacy, given that it was designed to distribute medication in a double-dummy fashion. In this study, 5-ASA therapy was well tolerated. Indeed, the adverse-effect profile reported in was similar to those reported in other long-term studies of 5-ASA. The majority of AEs were mild or moderate in severity^[12].

5-ASA compounds are very effective drugs for treating UC, and they are of particular value in maintenance therapy. However, long-term reduced compliance interferes with their effectiveness. A recent study has reported that local therapy, taking too many tablets, or inconvenient dosing regimens are the reasons for non-compliance, especially in maintenance therapy^[4,13,14]. In fact, patients with UC in remission who are not compliant with 5-ASA therapy have a fivefold greater risk of relapse than compliant patients^[4].

Patient adherence to medication has been emphasized recently in a Cochrane meta-analysis that suggests that future trials of ASA in UC should explore patient compliance rather than drug efficacy^[15]. This is the main reason why the doctor should tailor therapy for the individual patient by reducing the number of tablets at the effective dose to as few as possible, and by stressing to him/her the importance of following the drug's instructions. On the other side, the industry should improve patient compliance by reducing the number of tablets to be taken, and the frequency of daily dosage. 5-ASA MMx has been designed to reach these goals to make use of some properties of the drug: i.e., its long-term residence in the colon, the higher 5-ASA content in each tablet, and a delivery system that splits the active moiety along the entire colon.

CONCLUSION

5-ASA MMx has been shown to be at least as effective as the most employed 5-ASA compound Asacol[®], for inducing and maintaining remission of UC. 5-ASA MMx has three useful characteristics that should add to this formulation some advantage over the other 5-ASA compounds: (1) the 1.2-g content of each tablet decreases the number of tablets to be taken; (2) the long residence in the colon allows once-daily dosing; and (3) delivery of 5-ASA to the entire inflamed colon should produce efficacy comparable to that of a combination of tablets and enema.

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EDITORIAL

Molecular mechanisms of insulin resistance in chronic hepatitis C

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Supported by Australian National Health and Medical Research Council and the Robert W Storr Bequest to the University of Sydney

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Received: July 2, 2009 Revised: August 13, 2009

Accepted: August 20, 2009

Published online: September 21, 2009

Abstract

It is now widely recognized that chronic hepatitis C (CHC) is associated with insulin resistance (IR) and type 2 diabetes, so can be considered a metabolic disease. IR is most strongly associated with hepatitis C virus (HCV) genotype 1, in contrast to hepatic steatosis, which is associated with genotype 3 infection. Apart from the well-described complications of diabetes, IR in CHC predicts faster progression to fibrosis and cirrhosis that may culminate in liver failure and hepatocellular carcinoma. More recently, it has been recognized that IR in CHC predicts a poor response to antiviral therapy. The molecular mechanisms for the association between IR and HCV infection are not well defined. This review will elaborate on the clinical associations between CHC and IR and summarize current knowledge regarding the molecular mechanisms that potentially mediate HCV-associated IR.

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Key words: Hepatitis C virus; Insulin resistance; Treatment response; Interferon

Peer reviewers: Dr. Sk Md Fazle Akbar, Assistant Professor, Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791-0295, Japan; Christa Buechler, PhD, Regensburg University Medical Center, Internal Medicine I, Franz Josef Strauss Allee 11, 93042 Regensburg, Germany

Douglas MW, George J. Molecular mechanisms of insulin

resistance in chronic hepatitis C. *World J Gastroenterol* 2009; 15(35): 4356-4364 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4356.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4356>

INTRODUCTION

Infection with the hepatitis C virus (HCV) is a leading cause of chronic liver disease, with over 3% of the world's population (180 million people) infected and 130 million at risk of cirrhosis^[1]. The majority of infected individuals (60%-80%) develop chronic hepatitis C (CHC), which is associated with progressive liver fibrosis and a 3%-9% risk of cirrhosis after 20 years as shown in community-based studies^[2]. CHC is also associated with significant morbidity and mortality, accounting for 50%-76% of all liver cancer cases worldwide, and two thirds of liver transplants in the developed world^[1].

It is now widely recognized that CHC is associated with insulin resistance (IR) and type 2 diabetes (T2DM), so can be considered a metabolic disease. Apart from the well-described complications of diabetes, IR in CHC predicts faster progression to fibrosis and cirrhosis that may culminate in liver failure and hepatocellular carcinoma (HCC). More recently, it has been recognized that IR in CHC predicts a poor response to antiviral therapy. The molecular mechanisms for the association between IR and HCV infection are not well defined. This review will elaborate on the clinical associations between CHC and IR and summarize current knowledge regarding the molecular mechanisms that potentially mediate HCV-associated IR.

INSULIN SIGNALING AND IR

Insulin is an anabolic hormone secreted by pancreatic β -cells that is required for the maintenance of glucose homeostasis. It inhibits hepatic glucose production and increases peripheral glucose uptake and glycogen synthesis. The principal signaling pathway (Figure 1) involves sequential activation of the insulin receptor, insulin receptor substrates (IRS), phosphatidylinositol-3-kinase (PI3K), Akt and protein kinase C isoforms ζ and λ ^[3,4]. Akt is phosphorylated initially at serine 473 by phosphoinositide-dependent kinase (PDK)2 and

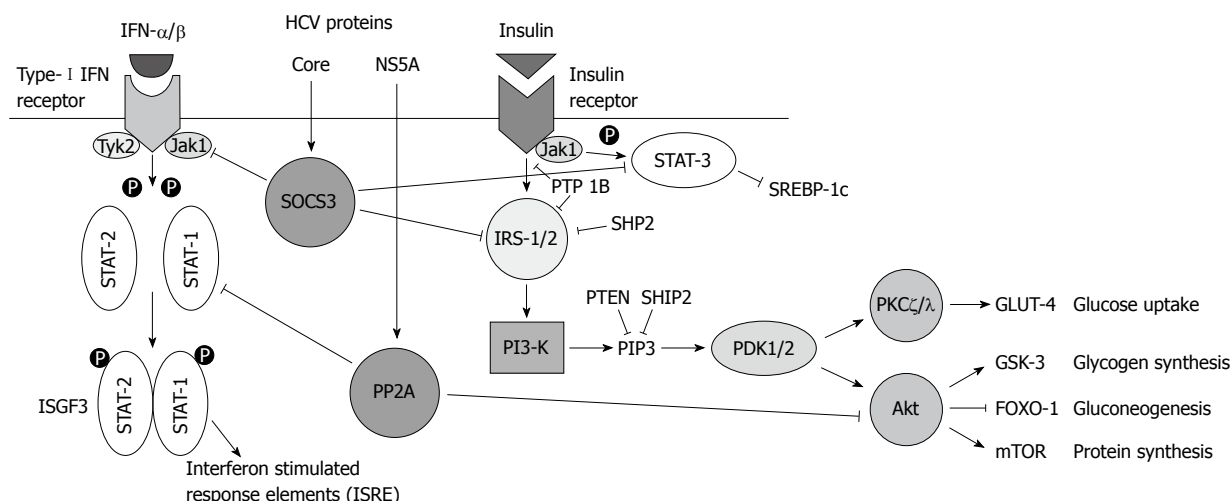


Figure 1 Hepatitis C virus core and NS5A proteins may inhibit insulin and interferon signaling.

subsequently at threonine 308 by PDK1. Activated Akt promotes storage of excess glucose as glycogen by phosphorylating glycogen synthase kinase and suppresses gluconeogenesis by inhibiting phosphoenol-pyruvate carboxykinase and glucose-6 phosphatase. In striated muscle and adipose tissue, activated Akt promotes translocation of the glucose transporter GLUT4 to the plasma membrane, promoting glucose uptake^[5].

Insulin promotes lipid synthesis *via* sterol regulatory element-binding protein 1c and fatty acid synthase, although importantly the pathways are probably different from those mediating insulin-stimulated glucose homeostasis^[5]. With regard to protein, insulin promotes cell survival and protein synthesis, mediated by the mammalian target of rapamycin (mTOR) pathway^[3].

Insulin resistance plays a fundamental role in the pathogenesis of T2DM. It results from defects at any level of the ligand-receptor-response pathway, including well-characterized defects at the level of the insulin receptor or IRS molecules, although these account for only a small minority of cases of IR in clinical practice^[3,4]. These defects can result from either reduced levels of signaling proteins, or modulation of their activity by phosphorylation. For example, IRS-1 is activated by phosphorylation of tyrosine residues, but inhibited by phosphorylation of key serine residues including Ser307, Ser318, Ser636 and Ser639^[6]. Negative feedback loops down-regulate the pathway in response to chronic glucose supply, including inhibition of PI3K by phosphatase and tensin homolog.

Two IRS proteins are important in human liver: IRS-1 and IRS-2^[7]. They appear to have complementary but overlapping roles in that IRS-1 knockout mice exhibit growth retardation and IR, while IRS-2 knockouts develop T2DM due to β -cell failure and hepatic IR. While both knockouts are insulin-resistant, IRS-1 knockout mice have reduced peripheral glucose uptake, while IRS-2 knockouts have a more complex phenotype, with both peripheral and central IR^[8]. This has led to the traditional view that IRS-2 is more important for insulin signaling and glucose homeostasis

in the liver^[9]. However, recent evidence suggests that IRS-1 may be more important for glucose homeostasis, while IRS-2 is more important for lipid metabolism^[10]. Therefore, changes affecting either IRS could contribute to HCV-induced hepatic IR.

The roles of mTOR in insulin signaling are multiple and complex. Early studies showed that insulin signaling *via* Akt caused phosphorylation and activation of mTOR^[11]. However it is now known that mTOR is present in at least two different mTOR complexes and plays multiple roles in insulin signaling, as reviewed recently^[12,13]. The mTOR-raptor complex 1 (mTORC1) appears to mediate the downstream effects of insulin on cell growth and proliferation^[12] and also provides negative feedback of insulin signaling by phosphorylating IRS-1 at inhibitory serine residues 636 and 639^[14]. In contrast, mTOR associates with rictor to form a second complex mTORC2^[12], the elusive “PDK2” that phosphorylates Akt at serine 473 in response to insulin^[15]. mTORC2 is much less sensitive to rapamycin inhibition than mTORC1^[12].

CLINICAL ASSOCIATIONS OF HCV AND IR

CHC and T2DM

It is now over 10 years since an association between HCV and diabetes was first described by Allison *et al*^[16] who noted that people with cirrhosis and HCV had T2DM more commonly than those with cirrhosis from other causes. A subsequent study of cirrhotic patients confirmed that T2DM was present in 21% of patients with cirrhosis due to CHC but was present in only 12% of patients with cirrhosis and chronic hepatitis B (CHB)^[17]. In the latter report, T2DM was particularly associated with HCV genotype 2a^[17].

Significantly, subsequent case control studies have confirmed that T2DM is associated with CHC even in the absence of cirrhosis^[18-20]. Most recently, a large cross-sectional study of over 9000 individuals in the USA found that in persons over 40 years of age, those with HCV infection were over three times more likely to

have T2DM than those without^[21]. Of relevance, there was no association between CHC and type 1 diabetes, and no association of hepatitis B virus infection with T2DM, suggesting a virus-specific association of HCV with T2DM. Interestingly, the association of T2DM was with HCV genotype 1b. In a subsequent large cohort study, it was noted that HCV-associated T2DM mainly occurred in patients with other risk factors for diabetes, such as older age and a high body mass index^[22]. Thus, among patients classified as “high risk” for T2DM, CHC increased diabetes risk more than 11-fold after 9 years^[22], although further studies are required to firmly establish causality.

CHC and IR

Insulin resistance is present in > 90% of individuals before the onset of frank T2DM. While the euglycemic hyperinsulinemic clamp^[23] is the “gold standard” for measuring glucose utilization and insulin sensitivity or resistance, a common clinical approximation is obtained using the homeostasis model of IR (HOMA-IR), calculated by the following equation: $\text{HOMA-IR} = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (}\mu\text{U/mL)} / 22.5$ ^[24,25]. Typically, a HOMA-IR value > 2 is used to signify significant IR^[26].

In 2003, Hui *et al*^[27] first reported that IR is increased in patients infected with HCV, particularly genotype 1 and that this may form the basis for the earlier observations regarding the association with T2DM. Subsequent studies have confirmed this association, including for genotype 4^[28] and possibly also genotype 2a^[29]. Since liver fibrosis irrespective of etiology can of itself cause hyperinsulinemia, it is important to exclude patients with advanced fibrosis in any assessment of the relationship between HCV and IR. This was undertaken in the study by Hui *et al*^[27], who demonstrated that even patients with minimal fibrosis (F0 or F1) had a mean HOMA-IR of 2.4 compared to 1.9 for matched uninfected controls ($P = 0.002$).

Studies have shown that in people with HCV and IR who respond to treatment there is a reduction in HOMA-IR^[26]. This improvement in insulin sensitivity is maintained for people with a sustained virological response (SVR)^[26,30], and results in a reduced risk of subsequent diabetes^[30,31]. However other groups have shown that the apparent reduction in diabetes risk may actually reflect the lower baseline risk of diabetes in patients who respond to interferon treatment, rather than an effect of treatment *per se*^[32]. It is also worth noting that not all patients with CHC develop IR, suggesting a complex interaction between virus and host factors that is only partially understood.

In contrast to the specific association of IR with CHC, IR is not associated with CHB. A recent study found that in patients with CHB the HOMA-IR reflected their overall metabolic profile, but was not increased in people with CHB compared with matched healthy controls^[33]. Another recent prospective study comparing patients with CHB and CHC confirmed

that IR is a specific feature of hepatitis C genotype 1 and 4 infection, but not CHB; it was present in 35% of patients with CHC compared with only 5% with CHB^[28].

Complications of HCV-induced IR

As well as predisposing to T2DM, the presence of IR in CHC predicts non-response to antiviral therapy^[26], both for genotype 1^[26,34-36] and genotypes 2 and 3 infection^[37]. Central obesity, which can be associated with IR, has also been shown to predict non-response^[38]. Furthermore, HCV-infected patients who respond to antiviral therapy show improved insulin sensitivity^[39]. Similarly, improvements in CHC-induced steatosis have been observed following SVR^[40]. Interestingly, IR has been associated in several studies with elevated HCV viral loads^[28,41,42]. One possible mechanism involves p21-activated kinase 1, which suppresses HCV replication and is stimulated by PI3K/Akt signaling, *via* mTOR^[43]. HCV-associated IR could decrease PI3K/Akt signaling and thus favor viral replication, although the mechanism by which HCV induces IR is unclear.

The interactions between HCV, IR, steatosis and hepatic fibrosis are complex and genotype specific. In CHC due to genotype 1, IR is associated with hepatic steatosis. In this setting, IR is either virus-mediated or due to host metabolic factors such as visceral obesity^[44]. A similar association has been shown for genotype 4^[45]. In contrast, steatosis in HCV genotype 3 infection is predominantly a direct effect of the virus, occurring in the absence of other metabolic risk factors^[46,47]. It should be noted however, that in people with genotype 3 CHC and obesity, a proportion of the hepatic steatosis will be secondary to their metabolic dysregulation. In patients infected with HCV, steatosis and IR are predictive factors for the later progression to hepatic fibrosis and cirrhosis^[28,48-51]; the latter predisposes to HCC. Likewise, diabetes mellitus itself has recently been shown to increase the risk of HCC^[52]. While CHC-induced steatosis has been reported as a risk factor for hepatic fibrosis^[53], subsequent studies suggest that IR is most important in this relationship, both for genotypes 1 and 3^[46,48].

MECHANISMS OF HCV-INDUCED IR

Inflammation and IR

Chronic inflammation plays a significant role in IR associated with metabolic liver disease, due to increased levels of interleukin (IL)-1, tumor necrosis factor (TNF)- α , IL-6 and leptin, and reduced levels of adiponectin^[54]. Diet-induced IR in the context of obesity partly involves the inflammatory mediator I κ B kinase β (IKK β)^[55]. Inflammatory cytokines such as IL-1, TNF- α and free fatty acids stimulate IKK β . This induces proteasomal degradation of I κ B, allowing nuclear translocation of the downstream effector molecule NF κ B to stimulate secretion of IL-6^[56]. IKK β also induces IR by inhibitory phosphorylation of the insulin signaling molecule IRS-1 at serine 312^[57]. High doses

of aspirin and other salicylates inhibit IKK β and can reduce IR in both rats and humans^[58-60]. IR can similarly occur in other inflammatory conditions associated with elevated TNF- α , including inflammatory bowel disease^[61], rheumatoid arthritis^[62] and psoriasis^[63].

Based on these well-described associations, it was initially proposed that IR in CHC may arise as a non-specific consequence of hepatic inflammation, possibly mediated by IKK β . In support of this hypothesis, expression of HCV core protein in transgenic mice (genotype 1b) induces hepatic IR^[64]. When fed a high-fat diet, these mice develop frank diabetes and hepatic steatosis that is associated with elevated circulating levels of TNF- α . The IR is reversed by administering antibodies against TNF- α , but the mechanism of this effect has not been well defined.

Early human studies were conflicting but more recent studies have shown that HCV-induced IR is NOT due to alterations in serum inflammatory cytokines or adipokines^[65]. Rather, IR in CHC seems to be due to direct virus-specific effects on insulin signaling. Thus, initial studies noted increased serum levels of TNF- α in subjects with CHC^[66-68] or showed a correlation with IR^[69] but did not adequately correct for potential confounders. A later well-controlled study compared 154 HCV-infected non-diabetic males with 75 matched uninfected controls^[65]. In that study, as expected, serum levels of TNF- α and IL-6 were higher in HCV-infected patients than controls but levels did not correlate with IR^[65]. Serum levels of the adipocytokines leptin and adiponectin were likewise independently associated with IR (adiponectin inversely), but not with HCV infection itself. The authors therefore concluded that these adipocytokines could not account for the increased IR seen in HCV-infected subjects^[65].

Direct effects of HCV in modulating insulin signaling HCV core protein

Although it was initially suggested that IR associated with CHC may be due to chronic inflammation, it is now known that HCV can induce IR directly, through specific viral effects^[70]. Much of the published literature in this area has focused on the HCV core protein, which has been proposed to cause IR in hepatocytes by reducing the level or activity of molecules involved in insulin signaling, particularly IRS-1 and IRS-2. However, there is considerable disagreement concerning which of these molecules is more important, and whether altered signaling results from changes in IRS expression, degradation, or altered activity^[64,71-75].

For example, one study found reduced activation (reduced tyrosine phosphorylation) of IRS-1 in liver biopsies from HCV-infected patients and reduced association of IRS-1 with its downstream effector PI3K but increased expression of IRS-1 protein^[71]. In contrast, another report demonstrated reduced expression of both IRS-1 and IRS-2 in patient samples and in livers from transgenic mice expressing HCV core protein^[73]. It was proposed that core protein stimulated increased

levels of the molecule suppressor of cytokine signaling (SOCS) 3, leading to ubiquitination and proteasomal degradation of IRS-1 and IRS-2^[73] (Figure 1). This is consistent with data showing IR in mice following over-expression of SOCS1 or SOCS3^[76]. In support of their hypothesis, the same group found that in patients who responded to antiviral therapy hepatic levels of both IRS-1 and IRS-2 were increased, along with improved clinical insulin sensitivity^[39]. One clinical study showed higher levels of SOCS3 in peripheral lymphocytes from people infected with HCV genotype 1 rather than genotype 2 and found that the level of SOCS3 was the best predictor of response to interferon therapy^[77]. In a similar study from the same group, polymorphisms in the SOCS3 gene were shown to correlate with clinical response to interferon^[78]. SOCS3 mRNA levels were higher in obese subjects with CHC than lean subjects and may contribute to their reduced response to IFN- α treatment^[79].

The effects of HCV genotype on insulin signaling are less well understood but are important given the clinical association of genotypes 1 and 4 with IR^[27,28]. One study compared the effects of over-expressing genotype 1b and 3a core proteins in the Huh7 hepatoma cell line^[75]. No difference in SOCS3 expression was detected, but cells expressing genotype 3a core contained higher levels of SOCS7 than cells expressing genotype 1b core, as well as reduced levels of IRS-1^[75]. In contrast, cells expressing genotype 1b core had a smaller reduction in the amount of IRS-1, but increased phosphorylation of IRS-1 at inhibitory serine residues (636/639), as well as increased mTOR activity^[75]. The authors therefore speculated that IR in the context of HCV genotype 1 infection is due to core-induced induction of the TORC1 mTOR/raptor complex, resulting in reduced IRS-1 signaling^[75]. A different group has shown reduced insulin signaling in core-expressing cells, due to JNK-mediated inhibitory phosphorylation of IRS-1 at serine 312^[72]. Although intriguing, the relevance of IRS-1 serine phosphorylation to clinical HCV-induced IR has yet to be confirmed.

PA28 γ is an inducer of late proteasome activity that may play a role in HCV-induced IR as it is essential for the development of IR in HCV core transgenic mice^[74]. Core transgenic mice display reduced insulin sensitivity, reduced activation (tyrosine phosphorylation) of IRS-1 and reduced expression (mRNA and protein) of IRS-2; factors which are restored to normal following knockout of the PA28 γ gene^[74]. TNF- α expression was increased in the livers of core transgenic mice and in human hepatoma cell lines expressing core protein, but knocking out or silencing PA28 γ returned TNF- α expression to normal^[74]. PA28 γ has also been shown to play a critical role in the development of steatosis and HCC^[80], and may provide a useful link between the different pathways affected by HCV.

HCV NS5A

Another molecule that may play a role in HCV-induced

IR is protein phosphatase 2A (PP2A). PP2A can affect several cell pathways and is upregulated in HCV infection, possibly due to increased endoplasmic reticulum (ER) stress^[81] or directly by the HCV non-structural protein NS5A^[82] (Figure 1). PP2A has been shown to mediate HCV-associated IR by dephosphorylating and thus inactivating Akt^[83]. In that study, PP2A levels were increased in HCV protein-expressing cell lines, the livers of transgenic mice expressing HCV proteins and in liver biopsies from HCV-infected patients. Impaired insulin signaling was demonstrated in each model, with reduced insulin-stimulated phosphorylation of Akt^[83]. However, the authors were unable to show a correlation between reduced Akt signaling and IR in HCV-infected patients, as measured by HOMA-IR^[83]. Interestingly, PP2A has been shown to inhibit interferon signaling and this has been proposed as another potential link between IR and reduced clinical response to interferon treatment in HCV-infected patients^[84].

Peroxisome proliferator activated receptor (PPAR)- α and PPAR- γ

PPARs are nuclear receptors that modulate lipid and glucose metabolism, as reviewed recently^[85]. PPARs form heterodimeric complexes with the retinoid X receptor (RXR) and bind to PPAR response elements (PPRE) on PPAR regulated genes, inhibiting their expression. Binding of PPAR ligands, including unsaturated fatty acids, eicosanoids, oxidised low density lipoprotein (LDL) and very LDL, causes dissociation of the PPAR-RXR complex, derepression and increased gene expression^[85]. In this way PPARs react to lipid excess by stimulating differentiation of adipocytes, oxidation of fatty acids and glucose metabolism.

PPAR- α is the major PPAR isoform present in liver and is also found in brown fat and heart. It regulates cell energy by stimulating oxidation of fatty acids in mitochondria and peroxisomes^[85]. Along with PPAR- δ , it stimulates expression of human adipose differentiation-related protein, thus promoting storage of cellular lipid in lipid droplets^[86]. PPAR- γ is the dominant isoform in adipose tissue, colon, myeloid cells and placenta, where it stimulates adipocyte differentiation and lipid storage^[85].

As well as their effects on lipid metabolism, PPARs may also play a role in HCV-induced IR^[87]. Liver biopsies from patients with CHC show reduced levels of PPAR- γ and PPAR- α mRNA^[88,89]. In an *in vitro* model of HCV-induced hepatic IR, cells expressing genotype 3 core protein, but not genotype 1b, had reduced levels of PPAR γ mRNA^[89]. In a follow-up study, treatment of genotype 3a core-expressing cells with the PPAR- γ agonist rosiglitazone improved insulin signaling^[75]. Interestingly, PPAR- α is required for HCV core-induced steatosis in transgenic mice^[90], suggesting overlapping mechanisms for IR and steatosis in people with CHC.

Oxidative stress and IR

Oxidative stress may contribute to IR in HCV-infected people, as well as to steatosis. *In vitro* studies demonstrate

increased mitochondrial reactive oxygen stress in hepatoma cells over-expressing core protein^[91] and in HCV core transgenic mice^[91,92]. Further, in HCV-infected patient serum, thioredoxin, a marker for oxidative stress, has been shown to correlate with clinical IR, independent of obesity^[93]. However the interactions between oxidative stress, IR, metabolic syndrome and steatosis are complex, with each potentially influencing the other. A recent study showed that in patients infected with HCV genotype non-3, HOMA-IR ($P < 0.01$), fibrosis ($P < 0.01$) and oxidative stress ($P < 0.05$) were independently associated with steatosis, whereas steatosis was independently associated with oxidative stress ($P < 0.03$) and HOMA-IR ($P < 0.02$)^[94]. The authors concluded that in genotype non-3 infection "oxidative stress and IR contribute to steatosis, which in turn exacerbates both IR and oxidative stress and accelerates the progression of fibrosis"^[94].

Treatment of IR in people infected with HCV

It has been shown that response to interferon-based treatment can be improved by diet-induced weight loss, which improves insulin sensitivity^[95]. Given the association between IR and poor treatment response in CHC, clinical trials of insulin-sensitizing drugs have been proposed to improve treatment response^[29]. Since the mechanisms of HCV-induced IR are not well understood it is not clear whether it is better to use thiazolidinediones that target PPAR- γ , or metformin, which activates AMP-activated protein kinase^[96] and may also stimulate the insulin receptor to signal *via* IRS-2^[97].

A recent pilot study adding the PPAR- γ agonist pioglitazone (15 mg) to pegylated interferon alpha and ribavirin was undertaken in patients with HCV-induced IR who had previously failed standard treatment^[98]. Although most of the treated patients showed improvement in their HOMA-IR on pioglitazone, none had a satisfactory virological response after 12 wk and the trial was terminated. A recent case report suggests that reducing IR prior to antiviral therapy, by pre-treating with insulin sensitizing drugs, may improve outcomes^[99]. The authors administered a high dose of pioglitazone (45 mg) to a patient with genotype 3a CHC who had previously failed antiviral treatment. After 5 mo of pioglitazone, the patient's HOMA-IR reduced from 4.8 to 1.3 and subsequent treatment with pegylated interferon and ribavirin for 48 wk produced a SVR^[99]. It is not clear whether this outcome was due to the higher dose of pioglitazone, the sequential treatment approach or the patient's genotype 3 infection^[100]. Several similar studies are underway to address these issues and their results are eagerly awaited.

CONCLUSION

There is increasing evidence that CHC is a metabolic disease, strongly associated with IR and T2DM. Insulin resistance is most strongly associated with HCV genotype 1, in contrast to hepatic steatosis which is associated with genotype 3 infection. Although the precise mechanisms of HCV-associated IR are unclear, several

possibilities have been suggested and the mechanisms may be multi-factorial, including both virus and host factors. Early studies in transgenic mice suggested IR may result from chronic inflammation and elevated TNF- α , but subsequent human studies have suggested a virus-specific effect. HCV core protein has the most evidence to support its role in IR, with likely effects on insulin signaling at the level of IRS. Core-induced increases in SOCS3 or SOCS7 expression may cause IRS destruction, probably requiring PA28 γ , but other proposed alternatives include feedback inhibition of IRS-1 by activated mTOR or JNK. Insulin resistance may also result from core-induced alterations in PPAR- α and PPAR- γ , especially in genotype 3 infection. PP2A can cause IR and in CHC its activity may be increased, either in response to ER stress or to the HCV non-structural protein NS5A, which interacts with many cellular pathways.

Since IR in CHC is associated with a reduced response to antiviral treatment, clinical trials are underway to determine whether reducing IR improves treatment outcomes. Preliminary data from pilot studies of PPAR- γ agonists have been disappointing, but case reports suggest that more aggressive treatment may be successful, particularly if IR is reduced prior to commencing antiviral therapy. Trials of other insulin-sensitizing drugs including metformin are also in progress and their results are eagerly awaited. Since the underlying mechanisms of HCV-induced IR are still not clear, ongoing research is essential to guide a more rational, targeted approach to therapy.

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S- Editor Tian L L- Editor Logan S E- Editor Zheng XM

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Treatment of malignant gastric outlet obstruction with endoscopically placed self-expandable metal stents

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Received: June 28, 2009 Revised: July 16, 2009

Accepted: July 23, 2009

Published online: September 21, 2009

Endoscopic self-expandable metal stent; Palliative treatment; Endoscopy

Peer reviewer: William Dickey, Professor, Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Gaidos JKJ, Draganov PV. Treatment of malignant gastric outlet obstruction with endoscopically placed self-expandable metal stents. *World J Gastroenterol* 2009; 15(35): 4365-4371 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4365.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4365>

Abstract

Malignant gastroduodenal obstruction can occur in up to 20% of patients with primary pancreatic, gastric or duodenal carcinomas. Presenting symptoms include nausea, vomiting, abdominal distention, pain and decreased oral intake which can lead to dehydration, malnutrition, and poor quality of life. Endoscopic stent placement has become the primary therapeutic modality because it is safe, minimally invasive, and a cost-effective option for palliation. Stents can be successfully deployed in the majority of patients. Stent placement appears to lead to a shorter time to symptomatic improvement, shorter time to resumption of an oral diet, and shorter hospital stays as compared with surgical options. Recurrence of the obstructive symptoms resulting from stent occlusion, due to tumor ingrowth or overgrowth, can be successfully treated with repeat endoscopic stent placement in the majority of the cases. Both endoscopic stenting and surgical bypass are considered palliative treatments and, to date, no improvement in survival with either modality has been demonstrated. A tailored therapeutic approach, taking into consideration patient preferences and involving a multidisciplinary team including the therapeutic endoscopist, surgeon, medical oncologist, radiation therapist, and interventional radiologist, should be considered in all cases.

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Key words: Malignant gastric outlet obstruction;

INTRODUCTION

Malignant gastroduodenal obstruction can occur in up to 20% of patients with primary pancreatic, gastric or duodenal carcinomas, with pancreatic cancer being the most common cause^[1]. Obstruction is a late occurrence in patients with advanced disease. Presenting symptoms include nausea, vomiting, abdominal distention, pain and poor oral intake which can lead to dehydration, malnutrition, and poor quality of life.

Traditionally, malignant gastric outlet obstruction has been treated surgically, usually by creating a gastrojejunostomy. More recently, the use of endoscopically placed self-expandable metal stents (SEMS) has become a routine practice^[2].

Radiologists were the first to offer an alternative to surgery by demonstrating the feasibility of stent placement using a peroral or percutaneous approach under fluoroscopic guidance^[3-5], but this approach never gained popularity due to its invasiveness and limited success rate^[4]. Technological advances have led to the development of self-expandable metallic stents that can pass through the operating channel of a therapeutic endoscope, allowing for endoscopic placement with fluoroscopic guidance, first described by Truong *et al*^[6] in 1992. Endoscopic treatment has become more successful as this approach has increased in popularity.

TECHNICAL CONSIDERATIONS

There are limitations in the ability to successfully place metal stents, including the inability to pass the guidewire

Table 1 Outcomes and complications of endoscopic stent placement

Author, yr	No. of patients	Stent used	Technical success (%)	Clinical outcome (%)	Complications, early/late
Cho <i>et al</i> ^[13] , 2009	75	Hanaro and Niti-S, covered and uncovered	98	87	3 (1 pneumonia, 1 perforation, 1 stent kinking)/35 (23 ingrowth, 2 overgrowth, 8 migration, 2 food impactions)
Nassif <i>et al</i> ^[9] , 2003	63	Wallstent (36), Choostent (32)	95	92	9 (1 perforation, 8 stent dysfunction)/18 (1 perforation, 13 obstruction, 4 migration)
Maetani <i>et al</i> ^[20] , 2009	60	Uncovered (31) and covered (29) Ultraflex	100	90.3 (uncovered), 86.2 (covered)	2 (mild pancreatitis)/10 (4 obstructions, 2 migrations, 2 stent fractures, 1 perforation, 1 bleed)
Kim <i>et al</i> ^[14] , 2007	53	NiTi-S Pyloric	100	81.1	5 (tumor ingrowth)/12 (9 tumor ingrowth, 1 tumor overgrowth, 2 migration)
Phillips <i>et al</i> ^[21] , 2008	46	Wallstent (40), Alimaxx (4), Bard (2)	100	91	5 (2 stent migration, 2 delayed-onset obstructive symptoms, 1 stent fracture)/2 (1 duodenal perforation, 1 aortoenteric fistula)
Lopes <i>et al</i> ^[16] , 2008	44	Choostent (14 uncovered, 1 covered), Hanarostent (21 uncovered, 1 covered), uncovered Wallstent (21)	100	Not measured	6 (1 perforation, 1 migration, 3 obstructions, 1 hemorrhage)/11 (8 obstructions, 2 migrations, 1 fistula)
Mosler <i>et al</i> ^[8] , 2005	36	Gianturco Z-stent, Ultraflex, Endocoil, enteral Wallstent	92	75	3 (2 stent migrations, 1 impaction)/13 (5 migrations, 4 tumor ingrowth, 2 erosion/perforation, 2 impactions)

through severely narrowed stenoses, anatomic difficulties such as a dilated stomach which may lead to significant looping, or complicated post-surgical anatomy that limits passing of the endoscope or the guidewire to the site of obstruction^[7]. It may be useful to obtain imaging of the obstructed area, such as performing an upper GI barium study, to try to assess the patient's anatomy, the stricture length, and degree of obstruction, if possible.

After administering conscious sedation, the area of stenosis is reached using a therapeutic upper endoscope. If the length of the stenosis is not known, then this can be determined by advancing the endoscope through the stenotic area or, if the stricture is not traversable, then it can be measured fluoroscopically. A guidewire is then advanced through the working channel of the endoscope and passed at least 20 cm distal to the obstructing area. The stent length should be at least 3-4 cm longer than the stenosis to allow for an adequate stent margin after placement. Next, the SEMS delivery system is passed over the guidewire through the working channel of the endoscope and aligned so that the ends of the undeployed stent overlies both ends of the stenosis equally. Once the alignment is correct, the stent is then deployed distal end first, followed by the proximal end. Next, the stent placement and luminal patency are confirmed endoscopically and fluoroscopically.

Patients with malignant gastric outlet obstruction often have coexistent biliary obstruction, which may present before or after the symptoms of gastric outlet obstruction. Placement of a metal biliary stent should be considered prior to duodenal stenting in patients with existent or impending biliary obstruction. Once the duodenal stent is in place, access to the biliary tree becomes extremely limited and a percutaneous transhepatic approach is usually required^[7].

confirmed presence of an unresectable malignancy or malignant recurrence at an anastomotic surgical site causing symptomatic gastric outlet or duodenal obstruction, a single stenotic region, and an expected short survival period (usually less than six months). The presence of free perforation with signs of peritonitis and tension pneumoperitoneum are contraindications to endoscopic stent placement^[7]. The desired outcomes after placement of a SEMS include relief of obstructive symptoms, return to a normal diet with improved nutritional status and improvement in the patient's quality of life. No studies have shown any evidence of survival benefit associated with the relief of malignant obstruction.

Efficacy of endoscopic stent placement in most studies is defined by two measures: technical and clinical success. Technical success is defined by accurate stent placement with adequate expansion of the stent and evidence of luminal patency post-procedure, usually evaluated by performing a water-soluble or barium contrast study. Clinical success is determined by resolution of the patient's obstructive symptoms and the ability to resume a regular diet after stent placement and maintain adequate oral intake during follow-up. The results of the studies evaluating the use of SEMS in malignant gastric outlet obstruction are summarized in Table 1.

The reported technical success rates for endoscopically placed SEMS range from 92%-100%. Mosler *et al*^[8] reviewed outcomes in 36 patients who had undergone SEMS placement for malignant gastric outlet or proximal small bowel obstruction over a 13-year period. Initial stent placement was successful in 33/36 patients (92%); two patients had immediate stent migration and one stent impacted into the duodenal wall. The authors felt that their technical success rate was not as high as more recent studies since their retrospective review extended from January 1991 to March 2003 and a variety of SEMS types were used during the earlier years that were not specifically designed for enteral use, leading to a higher rate of

EFFICACY AND OUTCOMES

Indications for the placement of a SEMS include the

early complications. Nassif *et al*^[9] also reported a high technical success rate with 60/63 patients (95%) obtaining immediate radiographic evidence of luminal patency. It is mentioned that three patients required hydrostatic stent dilation during the initial procedure for insufficient expansion, however it is unclear if these three patients were included in the unsuccessful category. In the other studies with 100% technical success, this was determined based on achieving the technical goals in one endoscopic session, whether that required placement of several overlapping stents due to a long stricture site, adjustment of a migrated stent by realignment using forceps or complete removal with new stent placement, or using hydrostatic dilation to ensure adequate expansion^[10-16].

The use of fluoroscopic and endoscopic guidance allows for adequate stent and stenosis visualization to ensure proper stent placement during the procedure and improve technical success overall. However, in the study by Kaw *et al*^[17], successful stent placement was achieved in 32/33 patients (96.9%) due to complete obstruction distal to a previous surgical site with recurrence at the anastomosis and inability to pass the guidewire through the stricture in one patient. Complete obstruction remains a major limitation to endoscopic stent placement and surgical intervention is then required if possible.

Not surprisingly, clinical success rates are usually lower than the technical success, with reports ranging from 79%-91%, depending on the definition of success^[17,18]. Mosler *et al*^[8] defined clinical success as improvement in obstructive symptoms (i.e. nausea, vomiting, abdominal distention, and reflux), which occurred in 29/32 patients (90.6%), with no symptomatic improvement in three patients. Kim *et al*^[10] also used symptomatic improvement as the measure of clinical success in their assessment of outcomes after SEMS placement in 53 patients with gastric outlet obstruction due solely to gastric cancer, in which they reported a success rate of 81.8%.

Adler *et al*^[11] and Lindsay *et al*^[12] specifically studied clinical success, defined as the ability to resume a regular diet after SEMS placement. Adler *et al*^[11] reported the results of endoscopic treatment for malignant gastric outlet obstruction in 36 patients and found an improvement in 31/36 (86%), which was statistically significant ($P < 0.0001$), with 61% being able to consume a solid or soft diet without symptoms of obstruction. Importantly, 58% noted improvement in < 1 d with 86% showing improvement in 3 d or less. Lindsay *et al*^[12] reported an 80% clinical success rate in patients deemed unsuitable for surgical intervention, with a median survival of 7 wk, as 32/40 patients were able to resume a solid or soft diet after stent insertion.

Also looking at the clinical success rate, van Hooft *et al*^[19] reported symptomatic improvement in 51 patients with malignant gastric outlet obstruction followed prospectively at three tertiary referral centers. In this study, clinical improvement was determined by the change in the Gastric Outlet Obstruction scoring system before treatment compared with the score after endoscopic placement of the Wallflex enteral stent. There was a 98% (50/51 patients) technical success

rate and an 84% (43/51 patients) clinical success rate, with a statistically significant ($P < 0.001$) improvement in obstruction symptoms after treatment, as well as improvement in overall performance status ($P = 0.002$). Forty-six patients (90%) were able to resume oral intake within one day of stent placement. This study did not, however, find an improvement in global quality of life.

Masci *et al*^[18] prospectively assessed the duration of symptomatic improvement in a cohort of 38 patients with malignant upper gastrointestinal obstruction treated with endoscopic stent placement. At 30 d, follow-up was available for 34 patients and 79.4% of these patients were able to tolerate a solid or soft diet. At 90 d, 11 patients remained alive and 90.9% remained on a solid or soft diet. At 180 d, only five patients were alive, however all of them were eating a solid or soft diet. Telford *et al*^[15] reported a median duration of oral intake of 146 d after SEMS placement, which increased to 219 d with repeat stenting for recurrent obstruction, in patients with a median survival of 97 d. These articles not only highlight the duration of stent patency but the short survival period in these patients.

Kim *et al*^[14] and Cho *et al*^[13] evaluated clinical factors that contribute to longer durations of stent patency. Kim *et al*^[14] found that patients who received chemotherapy after stent placement were noted to have significantly prolonged stent patency. Cho *et al*^[13] reported similar findings, however they included the use of covered stents, in addition to chemotherapy after stent placement, as a significant prognostic factor contributing to stent patency.

COMPLICATIONS

Complication rates range from 11%-43% and can be reported as immediate, which occur within 24 h after placement of the SEMS, early or late^[9,18]. Each study determines the time frame that differentiates early from late complications, which can vary anywhere from < 96 h to within two weeks to be considered an early complication^[16,18]. Immediate and early complications include problems with sedation, stent obstruction, stent malposition, perforation, aspiration, and bleeding. Late complications include stent obstruction, bleeding, perforation, stent migration, and fistula formation^[7] (Table 1).

In the study by Cho *et al*^[13], 2/75 (2.6%) patients experienced immediate complications (1 aspiration pneumonia and 1 bowel perforation, which was successfully treated with surgical gastrojejunostomy) and 1 patient had a recurrence of obstructive symptoms due to tumor ingrowth within 1 wk of stent placement (1.3%), which was treated with repeat stenting. Late complications in this study included stent migration in 8/75 patients (6 treated with repeat stenting, 1 treated with palliative gastrojejunostomy, and 1 was asymptomatic so received no further treatment) and recurrence of obstructive symptoms in 25/75 (33.3%) due to tumor ingrowth in 23 and tumor overgrowth in 2 (14 treated with repeat stenting, 1 with palliative radiotherapy, no further intervention in the other 10 patients either due to mild

symptoms, poor condition or patient preference).

In the study by Kim *et al*^[10], restenosis occurring at less than 4 wk after stent placement was found in 5/43 patients (11.6%), which was successfully treated with placement of covered stents in the new stenotic area. Stent-related problems requiring treatment occurred in 17 patients (32.1%) during the follow-up period (mean 145 d, range 4-718 d). Two patients (3.8%) had recurrence of symptoms due to distal stent migration (both successfully treated with placement of a second stent overlapping the first), stent overgrowth occurred in one patient (1.9%) at 331 d after deployment, tumor ingrowth occurred in 14 patients (26.4%) at a mean of 78.4 d after stent placement. In this study the mean survival was 145 d and the median stent patency time was 187 d.

Ten early complications were reported in the study by Nassif *et al*^[9]. Eight patients (12.7%) had a primary duodenal stent dysfunction (treated with insertion of a second stent or with hydrostatic dilation), 1 patient who had previously undergone duodenal dilation for papillary cannulation experienced a duodenal perforation 24 h after stent placement (successfully treated with surgical bypass), and one patient developed mild post-endoscopic retrograde cholangiopancreatography (ERCP) acute pancreatitis after undergoing biliary stenting (which resolved with medical management). Eighteen patients (28%) experienced late complications in this study, with the majority due to stent obstruction secondary to tumor ingrowth (in 12 patients) or due to impaction of the proximal end of the stent into the duodenal bulb (in 1 patient). These were treated with repeat stenting (in 9 patients), surgical bypass (in two patients) and conservative management (in two patients). Stent migration occurred in four patients (6.3%); two patients were treated with repeat stenting and the other two patients were asymptomatic and required no further intervention. There was one case of peritonitis due to duodenal wall perforation that occurred 1 mo after stent placement in a patient who was being treated with chemotherapy and radiation for duodenal invasion by a Hodgkin's lymphoma, which was treated with surgical bypass.

Lopes *et al*^[16] assessed complications associated with endoscopic stenting for palliative treatment of malignant esophageal, gastroduodenal, and colonic obstruction. In their study population of 153 patients, 44 patients underwent duodenal stent placement, 84% for neoplastic obstruction and 16% for extrinsic compression. In this group of patients there were 6 (12%) procedure-related complications, including 1 perforation, 1 stent migration, 3 obstructions and 1 hemorrhage, which were all successfully treated endoscopically. The group of patients with gastroduodenal obstruction had a higher procedure-related complication rate than the esophageal (8.8%) or colonic (7.5%) stenting groups, primarily due to early obstruction. Eleven (22%) late complications were reported, including 8 due to obstruction from tumor ingrowth or overgrowth, 2 due to migration and 1 fistula, which were all treated with endoscopic placement of new stents; however one patient with late stent migration was treated with supportive care only.

More recently, Maetani *et al*^[20] compared the outcomes, complication rate and reintervention rate in patients who received uncovered (31 patients) *vs* covered (29 patients) stents for malignant gastric outlet obstruction. There was a 100% technical success rate in both groups and no significant difference in clinical success. In each group, there was one patient who experienced mild pancreatitis within 1 wk of stent placement, which was managed medically in both instances. In the uncovered stent group, late complications included one stent fracture, which did not require any additional intervention; one stent obstruction due to hyperplasia, which required repeat stent placement for treatment; one case of bleeding, which ceased spontaneously; and 1 fatal perforation that occurred during chemoradiation therapy 39 d after stent placement. In the covered stent group, late complications included 1 stent fracture that occurred after ERCP for management of biliary stent dysfunction, 3 stent obstructions (2 due to tumor overgrowth, 1 due to hyperplasia), and 2 stent migrations; all were treated with repeat endoscopic stent placement. This study concluded that reintervention is more commonly required after covered stent placement for management of complications.

Looking at the long term results and complications of enteral stent placement for unresectable cancer, Phillips *et al*^[21] reviewed the outcomes of 46 patients with malignant gastric outlet obstruction. All patients had successful stent placement and 42 patients (91%) showed clinical improvement. There were 5 early complications (defined as occurring in ≤ 30 d) including stent migration in 2 patients, both treated with endoscopic removal of the initial stent and stent replacement; 2 patients with delayed-onset obstructive symptoms with patent SEMS, both treated with percutaneous endoscopic gastrojejunostomy placement; and 1 patient with stent fracture, treated with stent removal and endoscopic dilation. Late complications consisted of 1 patient with a duodenal perforation, treated with emergent surgical repair, and 1 patient who developed an aortoenteric fistula from stent erosion in the setting of a previous pancreaticoduodenectomy, which was treated with an endovascular aortic stent followed by definitive repair. There were 4 patients who developed stent obstruction and recurrence of obstructive symptoms due to local tumor ingrowth. In this study, unlike most others, this was not included as a procedural complication as it was due to progression of the underlying primary disease and not a result of the intervention.

TYPES OF STENTS

Studies have evaluated the use of different stent designs, covered and uncovered, to try to determine which stent is best for different patient populations. Since recurrent obstruction due to tumor ingrowth with uncovered stents is common^[22-24] and the migration rate of covered stents used for malignant gastric outlet obstruction was found to be unacceptably high (21% and 26% in two small studies^[25,26]), Kim *et al*^[27] examined the rate of stent restenosis in 49 patients with malignant gastric outlet

obstruction in an effort to identify characteristics that would predict early restenosis. In all patients, uncovered stents were used and there was a 100% technical success. The patients were then divided into two cohorts, one with early restenosis and the other without early restenosis, for retrospective comparison with regard to host-related factors (i.e. age and gender) and lesion-related factors (i.e. primary illness, stenosis site and length). The only statistically significant difference between these groups was the site of stenosis, with early restenosis occurring more frequently at the post-operative anastomotic sites ($P < 0.05$, 95% CI 0.003-0.005). The median follow-up period was 17 wk (range 2-64 wk), median patient survival was 10 wk, while the median stent patency time was 8 wk. Placement of an additional uncovered stent, placement of a covered stent inside an occluded uncovered stent, and use of laser therapy have all been used to treat stent restenosis^[28-30]. A study by Jung *et al.*^[31] reported a decrease in stent migration rate and a decrease in recurrent obstruction caused by tumor ingrowth with coaxial placement of uncovered and covered expandable stents, however, the use of this technique with all patients is cost-prohibitive.

ENDOSCOPY WITH STENT PLACEMENT VS SURGICAL GASTROENTEROSTOMY

Multiple retrospective studies have compared the outcomes of surgical bypass and endoscopic stent placement for the palliative treatment of malignant gastric outlet obstruction. Early studies showed significant morbidity, including delayed gastric emptying post-operatively for up to 31 d, and a mortality of up to 18%, associated with gastrojejunostomy^[32]. More recently, Maetani *et al.*^[33] retrospectively reviewed the outcomes of 20 patients who underwent palliative enteral stenting compared with 19 matched patients who underwent surgical gastrojejunostomy and found that the only statistically significant difference between the groups was an earlier return to an oral diet in the endoscopically treated group at 1 d compared with 9 d in the surgical group ($P < 0.0001$). Otherwise, there was no difference with regard to patient survival, complication rates, technical or clinical success rates, and possibility of discharge. There was a difference in the length of hospital stay (15 d in the endoscopically treated group *vs* 30 d in the surgical group), however, due to the small study population, this difference was not statistically significant. In 2005, Maetani *et al.*^[34] reported another slightly larger comparative study, limited to patients with gastric outlet obstruction due to gastric cancer, and reported similar results with a significant difference in time to resumption of an oral diet as well as a shorter procedure time in the endoscopically treated groups, but no significant difference in the other measured outcomes (possibility of discharge, median post-operative hospital stays, survival periods, complication rates).

In a study by Mittal *et al.*^[35], 16 patients who had open gastrojejunostomy, 14 patients who had laparoscopic

gastrojejunostomy, and 16 patients who underwent endoscopic stenting were all matched for age, sex, ASA grade and level of obstruction in order to compare treatment outcomes, including time to starting free oral fluids and a light diet, length of hospital stay and patient survival. They found that oral intake could be tolerated in 6 d in the open gastrojejunostomy group, in 4 d in the laparoscopic gastrojejunostomy group, and on the same day following endoscopic stenting ($P < 0.001$). In addition they found a higher rate of complications in both surgical groups compared with the endoscopically treated group ($P = 0.016$). There were no differences in the total length of stay between the groups, which was attributed to a delay in referral for endoscopic stenting, however the length of stay after procedure was significantly less in the endoscopic stenting group at 2 d compared with 7 d in the laparoscopic gastrojejunostomy group and 10 d in the open gastrojejunostomy group ($P < 0.001$). Interestingly, survival was noted to be shortest in the endoscopically treated group, which was likely due to patient selection as patients with more advanced disease are referred for the least invasive treatment. In addition to confirming similar findings regarding outcomes in endoscopically *vs* surgically treated patients, this study highlights the lack of difference between open and laparoscopic gastrojejunostomy, the latter of which was previously felt to be superior.

In a prospective, randomized trial of 18 patients referred for treatment of malignant antro-pyloric strictures, patients were randomly assigned into two treatment groups consisting of endoscopic stenting or gastroenterostomy and were followed for 3 mo. Endoscopic treatment was found to have a significantly shorter operative time, with an earlier restoration of oral intake and shorter hospital stay, consistent with previously reported outcomes. There were no differences noted between the groups with regards to delayed gastric emptying, morbidity, mortality, and clinical outcomes at the 3-mo follow-up^[36]. Jeurnink *et al.*^[37] reviewed the outcomes of 95 patients who underwent gastrojejunostomy (42 patients) or endoscopic stent placement (53 patients), including resumption of an oral diet, persistent and recurrent obstructive symptoms, re-interventions, complications, hospital stay and survival. As with previous studies, there was a significantly shorter hospital stay ($P < 0.001$) and shorter time to resuming an oral diet ($P = 0.01$) with endoscopic treatment. This study, however, also found a significantly shorter time to late major complications ($P = 0.004$), shorter time to recurrent obstructive symptoms ($P = 0.002$), and shorter time to re-intervention ($P = 0.004$) in the patients who underwent endoscopic treatment. This study points out that there are benefits and limitations with each treatment option.

The one indisputable difference between these treatment options is cost. In 2001, Yim *et al.*^[38] compared the costs of palliative treatment with endoscopic enteral stenting *vs* gastrojejunostomy in patients with malignant gastric outlet obstruction due to pancreatic cancer and found the cost of endoscopic treatment was \$9921 *vs* \$28173 for surgical bypass. Adler *et al.*^[11] also conducted a cost analysis, including procedural and hospitalization

costs, comparing 10 patients who underwent endoscopic stenting with 10 patients who had surgical bypass for the treatment of malignant gastric outlet obstruction and found the median cost of endoscopic treatment was \$5970 compared to \$13445 in the surgical group ($P < 0.0001$). Mittal *et al*^[35] included a cost comparison in their study comparing three treatment groups and, as expected, found the surgical treatment options to be more expensive than endoscopic treatment with the cost of open gastrojejunostomy to be NZ\$13256, laparoscopic gastrojejunostomy to be NZ\$10938, and endoscopic stenting costing NZ\$5736. It has been consistently shown in these studies that surgical intervention is more costly than endoscopic treatment strategies.

CONCLUSION

In summary, endoscopic placement of SEMS is a safe, minimally invasive, and cost-effective option for palliation of malignant gastric outlet obstruction. SEMS can be successfully deployed in the majority of patients. Stent placement appears to lead to a shorter time to symptomatic improvement, shorter time to resumption of an oral diet, and shorter hospital stays as compared with surgical options. There is, however, a potential for the development of recurrence of the obstructive symptoms, most often due to stent obstruction from tumor ingrowth or overgrowth, which can, in the majority of cases, be successfully treated with repeat endoscopic stent placement. Both endoscopic stenting and surgical bypass are considered palliative treatments and, to date, no improvement in survival with either modality has been demonstrated. A tailored therapeutic approach, taking into consideration patient preferences and involving a multidisciplinary team including the therapeutic endoscopist, surgeon, medical oncologist, radiation therapist, and interventional radiologist, should be considered in all cases.

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TOPIC HIGHLIGHT

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Paraneoplastic dermatological manifestation of gastrointestinal malignancies

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Received: March 5, 2009 Revised: May 12, 2009

Accepted: May 19, 2009

Published online: September 21, 2009

<http://www.wjgnet.com/1007-9327/15/4372.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4372>

INTRODUCTION

Numerous cutaneous disorders have been associated with underlining malignancies of the gastrointestinal (GI) tract. In some cases the skin can be directly infiltrated by cancer cells that represent metastatic spread from a GI malignancy (e.g. a Sister Mary Joseph nodule). In other cases, the skin lesions are related to the underlying presence of malignancy, but they do not contain malignant cells and are referred to as paraneoplastic dermatological syndromes^[1]. Some of them, such as Muir-Torre, Peutz-Jeghers, and Cronkhite-Canada syndromes, are inherited and are caused by genetic factors, others however, have unknown etiologies and unpredictable expression and prognosis.

Dermatologists have the advantage of recognizing certain cutaneous signs, which hint at underlying visceral malignancies. From a practical perspective, such cutaneous manifestations might have an important diagnostic value if they are the sole expressions of otherwise asymptomatic carcinomas. The recognition of some typical paraneoplastic dermatologic disorders can lead to prompt diagnosis of the underlying GI malignancy, timely administration of therapy, and ultimately, better prognosis. In this review we will discuss the most common paraneoplastic dermatologic syndromes from the perspective of the practicing gastroenterologist (Table 1).

Abstract

Numerous dermatological disorders have been associated with underlining malignancies of the gastrointestinal (GI) tract. Such cutaneous manifestations might have an important diagnostic value if they are the sole expressions of otherwise asymptomatic carcinomas. The recognition of some typical paraneoplastic dermatologic disorders can lead to the prompt diagnosis of the underlying malignancy, timely administration of therapy, and ultimately, better prognosis. In this review we discuss the most common paraneoplastic dermatological syndromes from the perspective of the practicing gastroenterologist. We also outline a comprehensive practical approach for the evaluation for occult malignancy in patients presenting with cutaneous findings potentially associated with GI cancers.

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Key words: Paraneoplastic; Dermatological; Gastrointestinal; Cancer; Malignancy

Peer reviewer: Vincent W Yang, Professor and Director, 201 Whitehead Research Building, 615 Michael Street, Atlanta, GA 30322, United States

Dourmishev LA, Draganov PV. Paraneoplastic dermatological manifestation of gastrointestinal malignancies. *World J Gastroenterol* 2009; 15(35): 4372-4379 Available from: URL:

ACANTHOSIS NIGRICANS

Acanthosis nigricans (AN) is a classic example of a paraneoplastic dermatosis, and its frequent relation with GI tract malignancies was emphasized by Darier at the end of the 19th century^[2]. The disease initiates with symmetric skin hyperpigmentation in the axillary and inguinal folds, submammary region, around the mamilla, umbilical, and ano-genital regions. Later, the skin lesions can infiltrate and become slightly hyperkeratotic pigmented velvety plaques surrounded by acrochordons (Figure 1). The neck region is frequently

Table 1 Relationships between morphologic features of cutaneous paraneoplastic disorders and gastrointestinal malignancy

Dermatologic disorder	Cutaneous manifestations	Localization	Associated gastrointestinal malignancy
Acanthosis nigricans	Pigmented papillomatous plaques	Axillar and inguinal folds, submammary and mamilla region, ano-genital regions	Gastric carcinoma; Colorectal carcinoma
Palmo-plantar keratoderma	Diffuse epidermal thickening with rugose appearance	Palms, fingers and soles	Gastric carcinoma; Hepatic metastases
Acrokeratosis paraneoplastica (Bazex syndrome)	Erythematous psoriasiform plaques	Hands, feet, ears, nose, elbows and knees	Squamous cell carcinoma of oropharynx/esophagus; Adenocarcinoma
Leser-Trélat syndrome	Multiple seborrheic keratoses	Trunk and extremities	Gastric carcinoma; Colorectal carcinoma; Esophageal carcinoma; Pancreatic carcinoma
Muir-Torre syndrome	Sebaceous adenomas; Sebaceous carcinomas; Keratoacanthomas	Head, trunk and extremities	Colorectal carcinoma; Colorectal adenomatous polyps
Paraneoplastic dermatomyositis	Periorbital heliotrope erythema and edema; Gottron papules; Gottron sign	Eyelids, upper cheeks, forehead; Phalangeal joints; Elbows, knees	Colorectal carcinoma; Gastric carcinoma; Hepatocellular carcinoma
Paraneoplastic pemphigus	Erosions; Vesicles and blisters, Erythematous to violaceous patches papules, plaques	Oral cavity; Head, trunk and extremities	Castleman's disease; Sarcoma; Adenocarcinoma; Squamous cell carcinoma
Peutz-Jeghers syndrome	Hyperpigmented macules	Buccal mucosa, gums; Tips of the fingers and toes	Multiple intestinal polyps; Colorectal carcinoma; Pancreatic carcinoma; Gastric carcinoma; Small bowel carcinoma
Cronkhite-Canada syndrome	Hyperpigmented macules; Nail plate separation, discoloration and atrophy; Alopecia	Hands, palms, arms, neck and face; Fingernails and toenails; Scalp	Hamartomatous polyps; Colon carcinoma; Gastric carcinoma
Paraneoplastic hypertrichosis lanuginosa acquisita	Lanugo hairs	Head, trunk and extremities	Colorectal carcinoma; Pancreatic carcinoma

affected in childhood. Similar pigmented papillomatous lesions can be observed on the mucous membranes of the oral cavity, nasal, and laryngeal mucosa, and vulva. The areola of the nipple can also be affected. Nails are brittle and hyperkeratotic, and leukonychia have been reported. Noncicatrical alopecia in the axilla and pubic regions is also possible.

AN can be idiopathic when related to endocrine disorders (obesity, insulin resistance, or overt diabetes mellitus), and only in some cases is it associated with malignancy^[3]. Only 20 cases of oral malignant AN were reported in the English language literature from 1968 to 2002^[4]. In malignancy-associated acanthosis nigricans the lesions are often more extensive and severe than when the cause is benign, and skin irritation can be a prominent and distressing symptom. Moreover, the skin lesions usually appear before the onset of any other GI symptoms^[5]. Malignancy-associated acanthosis nigricans is frequently seen with GI cancers^[6-8].

Anderson *et al*^[8] presented a 66-year-old male patient with poorly differentiated, metastatic gastric adenocarcinoma, who complained of severe pruritus and developed severe AN on the chest and nipples. The patient was treated with 5-fluorouracil, cisplatin and epirubicin chemotherapy resulting in dramatic improvement of the dermatological disorder and quality of life; however, the patient died six months later due to lymphangitic carcinomatosis. The authors assumed that factors affecting epidermal proliferation are involved because of the reduction in papillomatosis and increase in cutaneous pigmentation after chemotherapy administration^[8]. Pentenero *et al*^[4] reported a case of a 53-year-old man with gastric adenocarcinoma who suddenly developed hyperkeratotic, verrucous, slightly pigmented, brownish papules in the axillae and thickened

mucosa with a velvety and papillomatous surface, without hyperpigmentation on the lips, buccal mucosa and palate. Skin biopsies, performed from the buccal mucosa and the axilla, confirmed the diagnosis of AN with mucosal localization^[4].

Palmo-plantar keratoderma or “tripe palms” is a recognized feature of acanthosis nigricans and presents with epidermal thickening with a rugose appearance and broadened rete ridges bounded by deep sulci of the palms and fingers^[4,9] (Figure 2). Breathnach and Wells describe five patients with palmo-plantar keratoderma and acanthosis nigricans associated with gastric adenocarcinoma and in two patients they found squamous cell carcinoma^[9].

The exact pathophysiological mechanism of the paraneoplastic AN has not been well defined, but it could be related to cancer byproducts. Transforming growth factor alpha, structurally related to epidermal growth factor (EGF), has been considered as possible causative agent^[3,4].

The prognosis of the malignancy associated acanthosis nigricans tends to be poor because the underlying malignancy appears to behave aggressively. The average survival time of patients with signs of paraneoplastic AN is two years, although cases in which patients have survived for more than 10 years have been reported. Importantly, older patients with new onset AN usually have associated internal malignancy and therefore targeted investigation should be carried out (see below).

ACROKERATOSIS PARANEOPLASTICA (BAZEX SYNDROME)

The first patient with this entity was described by

Bazex *et al*^[10] in 1965, as “paraneoplastic syndrome with hyperkeratosis of the extremities”. Clinical manifestations include erythematous or livid squamous plaques resembling psoriasis, which are symmetrically distributed in acral regions and affect mainly the hands, feet, ears, nose, elbows and knees^[11]. Skin biopsies usually reveal nonspecific findings, including hyperkeratosis, acanthosis, parakeratosis, vacuolar degeneration, pigment incontinence, and a perivascular infiltrate of lymphocytes and histiocytes and occasionally dyskeratotic keratinocytes^[11].

Bazex syndrome predominates in males over 40 years and is most commonly associated with squamous cell carcinoma (SCC) of the upper bronchial and GI tracts^[11,12]. Adenocarcinomas of the stomach^[13], colon^[14], biliary system and hepatocellular carcinoma^[11], are also described in the literature. In a retrospective study of the primary location of malignancies in 113 patients with Bazex syndrome, Bologna *et al*^[15] reported the following results: oropharynx and larynx (48.6%), lung (17.7%), unknown location (16%), esophagus (10.6%, one of them with an associated pyriform sinus carcinoma), and isolated cases in the prostate, liver, stomach, uterus, vulva, and bone marrow.

LESER-TRÉLAT SYNDROME

Ulysse Trélat (1884) and Edmund Leser (1901)^[16], both surgeons, were the first to propose that multiple seborrheic keratoses could be associated with internal malignancy. Denucé, who was a resident of Trélat, wrote in 1899 that the professor had often stressed the symptom of “Trélat’s nevi” associated with deep tumors of the abdomen and pelvis in his lectures^[17]. The indication of Leser-Trélat or Leser-Trélat syndrome (LTS) is an eruptive appearance of, or at least a sudden increase in, the number or size of multiple seborrheic keratoses in association with an internal malignancy^[18,19]. No evidence of dermatitis or erythroderma precedes the seborrheic keratoses appearance on the skin and pruritus is a leading symptom in about half of the cases^[20]. Seborrheic keratoses associated with malignancy show no clinical or histological differences compared to patients without neoplasia^[21]. The majority of patients with LTS have adenocarcinomas, most commonly of the stomach^[22,23], colon or rectum^[19,21,24-28], and less frequently carcinomas of esophagus^[29], duodenum^[30], pancreas^[31], gallbladder^[32] or hepatocellular carcinoma^[33].

We observed a 66-year-old man who presented with multiple flat, sharply demarcated, yellowish to brown lesions with a verrucous surface located mainly on the trunk arms and thighs, which were clinically very suggestive of seborrheic keratoses (Figure 3). The patient developed fever and night sweats. A barium enema detected a tumor in the rectum. Computed tomography (CT) showed no evidence of distant metastasis. The rectal lesion was removed surgically, and histology showed moderately differentiated rectal adenocarcinoma involving muscularis propria with no evidence of perirectal lymph node involvement. However, three months after

the operation, seven lung metastases were visualized by CT. During courses of 5-fluorouracil, oxaliplatin and capecitabine chemotherapy, the cutaneous lesions markedly diminished but did not completely disappear^[19]. We found 14 other published cases of Leser-Trélat syndrome associated with colorectal adenocarcinoma in the literature from 1972 to 2004^[19].

About two-thirds of patients with LTS can have other paraneoplastic disorders, the most frequent of which is acanthosis nigricans, which accounts for one third of these cases^[34]. Moreover, Andreev *et al*^[35] considered that Leser-Trélat syndrome represents a particular clinical variant of acanthosis nigricans. The pathogenesis of LTS remains unclear. As in malignant acanthosis nigricans, an increased epidermal staining for the transforming growth factor alpha receptor has been observed^[36].

MUIR-TORRE SYNDROME

In 1967 Muir *et al*^[37], and later Torre^[38], reported patients with multiple sebaceous neoplasms and visceral malignancies. Muir-Torre syndrome (MTS) is defined by the development of internal malignancy, most commonly colon cancer, in association with sebaceous adenomas and epitheliomas, sebaceous carcinomas and multiple or early-onset keratoacanthomas^[39]. The syndrome has autosomal dominant inheritance, and is considered as a subtype of hereditary nonpolyposis colorectal cancer syndrome (HNPCCS). Sixty percent of patients with MTS have a strong family history of visceral malignancy and show clinicopathological overlap with HNPCCS^[40]. The pathogenesis includes mutations in the DNA mismatch repair genes (MLH-1 or MSH-2)^[39]. HNPCC and MTS usually result from an inherited defect in one allele of either MLH1 or MSH2^[41]; however, Muir-Torre syndrome more commonly involves a mutation of MSH2, while HNPCC shows a roughly equal prevalence of MLH1 and MSH2^[42].

Sebaceous gland tumors in MTS include sebaceous adenoma, sebaceous carcinoma and keratoacanthoma with sebaceous differentiation^[40]. Sebaceous hyperplasia and ectopic sebaceous glands do not appear to be significant markers of the syndrome. Sebaceous adenoma is believed to be the most specific lesion of Muir-Torre syndrome and these can sometimes show cystic change or keratoacanthoma-like architecture^[42]. Between 24%-30% of patients with MTS have sebaceous carcinomas^[39,43].

Cancers of the GI tract comprise more than 60% of the visceral malignancies in MTS, and colorectal cancer is the predominant neoplasm^[39,44]. Tumors are located predominantly in the proximal colon (cecum to splenic flexure), in contrast to the general populace, whose colorectal tumors are usually distal to the splenic flexure^[44,45]. An association with colorectal adenomatous polyps was observed in 26% of patients with MTS^[45].

Recently, a 54-year-old Japanese man with MTS who developed a sebaceous carcinoma and concurrently adenocarcinoma of the colon was reported^[46]. A novel germline mutation of the *MSH2* gene with duplication of the genomic region involving exon 7 was identified^[46].

Multiple sebaceous tumors or sebaceous tumors



Figure 1 Hyperpigmented papillomatous plaques in left axial of a male patient with acanthosis nigricans.



Figure 2 "Tripe palms" keratoderma on the palms of the same patient.

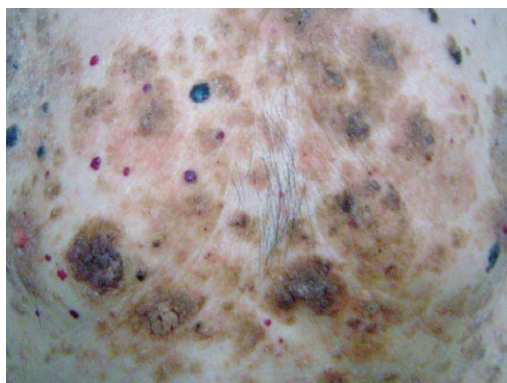


Figure 3 Multiple eruptive seborrheic keratoses on the trunk of a 66-year-old patient with rectal adenocarcinoma.

occurring before the age of 50 years are strong indicators of Muir-Torre syndrome. Moreover, MTS should be suspected in all cases when sebaceous gland tumors or multiple keratoacanthomas have been diagnosed. Some authors suggest that all patients with sebaceous-gland neoplasms should be screened for MTS in contrast to those with keratoacanthomas because they are less likely to be markers of MTS^[47]. All patients with MTS should undergo a colonoscopy to detect colorectal neoplasms.

PARANEOPLASTIC DERMATOMYOSITIS

The first report of paraneoplastic dermatomyositis (PDM) was by Stertz^[48], who in 1916 observed a patient with proximal muscle weakness, eyelid changes, muscle



Figure 4 Periorbital heliotrope erythema and edema in a patient with paraneoplastic dermatomyositis.



Figure 5 Violaceous plaques with erosions on the trunk of a patient with paraneoplastic pemphigus.

biopsy evidence of myositis, associated with gastric cancer. Dermatomyositis (DM) is a rare idiopathic inflammatory myopathy that presents clinically with proximal muscle weakness and characteristic cutaneous manifestations^[49]. Skin lesions can be classified as pathognomonic, characteristic, and compatible with DM^[50]. The more specific or pathognomonic manifestations of DM are the periorbital heliotrope rash, and erythematous maculopapular lesions covering bony prominences, described by Gottron in 1931 and named after him as Gottron papules and Gottron sign^[49,50]. The "Heliotrope" rash presents as a red to purple-colored confluent, macular erythema involving symmetrically the eyelids, upper cheeks, forehead, and the temples and is often associated with edema of the eyelids and periorbital tissues (Figure 4). Other characteristic skin lesions include: shawl sign (a violaceous erythema disposed in a "shawl" distribution over the neck, upper back, and shoulders), photosensitive poikiloderma, diffuse redness and shininess of the nail folds, "mechanic's hands" hyperkeratosis, cutaneous calcinosis, and scalp erythema^[49,51]. Other contemporary criteria for diagnosis of DM include the appearance of symmetric proximal muscle weakness, an elevation of serum skeletal-muscle enzymes levels, abnormal electromyography, features of inflammatory infiltration in muscle biopsy, autoantibodies against RNA synthetase antigens (Jo-1, PL-7, PL-12, and OJ) or against Mi2 or SRP antigens in patients' sera^[52].

The increased risk for developing cancer in DM patients has been convincingly demonstrated in several studies from Sweden^[53], Australia^[54], and Scotland^[55]. The malignancy can precede, occur concurrently with, or follow the diagnosis of dermatomyositis. In patients with dermatomyositis several predictive factors for the presence of underlying malignancy have been described including: age over 50 years, male gender, the presence of cutaneous necroses and ulcers, increased erythrocyte sedimentation rate and C-reactive protein, and highly elevated or normal serum creatinine kinase^[56-58].

Different types of tumors are observed in dermatomyositis patients. Adenocarcinomas are the most common and, in general, the frequency of each cancer type corresponds to those in the general population. In a study of 750 patients with polymyositis or dermatomyositis in Sweden, Sigurgeirsson *et al*^[53] reported that the colon (including the rectum) and the lungs were the most frequent cancer sites. Hatada *et al*^[59] mentioned that in Japan, gastric cancer was the most frequent malignant disease (25.4%) among patients with dermatomyositis. In South-Eastern Asia, the incidence of nasopharyngeal carcinoma is elevated in the male population with or without dermatomyositis^[60]. In another retrospective study, 12 patients with internal malignancy were described among group of 64 patients with polymyositis and 28 patients with dermatomyositis^[61]. Of those 12 patients, four had GI tract malignancies (two male patients, 74- and 75-year-old respectively had gastric carcinoma; another 51-year-old female had pharyngeal carcinoma and one female had pancreatic cancer)^[61]. From 1941 to 1988 only one case of paraneoplastic dermatomyositis associated with gastric cancer has been reported in Bulgaria^[62]. Since then, twelve additional PDM cases have been documented^[63]. Two of these 12 cases in our retrospective study of patients with PDM, had GI malignancy; a 54-year-old female who had rectal adenocarcinoma and a 64-year-old patient with pancreatic cancer^[59].

Many authors have stressed the importance of early investigation targeted at detecting malignancy in dermatomyositis. A detailed search for malignancy should be carried out during the first three to five years after the disease onset, however the optimal cancer-screening regimen necessary for patients with a recently diagnosed myositis remains uncertain.

PARANEOPLASTIC PEMPHIGUS

Paraneoplastic pemphigus (PNP) is an autoimmune bullous disease characterized by the production of various autoantibodies against plakin proteins in keratinocytes. The disease was described by Anhalt *et al*^[64] in 1990. Patients with paraneoplastic pemphigus have to fulfill at least four of the following five criteria: polymorphic eruption on skin and mucous membranes, histopathological features that include intraepidermal acantholysis and dyskeratosis with vacuolar changes, intraepidermal and/or basal membrane zone deposition of IgG and C3 on direct immunofluorescence (IF), and serum autoantibodies against 250, 230, 210 and 190 kDa

antigens (mainly desmoglein 1 and 3, periplakin and envoplakin) in immunoblotting^[64,65].

The mucosal involvement includes erosions of the oral cavity, conjunctiva, pharynx, anogenital areas and even the GI mucosa^[65,66]. Cutaneous manifestations of PNP are heterogeneous, and include vesicles and blisters, erythematous to violaceous maculae, papules, plaques, and even erythroderma (Figure 5). The morphology of the lesions resembles a variety of dermatological diseases, including pemphigus vulgaris, bullous pemphigoid, erythema multiforme, and lichen planus^[64,66,67].

Paraneoplastic pemphigus cases have been reported more often in patients with a history of lymphomas, chronic lymphocytic leukemia, poorly differentiated sarcoma and Castleman's disease. In a retrospective study of 163 cases with paraneoplastic pemphigus reported between 1990 and 2003 carcinomas were diagnosed in 14 cases; consisting of adenocarcinoma in seven, squamous cell carcinoma in two, multiple basal cell carcinomas in one, and bronchogenic carcinoma also in one patient^[68]. Ostezan *et al*^[69] presented a patient with severe mucocutaneous involvement of PNP associated with hepatocellular carcinoma. However, PNP is not always accompanied by neoplasia^[69], suggesting that other factors such as drugs and inflammatory diseases can trigger autoantibody formation related to PNP in the absence of a neoplasm^[70].

In 2001, Nguyen *et al*^[71] proposed a new term for this disease—"paraneoplastic autoimmune multiorgan syndrome" (PAMS), which, according to them, reflects the presence of target antigens and the pathologic damage frequently occurring in multiple organ systems including lung, kidney, and muscle.

PEUTZ-JEGHERS SYNDROME (HEREDITARY INTESTINAL POLYPOSIS SYNDROME)

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder described in 1921 by Peutz, who noted a relationship between the intestinal hamartomatous polyps and mucocutaneous macules in a Dutch family^[72]. The syndrome is caused by mutations in *STK11/LKB1*, serine/threonine kinase 11 genes, located on band 19p13.3^[73].

Cutaneous lesions consist of 1-5 mm diameter hyperpigmented macules, irregularly distributed over the buccal mucosa, gums, hard palate and lips, mainly on the lower lip. Lentigines usually appear during early childhood, and have a tendency to increase in size^[74]. Larger maculae (melanosis) are rarely seen over the back of the hands, the tips of the fingers and toes, and over the palms and soles^[75].

GI tract manifestations include numerous intestinal polyps in the jejunum, ileum and less frequently in the colon, rectum, stomach and duodenum that are typical hamartomas^[75]. Histology reveals pseudo invasion of the epithelial cells, forming benign glands surrounded by smooth muscle. About half of PJS patients die from cancer before the age of 60. The cumulative risk for

developing GI tract associated cancers in patients with PJS aged 15-64 years varies according to the localization from 0.5% for the esophagus, 29% for the stomach, 13% for the small intestine, 36% for the pancreas to 39% for the colon and rectum^[76].

CRONKHITE-CANADA SYNDROME

In 1955 Cronkhite *et al*^[77] described two women with acquired generalized GI polyps with features of hamartomatous polyps and epidermal changes.

Cutaneous lesions in patients with Cronkhite-Canada syndrome (CCS) include hyperpigmented macules ranging from a few millimeters to 10 cm in diameter, localized on the dorsal surface of the hands, palms, arms, neck, face and scalp^[74]. Fingernails and toenails have discoloration, atrophy, nail plate separation and shedding. Alopecia occurs rapidly and in some cases leads to total hair loss^[74,78].

GI lesions in CCS are hamartomatous polyps histologically revealing pseudopolypoid-inflammatory changes. Although considered a benign condition, in 1967, Gomes da Cruz^[79] reported an association of this syndrome with a cancer of the cecum and descending colon. Among 387 cases published in literature by the end of 2002, Cronkhite-Canada syndrome associated with colon cancer has been reported in 31 (8%) cases, and other 19 CCS patients (5%) had concomitant gastric cancer^[80].

Some authors propose phenotypic overlap between the features of CCS, and Peutz-Jegher syndrome, particular in the morphology of cutaneous and intestinal lesions^[81,82]. In contrast to PJS, however, no underlying genetic mechanism has been found so far in Cronkhite-Canada syndrome.

PARANEOPLASTIC HYPERTRICHOSIS LANUGINOSA ACQUISITA

In 1865, Turner reported a woman with breast cancer whose face and body in two or three weeks became covered with a thick crop of short and white downy hair^[83]. Lanugo hairs are long, thin and unpigmented, affecting the face and spreading in a caudal direction on the entire integument. Paraneoplastic hypertrichosis lanuginosa acquisita (PHLA) is predominant in women, and colorectal carcinoma is the most frequently associated malignancy, followed by lung and breast cancer^[84-86]. Patients usually have metastatic disease at the time of diagnosis and a poor prognosis^[86]. PHLA is associated with other paraneoplastic disorders such as acanthosis nigricans (which supports the hypothesis of tumor-produced cytokines stimulation over hair follicles).

EVALUATION OF PATIENTS WITH DERMATOLOGICAL MANIFESTATIONS ASSOCIATED WITH GI MALIGNANCY

The described dermatologic syndromes are not always

associated with malignancy, but in many cases can be idiopathic. Therefore the practicing physician is confronted with the great challenge of carrying out a comprehensive search for underlying cancer in a systematic and cost effective manner. There are no universally accepted algorithms for the scope of the evaluation in such a patient, but, in general, the work-up should be guided by the following principles: (1) Initial thorough medical history and physical examination (including rectal exam in both sexes, pelvic exam in women and prostate exam in men) followed by basic laboratory testing (complete blood count, erythrocyte sedimentation rate, serum chemistry panel, and urinalysis). At that point, targeted evaluation of any specific patient symptoms or laboratory abnormalities should be pursued (e.g. iron deficiency anemia should be investigated with colonoscopy and upper endoscopy); (2) If the patient is asymptomatic or has no risk factors for a particular type of cancer, age-appropriate cancer screening tests should be carried out (e.g. colonoscopy in patients older than 50); and (3) Limited additional testing, such as CT scan of the chest, abdomen, and pelvis, are recommended for patients with significantly increased risk of malignancy (e.g. smoking, positive family history for cancer). The role of screening with serum prostate specific antigen (PSA), CA125, and CA19-9 has not been well determined.

CONCLUSION

A wide variety of dermatologic signs have been associated with GI malignancy. Cutaneous manifestations might develop before the GI neoplasm is recognized and their prompt recognition can significantly aid in the diagnosis. Once one of the cutaneous lesions associated with GI cancer is diagnosed, an evaluation for underlying malignancy should be undertaken. The evaluation for cancer should start with thorough medical history, physical examination, and basic laboratory testing. In asymptomatic patients, age-appropriate cancer screening tests should be carried out. Targeted additional testing, such as CT scans of the chest, abdomen, and pelvis, is recommended for patients with significantly increased risk of malignancy.

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S- Editor Li LF L- Editor Stewart GJ E- Editor Lin YP



TOPIC HIGHLIGHT

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Squamous cell cancer of the rectum

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Received: April 14, 2009 Revised: May 12, 2009

Accepted: May 19, 2009

Published online: September 21, 2009

Key words: Rectal cancer; Squamous cell cancer; Endoscopic ultrasound; Chemoradiation; Surgery

Peer reviewer: Francis Seow-Choen, Professor, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

Dyson T, Draganov PV. Squamous cell cancer of the rectum. *World J Gastroenterol* 2009; 15(35): 4380-4386 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4380.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4380>

Abstract

Squamous cell carcinoma of the rectum is a rare malignancy. It appears to be associated with chronic inflammatory conditions and infections. The clear association seen between Human Papilloma Virus and various squamous cancers has not been firmly established for the squamous cell cancer of the rectum. The presentation is nonspecific and patients tend to present with advanced stage disease. Diagnosis relies on endoscopic examination with biopsy of the lesion. Distinction from squamous cell cancer of the anus can be difficult, but can be facilitated by immunohistochemical staining for cytokeratins. Staging of the cancer with endoscopic ultrasound and computed tomography provides essential information on prognosis and can guide therapy. At present, surgery remains the main therapeutic option; however recent advances have made chemoradiation a valuable therapeutic addition. Squamous cell carcinoma of the rectum is a distinct entity and it is of crucial importance for the practicing Gastroenterologist to be thoroughly familiar with this disease. Compared to adenocarcinoma of the rectum and squamous cell cancer of the anal canal, squamous cell carcinoma of the rectum has different epidemiology, etiology, pathogenesis, and prognosis but, most importantly, requires a different therapeutic approach. This review will examine and summarize the available information regarding this disease from the perspective of the practicing gastroenterologist.

INTRODUCTION

Squamous cell carcinoma of the gastrointestinal (GI) tract is a rare malignancy. When encountered, it usually involves the esophagus or the anal canal. Occasionally it can be associated with a GI tract fistula, lined by squamous mucosa. Squamous cell carcinoma of the rectum is extremely unusual and unlike squamous cell carcinoma of the esophagus and anal canal, little is known about the etiology, prognosis, and optimal treatment. This review will examine and summarize the available information regarding this disease from the perspective of the practicing gastroenterologist.

EPIDEMIOLOGY

Squamous cell carcinoma of the rectum is a rare entity and only case reports and relatively small case series have been published^[1-24] (Table 1). Schmidtman^[25] in 1919 described the first case of squamous cell carcinoma of the large intestine localized to the cecum. It was not until 1933 that the first case involving the rectum was subsequently described by Raiford^[1]. Since that time, 73 cases have been reported in the English language literature. Based on a review of this literature, the incidence of the disease is approximately 0.10 to 0.25 per 1000 colorectal cancers^[22,26-29]. Of all cases of squamous cell carcinoma of the large intestine, the rectum is the most frequent location for the disease, followed by the right colon^[29]. This is likely an underestimation due to reporting bias and histologic variability.

Given the rarity of the disease, strong epidemiological data regarding patient demographics, risk factors, natural history and optimal treatment is lacking. However, there have been several retrospective reviews that have tried to

Table 1 Reported cases of squamous cell carcinoma of the rectum

Study	Age	M/F	Surgery	Adjuvant	Outcome
Raiford ^[1] (1933)	43	F	Surgery		Dead at 17 mo
Catell <i>et al</i> ^[2] (1943)	63	M	LAR		Alive at 3.5 yr
Wiener <i>et al</i> ^[3] (1962)	52	F	APR		Dead at 1 yr
Cabrera <i>et al</i> ^[4] (1967)	62	F	APR		NR
	50	F			NR
Minkowitz ^[5] (1967)	49	F	Proctocolectomy		Dead at 5 mo
Williams <i>et al</i> ^[6] (1979)	45	M	APR		Dead at 9 mo
Vezeridis <i>et al</i> ^[7] (1983)	56	M	APR		IO death
	44	M	APR		Dead at 9 d
	61	F		EC	Dead at 4 mo
	66	F		5-FU & XRT	Dead at 15 mo
	62	F	APR	Bleo, Vin, Mtx-post	Dead at 13 mo
Lafreniere <i>et al</i> ^[8] (1985)	60	M	TAE	5FU/MC XRT-post	Alive at 2 yr
Pigott <i>et al</i> ^[9] (1987)	60	F	APR	XRT-post	Alive at 13 mo
Woods ^[10] (1987)	57	F	APR		Dead at 3 mo
Prener <i>et al</i> ^[11] (1988)	43	F	APR		Dead at 1 yr
	77	F	Polypectomy		Dead at 3 yr
	55	F	APR		Alive at 3 yr
	55	M	APR	XRT-post	Dead at 3 mo
	53	M	APR		Dead at 1 yr
Schneider <i>et al</i> ^[12] (1992)	44	M		5FU/MC & XRT	NR
	69	F	TAE	5FU/MC&XRT-post	Alive at 6 mo
Martinez-Gonzalez <i>et al</i> ^[13] (1996)	40	M	LAR	5FU & XRT-pre	Alive at 18 mo
Copur <i>et al</i> ^[14] (2001)	54	M	APR	Chemo& XRT-post	NR
Frizelle <i>et al</i> ^[15] (2001)			9 cases		
Sotlar <i>et al</i> ^[16] (2001)	87	M	LAR	5FU/MC & XRT	Dead at 20 mo
Gelas <i>et al</i> ^[17] (2002)	47	F	APR	XRT-pre	Alive at 16 yr
	63	M	APR	5FU/Cis & XRT-IO	Dead at 14 mo
	70	F	APR	XRT	Dead at 18 mo
	93	M	none	XRT	Dead at 4 mo
	45	F	LAR	5FU/Cis-pre & XRT/5FU/FA-post	Alive at 6 mo
	43	F	LAR	5FU/Cis & XRT	Alive at 2 yr
Anagnostopoulos <i>et al</i> ^[18] (2005)	75	M	APR	5FU/Cis-post	Alive at 14 mo
Lam <i>et al</i> ^[19] (2006)	44	F	LAR	XRT-pre	NR
Theodosopoulos <i>et al</i> ^[20] (2006)	39	F	APR	5FU/MC-pre/post & XRT	Alive at 18 mo
Pikarsky <i>et al</i> ^[21] (2006)	57	F		5FU/MC & XRT	Alive at 7 yr
Nahas <i>et al</i> ^[22] (2007)	58	F10/M2		Chemo/XRT-pre	Alive at 2.6 yr
				Chemo/XRT-pre	
			TAE	Chemo/XRT-pre	
			TAE	Chemo/XRT-pre	
			APR	Chemo/XRT-pre	
			LAR	Chemo/XRT-pre	
			APR	Chemo/XRT-pre	
			LAR	Chemo/XRT-pre	
			LAR	Chemo/XRT-pre	
			APR	Chem/XRT-post	
			TAE	Chem/XRT-post	
				Chemo-pre	
Kong <i>et al</i> ^[23] (2007)	48	F	TAE	5FU/MC & XRT-post	Alive at 3 yr
	53	F		Oxal, xelo, avastin, carbo, gemzar	
Clark <i>et al</i> ^[24] (2008)	75	M		5FU/Cis & XRT	Alive at 20 mo
	71	F		5FU/MC & XRT	Alive at 31 mo
	42	F		5FU/MC/Cis & XRT	Alive at 13 mo
	70	M		5FU/MC & XRT	Alive at 14 mo
	55	F	LAR	5FU/Cis & XRT	Alive at 19 mo
	45	F		Capecitabine/Cis & XRT	Alive at 23 mo
	71	F		5FU/Cis & XRT	Alive at 5 mo
Rasheed <i>et al</i> ^[53] (2009)	55	F		5FU/MC & XRT	Alive at 11 yr
	50	M		5FU/MC & XRT	Alive at 7 yr
	69	F		5FU/Cis & XRT	Alive at 4 yr
	61	M	APR	5FU/Cis & XRT	Alive at 4 yr
	58	M	APR	5FU/Cis & XRT	Alive at 2 yr
	41	F		5FU/Cis & XRT	Alive at 2 yr

NR: Not reported; F: Female; M: Male; LAR: Low anterior resection; APR: Abdominoperineal resection; TAE: Transanal excision; Chemo: Chemotherapy; XRT: Radiation therapy; IO: Intraoperative; 5FU: 5-fluorouracil; MC: Mitomycin C; Bleo: Bleomycin; Vin: Vincristine; Cis: Cisplatin; EC: Ethyl [bis (2,2-Dimethyl-L-aziridinyl) Phosphiny] carbamate; MTX: Methotrexate; Oxal: Oxaliplatin; Xelo: Xeloda; Carbo: Carboplatin; FA: Folic acid.

provide a framework for understanding this disease.

Squamous cell carcinoma of the rectum appears to affect individuals between the ages of 39 to 93 years old, with a mean age of 57 years. The disease tends to occur more frequently in women than in men. A review of available reports shows that 66% of cases occurred in women and 34% in men. Patients often present with advanced disease, Dukes C or Stage III^[8]. This might be due to a reporting bias based on the fact that most of these case reports come from tertiary care centers. There is no geographic or ethnic predilection that has been established for this disease, but it is interesting to note that Mel'nikov *et al*^[30] reported 107 cases of squamous cell cancer of the rectum in Russia in one center alone. The details of the study are unavailable, and it is unclear why this population would have such a seemingly high incidence of this malignancy. One plausible explanation is that some cases of anal squamous cell carcinoma might have been misclassified as originating in the rectum.

While no clear set of risk factors can be established, several associations have been observed. Some case reports have found squamous cell carcinoma in association with inflammatory processes involving the colon and rectum. Several cases have been reported in patients with ulcerative colitis^[27,31-33], while others have been found in association with infections including Schistosomiasis^[3], *Entamoeba histolytica*^[6] and human papilloma virus (HPV)^[16,23]. Adenocarcinoma has also been associated with squamous cell cancer of both the colon and rectum. Multiple studies have described either synchronous^[5,15,34] or metachronous lesions^[7,15,35,36] of adenocarcinoma occurring in the large intestine of patients with squamous cell cancer of the rectum. Additional coexisting diseases have been described including colonic duplication^[37,38], ovarian cancer^[39], prostate cancer^[7], endometrial cancer^[40], and breast cancer^[15].

PATHOGENESIS

With so few cases described, the exact mechanism behind the development of squamous cell cancer of the rectum remains elusive. Over the years, four hypotheses have developed regarding the pathophysiology of the disease. (1) Some suggest that inflammation or irritation, secondary to inflammatory bowel disease^[32,41,42], infection^[3,6,43] or radiation^[39,44], results in squamous metaplasia from which carcinoma develops^[10]; (2) Hicks and others^[45-47] have described the possibility of pluripotent stem cells capable of squamous differentiation. This theory is supported by the fact that squamous carcinoma is often found in the midst of poorly differentiated cells; (3) Michelassi *et al*^[48] have suggested that epithelial damage causes proliferation of uncommitted basal cells into squamous cells, which then undergo malignant transformation; (4) Histological reviews of adenocarcinomas have demonstrated areas of squamous differentiation, suggesting the possibility that these carcinomas may arise out of preexisting adenomas or adenocarcinomas^[6,46].

A clear association between HPV and squamous cell cancer of the anus has been established. Furthermore,

HPV has been associated with many squamous cell cancers including: skin, oral, vaginal, penile, esophageal and anal. The subclasses most commonly associated with virulent disease include HPV-16, 18, 31 and 33^[49]. The studies relating to HPV and squamous cell cancer of the rectum however, are few and varied in the methods of detection and the results obtained. Frizelle *et al*^[15] and Nahas *et al*^[22] evaluated a total of 11 squamous cell carcinoma patients for HPV using in situ hybridization and detected no HPV deoxyribonucleic acid (DNA) in any of the specimens. Audeau *et al*^[43] used immunohistochemistry to evaluate 20 patients with squamous cell cancer, adenosquamous cancer, and squamous metaplasia of the rectum, none of whom had detectable HPV. Polymerase chain reaction (PCR) has been considered the gold standard for detection of HPV. In two studies^[16,23], a total of four patients, have been evaluated *via* this methodology. All of those evaluated *via* PCR were HPV-16 positive. There have been concerns that the use of PCR for detection of HPV may lead to false positive results secondary to cross contamination. Indeed, two out of the above four patients with squamous cell cancer of the rectum evaluated *via* PCR were noted to have a history of cervical dysplasia, a condition known to be associated with HPV^[23]. At present we do not have firm evidence for a cause/effect relationship between infection with HPV and squamous cell cancer of the rectum.

DIAGNOSIS

Patients with squamous cell carcinoma of the rectum present with symptoms similar to those with adenocarcinoma of the rectum. The symptoms most frequently encountered are rectal bleeding, abdominal pain, change in bowel habits and weight loss^[8,43]. Patients usually experience symptoms for several weeks to months^[4,7].

Proctoscopy or colonoscopy with forceps biopsies of any visible abnormalities are the primary modalities for definitive diagnosis of rectal squamous cell carcinoma. The endoscopic appearance can range from a polyp to an ulcerated obstructing mass. Recent advances in endoscopy have been utilized to detect more subtle lesions. An example of this is narrow band imaging (NBI), a new endoscopic technique that highlights mucosa and underlying capillary networks. Fu *et al*^[42] describe the use of this technique in detection of squamous metaplasia in a patient with ulcerative colitis. If the metaplasia-dysplasia-carcinoma pathway is established as for other cancers, then NBI might play an important role in detecting premalignant lesions.

Occasionally, there can be difficulty either in distinguishing squamous cell cancer of the rectum from that of the anus or other small cell, poorly differentiated tumors on biopsy specimens. Immunohistochemistry has proved useful in characterizing these lesions. The most useful cytokeratins are CAM 5.2, AE1/AE3, and 34B12. CAM 5.2 helps to differentiate rectal from anal lesions. It characteristically stains rectal squamous

cell and adenocarcinoma but not anal squamous cell lesions^[22]. The cytokeratins AE1/AE3 stain positively for cells of squamous origin, helping to delineate less well-characterized lesions^[18,50].

In 1979, Williams *et al*^[6] established diagnostic criteria for squamous cell cancer involving the rectum which included: (1) absence of evidence of squamous cell carcinoma of any other part of the body, indicating possible metastasis; (2) careful proctoscopy to exclude proximal extension of anal squamous cell carcinoma; and (3) lack of a fistulous tract lined by squamous cells. The above criteria are required in conjunction with histology consistent with a squamous carcinoma^[51] without glandular differentiation^[52].

Squamous cell carcinoma antigen (SCC Ag) is a tumor marker that has been found to be associated with squamous carcinoma of the anus. Rasheed *et al*^[53], found SCC Ag to be elevated in three out of six patients with squamous cell carcinoma of the rectum. It was noted that after treatment with chemo- and/or radiation therapy, the SCC Ag normalized. In 2001, Comer *et al*^[27] found an elevated SCC Ag along with metastatic disease in a patient previously treated for rectal squamous cell cancer. Retreatment with chemotherapy and radiation resulted in an improvement in SCC Ag levels. Based on these observations, it appears that SCC Ag level is not suitable for initial diagnosis of squamous cell carcinoma of the rectum, but might be helpful to monitor disease response and progression.

Once the diagnosis of squamous cell carcinoma of the rectum has been established, the work-up should focus on staging of the tumor and evaluation for regional and distant metastasis. Trans-rectal endoscopic ultrasound (R-EUS) has become an integral part of the staging process of rectal cancer of all types. Accurate staging helps to determine appropriate surgical treatment (local excision *vs* radical resection) and the need for adjuvant therapy. The stage of the disease as determined by R-EUS is also predictive of patient survival. In squamous cell cancer of the anus, an increase in stage is associated with a decrease in five-year survival^[54]. While there are no large studies to support this for squamous cell cancer of the rectum, a review of available case reports supports a similar trend. R-EUS helps to determine the depth of tumor invasion. Superficial T1 lesions involve one or more of the first three echo layers (superficial mucosa, deep mucosa, and submucosa) of the rectal wall. Extension into the muscularis propria, the 4th echo layer, denotes a T2 lesion. Transmural invasion through the muscularis propria into the perirectal fat characterizes a T3 lesion, and T4 lesions involve invasion of surrounding organs^[55]. In addition to depth of tumor invasion, local nodal involvement can also be assessed. Size, echogenicity, shape and demarcation are felt to be helpful in distinguishing benign from malignant lymph nodes^[56], although this concept has yet to be validated in rectal cancer. Park *et al*^[57] evaluated the accuracy of R-EUS with fine needle aspiration (FNA) in the detection of rectal cancer. They found nodal involvement *via* only R-EUS in 33% of histologically confirmed nodes. When FNA was added

to the endosonographic examination, the accuracy of the test increased to 87%.

R-EUS should be performed in conjunction with computed tomography (CT) for complete staging. Several studies have compared the two modalities with regards to the staging of rectal cancer. The accuracy of R-EUS was superior to CT for evaluation of wall invasion (T staging), with a sensitivity of 67% to 93% *vs* 53% to 83%, respectively. R-EUS also outperformed CT for nodal staging, sensitivity 80% to 87% *vs* 57% to 72%^[55,58,59]. The current consensus is that R-EUS and CT are complimentary. R-EUS provides better tumor and local lymph node evaluation and CT has the advantage of detecting distant metastasis. Endorectal magnetic resonance imaging (MRI) has also been used for evaluation of local disease, allowing a larger area of view than R-EUS. In the limited studies available, endorectal MRI has yet to be shown to be superior to R-EUS^[60].

TREATMENT

The treatment paradigm for squamous cell carcinoma of the rectum primarily involves surgery. Secondary to the perceived aggressiveness and often late stage of presentation, surgery has always been thought to offer the best chance of a cure. Surgical techniques have been adopted from rectal adenocarcinoma treatment, despite the lack of randomized studies indicating the most appropriate therapy.

Surgery

Surgical options often depend upon tumor characteristics, such as size, location, depth of invasion, and local and distant metastasis. Additional considerations dictating surgical technique include the patient's body habitus and comorbidities^[61].

Local excision is appropriate in selected cases of stage T1 (invasion to the mucosa or submucosa) cancers or possibly stage T2 (invasion to the muscularis propria) lesions. There is growing evidence however, that T2 lesions particularly require close follow-up, as recurrence after local excision can be as high as 20%^[61]. In adenocarcinoma, low-risk lesions have been characterized as those that are well differentiated on histology and demonstrate no endovascular or lymphatic involvement^[62,63]. Clearly, this is in conjunction with no evidence of local or metastatic disease on R-EUS, CT or MRI^[61]. In 1985, Lafreniere *et al*^[8] reported a case of a 60-year-old successfully treated with local excision followed by chemotherapy and radiation. The patient was noted to be alive and well two years after the diagnosis.

For more advanced disease, low anterior resection (LAR) or abdominoperineal resection (APR) can be used for treatment, depending upon tumor location. For lesions in the proximal two-thirds of the rectum, LAR can be performed to remove the tumor-containing rectum. Preserving the anus allows for anastomosis of the descending colon with the distal rectum or anus thus maintaining rectal continuity. APR is performed not only for distal rectal lesions, but also for locally advanced

lesions, where disease free margins cannot be assured. APR allows for excision of anus and rectum as well as abdominal exploration for metastatic disease prior to creation of an ostomy. Overall, APR is associated with increased postoperative complications and poor long-term patient satisfaction^[64]. Review of the available case reports shows APRs have been performed more frequently; 2:1 compared to LAR. This is likely due to more advanced disease at diagnosis, which is frequently the case in squamous cell cancer of the rectum. Only seven of the 41 patients with adequately described surgical interventions underwent local resection^[8,12,22,23].

Chemoradiation therapy

Several studies have evaluated the utilization of chemoradiation therapy (CRT) for the treatment of squamous cell carcinoma of the rectum, as the primary therapy or in conjunction with surgery. Secondary to the infrequency in which cases are encountered, a standard protocol has yet to be established.

Several case reports have used CRT as the primary therapeutic intervention, usually in patients who are high-risk surgical candidates. The early studies^[7,13,29] showed suboptimal results with no significant change in mortality or maintenance of bowel continuity. Recently, Rasheed *et al*^[53] and Clark *et al*^[24] in two separate populations, evaluated the success of CRT in the treatment of squamous cell carcinoma of the rectum. Unlike previous cases^[7,13], these treatment regimens used primarily 5-fluorouracil based treatment along with either mitomycin-C or cisplatin. These were the same drug regimens that revolutionized the therapy squamous cell carcinoma of the anus^[65-68]. Around the same time, the benefits of radiotherapy combined with chemotherapy were also established, making CRT the treatment modality of choice for squamous malignancy involving the anus^[69-73]. Surgery was relegated to the role of salvage therapy^[54]. It was following this paradigm that CRT was adopted for treatment of squamous cell cancer of the rectum. Of the 13 patients treated with CRT by Rasheed and Clark, only three ended up with surgical resections. After histological evaluation, only one of the three resected specimens demonstrated residual tumor. The issue of which chemotherapeutic regimen to use in conjunction with radiotherapy, has not been evaluated in patients with squamous cell cancer of the rectum. Extrapolations can be made from trials in patients with anal canal cancer. A recent large prospective randomized study compared mitomycin C with cisplatin in patients with anal cancer^[74]. There was no difference in five-year survival, locoregional recurrence and distant metastasis between the two groups, but the patients receiving cisplatin had a higher rate of conversion to colostomy.

Endoscopy

Up to this point, endoscopy has not been reported as a treatment option for squamous cell cancer of the rectum, probably due to its rarity. Lee *et al*^[75] in 2000 reported a case of squamous cell metaplasia in the rectum successfully treated with argon plasma coagulation

Table 2 TMN classification for squamous cell rectal cancer

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
II A	T3	N0	M0
II B	T4	N0	M0
III A	T1-T2	N1	M0
III B	T3-T4	N1	M0
III C	Any T	N2	M0
IV	Any T	Any N	M1

(APC). The metaplasia-dysplasia-carcinoma relationship has not been established for squamous cell cancer of the rectum. If it is found to be similar to that of colon adenocarcinoma, then APC might provide a safe and easy alternative for the treatment of these lesions. Endoscopic mucosal resection (EMR) involves removal of lesions *via* the endoscopic resection of mucosa and submucosa. It has been used in the removal of superficial adenocarcinoma lesions, particularly in poor surgical candidates^[55]. When used in the treatment of adenocarcinoma, EMR shows a low-risk of recurrence and decreased need for surgery in appropriate populations^[54]. This might prove to be transferable technology to the treatment of rectal squamous cell carcinoma.

PROGNOSIS

The process of evaluating tumor spread is based on the TNM classification system used for squamous cell cancer of the anus: T = tumor invasion into the wall, N = nodal involvement, M = metastasis, (Table 2). From this information, the stage of disease can be established which is the most important predictor of prognosis^[76]. Frizelle's^[15] evaluation of 52 patients with squamous cell carcinoma of the colon revealed a prognosis similar to Stage I / II, node negative adenocarcinoma of the colon. However when nodal involvement occurred, squamous cell cancer demonstrated a worse prognosis stage for stage. Its overall prognosis is poor, secondary the tendency of rectal cancer to have local lymph node involvement compared to malignancy in other parts of the colon. This is in addition to the fact that it frequently presents at a late stage. Mixed squamous cell carcinoma and poor differentiation on histology have been found to be poor prognosticators^[15].

The overall five-year survival rate is 32%, with significant variation by stage; Duke B 50%, Duke C 33% and Duke D 0%. With the addition of adjuvant therapy, it is likely these numbers will improve. Studies have shown better outcomes with preoperative CRT. Nahas^[22] showed there was an increase in sphincter preserving surgeries from 67% to 71% when radiation therapy was added preoperatively.

CONCLUSION

Squamous cell carcinoma of the rectum is a rare malignancy. The available information for review is

clouded by a lack of uniformity in diagnosing staging and treating the disease. It is likely we will never be able to fully establish a firm relationship between it and adenocarcinoma of the colon, as found in other diseases, such as ulcerative colitis. Given the significant number of adenomas and adenocarcinomas reported in these patients, close follow-up for the development of colorectal cancer is warranted. It is likely that advances in technology and treatment of other diseases will also aid our success in management of this disease. Advances in CRT will likely supplant surgery as the primary intervention.

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Cytokeratin-18 fragments and biomarkers of the metabolic syndrome in nonalcoholic steatohepatitis

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Received: May 27, 2009 Revised: August 5, 2009

Accepted: August 12, 2009

Published online: September 21, 2009

Abstract

Nonalcoholic fatty liver disease (NAFLD) remains a leading cause of chronic liver disease. In the context of NAFLD, the presence of nonalcoholic steatohepatitis (NASH) portends an adverse prognosis with greater risk of liver fibrosis and cirrhosis. Although liver biopsy is the keystone of patient management in NAFLD, it is also increasingly clear that such evaluation has its limitations. The availability of biochemical markers of NAFLD and NASH has tremendous potential to radically alter management strategies for these conditions, as well as to monitor disease activity. Our article provides an overview of biomarker discovery and selection in the setting of NAFLD and highlights future directions in the field.

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Key words: Nonalcoholic steatohepatitis; Nonalcoholic fatty liver disease; Biomarkers; Liver biopsy

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Yilmaz Y, Kedrah AE, Ozdogan O. Cytokeratin-18 fragments and biomarkers of the metabolic syndrome in nonalcoholic steatohepatitis. *World J Gastroenterol* 2009; 15(35): 4387-4391 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4387.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4387>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease and its incidence is rising worldwide^[1-3]. The term NAFLD is used to describe a wide spectrum of fatty liver changes ranging from simple steatosis to nonalcoholic steatohepatitis (NASH)^[4]. Although simple steatosis usually follows a benign course, steatohepatitis is prone to progress to hepatic fibrosis and cirrhosis leading to excess morbidity and mortality^[5,6]. In this context, early identification of patients with NASH prior to the onset of advanced fibrosis would be helpful in guiding aggressive intervention. Liver biopsy remains the gold standard for obtaining an accurate diagnosis of NASH, as well as for differentiating this condition from simple steatosis. Unfortunately, biopsy is a costly and invasive diagnostic procedure, possibly subject to inter- and intra-observer variability^[7,8]. Thus it is becoming increasingly clear that although liver biopsy is the keystone of patient diagnosis and management in the setting of NAFLD, such evaluation has its limitations. Clinicians should therefore use additional tools to aid clinical assessment and to enhance their ability to identify the patient at risk for NASH and liver fibrosis. Biomarker use is one such tool to better identify subjects with NASH in the context of NAFLD, and hopefully to effectively prognosticate patients with this condition.

The development of NASH biomarkers can be theoretically achieved *via* two different strategies: the first strategy can be defined as “knowledge-based” (deductive method based on the current knowledge of NASH pathophysiology), while the second one is more “unbiased” (inductive strategy). The “knowledge-based” approach relies on a direct understanding of the pathophysiological processes that underlie the development of NASH as well as the evolution of its sequelae. It may consist of biochemical assays aiming to assess attractive novel candidate markers informed by the biology of the disease process. For instance, the understanding of the role played by hepatocyte apoptosis^[9] and insulin resistance^[10,11] in the pathobiology of liver injury has enabled the development of promising biomarkers of NASH, such as caspase-cleaved cytokeratin 18 fragments or numerous different adipokines. On the other hand, the “unbiased” approach involves the use of modern techniques including proteomics, metabolomics, and

bioinformatics that have allowed unbiased investigations of numerous putative markers that may be informative with regard to the various stages of NAFLD, including overt NASH and its sequelae^[12].

This article provides an overview of biomarker discovery and selection in the setting of NASH starting with some “knowledge-based” biomarkers. The list of biochemical markers provided in this review is not intended to be exhaustive; rather, a brief summary of some key biomarkers is provided.

BIOMARKERS OF HEPATOCYTE APOPTOSIS

To illustrate the opportunities and challenges related to the use of “knowledge-based” biomarkers of NASH, let us consider as an example biomarkers of hepatocyte apoptosis. Growing evidence has now accrued that hepatocellular apoptosis plays a central role in NAFLD progression^[9]. Interestingly, data from animal and human studies have suggested that apoptosis is prominent in NASH but not in simple steatosis^[13]. Cytokeratin 18 (CK-18) is the major intermediate filament protein in the liver and one of the most prominent substrates of caspases during hepatocyte apoptosis. Apoptotic cell death of hepatocytes is associated with release of caspase-cleaved CK-18 fragments into the bloodstream^[14], and several studies have demonstrated elevation of these molecules in the context of NAFLD. A pilot study by Wieckowska *et al*^[14] was the first to measure the levels of caspase-generated CK-18 fragments in patients with NAFLD. Results showed that caspase-cleaved CK-18 fragments were significantly higher in NAFLD patients than in controls, and that levels of these molecules correlated with the presence of liver fibrosis^[14]. In line with these results, the usefulness of CK-18 fragments for the diagnosis of NAFLD was subsequently confirmed in obese patients^[15], as well as in pediatric populations^[16]. Interestingly, caspase-generated CK-18 fragments released in NAFLD also serve as an indicator of hepatic inflammation. The increased apoptotic rate as a consequence of the hepatic inflammatory response is reflected by elevation of serum CK-18 fragments that may therefore distinguish NASH from simple steatosis^[17]. These results have been further confirmed; even in NAFLD patients with normal aminotransferase levels^[18].

Although CK-18 fragments can be used to identify patients with NASH and these molecules are currently being incorporated into multimarker schemes^[15], their routine use in patients with suspected NAFLD depends on the answers to several questions: Are the sensitivity and specificity of these tests similar to those obtained with liver biopsy? Where is the test being performed (Gastroenterology Department, Physician’s Office, Outpatient Unit)? What discrimination limits should be used? Is the test being performed for diagnosis or for prognosis? Answers to these questions are part of the scientific evaluation process that is critical for assessing

whether the information gained from CK-18 fragments is worth its cost to the healthcare system. Such answers require the performance of large studies to evaluate the relationship of caspase-cleaved CK-18 fragments with histological phenotypes of interest in the liver and, when applicable, the conduction of clinical trials to relate caspase-cleaved CK-18 fragments to disease risk and to therapeutic responses in patients with NAFLD.

BIOMARKERS OF THE METABOLIC SYNDROME

Although the pathogenesis of NAFLD is clearly multifactorial, this condition is currently considered as the hepatic manifestation of the metabolic syndrome. Comprehensive assessments of the role played by insulin resistance in the pathogenesis of NAFLD have also been published recently^[19-21]. The present review will not attempt to replicate these texts. On the other hand, we aim to provide here a concise overview of biomarkers of the metabolic syndrome (leptin, adiponectin, resistin, soluble RAGE) in the setting of NAFLD, including a display of the evidence linking them to NASH.

Leptin is a peptide hormone, mainly produced by adipocytes, that plays a central role in the regulation of body weight^[22]. In human liver, leptin has been shown to attenuate a number of insulin-induced activities ultimately resulting in insulin resistance^[23]. Furthermore, a proinflammatory and profibrogenic activity of leptin has been reported^[24]. Since leptin has been linked to metabolic abnormalities and insulin resistance, its potential role in NAFLD has been the focus of much investigation. Compared with controls, significantly higher levels of leptin have been observed in patients with NAFLD^[25,26] and in those with NASH^[27,28]. However, there are inconsistencies in published literature, with some authors showing an unaltered level of leptin in the setting of NAFLD^[29]. In addition, serum leptin levels showed no correlation with liver fibrosis^[30].

Adiponectin is an adipose tissue-specific protein whose receptors are expressed in several cell types including hepatocytes^[31]. Main functions of this molecule comprise of the downregulation of inflammatory processes, the promotion of lipolysis, and the prevention of lipid accumulation^[32]. Adiponectin is known to exert antifibrogenic and antiestrogenic effects in the liver^[33]. Hypoadiponectinemia has been suggested as contributing to insulin resistance and the metabolic syndrome^[34], and several studies have reported decreased adiponectin levels in patients with liver steatosis as compared with controls^[35-37]. Of note, some authors have also demonstrated that lower adiponectin levels are associated with more extensive necroinflammation in the setting of NAFLD and that they may contribute to the development of NASH^[38,39]. However, controversy surrounds the role of decreased adiponectin level as a reliable laboratory marker of NASH^[40].

Resistin is a 10 kDa protein of 94 amino acids highly expressed in the adipose tissue. It is a major determinant of hepatic insulin resistance induced by high-fat diet in animal models^[41]. Although human data regarding the role of this adipokine in insulin sensitivity and the metabolic syndrome are controversial^[42], preliminary evidence seems to suggest a potential role of this adipokine in the pathogenesis of NAFLD. Pagano *et al*^[43] have initially shown that patients with NAFLD are characterized by higher serum resistin levels in association with the NASH score, an index that takes into account necrosis, inflammation, and fibrosis in liver biopsies. However, no correlation was found between insulin resistance and hepatic steatosis score^[43]. These findings were recently replicated by Jiang *et al*^[44], who showed that serum resistin levels were significantly elevated in patients with NAFLD compared to controls. In contrast, Cho *et al*^[40] failed to show altered levels of this molecule in Korean male patients.

Besides classical adipokines, levels of soluble receptor for advanced glycation endproducts (sRAGE) have been recently linked to insulin resistance and several components of the metabolic syndrome^[45]. Recently, Yilmaz *et al*^[46] have investigated concentrations of sRAGE across the spectrum of NAFLD. Levels of sRAGE were significantly lower in patients with definite NASH and borderline NASH compared to controls. Interestingly, levels of sRAGE were significantly and inversely correlated with serum aminotransferase, indicating that lower concentrations of sRAGE are associated with the most severe forms of NAFLD^[46].

Altogether, our investigations indicate that a flurry of case-control studies relating biomarkers of the metabolic syndrome to NAFLD and NASH have been conducted in the past decade. Most of the available data are on adipokines, and much less is known about soluble RAGE. Of note, most studies to date also have been carried out in groups of people of Caucasian ancestry, and there are few data on African populations or African Americans. Furthermore, matching of cases and controls limits comparisons across sex and age demographics. Finally, the use of different assays across the studies also makes it difficult to define cut-off points. Additional larger studies of more diverse populations, including a full range of potential confounding variables, would be helpful at this juncture.

MISCELLANEOUS “KNOWLEDGE-BASED” BIOMARKERS

By using the “knowledge-based” approach, numerous other potential biomarkers of NAFLD and NASH have been explored in pilot cross-sectional studies. Angiopoietin-like protein 3 (ANGPTL3) is a liver-derived plasma protein that modulates plasma triglyceride clearance^[47], which has been recently investigated in the setting of NAFLD^[48]. Levels of ANGPTL3 have been found to be higher in patients with NASH than in those with simple fatty liver. Nonetheless, no association with

histological staging and pathological characteristics of NAFLD was seen^[48]. Pentraxin 3 (PTX3) is an acute-phase reactant that reflects the tissue inflammatory response^[49]. It has been recently demonstrated that plasma PTX3 levels may differentiate NASH patients from non-NASH subjects, and that higher plasma PTX3 levels are associated with severe stages of hepatic fibrosis^[50]. However, it is unknown whether any of these biochemical markers are involved in the causal chain of NAFLD progression, mediating the effects of other risk factors (e.g. insulin resistance or inflammation) or whether they merely reflect the presence of NAFLD. Additional basic and clinical research will likely shed more light on these issues.

PROTEOMIC APPROACHES

Proteomic approaches to the identification of NASH biomarkers rely principally on the unbiased comparative analysis of protein expression in normal and diseased liver tissues to identify aberrantly expressed proteins that may represent new biomarkers, as well as direct serum protein profiling^[12]. Proteomics methodologies include the isolation, identification, and quantification of proteins in biosamples by adsorption of proteins to activated surfaces (matrix-assisted laser desorption/ionization technology), or *via* peptide ionization procedures and mass spectroscopy. Mass spectrometry can yield a comprehensive profile of peptides and proteins in biosamples without the need for initial protein separation, thereby facilitating biomarker identification with reduced sample requirements and a high throughput^[12].

Only two studies to date have examined the NAFLD proteome. Younossi *et al*^[51] investigated, by means of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF), serum protein profiles of different subtypes of NAFLD and identified twelve significantly different protein peaks across the study groups. Using a similar SELDI-TOF technique, Trak-Smayra *et al*^[52] have searched for serum markers of steatosis and NASH in obese nonalcoholic patient candidates for bariatric surgery. The authors identified two peaks of 7558.4 and 7924.2 *m/z* that may distinguish between NASH patients and controls. Such peaks were identified as being the double charged ions of hemoglobin-alpha and hemoglobin-beta. There were also three peaks, the intensity of which increased significantly according to severity of liver lesions (steatosis and NASH), but no association was seen with either liver function tests or metabolic parameters^[52].

Although further research of the NAFLD/NASH proteome is certainly needed, the global analysis of protein expression represents an important paradigm shift from the traditional single-molecule approach to the evaluation of protein networks. The future availability of rapid, high-throughput analytical platforms are likely to facilitate molecular phenotyping of different subtypes (simple fatty liver, borderline NASH, definitive NASH) in the spectrum of NAFLD.

CONCLUSION

The need to carry out a biopsy to distinguish NASH from simple steatosis has impeded research to develop strategies for interventions to treat steatohepatitis. In this context, the overall expectation of a NASH biomarker is to enhance the ability of the clinician to optimally manage the patient without the use of liver biopsy^[53]. Theoretically, biomarkers may provide a powerful approach in the understanding of the spectrum of NAFLD, with potential applications in several areas including screening, diagnosis, prognosis, and therapeutic monitoring. Advances in proteomics, metabolomics, and bioinformatics have revolutionized unbiased investigations of several biomarkers that may be informative with regard to the various stages of NAFLD, including NASH and its potential sequelae (advanced fibrosis, hepatocellular carcinoma, end-stage liver disease). Obviously, a crucial prerequisite for the clinical use of biomarkers is elucidation of analytical features, standardization of analytical methods, assessment of performance characteristics, and demonstration of cost-effectiveness. A new biomarker of NASH will be of clinical value only if it is reproducibly obtained in a standardized fashion, it is easy to interpret by clinicians, and if it has high sensitivity and high specificity for identifying NASH, and for distinguishing this condition from benign simple fatty liver. Establishing the prognostic utility of a biomarker in the setting of NAFLD to identify patients that may progress to NASH and fibrosis is more challenging because it requires a prospective design, and serial liver biopsies are presently the gold standard. Although there is evidence to suggest that currently available biomarkers (and their combination) have an increasing ability to distinguish “case” (NASH) from “noncase” (not-NASH) in cross-sectional studies, the step ahead in this field is to differentiate by the use of a biochemical marker “those who will develop fibrosis and end-stage liver disease” from “those who will not” in longitudinal investigations. Hopefully, the ongoing research in NASH biomarker development will also mandate a systematic organization of data that may facilitate the online sharing of biomarker metadata among researchers. Over the next years, technological advances will likely facilitate the use of multimarker profiling to replace the gold standard for the diagnosis of NASH, liver biopsy, with a “biomarker biopsy”^[54].

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S- Editor Li LF L- Editor Logan S E- Editor Zheng XM



ORIGINAL ARTICLES

Molecular mechanism and functional consequences of lansoprazole-mediated heme oxygenase-1 induction

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Received: March 9, 2009 Revised: August 17, 2009

Accepted: August 24, 2009

Published online: September 21, 2009

in macrophages. In addition, lansoprazole-induced ferritin protein levels in both cell systems. Moreover, induction of the antioxidant proteins HO-1 and ferritin by lansoprazole was followed by a decrease in NADPH-mediated ROS formation. The radical scavenging properties of lansoprazole were diminished in the presence of the HO inhibitor, chromium mesoporphyrin IX. Induction of *HO-1* gene expression by lansoprazole was not related to oxidative stress or to the activation of the mitogen-activated protein kinase pathway. However, the phosphatidylinositol 3-kinase inhibitor LY294002 showed a concentration-dependent inhibition of HO-1 mRNA and promoter activity.

CONCLUSION: Activation of HO-1 and ferritin may account for the gastric protection of lansoprazole and is dependent on a pathway blocked by LY294002.

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Key words: Antioxidants; Ferritin; Heme oxygenase-1; Lansoprazole; Reactive oxygen species

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Schulz-Geske S, Erdmann K, Wong RJ, Stevenson DK, Schröder H, Grosser N. Molecular mechanism and functional consequences of lansoprazole-mediated heme oxygenase-1 induction. *World J Gastroenterol* 2009; 15(35): 4392-4401 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4392.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4392>

Abstract

AIM: To investigate the molecular mechanism and functional consequences of heme oxygenase-1 (HO-1) activation by lansoprazole in endothelial cells and macrophages.

METHODS: Expression of HO-1 mRNA was analyzed by Northern blotting. Western blotting was used to determine the HO-1 and ferritin protein levels. NADPH-dependent reactive oxygen species (ROS) formation was measured with lucigenin-enhanced chemiluminescence. HO-1 promoter activity in mouse fibroblasts, stably transfected with a 15-kb *HO-1* gene that drives expression of the reporter gene luciferase, was assessed using *in vivo* bioluminescence imaging.

RESULTS: Lansoprazole increased HO-1 mRNA levels in endothelial cells and HO-1 protein levels

INTRODUCTION

Peptic ulcer disease remains common worldwide. It is caused most frequently by stress, alcohol, *Helicobacter pylori* (*H. pylori*) infection, or the use of non-steroidal anti-inflammatory drugs (NSAIDs). It is believed that the integrity of the gastric mucosa depends on a delicate balance between aggressive and defensive mechanisms. Although the cellular and molecular bases of gastric mucosal defense are well understood, the mechanisms by which mucosal damage is mediated by aggressive factors

remain largely unclear. The increased production of reactive oxygen species (ROS), termed oxidative stress, is considered to be a major causative factor for mucosal lesions induced by stress^[1], NSAIDs^[2], and *H. pylori*^[3].

Proton pump inhibitors (PPIs) are the drugs of choice for the therapeutic control of gastroesophageal reflux disease, peptic ulcer disease, and eradication of *H. pylori*, and as mucosal protective agents when using NSAIDs. They have been shown to be more effective than other anti-secretory drugs (i.e. H₂ receptor antagonists) in the management of acid-related diseases^[4]. The anti-secretory action is mediated by their irreversible inhibition of H⁺/K⁺-ATPase, the terminal proton pump of parietal cells^[5]. However, emerging evidence suggests that the benefit of PPIs is not only mediated by their potent blockade of the gastric H⁺/K⁺-ATPase, but also by their ability to provide anti-inflammatory, anti-apoptotic, and antioxidative effects^[6,7]. By scavenging ROS and thus protecting the mucosa, these pleiotropic effects of PPIs may be responsible for maintaining the anatomical and functional integrity of the gastric mucosa. In addition to a variety of endogenous factors such as prostaglandins, nitric oxide, and sulfhydryl compounds, which have been proposed to account for the gastroprotective effects of PPIs^[7,8], we previously have shown that PPIs also induce the antioxidant protein heme oxygenase-1 (HO-1) in gastric epithelial and endothelial cells^[9].

The inducible stress protein HO-1 is the rate-limiting enzyme involved in heme breakdown to generate equimolar amounts of biliverdin, free iron, and CO^[10]. Biliverdin is subsequently converted by biliverdin reductase to bilirubin, which is a potent free radical scavenger and acts as a strong endogenous antioxidant^[11]. CO has been recognized as a protective agent in hemorrhagic shock and as a modulator of vascular tone. Moreover, it shows anti-apoptotic and anti-inflammatory activity^[12]. The HO-1-dependent release of free iron leads to the expression of a second antioxidant protein, ferritin^[13]. Ferritin rapidly sequesters free cytosolic iron, thus limiting the formation of oxygen-centered radicals *via* the Fenton reaction. Thereby, ferritin has emerged as a critical and fast-acting endogenous cytoprotectant that plays an important role in cellular antioxidant defense mechanisms^[13].

The activation of the *HO-1* gene is regulated primarily at the level of transcription involving various signaling pathways. In particular, phosphorylation-dependent signaling cascades that bind to the transcription factors regulating the *HO-1* gene seem to play a key role in *HO-1* gene stimulation. In an inducer- and cell-specific fashion, signaling pathways that are implicated in regulating *HO-1* gene expression are those important for proliferation and cell survival. Many studies have focused on the activation of the mitogen-activated protein kinases (MAPKs). Recently, other investigators have demonstrated a link between the phosphatidylinositol 3-kinase (PI3K) cell survival pathway and regulation of the *HO-1* gene. Some reports suggest a role of cAMP-dependent protein kinase A, protein kinase C, cGMP-

dependent PKG, or tyrosine kinases in HO-1 transcriptional regulation^[14].

The aim of this study was to elucidate the mechanism of gastric protection by PPIs beyond their effective acid reduction properties, using lansoprazole as a model compound. We focused on the activation of the antioxidant proteins, HO-1 and ferritin, by lansoprazole in cell systems that lack the actual PPI target, the H⁺/K⁺-ATPase pump. We then further assessed the underlying mechanism of the upregulation of HO-1 by lansoprazole.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), cell culture media, and penicillin and streptomycin were obtained from GIBCO (Eggenstein, Germany). Chemiluminescence Western Blotting Kit and D-luciferin were purchased from GE Healthcare (Freiburg, Germany) and BioSynth (Naperville, IL, USA), respectively. Wortmannin and primary HO-1 antibody were obtained from Axxora (Grünberg, Germany). PeqGOLD TriFast was purchased from Peqlab (Erlangen, Germany). Chromium mesoporphyrin IX (CrMP) was purchased from Frontier Scientific (Carnforth, UK). For HO-1 probes, the template was an *Eco*RI restriction fragment of the human HO-1 cDNA (clone 2/10), which was kindly provided by Dr. Rex Tyrrell (University of Bath, UK)^[15]. The polyclonal antibody against human ferritin and all other chemicals were obtained from Sigma (Taufkirchen, Germany). The stock solutions of lansoprazole (300 mmol/L), ranitidine (300 mmol/L), MAPK inhibitors [SB203580 (22 mmol/L), SB202190 (20 mmol/L), SP 600125 (45 mmol/L), PD 098059 (22.5 mmol/L)], and PI3K inhibitors [LY294002 (20 mmol/L), wortmannin (100 µmol/L)] were all prepared in dimethyl sulfoxide and stored at -20°C in the dark. The stock solution of CrMP (10 mmol/L) was prepared by dissolving 6.5 mg in 50 µL 2 mol/L NaOH, adjusted to 1.0 mL with sterile water, and stored at -20°C in the dark until use or for up to 12 mo.

Cell culture

Human endothelial-like ECV304 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). ECV304 cells have been used as a convenient model for the study of vascular endothelial cells^[16,17], because they show endothelium-like properties such as producing endothelium specific Weibel-Palade bodies, endothelium-related antigens and angiotensin-converting enzyme, in addition to having human-umbilical-vein-endothelial-cell-like morphology^[18,19]. Cells were grown in M199 medium that contained 10% FBS, streptomycin (100 µg/mL), and penicillin (100 U/mL). Murine bone-marrow-derived macrophages (J774), obtained from the American Type Culture Collection (Manassas, VA, USA), were maintained in Dulbecco's Minimal Essential Medium (DMEM) that contained 10% FBS, streptomycin (100 µg/mL), and penicillin (100 U/mL). Embryonic

mouse fibroblast cells (NIH3T3-HO-1-*luc*), stably transfected with a transgene that contained the full-length (15 kb) mouse HO-1 promoter that drives expression of the reporter gene luciferase, were grown in DMEM that contained 10% FBS, streptomycin (100 µg/mL), and penicillin (100 U/mL). All cells were maintained in a humidified incubator at 37°C and 5% CO₂.

HO-1 mRNA analysis

Sub-confluent endothelial cells in 150-mm culture dishes were incubated for 8 h in the presence of control medium, lansoprazole, or ranitidine. Superoxide dismutase (SOD), as well as PI3K and MAPK inhibitors, was added 20 min before lansoprazole treatment. Total RNA was extracted using Trizol reagent according to manufacturer's instructions (Peqlab). For Northern blotting, samples that contained equal amounts of RNA (20-30 µg) were separated on a 1% denaturing formaldehyde gel and then transferred onto a positively charged nylon membrane by vacuum (500 mbar). The transferred RNA was fixed by baking at 80°C for 30 min. After incubation, membranes were hybridized with a randomly primed ³²P-labeled human HO-1 cDNA probe^[15] overnight at 65°C. Equal loading of RNA was assessed by staining 18S and 28S rRNAs with ethidium bromide and by a second hybridization using a ³²P-labeled β-actin cDNA probe.

Formation of ROS

NADPH-dependent ROS formation was measured by monitoring lucigenin-derived chemiluminescence at 37°C using a Berthold LB96V luminometer according to previously published protocols^[20]. Cells were first cultured in six-well plates. After pretreatment with lansoprazole or ranitidine for 12 h, cells were washed and suspended in PBS and then lucigenin (5 µmol/L) and NADPH (10 µmol/L) were added. CrMP (3 µmol/L) was added 20 min before lansoprazole treatment. Chemiluminescence was measured in relative light units (RLU) every 5 min over a period of 20 min. Data were expressed as the mean of peak values in the 20-min measurement normalized to the maximal light emission (RLU_{max}%) of time-matched NADPH-treated control cells.

HO-1 and ferritin protein analysis

ECV304 cells and macrophages were cultured in 100-mm dishes as described above. After 4-24 h of incubation with control medium or lansoprazole, cells were washed and extracted as described previously^[21]. One hundred micrograms of HO-1 protein or 20 µg ferritin protein were separated by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and incubated with a polyclonal antibody to HO-1 or ferritin. Antigen/antibody complexes were visualized using a horseradish peroxidase chemiluminescence system according to the manufacturer's instructions. The densitometric quantitation was performed using Quantity One Basic software (Bio-Rad, USA).

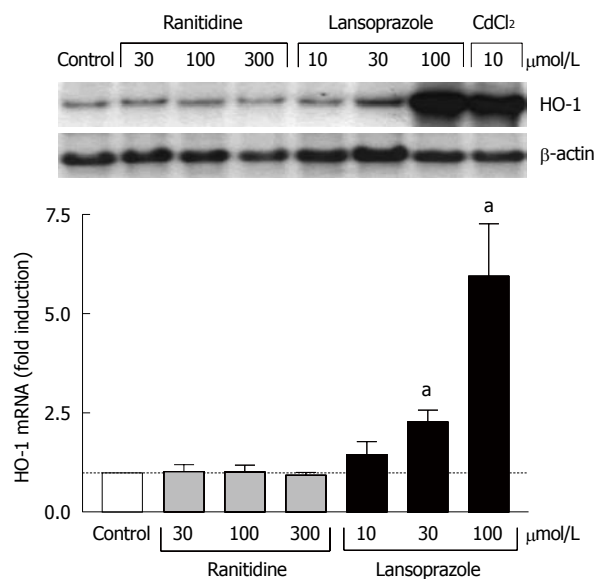


Figure 1 Effect of ranitidine and lansoprazole on HO-1 mRNA induction in endothelial cells after 8 h of incubation. Fold induction from control levels is shown as mean ± SD of three separate experiments. **P* < 0.05 vs control. Treatment with CdCl₂ was used as a positive control. Representative Northern blotting analysis is shown in the upper panel.

In vivo HO-1 promoter activity

NIH3T3-HO-1-*luc* cells, stably transfected with a 15-kb HO-1 gene upstream of the transcription initiation site that drives expression of the reporter gene luciferase, were treated with control medium or lansoprazole. PI3K and MAPK inhibitors were added 20 min before lansoprazole. After 24 h incubation, luciferin (300 µg/mL) was added to the cells. Light emission, used as an index of HO-1 promoter activity in living cells, was collected using the In Vivo Imaging System (IVIS™, Caliper Life Sciences, Alameda, CA, USA), quantitated using LivingImage software (Caliper Life Sciences), and expressed as photons emitted/5 min, as previously described^[22].

Statistical analysis

Results are expressed as mean ± SD. Data were analyzed using ANOVA and by Bonferroni's correction for multiple comparisons. All statistical calculations were performed using GraphPad Prism 3.02. Differences were considered significant at *P* < 0.05. Analyses were based on three to six independent experiments using different cell passages on different days.

RESULTS

Effect of lansoprazole and ranitidine on HO-1 mRNA levels

In endothelial cells, the effects of ranitidine (an H₂ receptor antagonist) and lansoprazole on HO-1 mRNA levels were compared following 8 h incubation with each compound. Compared to untreated control cells, ranitidine treatment did not affect HO-1 mRNA levels at any concentration (Figure 1). In contrast, lansoprazole treatment elevated HO-1 mRNA levels in a concentration-dependent manner with nearly a sixfold induction at 100 µmol/L. This increase in HO-1 mRNA levels was

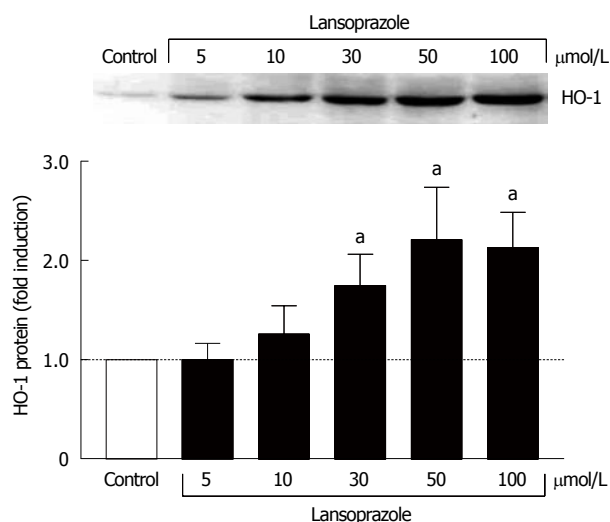


Figure 2 Effect of lansoprazole on HO-1 protein expression in macrophages (J774 cells) after 12 h of incubation. Fold induction from control levels is shown as mean \pm SD of three to six separate experiments. $^aP < 0.05$ vs control. Representative Western blotting analysis is shown in the upper panel.

comparable to the induction observed following treatment with the positive control CdCl_2 (10 $\mu\text{mol/L}$).

Effect of lansoprazole on HO-1 protein expression in macrophages

When we investigated the effect of lansoprazole (5–100 $\mu\text{mol/L}$) on HO-1 protein expression in J774 macrophages, a cell system contributing positively to the mucosal defense, a significant 1.75–2.2-fold induction in HO-1 protein levels was found following 12 h of incubation with lansoprazole at concentrations of 30, 50 and 100 $\mu\text{mol/L}$ (Figure 2).

Effect of ranitidine and lansoprazole on NADPH-mediated ROS formation

Activated phagocytes represent a major source of ROS production and are therefore used to investigate oxidative stress in cell systems. To analyze the free-radical scavenging effects of the anti-secretory drugs ranitidine and lansoprazole, macrophages were preincubated with these compounds for 12 h. Ranitidine (30 $\mu\text{mol/L}$) did not affect NADPH-mediated ROS formation (Figure 3). In contrast, lansoprazole (30 $\mu\text{mol/L}$) significantly decreased NADPH-mediated ROS production (20%). To examine whether HO-1 accounted for the cell protection mediated by lansoprazole, the selective HO inhibitor CrMP was used. CrMP (3 $\mu\text{mol/L}$) eliminated the antioxidant action of lansoprazole against NADPH-mediated free-radical formation. No change in NADPH-mediated ROS formation was observed in cells treated with CrMP alone.

Effect of lansoprazole on ferritin protein expression in endothelial cells and macrophages

Increased ferritin protein levels were found in tandem with increased HO-1 protein in macrophages in a concentration-dependent manner after 12 h of incubation with lansoprazole (Figure 4A). Ferritin protein expression

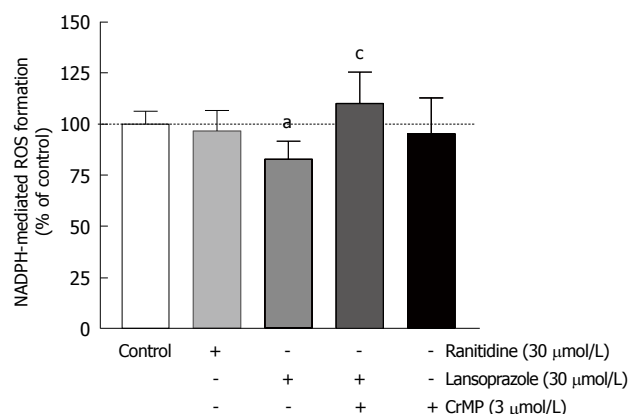


Figure 3 Lansoprazole, but not ranitidine, decreased NADPH-dependent ROS formation in macrophages. This effect was reversed in the presence of CrMP. Measurements of lucigenin-enhanced chemiluminescence were performed. $^aP < 0.05$ vs control; $^cP < 0.05$ vs lansoprazole alone. Data are shown as mean \pm SD of three to six separate experiments.

in endothelial cells also increased in a time-dependent manner following exposure to lansoprazole (Figure 4B). The induction of ferritin protein expression occurred at a lansoprazole concentration of 100 $\mu\text{mol/L}$, which significantly induced HO-1 mRNA and protein levels.

Regulation of the lansoprazole-induced HO-1 gene expression: effect of oxidative stress

Since HO-1 is highly induced by oxidative stress, we evaluated the role of superoxide anion in the PPI-mediated upregulation of HO-1 mRNA. Endothelial cells were treated with lansoprazole (30–100 $\mu\text{mol/L}$) in the presence of SOD for 8 h (Figure 5). Preincubation with SOD (15 U/mL) did not change the basal or lansoprazole-mediated increases in HO-1 mRNA levels. These results indicate that superoxide anion is not involved in the regulation of HO-1 expression by lansoprazole.

Effect of MAPK inhibitors on the lansoprazole-induced HO-1 gene activation

Induction of HO-1 mRNA in endothelial cells after treatment with lansoprazole for 8 h was not influenced by pretreatment with the ERK inhibitor PD098059 (10 $\mu\text{mol/L}$) or the JNK inhibitor SP600125 (10 $\mu\text{mol/L}$) (Figure 6A and B). At a concentration of 10 $\mu\text{mol/L}$, the p38 inhibitor SB203580 had no effect on HO-1 mRNA induction by lansoprazole (50 $\mu\text{mol/L}$). Higher concentrations of the inhibitor decreased HO-1 mRNA levels in comparison to that following treatment with 50 $\mu\text{mol/L}$ lansoprazole alone (Figure 6C). Thus, to further explore the role of p38 MAPK on PPI-mediated HO-1 gene activation, we treated endothelial cells with a second p38 MAPK inhibitor (α and β subunit), SB202190 (0.1–10 $\mu\text{mol/L}$). SB202190 did not affect the induction of HO-1 mRNA by lansoprazole (Figure 6C). Moreover, when we investigated the effect of both p38 MAPK inhibitors on HO-1 promoter activity using NIH3T3-HO-1-*luc* cells, incubation with 30 $\mu\text{mol/L}$ lansoprazole alone for 24 h significantly increased HO-1 promoter

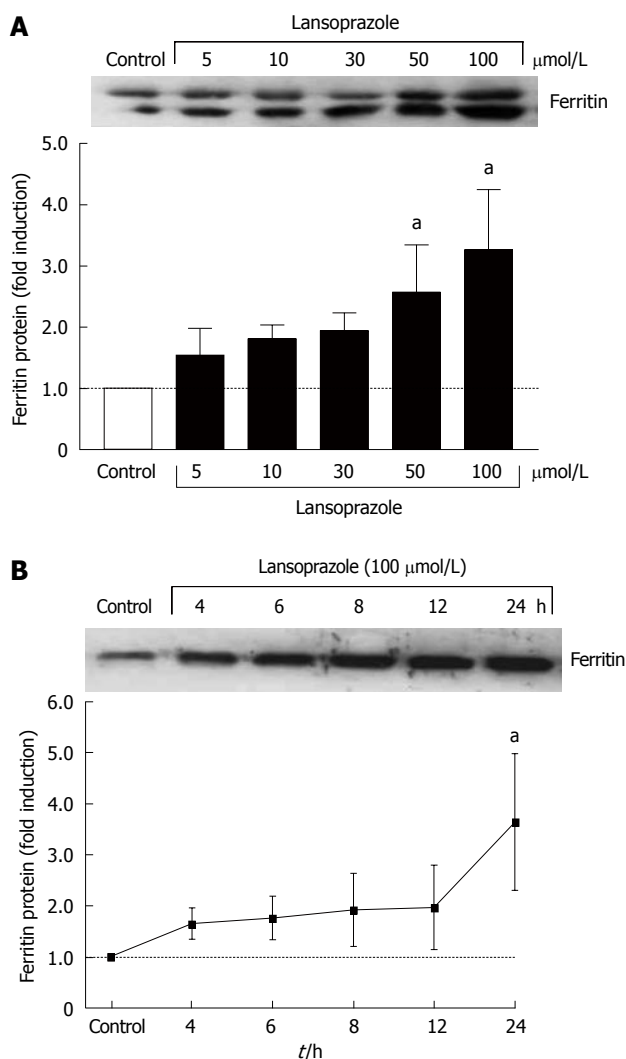


Figure 4 Lansoprazole increased ferritin protein expression in a concentration- and time-dependent manner in macrophages (A) and endothelial cells (B). Data are shown as mean \pm SD of three to six separate experiments. $^aP < 0.05$ vs control. A representative Western blotting analysis is shown in the upper panel.

activity by 1.7-fold compared with untreated control levels (data not shown). Neither p38 inhibitor diminished HO-1 promoter activation by lansoprazole (Table 1). All MAPK inhibitors alone did not affect HO-1 mRNA levels or HO-1 promoter activity.

Involvement of the PI3K pathway in the regulation of HO-1 induction by lansoprazole

Preincubation of endothelial cells with the PI3K inhibitor LY294002 (5-50 μmol/L) diminished the lansoprazole-induced (50 μmol/L) increase in HO-1 mRNA expression in a concentration-dependent manner (Figure 7A and C). Similar effects were observed in NIH3T3-HO-1-*luc* cells (Figure 8A and C). The lansoprazole-mediated (30 μmol/L) promoter activation was abolished by up to 50% in the presence of LY294002 (5-25 μmol/L) (Figure 8C). Preincubation with the fungal metabolite wortmannin (0.01 and 0.1 μmol/L) did not affect the lansoprazole-mediated increase in HO-1 mRNA and HO-1 promoter activity levels (Figure 7B and C, Figure 8B and C).

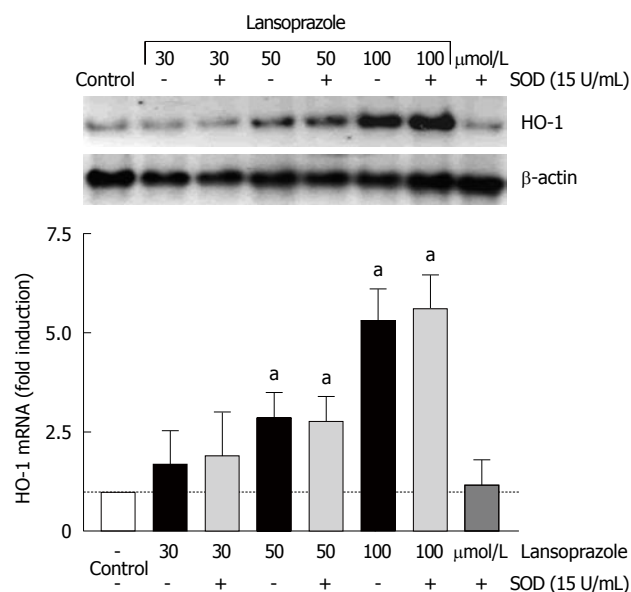


Figure 5 Effect of SOD on lansoprazole-induced HO-1 mRNA expression. After pretreatment with SOD for 20 min, endothelial cells were incubated with lansoprazole for 8 h. Fold induction compared to control is shown as mean \pm SD of three separate experiments. $^aP < 0.05$ vs control. A representative Northern blotting analysis is shown in the upper panel.

Table 1 Effect of p38 inhibitors SB203580 and SB202190 on lansoprazole-induced HO-1 promoter activity in NIH3T3-HO-1-*luc* cells (mean \pm SD, %)

Treatment	HO-1 promoter activity
Lansoprazole (30 μmol/L)	100.0 \pm 8.4
Lansoprazole (30 μmol/L) + SB203580 (10 μmol/L)	109.2 \pm 9.0
Lansoprazole (30 μmol/L) + SB203580 (20 μmol/L)	108.8 \pm 12.6
Lansoprazole (30 μmol/L) + SB203580 (30 μmol/L)	101.8 \pm 14.4
Lansoprazole (30 μmol/L) + SB202190 (0.1 μmol/L)	91.0 \pm 3.7
Lansoprazole (30 μmol/L) + SB202190 (1.0 μmol/L)	112.4 \pm 15.1

Change from HO-1 promoter activity measured in cells treated with 30 μmol/L lansoprazole.

Both PI3K inhibitors alone did not affect HO-1 promoter activity or transcriptional levels under identical experimental conditions.

DISCUSSION

Since ROS and inflammation are important causative factors for the development of mucosal damage, the pleiotropic effects of PPIs are of particular clinical interest for the control of gastroduodenal ulcers. Thus, investigation of the molecular mechanism of PPI-mediated gastric protection is required.

In this study, we used endothelial cells and macrophages, which play important roles in mucosal defense, to evaluate the effects of PPIs on the HO-1/ferritin system. The blood flow through the endothelial cells that innervate the mucosa is crucial for supplying nutrients, limiting damage, and facilitating repair. Macrophages play key roles in the mucosal immune system, sensing and in response to foreign materials^[23].

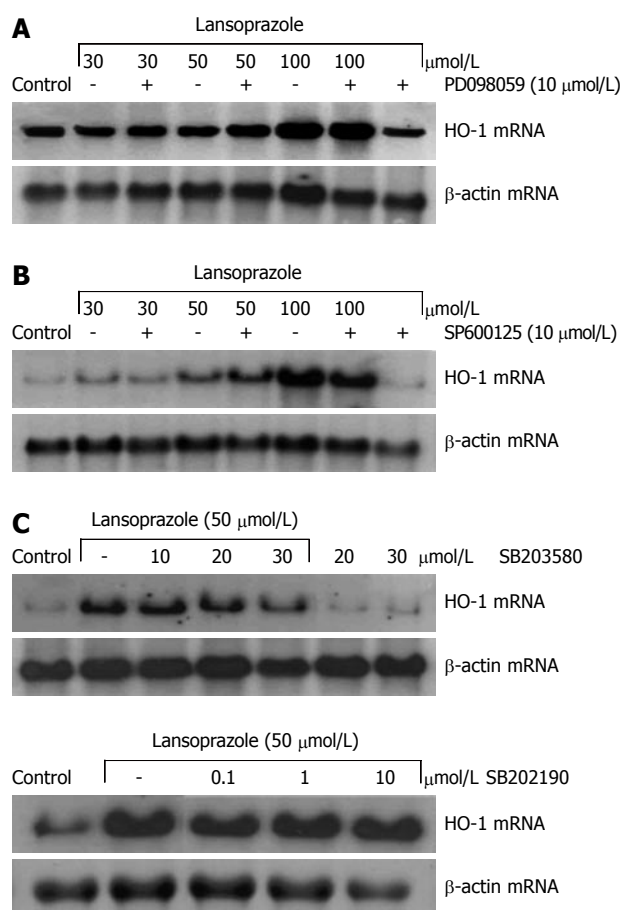


Figure 6 Effect of different MAPK inhibitors on lansoprazole-induced HO-1 mRNA expression. After pretreatment with ERK inhibitor PD098059 (A), JNK inhibitor SP600125 (B), or p38 inhibitors SB203580 and SB202190 (C) for 20 min, endothelial cells were incubated with lansoprazole for 8 h. The Northern blotting analysis shown is representative of three to six independent experiments.

Neither cell type expresses H^+/K^+ -ATPase, therefore, the observed induction of HO-1 and ferritin by PPIs is assumed to be independent of their antiseecretory properties. We have shown previously that PPIs induce HO-1 mRNA, protein and enzymatic activity in gastric epithelial cells and endothelial cells^[9]. In this study, we provided further evidence that activation of the *HO-1* gene by PPIs might account for their beneficial effects in the treatment of peptic ulcer disease. We demonstrated that the H_2 receptor antagonist ranitidine, in contrast to lansoprazole, failed to increase HO-1 mRNA levels. Moreover, lansoprazole also significantly upregulated HO-1 protein levels as a consequence of elevated HO-1 mRNA levels in J774 cells (data not shown). Macrophage-derived ROS production promotes the killing of microorganisms on one hand, but on the other hand, contributes to oxidative stress in inflammatory sites and alters basic cell functions such as adhesion and proliferation^[24,25]. In acute inflammatory illnesses, HO-1 mRNA levels are significantly elevated, which suggests that monocytes exert potent anti-inflammatory effects *via* HO-1 activation, and thereby regulate the production of pro-inflammatory cytokines to protect organs and cells from irreversible damage^[26].

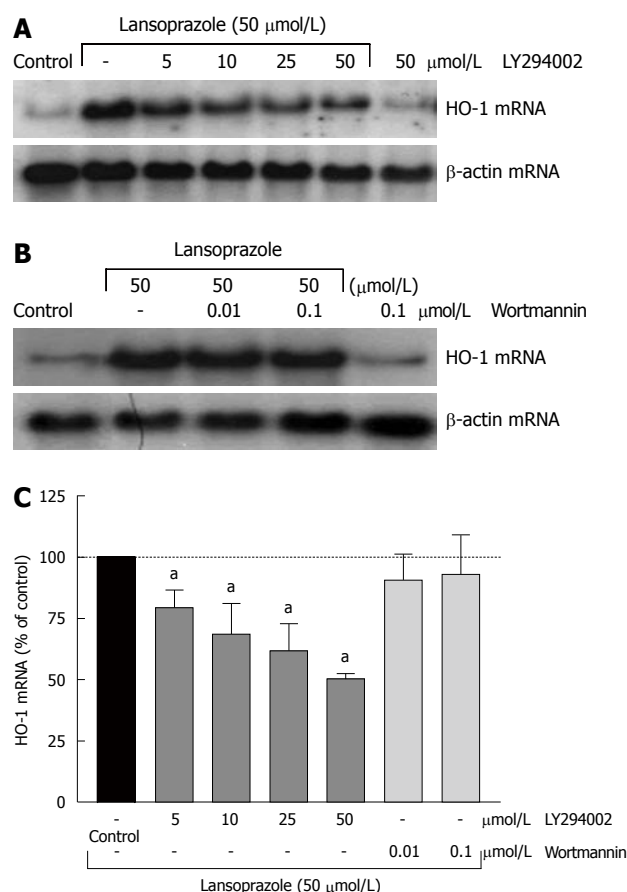


Figure 7 Effect of PI3K inhibitors LY294002 and wortmannin on lansoprazole-induced HO-1 mRNA expression. After pretreatment with PI3K inhibitors for 20 min, endothelial cells were incubated with lansoprazole for 8 h. Representative Northern blotting analysis is shown (A and B). Results are expressed as mean \pm SD of three to six separate experiments compared to lansoprazole (control = gene expression of cells stimulated with 50 μ mol/L lansoprazole = 100%) (C). ^a $P < 0.05$ vs lansoprazole alone.

Thus, upregulation of HO-1 expression by lansoprazole in macrophages could be beneficial in the management of mucosal inflammation.

In addition, we demonstrated that lansoprazole performed free-radical scavenging in macrophages. The protection against NADPH-mediated ROS production by lansoprazole occurred after 12 h of incubation and after washout of lansoprazole. Moreover, the inhibition of the antioxidant effect of lansoprazole in the presence of the HO inhibitor CrMP^[27] demonstrates that HO-1 and its enzymatic products are indeed of functional relevance for the antioxidant effects of lansoprazole.

All experiments in this study have been performed with lansoprazole, but studies using omeprazole have shown similar effects on HO-1 mRNA and HO-1 and ferritin protein expression, as well as on the reduction of ROS (data not shown). Taken together, these data indicate that the induction of HO-1 might be responsible, at least in part, for the antioxidative action of PPIs and their advantage over H_2 -antagonists in the therapy of ulcer disease and NSAID-related mucosal damage.

Our data support other work that has shown that

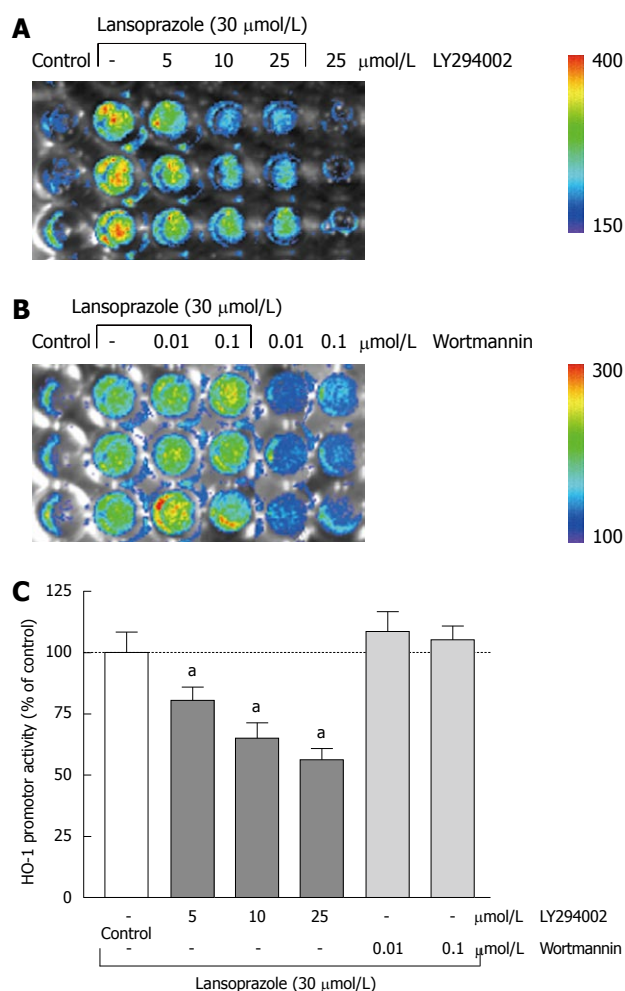


Figure 8 Effect of PI3K-inhibitors LY294002 and wortmannin on lansoprazole-induced HO-1 promoter activity in stable transfected cell lines. Representative images of three to six separate experiments are shown (A and B). Light emission (shown by the rainbow bars: blue, least intense; red, most intense) was measured in NIH3T3-HO-1-luc cells with the IVIS. Results are expressed as mean \pm SD of three to six separate experiments compared to lansoprazole (control = HO-1 promoter activity of cells stimulated with 30 $\mu\text{mol/L}$ lansoprazole = 100%) (C). ^a $P < 0.05$ vs lansoprazole alone.

HO-1 might be a mediator of gastroprotective pathways. Immunohistochemical studies have demonstrated that HO-1 is expressed constitutively in normal gastric and colonic mucosa and its upregulation occurs in these tissues during inflammation or the healing phase of gastric ulcers^[28,29].

In the present study, significant HO-1 induction following treatment with lansoprazole in endothelial cells and macrophages, as well as antioxidant protection occurred at a concentration of 30 $\mu\text{mol/L}$. Plasma concentrations after oral administration of lansoprazole (30 mg/d) ranged from 2.2 to 5.6 $\mu\text{mol/L}$ ^[30]. Depending on the dose (30-90 mg/d) and the route of administration, higher plasma concentration levels (e.g. 15-21 $\mu\text{mol/L}$) may be attainable^[31,32]. These concentrations approximate the concentrations found to be effective at increasing the level of HO-1 expression in the present study. Furthermore, Blandizzi and co-workers have suggested that the ED₅₀ of lansoprazole needed to provide gastroprotection is 2-4 times higher compared to that needed for

inhibition of acid secretion^[7].

The anti-inflammatory and antiproliferative actions of the HO-1 product CO, as well as the potent antioxidant effect of bilirubin, are thought to contribute to the overall protective effect of HO-1^[12]. CO has also been shown to provide vasodilatory activities through the activation of soluble guanylyl cyclase^[33]. Omeprazole and lansoprazole induce relaxation of isolated human arteries^[34] at the same concentration range (30-300 $\mu\text{mol/L}$ lansoprazole) that we have found to be effective in the induction of HO-1. Although Naseri and co-workers have suggested the regulation of intracellular Ca^{2+} as an underlying mechanism, it might also be possible that the generation of CO by HO-1 activity contributes to the vasodilatory effect of PPIs.

The third direct product of heme metabolism, free Fe^{2+} , leads to the induction of a multimeric iron-chelating protein, ferritin^[35]. In the present study, the ferritin protein induction by lansoprazole occurred in a concentration- and time-dependent manner in macrophages and endothelial cells. The activation of ferritin protein synthesis was obtained after long incubations (12-24 h) and at concentrations that have also been shown to result in HO-1 mRNA and protein increase, which suggests ferritin induction as a functional consequence of elevated HO-1 activity.

Besides induction through its physiological substrate heme, HO-1 gene expression can also be stimulated by a variety of stress inducers including, but not limited to, heavy metals, ultraviolet irradiation, endotoxin, and oxidants such as hydrogen peroxide^[14]. In contrast, antioxidants such as α -tocopherol and allopurinol prevent the upregulation of HO-1^[36]. Thus, we investigated the role of ROS in PPI-mediated HO-1 induction. Pretreatment with the antioxidant enzyme SOD did not affect lansoprazole-mediated HO-1 induction, precluding a mediator role of superoxide.

Recently, it has been shown by Yeo and co-workers that PPIs influence MAPK-dependent pathways^[37]. However, in the present study, the ERK inhibitor PD098059 and the JNK inhibitor SP600125 had no inhibitory effect on lansoprazole-induced HO-1 mRNA. All inhibitors have been used in concentrations previously shown to be effective in the inhibition of the final kinase of the respective MAPK cascade^[38,39]. The p38 kinase inhibitor (α - and β -subunit) SB203580, at a concentration of 30 $\mu\text{mol/L}$, impaired the lansoprazole-mediated HO-1 mRNA induction. SB 203580 has been shown to inhibit its established targets, the α - and β -subunit of p38 with IC₅₀ values of 50 and 500 nmol/L, respectively. Yet, other kinases like lymphocyte-specific protein tyrosine kinase, glycogen synthase kinase-3 β (GSK3 β) and Akt1 (protein kinase B α) were also inhibited by SB 203580, with IC₅₀ values that were 100 \pm 500-fold higher than that for p38^[40]. As SB203580 interacts with other kinases at higher concentrations, we determined the effect of a second p38 inhibitor, SB202190, on HO-1 mRNA induction by lansoprazole. SB202190 was without effect on the lansoprazole-mediated increase in HO-1 mRNA. In agreement with

this, both p38 inhibitors have been without effect on lansoprazole-induced HO-1 promoter activity in NIH3T3-HO-1-*luc*-cells, which indicates that MAPK activation is not involved in PPI-mediated HO-1 induction.

The PI3K inhibitor LY294002 diminished the increase of HO-1 mRNA level and HO-1 promoter activity induced by lansoprazole in a concentration-dependent manner. Surprisingly, a second PI3K inhibitor, wortmannin, did not affect HO-1 expression. Although both PI3K inhibitors have been shown to block the phosphorylation of PI3K completely at the concentrations used in our study^[41,42], the potency to interact with other kinases is different. An inhibitory effect by LY294002 but not by wortmannin on HO-1 induction has already been described by other groups, which suggests that PI3K is not involved in *HO-1* gene activation in these particular cases^[43,44]. LY294002 is also known to block casein kinase 2 (CK2) and GSK3 β by up to 50% at a concentration of 50 $\mu\text{mol/L}$ ^[40]. Moreover, the phosphorylation of PI3K downstream kinase p70^{s6k} is inhibited almost completely at a concentration of 10–25 $\mu\text{mol/L}$ LY294002, whereas the inhibition of Akt phosphorylation requires LY294002 doses of up to 150–200 $\mu\text{mol/L}$ ^[45]. Therefore, a different influence of special PI3K downstream kinases or an involvement of CK2 or GSK3 β on the PPI-mediated *HO-1* gene activation is conceivable. Indeed there is evidence that CK2 might exert a significant influence on *HO-1* gene activation by the phorbol 12-myristate 13-acetate^[46]. Further studies are needed to clarify the influence of GSK3 β , CK2 and PI3K downstream kinases on HO-1 induction by PPIs.

In summary, we demonstrated that lansoprazole is a potent inducer of the antioxidant proteins HO-1 and ferritin. The induction of HO-1 by lansoprazole is independent of oxidative stress, and involves a signaling pathway that is blocked by LY294002. The activation of the HO-1/ferritin pathway occurs in endothelial cells and macrophages; cells relevant to the mucosal microcirculation and the mucosal immune system. Neither cells express H⁺/K⁺-ATPase, therefore, the observed induction of HO-1 and ferritin by lansoprazole can be assumed to occur independently of the antiseecretory effect. It is plausible that HO-1 and its enzymatic products are responsible for, or contribute to, the gastric protection seen with lansoprazole and other PPIs.

ACKNOWLEDGMENTS

We thank Hendrik J Vreman, PhD (Department of Pediatrics, Stanford University School of Medicine, Stanford, CA, USA) for valuable discussions and for critically reviewing the manuscript, and Petra Schwartz for her excellent technical assistance.

COMMENTS

Background

Proton pump inhibitors (PPIs) irreversibly inhibit the gastric proton pump

in parietal cells and are therefore potent antiseecretory drugs. However, comparisons with other acid-reducing agents suggest that the gastroprotective effect of PPIs cannot be explained solely by their antisecretory action. Recent studies have revealed anti-apoptotic, anti-inflammatory, and free-radical scavenging properties of PPIs. The inducible stress protein, heme oxygenase-1 (HO-1), catalyzes the degradation of heme. As a result of the antioxidative, anti-inflammatory, and vasodilatory effects of its products, bilirubin and CO, HO-1 is believed to be a mediator of cytoprotective pathways.

Research frontiers

The pathogenesis of gastric ulcer disease is generally characterized by a decrease in mucosal defense and an increase in aggressive mechanisms. Although the cellular and molecular basis of the gastric mucosal defense are well understood, the mechanisms of mucosal damage mediated by aggressive factors remain largely unclear. The increased production of reactive oxygen species (ROS), termed oxidative stress, is considered to play a crucial role in the development of mucosal damage.

Innovations and breakthroughs

The induction of the antioxidant proteins HO-1 and ferritin by PPIs occurs independently of their antisecretory effects. It is plausible that HO-1 and its enzymatic products are responsible for, or at least contribute to, the gastric protection observed with PPI treatment. There is evidence that PPIs activate the *HO-1* gene through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway.

Applications

The present results suggest that the cytoprotective effects of PPIs can be ascribed to a reduction of oxidative stress via induction of the HO-1/ferritin system in endothelial cells and macrophages. PPIs might be beneficial for cardiac patients and patients on daily pain medication by enhancing gastric mucosal protection and reducing the risk of gastrointestinal bleeding caused by chronic use of aspirin and other NSAIDs. Moreover, PPI-dependent HO-1 induction in endothelial cells of the systemic circulation might exert vascular-protective actions.

Terminology

HO-1 is also known as heat shock protein 32. The enzyme catalyzes the degradation of heme and leads to the accumulation of free iron, CO, and bilirubin. HO-1 is the inducible of two isoforms of the enzyme. PI3K catalyzes the production of the plasma membrane lipid phosphatidylinositol-3,4,5-trisphosphate. Signaling pathways downstream of PI3K affect cell growth, survival and movement.

Peer review

The manuscript investigated molecular mechanism(s) for lansoprazole-induced gastric protection. The authors used endothelial cells and macrophages as models for cytoprotection and showed that lansoprazole induced HO-1 and ferritin and thereby decreased ROS formation in those cell systems. This *HO-1* gene activation involved the PI3K pathway. The authors' hypothesis that lansoprazole upregulates HO-1 and thereby protects gastric mucosa is provocative.

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S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zheng XM



ORIGINAL ARTICLES

Identification of the layered morphology of the esophageal wall by optical coherence tomography

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Received: April 13, 2009 Revised: July 21, 2009

Accepted: July 28, 2009

Published online: September 21, 2009

propria with deeper structures of the esophageal wall.

CONCLUSION: We demonstrated that the OCT image of the normal esophageal wall showed a five-layered morphology, which corresponds to histological esophageal wall components.

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Key words: Optical coherence tomography; Esophagus; Muscularis mucosa; Esophageal squamous cell carcinoma; Endoscopic ultrasonography

Peer reviewers: Anders E Lehmann, PhD, Associate Professor, Senior Principal Scientist, Bioscience, AstraZeneca R&D Mölndal, Mölndal, Sweden; Michael F Vaezi, Professor, Department of Gastroenterology and Hepatology, Vanderbilt University Medical Center, 1501 TVC, Nashville, TN 37232-5280, United States

Yokosawa S, Koike T, Kitagawa Y, Hatta W, Uno K, Abe Y, Iijima K, Imatani A, Ohara S, Shimosegawa T. Identification of the layered morphology of the esophageal wall by optical coherence tomography. *World J Gastroenterol* 2009; 15(35): 4402-4409 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4402.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4402>

Abstract

AIM: To assess each layer of the optical coherence tomography (OCT) image of the esophageal wall with reference to the histological structure.

METHODS: Resected specimens of fresh pig esophagus was used as a model for the esophageal wall. We injected cyanoacrylate adhesive into the specimens to create a marker, and scanned them using a miniature OCT probe. The localization of these markers was assessed in the OCT images. Then we compared the OCT-imaged morphology with the corresponding histological section, guided by the cyanoacrylate adhesive markers. We prepared a second set of experiments using nylon sutures as markers.

RESULTS: The OCT image of the esophageal specimen has a clear five-layered morphology. First, it consisted of a relatively less reflective layer; second, a more reflective layer; third, a less reflective layer; fourth, a more reflective layer; and fifth, a less reflective layer. Comparing the OCT images with marked histological sections showed that the first layer corresponded to stratified squamous epithelium; the second to lamina propria; the third to muscularis mucosa; fourth, submucosa; and fifth, muscularis

INTRODUCTION

The recent widespread use of endoscopy has enabled us to detect early stage superficial esophageal squamous cell carcinoma (SESCC). Endoscopic resection can be performed for these SESCO with the advantage of it being a minimally invasive treatment for patients^[1-5]. According to a draft of subclassifications of SESCO that was formulated by the Japanese Society for Esophageal Disease^[6], the depth of invasion of SESCO is defined as follows: epithelial layer (T1a-EP), proper mucosal layer (T1a-LPM), muscularis mucosa (T1a-MM), upper third of the submucosal layer (SM1), middle third of the submucosal layer (SM2), lower third of the submucosal layer (SM3). Based on this subclassification, endoscopic resection is indicated only for “T1a-EP” or “T1a-LPM” cancers because their frequency of lymph node metastasis is extremely rare. On the other hand, it was reported that

lymph node metastasis was observed in 8% of “T1a-MM” cancers and 13% of “SM1” cancers^[7], and that endoscopic resection may be insufficient as a radical therapy for these “T1a-MM” or “SM1” cancers^[6-10]. Therefore, an accurate diagnosis of depth of cancer invasion is important in the decision for an appropriate treatment for the SESCC patients, especially for the patients with “T1a-MM” or “SM1” cancers. Until now, endoscopic ultrasonography (EUS) has been the standard examination for the assessment of depth of cancer invasion in SESCC. However, its accuracy for diagnosing “T1a-MM” and “SM1” cancers is inadequate^[11].

A new imaging modality, optical coherence tomography (OCT)^[12] is a non-invasive optical imaging technology that provides high resolution, cross-sectional images of biological tissue in real time. OCT is analogous to B-scan ultrasonography (US); however, it measures reflected infrared light rather than acoustical waves. The resolution of OCT imaging (10-20 μm) is 5 to 25 times higher than that of high frequency US. Therefore, OCT is a currently available clinical device with the highest resolution for intraluminal tomographic imaging^[13].

Several *in vivo* and *in vitro* studies have reported the feasibility of OCT imaging in the GI tract^[14-28]. For the normal esophageal wall, it was reported that the OCT image was delineated as a layered morphology. However, there are few studies concerning the histological interpretation with regard to the layered morphology of the OCT image and these studies differ in their interpretation.

The aim of this study was to ascertain which layer of the OCT image corresponded to each component of the esophageal wall.

MATERIALS AND METHODS

We employed an OCT system developed by Light Lab Imaging (Boston, USA) and HOYA (Tokyo, Japan) (Figure 1). The OCT images were obtained using a superluminescent diode light source with a center wavelength of 1300 nm, a bandwidth of 50 nm, and power output 10 mW, resulting in a 10-20 μm axial image resolution. The lateral or transverse resolution was determined by the diffraction limit of the OCT endoscopic catheter. The spot diameter that resulted from the diffraction of the light was selected to be comparable to the axial OCT resolution while maintaining an appropriate depth of focus. By scanning the interrogating beam across the tissue surface, a series of tomograms were obtained and constructed into a two-dimensional image.

We used a 1.5 mm diameter prototype OCT probe which could be inserted through the accessory port of an endoscope and provide a 360-degree radial scan. Since the OCT beam is invisible, the position of the beam on samples was monitored using a coincident, visible-light, guiding beam (670 nm). OCT images were typically displayed in gray-scale. That is, the image could be configured to represent highly reflective signals as white, and low reflective signals as black.

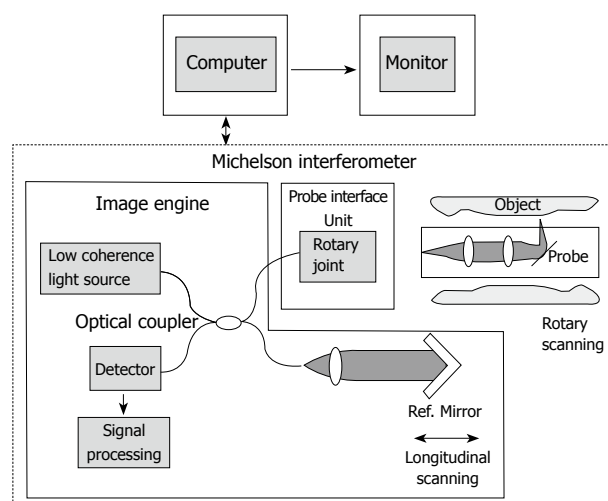


Figure 1 Schema of the OCT system (Light Lab Imaging, Boston, USA, and HOYA, Tokyo, Japan) used in the present study. Near infrared light is generated from the light source, and then is split evenly. One beam is directed to the tissue sample and the other to a reference mirror. The light is reflected from both the sample and mirror. The reflected light beams are recombined in a beam splitter.

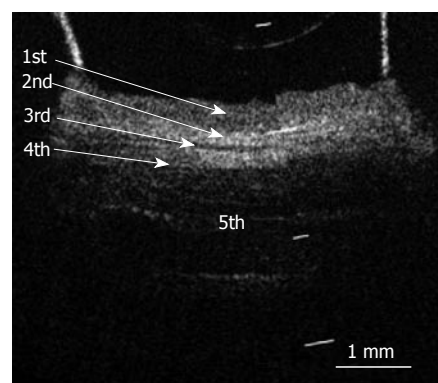


Figure 2 OCT image of a fresh pig esophageal wall specimen. OCT image of the esophageal wall delineated a five-layered morphology (arrows) consisting of a relatively less reflective layer (the first layer), a more reflective layer (the second layer), a less reflective layer (the third layer), a more reflective layer (the fourth layer), and a less reflective layer (the fifth layer). The white bar represents 1 mm.

We assessed the layered morphology of OCT images of the esophageal wall, referring to previous studies for the identification of the layered morphology of the GI tract wall with EUS^[29,30]. We employed fresh pig esophageal wall as tissue specimens, because the histological structure of the pig esophageal wall is similar to that of humans. The tissue specimens were used within two hours, because the inherent optical property of the specimens may change with time. Excess blood and mucus were carefully removed by washing with saline. Specimens were then stretched and pinned onto a rubber plate with the luminal surfaces exposed. The position for the OCT imaging was marked on the specimen using two needles pinned through the specimen about 2 mm apart. To create a marker for identifying the layered morphology of the OCT image, we injected a small amount of cyanoacrylate adhesive

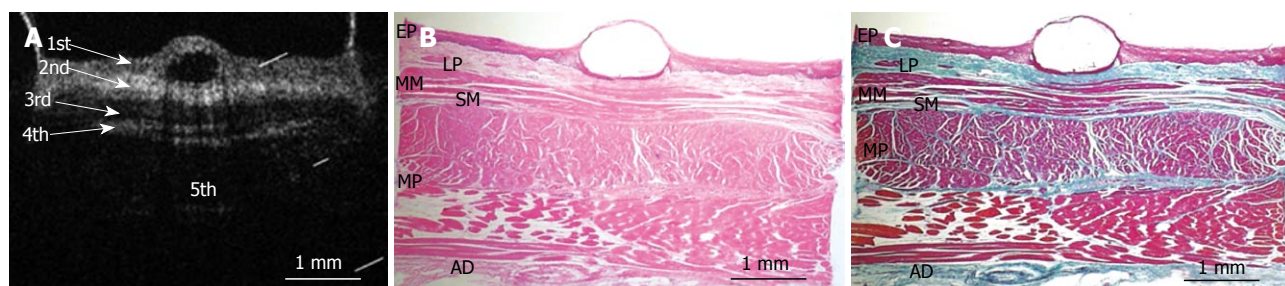


Figure 3 OCT image and corresponding histological sections of esophageal wall specimen injected with cyanoacrylate adhesive into the first layer. A: OCT image of the esophageal wall specimen injected with cyanoacrylate adhesive into the first layer (relatively less reflective layer). The injection location of cyanoacrylate adhesives into the first layer is clearly recognized. B: Corresponding histological section. The cyanoacrylate adhesive is observed in the stratified squamous epithelium (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm. EP: Stratified squamous epithelium; LP: Lamina propria; MM: Muscularis mucosa; SM: Submucosa; MP: Muscularis propria; AD: Adventitious tissue.

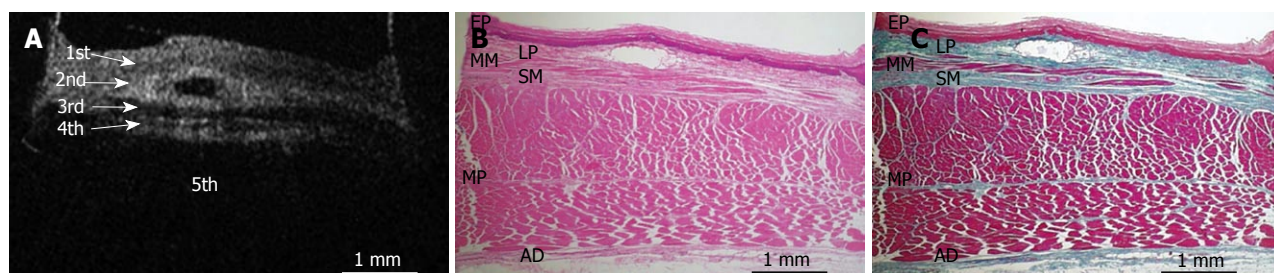


Figure 4 OCT image and corresponding histological sections of esophageal wall specimen injected with cyanoacrylate adhesive into the second layer. A: OCT image of the esophageal wall specimen injected with cyanoacrylate adhesive into the second layer (more reflective layer). The injection location of cyanoacrylate adhesive into the 2nd layer is clearly recognized. B: Corresponding histological section. The cyanoacrylate adhesive is observed in the lamina propria (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

with a needle (24 G, diameter 400 μm) into the tissue specimen between the two needles. After the injection, we scanned the position between the two needles with the OCT probe.

The specimens were subjected to routine histological processing. Briefly, the specimens were immersed in 10% buffered formalin for 48 h and processed for standard paraffin embedding. Five-micron-thick sections were cut at the marked position and stained with Hematoxylin-Eosin (HE) and Elastica-Masson (EM). Finally, the layered morphology of OCT images was compared with that of each marked histological section.

We performed another experiment using nylon sutures as a marker instead of injecting cyanoacrylate adhesive. Thin nylon sutures (surgical suture with a needle: diameter 70 μm approximately) were passed through each layer of the specimens to create a marker on each layer imaged by OCT.

RESULTS

The OCT image of normal esophageal wall

Figure 2 shows an OCT image of the normal pig esophagus specimen. In the OCT image, the five-layered morphology of the esophageal wall is clearly delineated. The layers were arranged outward as follows: a relatively less reflective layer (the first layer), a more reflective layer (the second layer), a less reflective layer (the third layer), a more reflective layer (the fourth layer), and a less reflective layer (the fifth layer).

Comparison of the OCT image with histology using cyanoacrylate adhesive as a marker

Figures 3-7 show OCT images and corresponding histology of the normal pig esophageal wall injected with cyanoacrylate adhesive. The OCT image after injection with cyanoacrylate adhesive into the first layer and corresponding histology are shown in Figure 3. The position injected with cyanoacrylate adhesive was recognized as a non-reflective spot. The non-reflective spot was observed in the first layer of the OCT image. In the corresponding histological section, the cyanoacrylate adhesive was observed in stratified squamous epithelium. Therefore, we confirmed that the first (relatively less reflective) layer of the OCT image corresponded to stratified squamous epithelium. The second (more reflective) layer corresponded to lamina propria (Figure 4), the third (less reflective) layer corresponded to muscularis mucosa (Figure 5), the fourth (more reflective) layer corresponded to submucosa (Figure 6), and the fifth (less reflective) layer corresponded to muscularis propria with deeper structures of the esophageal wall (Figure 7).

Comparison of the OCT image with histology using thin nylon suture as a marker

Figures 8-12 show OCT images and corresponding histology using thin nylon sutures as markers. In Figure 8, the thin nylon suture was recognized as a more reflective dot in the first layer of the OCT image. In the corresponding histological section, the fragment of

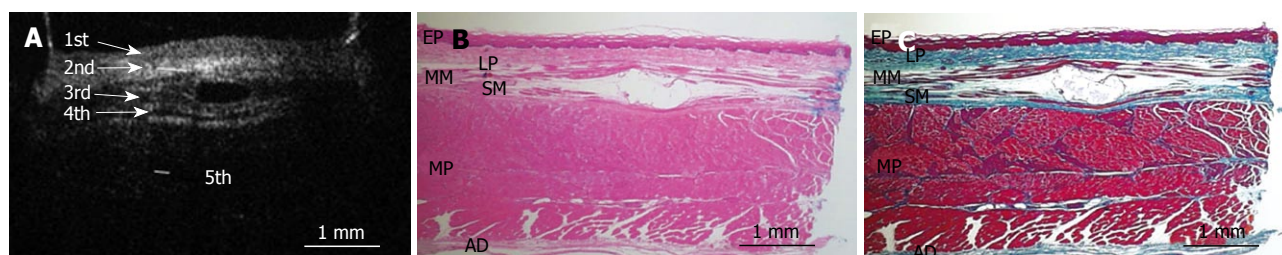


Figure 5 OCT image and corresponding histological sections of esophageal wall specimen injected with cyanoacrylate adhesive into the third layer. A: OCT image of esophageal wall injected with cyanoacrylate adhesive into the third layer (less reflective layer). The injection location of cyanoacrylate adhesive into the third layer is clearly recognized. B: Corresponding histological section. The cyanoacrylate adhesive is observed in the muscularis mucosa (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

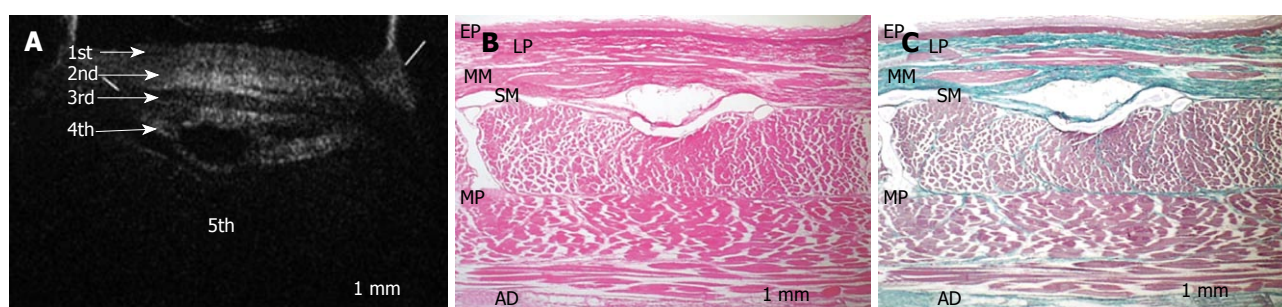


Figure 6 OCT image and corresponding histological sections of esophageal wall specimen injected with cyanoacrylate adhesive into the fourth layer. A: OCT image of esophageal wall specimen injected with cyanoacrylate adhesive into the fourth layer (more reflective layer). The injection location of cyanoacrylate adhesive into the fourth layer is clearly recognized. B: Corresponding histological section. The cyanoacrylate adhesive is observed in submucosa (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

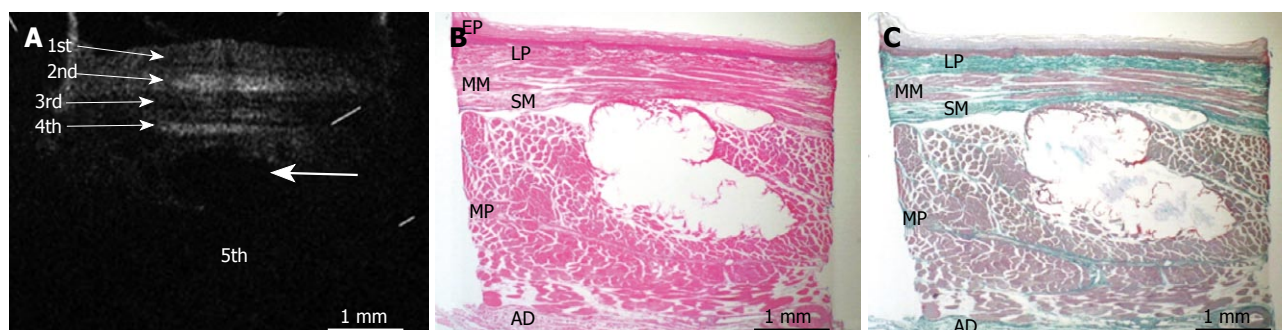


Figure 7 OCT image and corresponding histological sections of esophageal wall specimen injected with cyanoacrylate adhesive into the fifth layer. A: OCT image of esophageal wall specimen injected with cyanoacrylate adhesive into the fifth layer (less reflective layer). The large white arrow indicates the injection location for cyanoacrylate adhesive. B: Corresponding histological section. The cyanoacrylate adhesive is observed in the muscularis propria (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

thin nylon suture was observed in stratified squamous epithelium. Therefore, we confirmed that the first (less reflective) layer of the OCT image corresponded to stratified squamous epithelium. The second (more reflective) layer corresponded to the lamina propria (Figure 9), the third (less reflective) layer corresponded to the muscularis mucosa (Figure 10), the fourth (more reflective) layer corresponded to the submucosa (Figure 11), and the fifth (less reflective) layer corresponded to the muscularis propria with deeper structures of the esophageal wall (Figure 12).

Figure 13 presents a schema of the OCT image of the normal esophageal wall with a delineated five-layered morphology, consisting of relatively less reflective stratified squamous epithelium, more reflective

lamina propria, less reflective muscularis mucosa, more reflective submucosa, and less reflective muscularis propria with deeper structures of the esophageal wall.

DISCUSSION

We have shown that OCT can clearly delineate the five-layered morphology of the normal pig esophageal wall *in vitro*. In addition, we confirmed that the layered morphology imaged by OCT matches the histological structure of the esophageal wall.

Sergeev *et al.*^[14] were the first to report that they obtained OCT images of the human esophageal wall and stomach. Also, several studies demonstrated the utility of OCT for the diagnosis of Barrett's esophagus

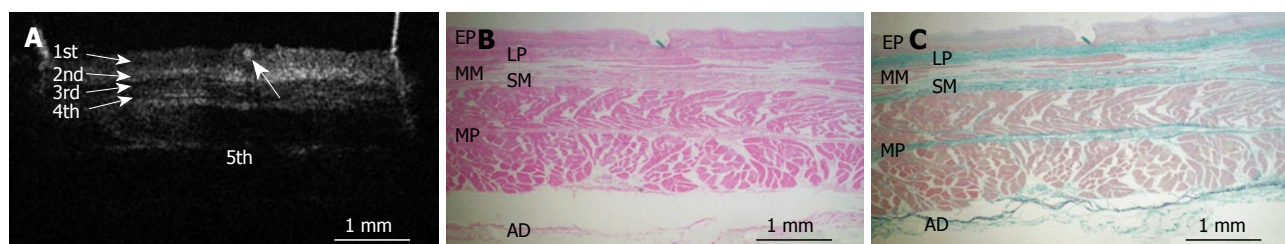


Figure 8 OCT image and corresponding histological sections of esophageal wall specimen with a thin nylon suture in the first layer. A: OCT image of esophageal wall specimen with a thin nylon suture in the first layer (relatively less reflective layer). The marker of the thin nylon suture (large white arrow) is recognized in the 1st layer. B: Corresponding histological section. The fragment of nylon suture is observed in the stratified squamous epithelium. (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

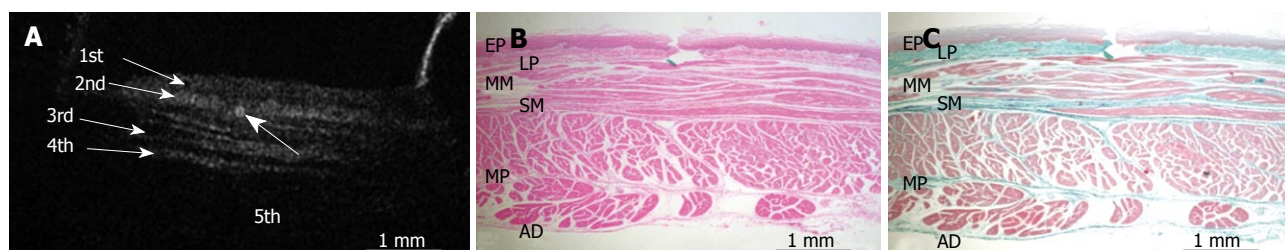


Figure 9 OCT image and corresponding histological sections of esophageal wall specimen with a thin nylon suture in the second layer. A: OCT image of esophageal wall specimen with a thin nylon suture in the second layer (more reflective layer). The marker of the thin nylon suture (large white arrow) is recognized in the second layer. B: Corresponding histological section. The fragment of thin nylon suture is observed in the lamina propria. (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

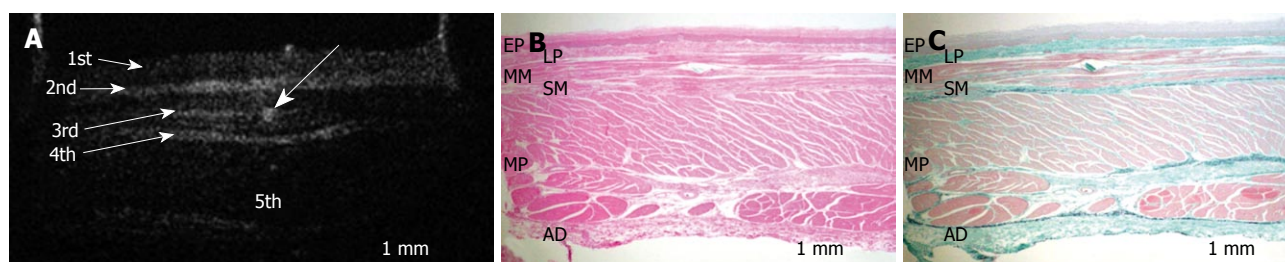


Figure 10 OCT image and corresponding histological sections of esophageal wall specimen with a thin nylon suture in the third layer. A: OCT image of esophageal wall specimen with a thin nylon suture in the third layer (more reflective layer). The marker of thin nylon suture (large white arrow) is recognized in the third layer. B: Corresponding histological section. The fragment of thin nylon suture is observed in the muscularis mucosa. (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

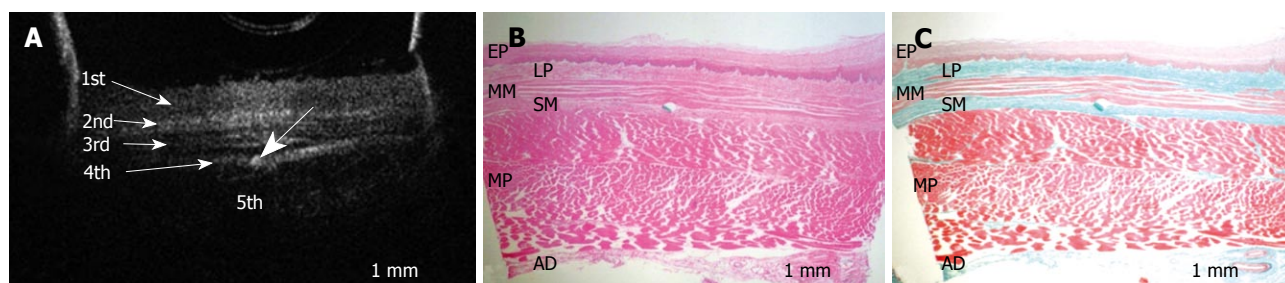


Figure 11 OCT image and corresponding histological sections of esophageal wall specimen with a thin nylon suture in the fourth layer. A: OCT image of esophageal wall specimen with a thin nylon suture in the fourth layer (more reflective layer). The marker of thin nylon suture (large white arrow) is recognized in the fourth layer. B: Corresponding histological section. The fragment of nylon suture is observed in the submucosa. (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

(BE) because the high resolution of OCT images could distinguish BE from stratified squamous epithelium or gastric mucosa^[15-22].

In the normal squamous stratified epithelium of the

human esophageal wall, several studies demonstrated *in vivo* that the OCT image of the esophageal wall was delineated as a five-layered morphology, and that the muscularis mucosa was delineated as a less reflective

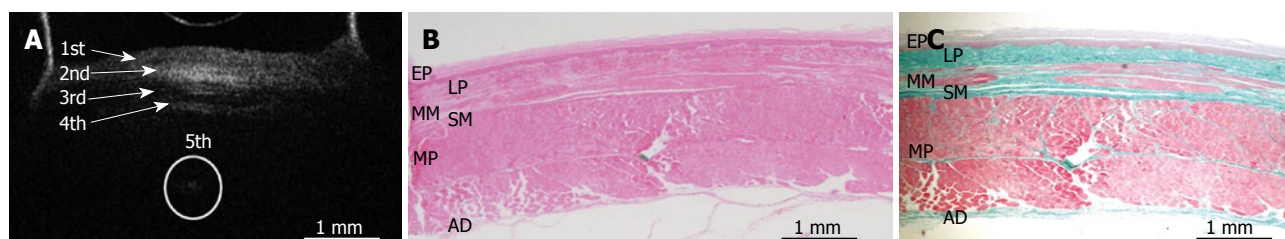


Figure 12 OCT image and corresponding histological sections of esophageal wall specimen with a thin nylon suture in the fifth layer. A: OCT image of esophageal wall specimen with a thin nylon suture in the fifth layer (more reflective layer). The marker of thin nylon suture (indicated within white circle) is recognized in the fifth layer. B: Corresponding histological section. The fragment of nylon suture is observed in the muscularis propria. (HE, original magnification $\times 4$). C: Corresponding histological section (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

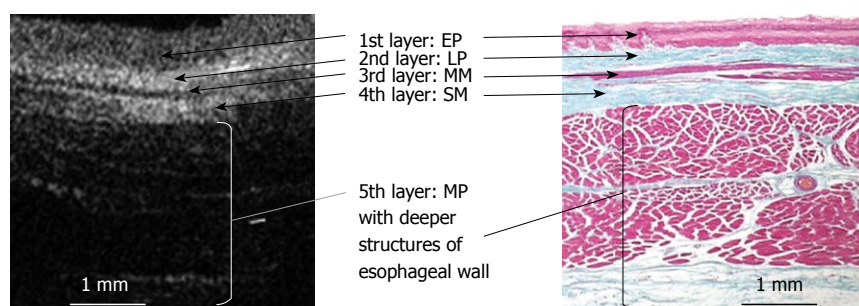


Figure 13 A schema of the correspondence between the OCT image of normal esophagus and histology. The OCT image of normal esophageal wall delineates a five-layered morphology, consisting of relatively less reflective stratified squamous epithelium (the first layer), more reflective lamina propria (the second layer), less reflective muscularis mucosa (the third layer), more reflective submucosa (the fourth layer), and less reflective muscularis propria with deeper structures of esophageal wall (the fifth layer). (EM, original magnification $\times 4$). The white and black bars represent 1 mm.

layer^[14-17,23]. However, Sivak *et al*^[24] reported that the esophageal wall was observed as an eight-layered morphology, and that the muscularis mucosa appeared to be a relatively thick triple layer (two more reflective layers separated by one of less reflectivity). Das *et al*^[25] reported that muscularis mucosa was delineated as a more reflective multilayered structure. Jäckle *et al*^[23] attempted to interpret *in vivo* OCT images compared with the histological structure using vessels or glands in the biopsy or mucosectomy specimens of the esophageal wall as markers, resulting in the association of the main structures of *in vitro* OCT images with epithelium, lamina propria and muscularis mucosa. However, it has proved difficult to determine all the layers of the esophageal wall visualized by OCT using only biopsy and mucosectomy specimens. Therefore, correlation of the layered morphology of the *in vivo* OCT image with the histological structure of esophageal wall has not been sufficiently clarified.

On the other hand, *in vitro* studies of surgically resected specimens or autopsies can compare OCT images of the esophageal wall with full thickness sections. Cilesiz *et al*^[26] reported that OCT images of esophageal wall were observed to closely agree with *in vivo* data. But, even for *in vitro* studies, the interpretation of OCT images of esophageal wall is still under discussion. Tearney *et al*^[27] suggested that muscularis mucosa was more reflective than stratified squamous epithelium. Other studies reported that stratified squamous epithelium presented as a more reflective layer than lamina propria^[18,28]. In these studies, the layered morphology of the OCT image was interpreted without a definitive marker for orientation of the histological structure of the esophageal wall.

Considering the above-mentioned matters, we used fresh pig esophageal wall specimens and histologically

assessed the layered morphology imaged by OCT using both cyanoacrylate adhesives and nylon sutures as markers. Under these conditions, we demonstrated that the OCT image of the normal esophageal wall clearly delineated the five-layered morphology, and that the layered morphology consisted of relatively less reflective stratified squamous epithelium, more reflective lamina propria, less reflective muscularis mucosa, more reflective submucosa, and less reflective muscularis propria with deeper structures of esophageal wall.

The mechanism by which OCT images delineate five-layered morphology may be explained by the difference of reflectivity of light through the esophageal tissues, which have different optical properties. Generally, the structure of epithelium, muscularis mucosa, and muscularis propria is uniform and orderly consisting of only epithelial cells or smooth muscle fibers. In this study, OCT images delineated them as less reflective layers because the light passes through them with less obstruction. In contrast, lamina propria and submucosa consist of various mixed components such as connective tissue, vessels, lymph follicles, and glands. The OCT images of these were delineated as more reflective layers due to obstruction of the passage of light.

Concerning the diagnosis for depth of invasion of SESCC, Murata *et al*^[11] reported that accuracy assessed by EUS was 81% for both “T1a-EP” and “T1a-LPM” cancers, but that the accuracy for both “T1a-MM” and “SM1” cancers was only 60%, suggesting that it was hard to make a diagnosis for the “T1a-MM” and “SM1” cancers by EUS. In our study, OCT clearly imaged the muscularis mucosa which is a boundary line between “T1a-MM” and “SM1” cancer. Therefore, OCT may provide us with the opportunity to select more appropriate treatment for SESCC patients than

does EUS. Das *et al*^[25] reported that the resolution of the OCT image was superior to that of high-frequency EUS; however, the depth of penetration with OCT was limited to mucosa and submucosa when compared with the high-frequency EUS. Further investigation is necessary to evaluate the feasibility of diagnosing the depth of invasion in SESCC.

In conclusion, we have demonstrated that the OCT image of the normal esophageal wall clearly delineates a five-layered structure, and we have identified the layered morphology which consists of relatively less reflective stratified squamous epithelium, more reflective lamina propria, less reflective muscularis mucosa, more reflective submucosa, and less reflective muscularis propria with deeper structures of the esophageal wall.

ACKNOWLEDGMENTS

We thank Masashi Kitatsuji and Nobuyuki Saita, HOYA CORPORATION PENTAX Life Care Division, for invaluable assistance.

COMMENTS

Background

In the normal esophageal wall, it has been reported that the optical coherence tomography (OCT) image is delineated as a layered morphology. But there are few studies concerning the histological interpretation of the layered morphology of the OCT image and these studies differ in their interpretation.

Research frontiers

To ascertain which layer of the OCT image corresponded to each component of the esophageal wall.

Innovations and breakthroughs

Comparing the OCT images with marked histological sections showed that the first layer corresponded to stratified squamous epithelium; the second to lamina propria; the third to muscularis mucosa; fourth, submucosa; and fifth, muscularis propria with deeper structures of the esophageal wall.

Applications

OCT images may contribute to the accurate diagnosis of the depth of cancer invasion, and thus help when choosing therapeutic options for superficial esophageal squamous cell carcinoma (SESCC).

Peer review

The authors demonstrated that the OCT image of the normal esophageal wall clearly delineates a five-layered structure, and identified the layered morphology consisting of relatively less reflective stratified squamous epithelium, more reflective lamina propria, less reflective muscularis mucosa, more reflective submucosa, and less reflective muscularis propria with deeper structures of the esophageal wall. Therefore, OCT images may contribute to the accurate diagnosis for the depth of cancer invasion, when choosing therapeutic options for SESCC.

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S- Editor Tian L L- Editor Logan S E- Editor Yin DH



ORIGINAL ARTICLES

Influence of paeonol on expression of COX-2 and p27 in HT-29 cells

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Received: June 3, 2009

Revised: July 28, 2009

Accepted: August 4, 2009

Published online: September 21, 2009

Key words: Paeonol; HT-29 cell line; Cyclooxygenase-2; p27

Peer reviewer: Jian-Ying Wang, Professor, University of Maryland School of Medicine, Baltimore VA Medical Center (112), 10N. Greene St, Baltimore, MD 21201, United States

Ye JM, Deng T, Zhang JB. Influence of paeonol on expression of COX-2 and p27 in HT-29 cells. *World J Gastroenterol* 2009; 15(35): 4410-4414 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4410.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4410>

Abstract

AIM: To investigate the effect of paeonol on controlling the proliferation of colorectal cancer cell line HT-29 and to discuss its possible mechanism.

METHODS: The inhibitory effect of paeonol on proliferation of HT-29 cells was detected by MTT assay. The results of apoptosis were measured by flow cytometry. Immunocytochemical staining was performed to detect the expression of cyclooxygenase-2 (COX-2) and protein p27 in HT-29 cells treated with paeonol at different concentrations. Reverse transcription-polymerase chain reaction (RT-PCR) was used for mRNA analysis.

RESULTS: From the data of both MTT and flow cytometry, we observed that cell proliferation was inhibited by different concentrations of paeonol. By immunocytochemical staining, we found that HT-29 cells treated with paeonol (0.024-1.504 mmol/L) reflected reduced expression of COX-2 and increased expression of p27 in a dose-dependent manner. RT-PCR showed that paeonol down-regulated COX-2 and up-regulated p27 in a dose- and time-dependent manner in HT-29 cells.

CONCLUSION: One of the apoptotic mechanisms of paeonol is down-regulation of COX-2. p27 is up-regulated simultaneously and plays an important part in controlling cell proliferation and is a crucial factor in the Fas/FasL apoptosis pathway.

INTRODUCTION

It has been confirmed that inhibition of expression of cyclooxygenase-2 (COX-2) has antitumor activity against gastrointestinal carcinoma^[1]. Selective COX-2 inhibitor celecoxib can prevent and reduce the incidence of colorectal cancer to some extent^[2]. However, a recent study has shown that cardiovascular toxicity is caused when selective COX-2 inhibitors are used in the long term^[3]. Consequently, it is considerable importance that a more effective and less toxic selective COX-2 inhibitor, which can be used for the treatment of colorectal carcinoma, is found.

Paeonol is a herb that is the main active component of peony bark. It has been shown to have many pharmacological activities, such as antifebrile, analgesic, antipyretic, anti-atherosclerotic, anti-platelet and antioxidative activity^[4,5]. It has also been demonstrated that paeonol has antitumor effects in animal experiments^[6]. Our recent study on apoptosis after addition of paeonol in human colorectal carcinoma cell line HT-29 shows that paeonol has antitumor activity, by regulating the expression of Fas, Bcl-2, Bax and p53 and finally causing apoptosis^[7,8]. However, the pathway of paeonol-induced apoptosis is still confusing, which emphasizes the necessity of identifying its antitumor mechanism.

In the present study, the expression of COX-2 and p27 was detected and compared, and one of the antitumor mechanisms of paeonol was explored.

MATERIALS AND METHODS

Materials

Human colorectal carcinoma cell line HT-29 was purchased from the Cancer Research Institute of the

Wuhan University in China. Paeonol (Tianzhen Pharmaceutical Company of Ningbo, China), 5 mg/mL, RPMI 1640 (Hyclone Company, USA), calf serum, DABC immunoassay kit (Beijing Zhongshan Company, China), mouse anti-p27, rabbit anti-COX-2, GAPDH, anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were purchased as indicated.

Methods

MTT assay^[9]: Cell proliferation was measured using the MTT assay. Cells were plated in triplicate at 5×10^3 cells/well in 96-well plates, and treated with increasing concentrations of paeonol for culture of 24, 48, 72 and 96 h. Twenty microliters of 5 mg/mL MTT (Amresco, OH, USA) was added to each well and the cells were cultured at 37°C for an additional 4 h. The resulting formazan crystals were solubilized by the addition of 150 μ L DMSO to each well. The optical density at 570 nm was measured and the percentage cell viability was calculated using the following formula: percentage cell viability = (absorbance of experimental well - absorbance of blank)/(absorbance of untreated control well - absorbance of blank) \times 100% (Figure 1).

Immunocytochemistry: HT-29 cells (5×10^5 - 7×10^5) were seeded into 24-well plates and treated with paeonol at a concentration of 0, 0.094, 0.376 or 1.504 mmol/L for 48 h, in triplicate, and then cells were trypsinized, and rinsed with PBS. Immunocytochemical staining was carried out using the DABC immunoassay kit according to the manufacturer's instructions. Immunostaining was observed by immunofluorescence microscopy. Positive staining for COX-2 was located mainly in the cytosol, and p27 in the nucleus. Immunocytochemical staining was classified as follows^[10]: number of positive cells < 10% was deemed as negative (-), 10%-25% as (+), 25%-50% as (++), and > 50% as (+++).

Flow cytometry: HT-29 cells (5 - 7×10^5) were seeded in six-well plates. Paeonol at 0, 0.094, 0.376 or 1.504 mmol/L was added to each well, in triplicate. Cells were incubated for another 48 h. Cells (5×10^6 /L) were collected and detected by flow cytometry.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR): Total RNA was isolated using the Trizol reagent (Invitrogen, CA, USA) and 1 μ g RNA was used as a template for the synthesis of cDNA using the RversetAid First Strand cDNA Aynthesis Kit (Fermentas, ME, USA) according to the manufacturer's instructions. PCR analysis was performed in a final volume of 25 μ L using PCR Master Mix (Fermentas). COX-2-specific primer sequences (forward: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'; reverse: 5'-AFATCATCTCTGCCTGAGTATCTT-3'); p27-specific primer sequences (forward: 5'-CGTGCAGTGTCTAACGG-3'; reverse: 5'-CGGATCAGTCTTTGGGTC-3'); GAPDH-specific sequences (forward: 5'-ACGGATTGTGGTCGTATTGGG-3'; reverse: 5'-TC

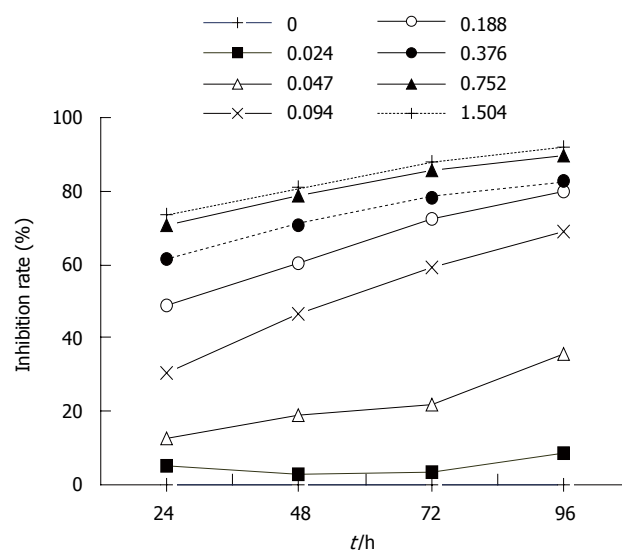


Figure 1 Effect of different concentrations (0-1.504 mmol/L) of paeonol on proliferation of HT-29 cells. The inhibition of cell proliferation showed a dose- as well as time-dependent increase.

CTGGAAGATGGTGTGATGGG-5') were designed using the Primer Premier 3 (Applied Biosystems, CA, USA). PCR products were separated in 1.5% agarose gels, stained with ethidium bromide and photographed.

Statistical analysis

Statistics were calculated with the SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). All determinations were repeated in triplicate. Data are presented as means \pm SD. Significance for comparison of samples was determined by Student's *t* test and χ^2 test. $P < 0.05$ were considered to be statistically significant throughout the study.

RESULTS

Inhibition of cell proliferation by paeonol in a dose- and time-dependent manner

HT29 cells were treated with paeonol at different concentrations for 0, 24, 48 and 96 h, and the rate of inhibition was determined by MTT assay. We observed that growth of HT-29 cells was suppressed in a dose- and time-dependent manner (Figure 1).

Effects of paeonol on cell apoptosis

In order to confirm the results obtained from the MTT assay and to elucidate the mechanisms of action of paeonol, we analyzed apoptosis using DABC immunocytochemistry (Figure 2A and B) and flow cytometry (Figure 3). HT-29 cells were treated with 0, 0.094, 0.376 or 1.504 mmol/L paeonol and examined after 48 h. Paeonol lowered the proportion of COX-2-positive cells, while increasing the level of p27, in a dose-dependent fashion (Tables 1 and 2).

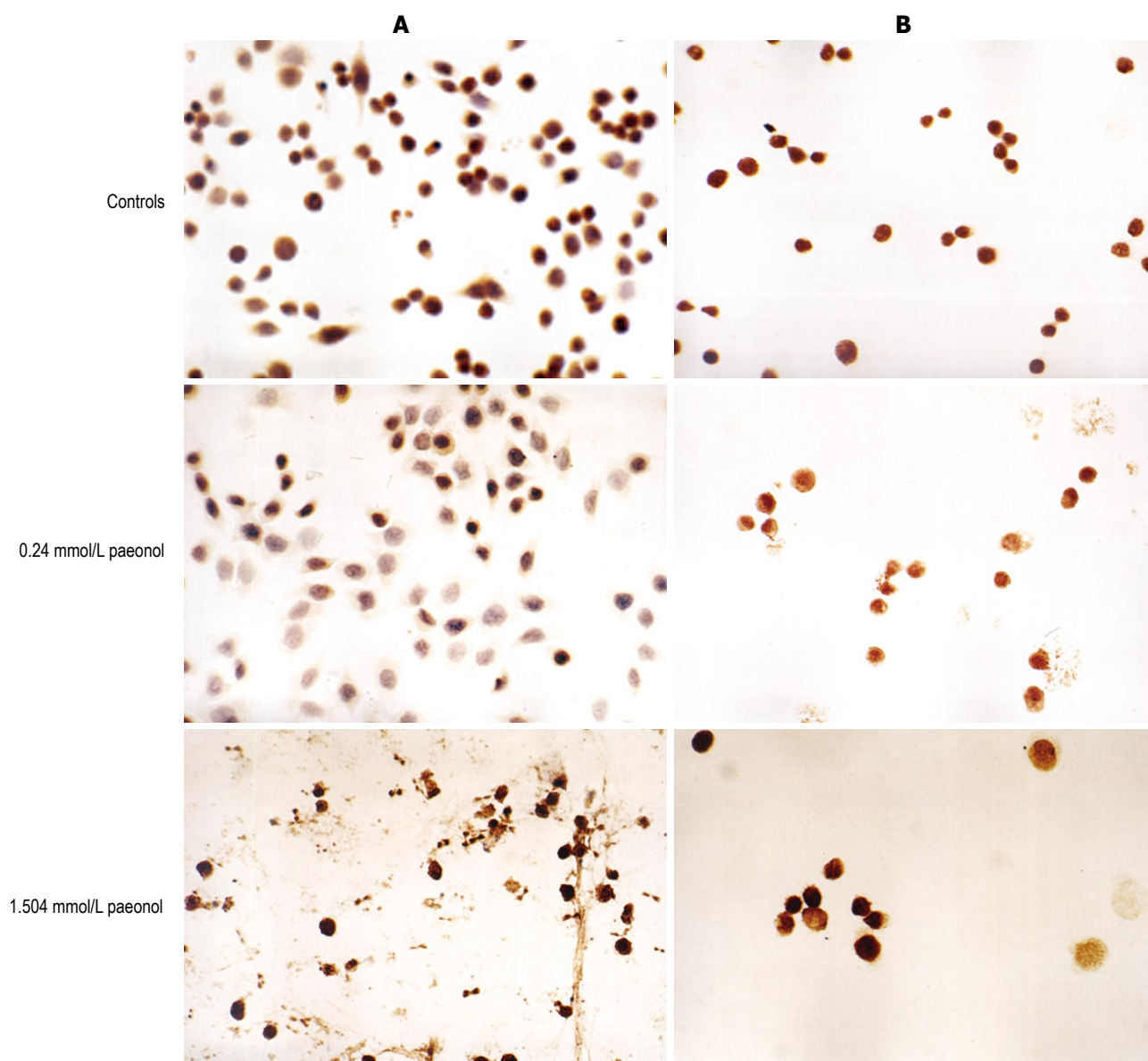
Cell cycle distribution was assessed by flow cytometry. As shown in Figure 2B, the rate of apoptosis increased significantly as higher concentrations of paeonol were used, which reached 34.5% when paeonol was

Table 1 Expression of COX-2 protein in HT-29 cells after treatment with paeonol

Paeonol concentration (mmol/L)	Staining intensity	Positivity rate (%)
0	+++	98.21 ± 0.835
0.094	+++	95.82 ± 0.751 ^a
0.376	++	47.39 ± 0.831 ^a
1.504	+	23.60 ± 1.390 ^a

^a*P* < 0.05 vs untreated control cells.**Table 2** Rate of apoptosis, cell cycle and expression of p27 after treatment with paeonol

Paeonol concentration (mmol/L)	p27 staining intensity	p27 positive rate	Rate of apoptosis	G ₀ /G ₁ phase	S phase	G ₂ /M phase
0	-	0.46	2.3	61.3	20.3	18.4
0.094	+	18.18 ^b	7.6 ^b	28.7 ^b	55.8 ^b	16.1
0.376	++	46.70 ^b	16.1 ^b	50.7 ^b	35.8 ^b	13.5 ^b
1.504	+++	86.86 ^b	34.5 ^b	38.0 ^b	45.6 ^b	16.4

^b*P* < 0.01 vs untreated control cells.**Figure 2** Expression of COX-2 (A) and p27 (B) in HT-29 cells induced by paeonol (× 400).

1.054 mmol/L. Paeonol caused S-phase arrest and thus inhibited transition to the G₀ and G₂/M phases in a dose-dependent manner (*P* < 0.01) (Table 2).

The results suggested that apoptosis was the main mechanism by which paeonol inhibited the proliferation of HT-29 cells, and COX-2 and p27 were involved in the changes.

Dose- and time-dependent effect of paeonol on COX-2 and p27

It has already been reported that COX-2 plays a critical role in controlling the growth as well as apoptosis in carcinogenesis. To elucidate the interaction between COX-2 and paeonol, HT-29 cells were exposed to 0, 0.094, 0.376 or 1.504 mmol/L paeonol for 48 h, and

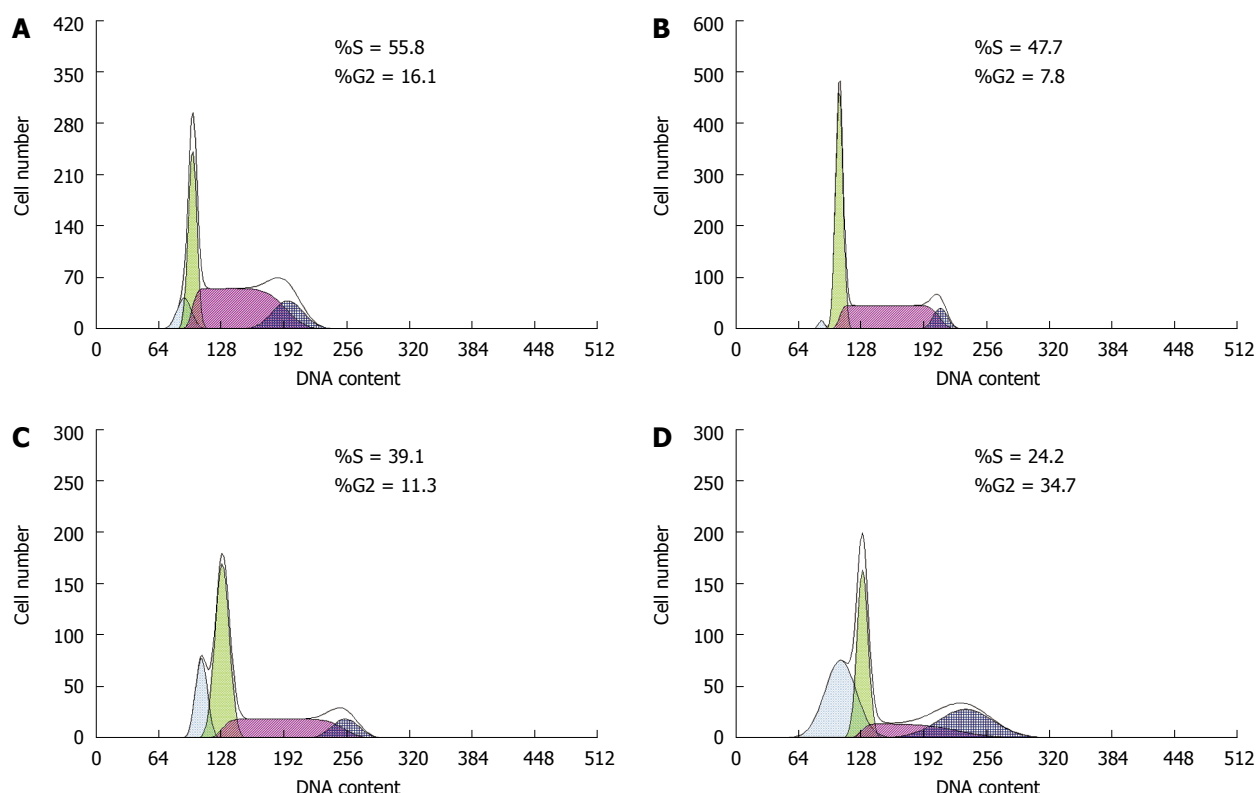


Figure 3 Flow cytometry detects apoptosis of HT-29 cells induced by paeonol. A: Controls; B: 0.094 mmol/L paeonol; C: 0.376 mmol/L paeonol; D: 1.504 mmol/L paeonol.

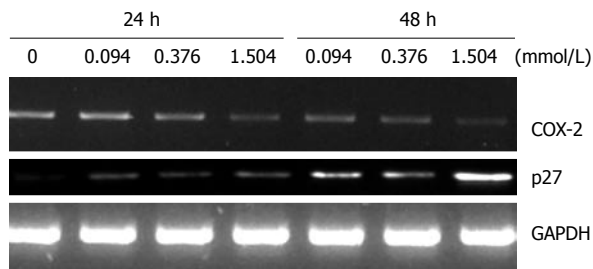


Figure 4 Changes in COX-2 and p27 induced by paeonol. After exposure to different concentrations for 24 and 48 h, HT-29 cells were observed to have a dose-dependent decrease in COX-2, but an increase in p27. GAPDH acted as an internal control.

expression of COX-2 and p27 was detected, as shown in Figure 4. We observed that the level of COX-2 mRNA from cells treated with 0.094 mmol/L paeonol was lower than in the controls. Treatment with 1.504 mmol/L paeonol caused a further decrease in COX-2 expression, which indicated a dose-dependent decrease in COX-2 expression.

p27, which also plays a critical role in apoptosis, showed increased mRNA expression as the concentration of paeonol was increased (Figure 4), which indicated a dose- and time-dependent response.

DISCUSSION

COX-2 is an key inducible enzyme that regulates prostaglandin E2 (PGE2) synthesis^[11]. It has a low expression level in normal tissue, while its expression in

multiple malignant cancer cells is exceedingly high^[12,13]. By promoting the proliferation of cancer cells and suppressing the expression of certain angiogenesis genes, PGE2 contributes to reducing apoptosis and increasing tumor invasiveness^[14,15]. We found in the present study that paeonol had an inhibitory effect on the expression of COX-2 in HT-29 cells, which was dose- as well as time-dependent. The results show that one of the possible pharmacological effects of paeonol is inhibiting the expression of COX-2.

p27 is an anti-oncogene which is one of the cyclin-dependent kinase inhibitors, and it belongs to the p21 family. It inhibits cell proliferation by blocking the cell cycle^[16,17]. We discovered in the present study that paeonol promoted the expression of p27 in HT-29 cells in a dose-dependent manner ($P < 0.01$). The cell cycle was blocked in S phase, and the percentages of G₀/G₁ and G₂/M cells decreased significantly after paeonol treatment. From the cytometry figures, we observed that there was an obvious apoptosis peak after adding paeonol to HT-29 cells at a concentration of 1.504 mmol/L, and the rate of apoptosis was higher than that in the controls. This shows that paeonol can cause apoptosis by promoting the expression of p27, which blocks the cell cycle in S phase and interferes with DNA synthesis.

We also discovered that not only does paeonol down-regulate the expression of COX-2, but it also promotes the expression of p27 at the same time, which suggests that they are on the same apoptotic pathway. It has been reported that COX-2 is one of the upstream factors that controls Bcl-2 expression^[18]. It has already

been demonstrated in our previous study that paeonol can decrease the expression of Bcl-2 and increase the expression of Fas and caspase-8 in HT-29 cells, and we drew the conclusion that Fas/FasL is a central pathway that leads to paeonol-induced apoptosis^[19]. In the present study, we found that COX-2 and p27 were correlated negatively after paeonol treatment, and consequently, we deduced that COX-2 and p27 may cooperate in controlling the proliferation of colorectal carcinoma cells. That is, when activated, COX-2 inhibits expression of p27 and promotes cell proliferation. Another inference we can make from this study is that p27 plays an important part in the control of Fas/FasL apoptosis and promotes apoptosis when its expression is up-regulated.

To identify the apoptotic mechanism of paeonol more clearly, further investigations are needed. We expect that paeonol may replace nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors because of their toxic effects, and form a new strategy for the therapy of colorectal carcinoma.

COMMENTS

Background

Paeonol has been shown to have many pharmacological activities, which include an antitumor effect in animal experiments. The authors' previous work on the expression of apoptosis and apoptosis-related genes after treating human colorectal cell line HT-29 with paeonol has shown that paeonol has antitumor activity, by regulating the expression of apoptosis genes Fas, Bcl-2, Bax and p53, and inducing apoptosis. However, until now, the mechanism of action of paeonol has remained unclear. In the present study, the mechanism of the antitumor effect of paeonol was explored, and the expression of cyclooxygenase-2 (COX-2) and protein p27 was detected and discussed.

Research frontiers

Colorectal carcinoma is common all around the world and has a high incidence among all the carcinomas. In research on paeonol, the hot topics are to identify the mechanism of its antitumor action, and to establish the possible pathways by which paeonol induces apoptosis in the HT-29 cell line.

Innovations and breakthroughs

The authors discovered that p27 also played some role in regulating COX-2, which indicates that p27 may play a part in the control of the Fas/FasL apoptosis pathway and promote apoptosis when it is up-regulated.

Applications

The authors expect that paeonol may replace nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors because of their toxic effects and form a new therapeutic strategy for colorectal carcinoma.

Peer review

This study determined the changes in expression pattern of COX-2 and p27 following paeonol treatment of HT-29 cells. The results showed that there was a significant decrease in COX-2 expression that was associated with an increase in p27 activity in a dose-dependent manner. The authors also conducted flow cytometry analysis to differentiate the growth phases after paeonol administration.

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S- Editor Li LF L- Editor Kerr C E- Editor Ma WH

Capecitabine treatment patterns in patients with gastroesophageal cancer in the United States

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Received: July 27, 2009 Revised: August 21, 2009

Accepted: August 28, 2009

Published online: September 21, 2009

Abstract

AIM: To assess the use of capecitabine-based therapy and associated complication rates in patients with gastroesophageal cancer (GEC) in a real-world treatment setting.

METHODS: Patients with claims between 2004 and 2005 were identified from the Thomson Reuters MarketScan® databases. Capecitabine regimens were compared with 5-fluorouracil (5-FU) and other chemotherapy regimens, and were stratified by treatment setting.

RESULTS: We identified 1013 patients with GEC: approximately half had treatment initiated with a 5-FU regimen, whereas 11% had therapy initiated with a capecitabine regimen. The mean capecitabine dose overall was 2382 ± 1118 mg/d, and capecitabine was used as monotherapy more often than in combination. Overall, 5-FU regimens were the most common treatment option in neoadjuvant and adjuvant settings, while other non-capecitabine regimens were used more widely in first- and second-line settings. The overall unadjusted complication rate for capecitabine regimens was about half of that seen with 5-FU regimens. In multivariate analyses, capecitabine recipients had a 51% (95% CI: 26%-81%) lower risk of developing any complication than 5-FU recipients did. The risk of developing bone marrow, constitutional, gastrointestinal tract, infectious, or skin complications was lower

with capecitabine therapy than with 5-FU.

CONCLUSION: Capecitabine appeared to have a favorable side effect profile compared with 5-FU, which indicates that it may be a treatment option for GEC.

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Key words: Capecitabine; 5-fluorouracil; Hand-foot syndrome; Gastroesophageal cancer

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Saif MW, Shi N, Zelt S. Capecitabine treatment patterns in patients with gastroesophageal cancer in the United States. *World J Gastroenterol* 2009; 15(35): 4415-4422 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4415.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4415>

INTRODUCTION

Gastroesophageal cancer (GEC), which includes cancers that originate in the esophagus, gastroesophageal junction and stomach, is common in many countries. For example, gastric cancer is, by some estimates, the fourth most common cancer in the world, and esophageal cancer is eighth^[1]. The incidence of GEC shows a marked geographic variation, with high-prevalence areas including Asia, Southern and Eastern Africa, and South America^[2,3].

GEC is relatively uncommon in the United States compared with colorectal, lung, breast, and prostate cancers; however, the incidence of esophageal cancer (both squamous cell and adenocarcinoma) appears to be increasing^[4-6]. It is estimated that in 2008, 37 970 cases of GEC will have been diagnosed and 25 160 deaths will have been attributed to these cancers^[4].

A patient's outcome depends on cancer stage at diagnosis; approximately 50% of patients with gastric or esophageal cancer have advanced disease (at least extending beyond the locoregional confines) at diagnosis^[2,3]. The disease is highly metastatic; as a result, 70%-80% of resected specimens will have metastasized to the regional lymph nodes at the time of resection. The disease is associated with a poor prognosis: overall 5-year survival has been estimated at < 10%^[7].

Patients with resectable disease are candidates for adjuvant chemotherapy. 5-fluorouracil (5-FU) plus leucovorin and radiotherapy significantly improve median overall survival compared with surgery alone in patients with resected adenoma of the stomach or gastroesophageal junction (36 mo *vs* 27 mo; $P = 0.005$)^[8]. Regimens that may be considered for preoperative use or as definitive chemoradiation therapy for localized esophageal carcinoma include fluoropyrimidine-cisplatin, taxane-based, and oxaliplatin, and irinotecan-based regimens^[2].

Chemotherapy should also be considered for patients with metastatic disease. A meta-analysis of randomized trials in a total of 390 patients with advanced gastric cancer showed a greater 1-year survival rate and improved quality of life with chemotherapy than with supportive care^[9]. In this analysis, the 1-year survival rate achieved with supportive care was 8%, compared with 20% with chemotherapy ($P = 0.05$), and 30% of the patients in the chemotherapy group attained a 6-mo symptom-free period, *vs* 12% in the supportive care group ($P < 0.01$).

Chemotherapy options for advanced gastric or esophageal cancer include 5-FU/leucovorin, fluoropyrimidine-, cisplatin-, oxaliplatin-, and irinotecan-based combinations, and taxane-based regimens^[2].

Another treatment option currently being investigated in patients with GEC is the fluoropyrimidine capecitabine. Capecitabine has been used widely to treat patients with colorectal and breast cancer, and has a well-established safety profile. Capecitabine is administered orally, therefore, there is no requirement for intravenous access, and the associated complications and morbidity seen with IV 5-FU are absent.

Two recent phase III trials have suggested that survival with capecitabine-based regimens compares favorably with that with 5-FU-based regimens as first-line therapy for GEC^[10,11]. The REAL-2 study randomized 1002 patients with advanced GEC to receive first-line triple therapy with epirubicin and cisplatin plus either 5-FU (ECF) or capecitabine (ECX), or triple therapy with epirubicin and oxaliplatin plus either 5-FU (EOF) or capecitabine (EOX). The primary endpoint was non-inferiority in overall survival for the regimens that contained capecitabine as compared with 5-FU, and oxaliplatin as compared with cisplatin. Capecitabine-containing regimens demonstrated non-inferiority in overall survival compared with 5-FU-containing regimens. Median survival times in the ECF, ECX, EOF, and EOX groups were 9.9, 9.9, 9.3, and 11.2 mo, respectively.

In the other phase III trial, Kang and colleagues compared first-line doublet therapy with capecitabine/cisplatin *vs* 5-FU/cisplatin in 316 patients with advanced gastric cancer. The primary endpoint was non-inferiority in progression-free survival (PFS) between the two regimens. Non-inferiority was reached, with the capecitabine regimen achieving a median PFS of 5.6 mo *vs* 5.0 mo with the 5-FU regimen (unadjusted HR 0.81; 95% CI, 0.63-1.04; $P < 0.001$ *vs* non-inferiority margin of 1.25).

The current study was undertaken to assess the

usage of capecitabine-based therapies and associated complication rates in patients with GEC in a real-world treatment setting. Capecitabine is currently not approved by the US FDA for this indication.

MATERIALS AND METHODS

Data source

This retrospective analysis used claims data from the Thomson Reuters Healthcare MarketScan[®] Commercial Claims and Encounters and Medicare Supplemental and Coordination of Benefits databases to determine treatment patterns in patients with GEC. These databases contain data from 25 million individuals of all ages who are covered in the United States under employer- and government-funded (Medicare) health insurance plans, including a variety of fee-for-service and capitated provider reimbursement schemes. The MarketScan databases capture information on inpatient and outpatient health care claims, as well as outpatient pharmacy-dispensed drug claims. In compliance with the Health Insurance Portability and Accountability Act (HIPAA), patient data included in the analysis were statistically de-identified and therefore exempt from Institutional Review Board approval.

Patient selection

Patients included in the current analysis were required to have had at least three claims with a primary or secondary diagnosis of stomach or esophageal cancer [*International Classification of Diseases, Ninth Revision* (ICD-9) diagnosis codes 150 or 151] between January 1, 2000 and December 31, 2005. Because predominant treatments change over time and this study sought to compare current chemotherapy regimens, only patients with at least one claim for an identifiable chemotherapy agent between 2004 and 2005 were retained.

Patients were required to have been enrolled in the plan for at least 6 mo before the first chemotherapy administration (index date) in 2004 or 2005, and for at least 30 d after index. Patients with index dates between January 1, 2004 and February 14, 2004 were required to have had no claims for chemotherapy for at least 45 d before the index date.

Availability of data for both medical and pharmacy coverage was also required. Patients with only a chemotherapy diagnosis or revenue codes were excluded. Patients were followed from diagnosis until death, disenrollment, or study end (December 31, 2005).

Patient characteristics

Patient-level demographic variables measured as of the index date included age, geographic region, area of residence (urban *vs* rural), payer type (commercial *vs* Medicare), and length of follow-up (mo).

Clinical variables evaluated during the 6-mo pre-index period included: total direct health care costs; the Charlson Comorbidity Index (CCI); the Chronic Disease Score (CDS); select comorbidities (anemia, anxiety or depression, cerebrovascular disease, congestive heart failure, chronic

obstructive pulmonary disease, diabetes, hypertension, or hypercalcemia); and pre-index treatments (radiotherapy, surgery). Other variables included the year of first cancer diagnosis (between 2000 and 2005), year of first chemotherapy (between 2000 and 2005), and the presence of metastases at any point during the pre-index period.

Treatment episodes

A treatment episode-level analysis file was created, which contained all chemotherapy treatments identified from the claims database. A treatment episode was defined as the time from treatment initiation until the addition of a new agent not seen in the first 30 d, or until a gap of ≥ 45 d in treatment. Neoadjuvant treatment regimens ended on the date of surgery. Patients could contribute more than one treatment episode to the study if they received multiple chemotherapy regimens during the 2004-2005 study period.

Each chemotherapy episode was categorized according to treatment regimen and setting. Treatment regimens of interest included capecitabine monotherapy; capecitabine in combination with a platinum agent; capecitabine in combination with a platinum and a non-platinum agent; capecitabine in combination with a non-platinum agent; 5-FU monotherapy; 5-FU in combination with a platinum agent; 5-FU in combination with a platinum and a non-platinum agent; 5-FU in combination with a non-platinum agent; and other chemotherapy. Treatment settings included neoadjuvant (90 d before surgery), adjuvant (within 90 d after surgery), first line (> 90 d after surgery), and second line (any subsequent treatment regimens).

Complications of chemotherapy

Complications of chemotherapy were identified at the treatment episode level, using diagnosis codes, procedure codes, or pharmacological treatment specific to adverse events (Table 1). Adverse events were grouped into six categories: bone marrow complications (anemia, neutropenia, secondary thrombocytopenia); constitutional symptoms (asthenia, cough, fever, headache, insomnia, night sweats); gastrointestinal tract symptoms (constipation, diarrhea, esophagitis, gastritis, mucositis, nausea and vomiting, weight loss); infection (including central-line infection); skin complications (alopecia, dermatitis); and “other” (central-line thrombosis, pneumothorax). The complication rate was standardized per 1000 person-months on treatment. Person-month accumulation was based on time to event for treatment episodes with a complication event, and treatment episode length for those without an event.

Statistical analysis

Descriptive analyses of patient demographics, clinical characteristics, complication rates, and treatment characteristics were performed for each individual treatment regimen. Categorical variables were summarized in frequency tables. Continuous and other numeric variables were summarized by presenting the number of

Table 1 Definition of complications

Complication	ICD-9-CM	Procedure codes	Treatment
Anemia	281.xx, 283.xx, 284.xx, 285.xx		
Alopecia	704.0x, A9282		
Asthenia	780.7x	HCPSC codes G9029-G9032	
Constipation	546.0x		Therapeutic class 150-156
Cough	786.2x, 786.3x, 786.4x		Therapeutic class 128, 131
Dehydration	276.50, 276.51		
Dermatitis	693.0x, 693.8x, 693.9x		
Diarrhea	007.xx, 009.x, 787.91		Therapeutic class 148
Esophagitis	530.1x		
Fever	780.6x		
Gastritis	535.xx		
Headache	784.0x		
Infection	001.xx-018.xx, 030.xx-041.xx, 045.xx-057.xx, 070.xx-079.xx, 110.xx-118.xx, 130.xx-136.xx, 480.xx-486.xx, 995.91, 995.92		Therapeutic class 2-20
Insomnia	780.51, 780.52		Therapeutic class 74, Eszopiclone, Zaleplon, Zolpidem Tartrate
Mucositis	528.0x, 528.1x, 528.2x, 528.3x, 528.6, 529.0x, 054.2x		
Nausea and vomiting	787.0x,	CPT codes G9022, G9023, G9024, or HCPSC codes for antiemetics	Therapeutic class 160
Neutropenia	288.0x	HCPSC codes for neutropenia treatment	
Night sweats	780.8x		
Weight loss	783.2x, 783.0x		
Complications of vascular access devices	999.3x, 996.62, 996.74, 512.0x, 512.1x, 512.8x, 287.4x		

observations, mean, and standard deviation.

Multivariate analyses were performed to adjust for differences in patient demographic and clinical factors that confounded the complication rates from the descriptive analysis. Cox proportional hazard models were used to estimate time to first complication event for each of the six complication categories described above. Covariates employed in the models included patients' age, sex, region, cancer type, presence or absence of metastases during each treatment episode, treatment setting, cancer diagnosis year, pre-index anemia status, pre-index CCI score, and pre-index surgery status.

Complications that occurred in patients treated with capecitabine were compared to those occurring in patients who received 5-FU regimens (i.e. capecitabine monotherapy was compared with 5-FU monotherapy;

Table 2 Baseline demographic and clinical characteristics¹ of eligible patients with GEC by index treatment regimen (mean \pm SD) *n* (%)

	5-FU (<i>n</i> = 257)	CAP (<i>n</i> = 105)	5-FU combination (<i>n</i> = 344)	CAP combination (<i>n</i> = 76)	Any 5-FU (<i>n</i> = 540)	Any CAP (<i>n</i> = 166)	Total (<i>n</i> = 1013) ¹
Demographics ²							
Female	180 (70.0)	77 (73.3)	264 (76.7)	61 (80.3)	395 (73.2)	127 (76.5)	746 (73.6)
Mean age (yr)	63.2 (12.1)	63.6 (12.4)	60.3 (11.6)	60.4 (12.0)	61.7 (11.9)	63.0 (12.3)	62.6 (11.8)
Covered by Medicare	127 (49.4)	50 (47.6)	126 (36.6)	30 (39.5)	231 (42.8)	78 (47.0)	465 (45.9)
Residing in urban area	202 (78.6)	83 (79.1)	259 (75.3)	64 (84.2)	416 (77.0)	136 (81.9)	784 (77.4)
Clinical characteristics ³							
CCI	5.5 (3.2)	5.9 (3.0)	4.9 (3.0)	5.1 (3.1)	5.1 (3.1)	5.5 (3.0)	5.2 (3.1)
CDS	4.2 (3.5)	4.5 (3.7)	4.6 (3.4)	4.3 (3.5)	4.4 (3.4)	4.6 (3.6)	4.6 (3.5)
Metastatic disease ⁴	161 (62.7)	55 (52.4)	248 (72.1)	47 (61.8)	364 (67.4)	97 (58.4)	675 (66.6)
Previous treatment							
Surgery	125 (48.6)	27 (25.7)	46 (13.4)	11 (14.5)	154 (28.5)	34 (20.5)	239 (23.6)
Radiotherapy	115 (44.8)	25 (23.8)	162 (47.1)	15 (19.7)	252 (46.7)	40 (24.1)	410 (40.5)
Baseline expenditure (\$) ⁵	5479 (4730)	4965 (6277)	4598 (5019)	4178 (4005)	5014 (5061)	4607 (5573)	4763 (5193)

Marketscan Commercial and Medicare Databases: 2000-2005. CAP: Capecitabine. ¹One patient may receive multiple regimens; therefore, the sum of patients in each episode will exceed the total number of study-eligible patients; ²Demographics measured on the date the first study-eligible chemotherapy was administered; ³Clinical characteristics measured in the 6 mo before the first study-eligible chemotherapy, unless otherwise specified; ⁴Measured using all available data prior to episode onset; ⁵Mean per month over 6 mo pre-index.

capecitabine combination regimens were compared with 5-FU combination regimens; and “any capecitabine” regimens were compared with “any 5-FU” regimens).

RESULTS

Demographic and clinical characteristics

A total of 1013 patients with GEC were identified from the database and met the inclusion criteria for this analysis. These patients had 1349 treatment episodes during 2004 and 2005. Their characteristics are summarized in Table 2. There were more females than males in the selected population (73.6% of total patients), and the average age was 62.6 (\pm 11.8) years (most patients were in the 50-64-year age group). Patients were followed-up on average for 9.9 (\pm 6.9) mo after the initiation of the index chemotherapy.

Approximately two-thirds of patients were classified as having non-metastatic disease at the time of index treatment. Patients had high comorbidity scores at baseline (mean CCI score 5.2 ± 3.0 ; mean CDS 4.6 ± 3.5). Hypertension was the most common comorbidity and occurred in 30.6% of patients during the 6-mo pre-index period. Other common comorbidities included anemia, diabetes, and coronary artery disease.

Radiotherapy was used in 40.5% of patients in the 6-mo pre-index period, and 23.6% of patients underwent surgery.

Treatment patterns

Comparison of index treatment patterns: Approximately half of all patients included in the current analysis received a 5-FU regimen as their index treatment (540 of 1013; 53.3%) (Table 2). 5-FU combination therapy was initiated in 33.9% of patients, and 25.3% received 5-FU monotherapy. In comparison, 16.4% of patients had therapy initiated with a capecitabine regimen (166 of 1013); 10.4% received capecitabine monotherapy, and

7.5% received capecitabine combination therapy.

Patients who received capecitabine as index treatment were on average 1.5 ± 0.4 years older than those who were treated with 5-FU-based regimens (mean age: 63.0 ± 12.3 years *vs* 61.7 ± 11.9 years). Aggregate measures of comorbidity and chronic disease were similar between patients given 5-FU- and capecitabine-based regimens (Table 2). Patients who received capecitabine-based therapy were followed for about 1 mo less than those given 5-FU-based regimens (mean follow-up 9.1 ± 6.4 mo *vs* 10.3 ± 6.9 mo).

Comparison of treatment patterns by drug regimen and treatment setting:

Combined data for all treatment episodes (index and subsequent treatments) showed that 5-FU regimens remained the most widely used treatments (53.3% of patients) and 5-FU monotherapy was the most popular 5-FU regimen (25.4% of patients) (Figure 1 and Table 3). Overall, 16.4% of patients received capecitabine across all treatment settings: capecitabine monotherapy was the preferred regimen, given to 10.4% of patients (1.9% received a capecitabine-platinum combination; 3.1% received a capecitabine-platinum-non-platinum combination; and 3.2% received a capecitabine-non-platinum combination). Overall, 51.7% of patients received other chemotherapy regimens.

First-line treatment was the most common treatment setting in the selected population (73.3%), followed by second-line therapy (34.9%), adjuvant therapy (21.0%), and neoadjuvant therapy (8.3%) (Table 3). Patients could have more than one treatment episode; therefore, the sum of the percentage of patients receiving each treatment exceeded 100%.

5-FU was used widely in the neoadjuvant and adjuvant settings (in 60.7% and 65.7% of patients, respectively) (Figure 2 and Table 3). Capecitabine was used infrequently in the neoadjuvant setting (4.8% of patients), but was used more often in the adjuvant setting (11.3% of patients).

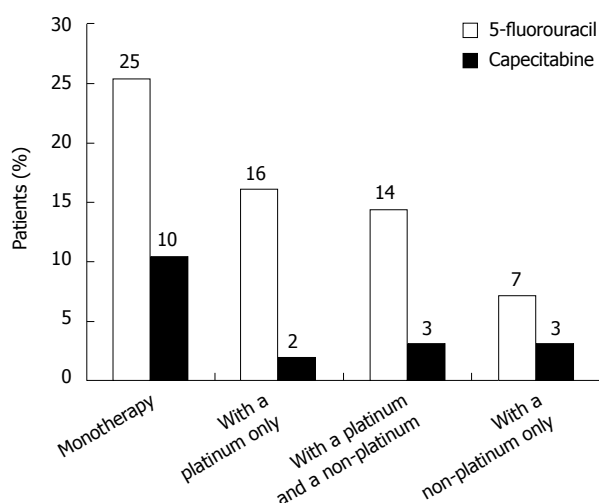
Table 3 Distribution of patients by chemotherapy regimen and treatment setting¹ *n* (%)

Chemotherapy regimen	Neoadjuvant (<i>n</i> = 84)	Adjuvant (<i>n</i> = 213)	First-line (<i>n</i> = 743)	Second-line (<i>n</i> = 354)	Any setting (<i>n</i> = 1013)
All 5-FU-based regimens	51 (60.7)	140 (65.7)	319 (42.9)	139 (39.3)	540 (53.5)
5-FU monotherapy	16 (19.0)	104 (48.8)	89 (12.0)	68 (19.2)	257 (25.4)
5-FU + platinum	24 (28.6)	19 (8.9)	104 (14.0)	25 (7.1)	163 (16.1)
5-FU + platinum + non-platinum	14 (16.7)	18 (8.5)	96 (12.9)	31 (8.8)	145 (14.3)
5-FU + non-platinum	2 (2.4)	5 (2.3)	30 (4.0)	40 (11.3)	73 (7.2)
Capecitabine-based regimens	4 (4.8)	24 (11.3)	90 (12.1)	72 (20.3)	166 (16.4)
Capecitabine monotherapy	2 (2.4)	18 (8.5)	50 (6.7)	42 (11.9)	105 (10.4)
Capecitabine + platinum	2 (2.4)	1 (0.5)	11 (1.5)	8 (2.3)	19 (1.9)
Capecitabine + platinum + non-platinum	0 (0.0)	6 (2.8)	16 (2.2)	11 (3.1)	31 (3.1)
Capecitabine + non-platinum	0 (0.0)	1 (0.5)	13 (1.7)	19 (5.4)	32 (3.2)

¹Patients may be treated with multiple regimens in more than one treatment setting. Accordingly, percentages may exceed 100%.

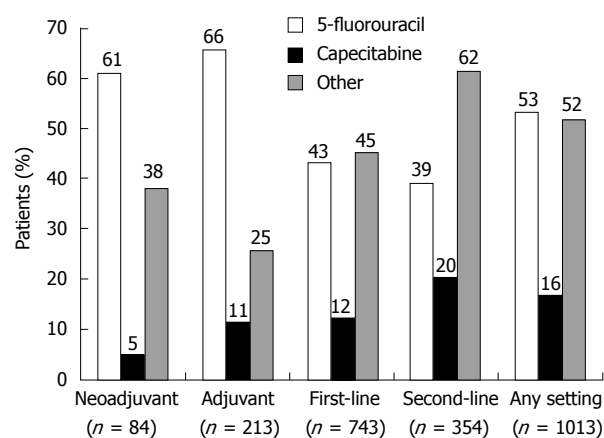
Table 4 Frequency of complications (rate per 1000 person-months) in patients with GEC, by treatment regimen

Complication	5-FU monotherapy	Capecitabine monotherapy	5-FU combination therapy	Capecitabine combination therapy	Any 5-FU regimen	Any capecitabine regimen
Bone marrow	233	110	349	205	307	151
Constitutional symptom	254	108	323	295	293	183
Gastrointestinal tract symptoms	465	183	577	284	529	224
Infection	199	114	268	150	238	131
Skin complications	0	0	2	0	1	0
Other	14	14	22	32	19	22
Any complication	764	336	835	460	806	387

**Figure 1** Chemotherapy treatment patterns across all settings in patients with GEC (*n* = 1013).

Other chemotherapy regimens were used in 38.1% and 25.4% of patients in these respective treatment settings.

In the first-line setting, 42.9% and 12.1% of patients, respectively, were treated with 5-FU or capecitabine regimens. Although still a popular choice, 5-FU was used less often in the second-line setting than in other settings (in 39.3% of patients), and other chemotherapy regimens became the most popular choice (used in 61.6% of patients). The second-line treatment setting was the most popular choice for capecitabine-based therapies (given to 20.3% of patients). Capecitabine monotherapy was used in 11.9% of patients in this setting; cisplatin plus a

**Figure 2** Distribution of chemotherapy by treatment setting.

non-platinum compound was used in 5.4% of patients (Table 3).

Capecitabine dosage regimens: The mean overall capecitabine dose was 2382 (\pm 1118) mg/d; a lower dose was used in combination regimens than in monotherapy [mean overall dose: 2349 (\pm 1052) *vs* 2410 (\pm 1175) mg/d, respectively].

Complication rates: Capecitabine compared favorably with 5-FU in terms of crude (unadjusted) complication rates (Table 4). The unadjusted complication rate (per 1000 person-months) for capecitabine-based regimens was half that for 5-FU-based regimens (387 *vs* 806 per 1000 person-months). Patients who received

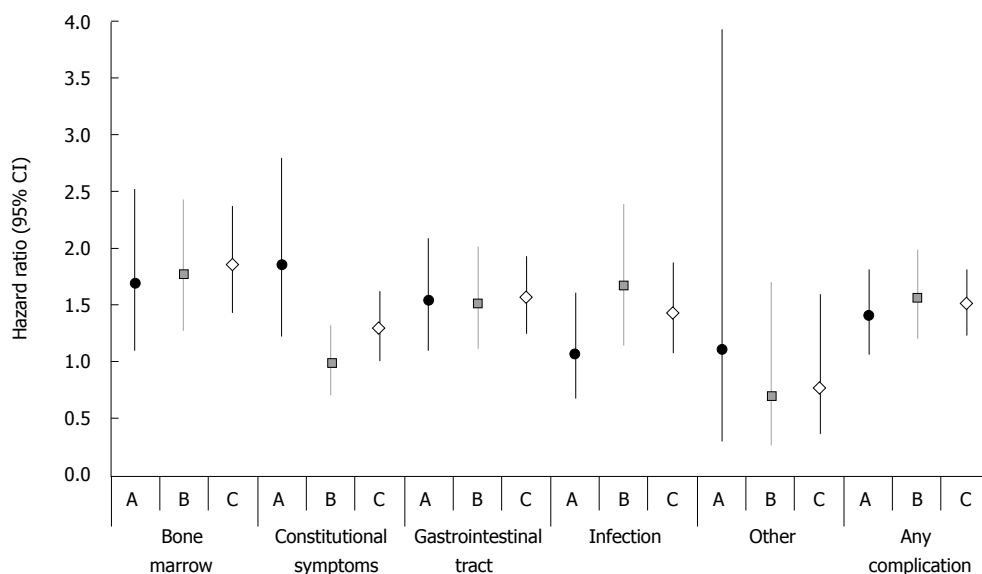


Figure 3 Adjusted complication hazard ratio: 5-FU vs capecitabine regimens. A: 5-FU monotherapy compared with capecitabine monotherapy; B: 5-FU combination compared with capecitabine combination; C: Any 5-FU regimen compared with any capecitabine regimen. Complications were determined per 1000 person-months.

capecitabine monotherapy were 56% less likely to have a complication during treatment than patients given 5-FU monotherapy (336 *vs* 764 per 1000 person-months), and patients who received capecitabine combination therapy were 45% less likely to have a complication during treatment than those given 5-FU combination therapy (460 *vs* 835 per 1000 person-months).

Complication rates were lower with capecitabine regimens than with 5-FU across four individual categories: bone marrow (151 *vs* 307; 51% lower); constitutional symptoms (183 *vs* 293; 37% lower); gastrointestinal tract (224 *vs* 529; 58% lower); and infection (131 *vs* 238; 45% lower). Skin complications were rare in both comparison cohorts.

Relative risk of complications: The results of multivariate analyses supported the findings of the crude rate calculations (Figure 3). Cox proportional hazard models showed that, overall, 5-FU therapy was associated with a significantly higher risk for any complication than capecitabine (adjusted HR: 1.51, 95% CI: 1.26-1.81) (Figure 3). Specifically, the higher risk of developing a complication associated with 5-FU therapy was observed for bone marrow events (HR: 1.84, 95% CI: 1.42-2.37), constitutional symptoms (HR: 1.28, 95% CI: 1.01-1.63), and gastrointestinal tract events (HR: 1.56, 95% CI: 1.27-1.93). No difference was detected in the risk for infection or "other" complications, possibly because of the low number of reported events (skin: 1 *vs* 0; "other": 19 *vs* 22, for capecitabine and 5-FU regimens, respectively).

The risk of complication with 5-FU monotherapy was markedly higher than with capecitabine monotherapy for bone marrow (HR: 1.68, 95% CI: 1.11-2.53), constitutional symptoms (HR: 1.85, 95% CI: 1.23-2.79), and gastrointestinal tract complications (HR: 1.52, 95% CI: 1.10-2.09). The overall risk for any complication also differed significantly between the two monotherapy treatment groups (HR: 1.39, 95% CI: 1.07-1.81) (Figure 3).

Similar to monotherapy, the difference in overall

risk of any complication was significantly higher with 5-FU combination regimens than with capecitabine combination regimens (HR: 1.55, 95% CI: 1.21-1.99). The risk of bone marrow complications was 76% higher (HR: 1.76, 95% CI: 1.27-2.44), and the risk of developing a gastrointestinal tract event was higher (HR: 1.50, 95% CI: 1.13-2.01) with 5-FU than with capecitabine combination regimens (Figure 3).

DISCUSSION

Of the 1013 patients with GEC identified in this retrospective database analysis, approximately 50% had treatment initiated with a 5-FU regimen, whereas only 11% had therapy initiated with a capecitabine regimen. The mean capecitabine dose overall was 2382 (SD \pm 1118) mg/d, and capecitabine was used as monotherapy more often than in combination. Overall, 5-FU regimens were the most common treatment option in the neoadjuvant and adjuvant settings, whereas other non-capecitabine regimens were used more widely in the first- and second-line settings.

The retrospective analysis of complication rates with capecitabine and 5-FU therapies indicates that capecitabine-based therapy may be a better tolerated treatment option than 5-FU in patients with GEC. The overall unadjusted complication rate for capecitabine regimens was about half of that seen with 5-FU regimens: in multivariate analyses, capecitabine recipients had a 51% lower risk of developing any complication than 5-FU recipients did. Lower complication rates were observed in bone marrow, constitutional, gastrointestinal tract, infection, and skin complications. Overall, patients who received capecitabine therapy (monotherapy or combination) were 38% less likely than their 5-FU counterparts to have a complication associated with therapy.

The two recent phase 3 trials of first-line capecitabine in GEC that were described earlier^[10,11] have demonstrated non-inferiority in survival parameters for capecitabine-

vs 5-FU-based regimens, as well as similar tolerability between the regimens. Although the incidence of grade 3/4 neutropenia was higher with the ECX regimen compared with ECF (51.1% *vs* 41.7%), in the study by Cunningham and colleagues, the incidence of these events was similar or slightly lower with the capecitabine regimen EOX *vs* EOF (27.6% *vs* 29.9%)^[10], and capecitabine/cisplatin *vs* 5-FU/cisplatin (16% *vs* 19%) in the study by Kang and colleagues^[11]. Other common treatment-related grade 3/4 adverse events in the latter study were vomiting (7% *vs* 8%) and stomatitis (2% *vs* 6%) with capecitabine/cisplatin *vs* 5-FU/cisplatin, respectively. Thus, the clinical efficacy of capecitabine demonstrated in the two phase three studies, combined with the lower complication rate observed in the current retrospective analysis, support capecitabine use for the treatment of patients with GEC. Capecitabine therapy also has the advantage of convenient oral administration, which eliminates the need for intravenous access and associated complications.

The treatment patterns observed in our analysis correspond with standard recommended treatment protocols for GEC: 5-FU-based regimens were used in about 50% of patients and other non-capecitabine chemotherapy regimens were also a common choice. Although not approved for this indication, there was considerable use of capecitabine for the treatment of GEC in the study period between 2004 and 2005. Overall, 16% of patients received a capecitabine-based regimen. Monotherapy was the preferred capecitabine regimen, and was given to 10% of patients overall across all treatment settings (1.9% received capecitabine plus a platinum agent; 3.1% received a capecitabine-platinum-non-platinum combination; 3.2% received a capecitabine-non-platinum combination). Capecitabine was most likely used in the second-line setting, and least likely used as neoadjuvant therapy.

In interpreting our findings, several limitations of the analysis should be considered. Although the current study was based on a large, diverse sample of patients with GEC, it was not a random population sample, and may not represent the United States population as a whole. Moreover, diagnostic information is recorded by physicians and hospitals to support their claims for reimbursement for particular services, but additional clinical information is limited. We relied exclusively on billing and coding by health care providers to identify cancer type, treatment setting, treatment regimen, treatment episode, and complication events. As such, particular conditions common among patients undergoing chemotherapy may not have been recorded on a claim unless deemed clinically relevant. In addition, clinical measures were not used to confirm the presence of complications. We also presumed that the use of known treatment for a complication was evidence of its existence. However, as prophylaxis is often used for specific events, the frequency of complications reported in the current study may have been overstated. Alternatively, the frequency of complications may have been understated because patients may have experienced complications that did not result in the generation of a

health care claim for reimbursement. For example, hand-foot syndrome was recorded using the ICD-9 code 693, which includes dermatitis caused by substances taken internally. To ensure that hand-foot syndrome was not being miscoded, we conducted a sensitivity analysis using all ICD-9 codes indicative of an inflammatory dermatologic reaction (codes 690-698), but rates still remained low. Cases of hand-foot syndrome may not have been clinically significant to warrant coding. Finally, a key problem that often plagues observational studies is the lack of randomization in assigning individuals to either treatment or control groups. Given this concern, the estimation of the effects of treatment may have been biased by the existence of confounding factors. The current study used multivariate models to adjust for these pretreatment differences. Given the strength of the selection bias observed in the current study, it may be desirable for future studies to incorporate a propensity model approach in combination with multivariate adjustment.

In conclusion, in the United States, 5-FU-based regimens represented approximately 50% of the regimens used to treat patients with GEC between 2004 and 2005, which reflects current treatment recommendations for this indication. Capecitabine-containing regimens represented 16% of all regimens used for this indication during this time period, and were used most frequently in the second-line setting. Capecitabine appeared to have a favorable side-effect profile compared with 5-FU and, thus, could be a useful treatment option for patients with GEC.

COMMENTS

Background

Capecitabine is an oral cytotoxic agent with comparable efficacy to and a better safety profile than intravenous 5-fluorouracil (5-FU), as seen in clinical trials of gastroesophageal cancer (GEC). This study compared the occurrence of select adverse events (AEs) in patients treated with capecitabine and 5-FU in a real-world setting.

Research frontiers

Although two recent phase III trials have suggested that survival with capecitabine-based regimens compares favorably with that with 5-FU-based regimens as first-line therapy for GEC (REAL-2 study and Kang *et al* study), data are lacking for the use of capecitabine in patients with GEC in a real-world treatment setting. This study was undertaken to assess the usage of capecitabine-based therapy and associated complication rates in patients with GEC in a real-world treatment setting.

Innovations and breakthroughs

The complication rate of a capecitabine regimen was nearly half that of 5-FU (387/1000 *vs* 806/1000 person-months). These findings held when comparing monotherapy (336/1000 *vs* 764/1000 person-months) and combination (460/1000 *vs* 835/1000 person-months) regimens. After adjusting for differences in demographic and clinical profile, patients on capecitabine monotherapy had significantly lower risk for any complication compared with patients on 5-FU alone (HR: 1.39, 95% CI: 1.07-1.81). Patients on a capecitabine combination regimen had significantly lower risk of complication compared with patients on a 5-FU combination regimen (HR: 1.55, 95% CI: 1.21-1.99).

Applications

Capecitabine is currently not approved by the US FDA for treatment of GEC. Consistent with trial data, in this real-world setting, capecitabine alone and in combination with other agents had lower rates of AEs than 5-FU did. These findings support that, in the treatment of GEC, capecitabine regimens produce a similarly favorable safety profile in the real-world setting as in controlled

clinical trials.

Terminology

Charlson Comorbidity Index (CCI): The CCI predicts the 1-year mortality for a patient who may have comorbid conditions, such as heart disease, diabetes, or cancer (a total of 17 conditions). Each condition is assigned a score of 1, 2, 3 or 6 depending on the associated risk of dying from this condition. Scores for individual comorbidities are added and the sum is used as a predictor for mortality. The CCI aids in directing how aggressively a condition should be treated (e.g. although a patient may have cancer, additional comorbidities may be severe enough that the costs and risks of treatment may outweigh the short-term benefit from treatment of the cancer). Chronic Disease Score (CDS): The CDS is a risk-adjustment measure based on age, sex, and history of dispensed drugs.

Peer review

It is useful to publish the practice of the utilization of these drugs. This was a retrospective study and although there are biases related to the data collection and lack of control related to the selection of patients for different treatments, these limitations are recognized and the study makes an important contribution to our knowledge of the potential of capecitabine in the treatment of GEC.

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S- Editor Tian L L- Editor Kerr C E- Editor Ma WH



A Brazilian experience of the self transglutaminase-based test for celiac disease case finding and diet monitoring

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Supported by Pontifical Catholic University of Paraná
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Received: July 3, 2009 Revised: August 25, 2009
Accepted: September 1, 2009
Published online: September 21, 2009

CONCLUSION: The point-of-care test was as reliable as conventional serological tests in detecting CD cases and in CD diet monitoring.

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Key words: Celiac disease; Gluten; Relatives; Dyspepsia; Diarrhea

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Kotze LM, Brambila Rodrigues AP, Kotze LR, Nisihara RM. A Brazilian experience of the self transglutaminase-based test for celiac disease case finding and diet monitoring. *World J Gastroenterol* 2009; 15(35): 4423-4428 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4423.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4423>

Abstract

AIM: To evaluate the effectiveness of a rapid and easy fingertip whole blood point-of-care test for celiac disease (CD) case finding and diet monitoring.

METHODS: Three hundred individuals, 206 females (68.7%) and 94 males (31.3%), were submitted to a rapid and easy immunoglobulin-A-class fingertip whole blood point-of-care test in the doctor's office in order to make immediate clinical decisions: 13 healthy controls, 6 with CD suspicion, 46 treated celiacs, 84 relatives of the celiac patients, 69 patients with dyspepsia, 64 with irritable bowel syndrome (IBS), 8 with Crohn's disease and 9 with other causes of diarrhea.

RESULTS: Upper gastrointestinal endoscopy with duodenal biopsies was performed in patients with CD suspicion and in individuals with positive test outcome: in 83.3% (5/6) of the patients with CD suspicion, in 100% of the patients that admitted gluten-free diet transgressions (6/6), in 3.8% of first-degree relatives (3/79) and in 2.9% of patients with dyspepsia (2/69). In all these individuals duodenal biopsies confirmed CD (Marsh's histological classification). The studied test showed good correlation with serologic antibodies, endoscopic and histological findings.

INTRODUCTION

Celiac disease (CD) is a multifactorial disorder that occurs in genetically susceptible individuals. CD is considered an immune-mediated enteropathy caused by a permanent intolerance of the small bowel mucosa to gluten, and affects approximately 1% of the world population^[1]. In our geographic area the estimated prevalence is 1/475^[2]. Individuals with CD can be asymptomatic or can present a variety of gastrointestinal symptoms or extra-intestinal manifestations and often the diagnoses of dyspepsia or irritable bowel syndrome (IBS) are suggested^[3]. The diagnosis of CD is based on serologic and histopathologic findings. Antiendomysial (EMA) and anti-tissue transglutaminase antibodies, class IgA, are considered highly specific serological markers for CD. Biopsies of the proximal small bowel in positive individuals for these antibodies confirm the diagnosis of CD^[1].

As CD is a disorder that can be ruled out in the differential diagnosis of several gastrointestinal diseases, non-invasive tests are recommended as a first-step to screening patients before small intestinal biopsy to confirm CD^[1,4]. Also, gluten-triggered tissue autoantibodies can be determined to aid in monitoring the diet and for case findings of CD in at-risk groups such as relatives^[5] and in patients with other autoimmune

Table 1 Demographic data of all the studied individuals¹

Group	Number	Female	Male	Median age	Range	Positivity
Healthy control	13	6	7	40.0	7-75	0
Celiac at diagnosis	6	4	2	41.2	28-61	5 (83.3%) ¹
GFD adherence	40	29	11	39.6	6-76	0
GFD transgression	6	4	2	24.0	10-38	6 (100.0%) ¹
First-degree relatives	79	56	23	30.7	5-79	3 (3.8%) ¹
Second-degree relatives	6	3	3	33.5	9-60	0
Dyspepsia	69	46	23	40.6	12-78	2 (2.9%) ¹
Irritable bowel syndrome	64	46	18	46.8	12-81	0
Crohn's disease	8	5	3	35.6	27-45	0
Diarrhea	9	7	1	46.6	14-63	0
Total	300	206	94			

¹In bold the number of individuals with a positive test and the respective percentages. GFD: Gluten-free diet.

diseases^[6-8].

The conventional tests, EMA determined by indirect immunofluorescence and anti-tTG performed by ELISA, require sera samples, are laborious and time-consuming, need special laboratorial centers, are expensive and the results are available only after a time lag. So, a rapid test, with a result which can be available immediately, will help the physicians to make decisions at their own offices^[9].

The aim of this study was to evaluate the effectiveness of a rapid and easy immunoglobulin-A-class whole blood point-of-care test for celiac autoantibody detection in patients routinely seen in the clinic, to enable immediate clinical decisions.

MATERIALS AND METHODS

Patients

The patients were tested at the Gastroenterology and Endoscopy Services, Pontifical Catholic University of Paraná, and also tested on site in the author's office, Curitiba city, State of Paraná, South of Brazil. The study was approved by the Research Ethic Committee of the institution. All the participants gave informed consent.

A total of 300 consecutive Caucasoid individuals born in southern Brazil were studied, 206 females (68.7%) and 94 males (31.3%) divided by groups: 13 controls not relatives of the celiac patients; 6 with CD suspicion; 46 treated celiacs, 40 with and 6 without compliance to a gluten-free diet (GFD); 85 relatives of the celiac patients, 79 first- and 6 second-degree relatives; 69 patients with clinical diagnosis of dyspepsia; 64 with irritable bowel syndrome; 8 patients with Crohn's disease; 9 with other causes of diarrhea (4 lactose intolerance, 2 collagenous colitis, 1 diarrhea/possible gastroenteritis, 1 severe psoriasis, 1 AIDS). Table 1 shows the demographic data of the studied groups.

Methods

The diagnosis of CD was based on serologic tests and on the findings of a small intestinal mucosal biopsy. Because some tests are operator-dependent, the same professional (RMN) performed all the serologic tests: Antiendomysial antibodies (IgA EmA) by indirect

immunofluorescence assay using human umbilical cord as substrate, according to Volta *et al*^[10], sera considered positive if fluorescence was observed at a dilution of 1/2.5 or higher; serum IgA anti-tissue transglutaminase antibodies (anti-tTG) titres measured by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (INOVA Diagnostics Inc., USA), based on the method described by Dieterich *et al*^[11], results higher than 20 U were considered positive.

Upper gastrointestinal endoscopy was done by the same physician (APBR) with attention to the classical endoscopic markers of CD: erosions, scalloping of folds, decrease and atrophy of duodenal folds, mosaic patterns^[12,13]. The histological analysis was performed by the same pathologist (LRK), familiar with the spectrum of mucosal changes in CD, using Marsh's classification^[14] in Hematoxylin-Eosin stained fragments. These were, briefly, type I (infiltrative) with increased number of intraepithelial lymphocytes; type II (hyperplastic) with increased number of these cells and crypt hyperplasia; type III (destructive) with villous atrophy, A: partial, B: subtotal and C: total; type IV (hypoplastic) with flat atrophic mucosa. The number of intraepithelial lymphocytes (IEL) was counted according to Ferguson and Murray^[15]; 24% was considered normal for the Brazilian population, according to previous study^[16].

All the individuals (patients and relatives) were submitted to a rapid test for celiac-specific immunoglobulin A class tissue transglutaminase antibody detection performed from fresh fingertip whole blood sample (*Biocard Celiac-Test*TM, ANI Biotech, Vantaa, Finland). The basic concept is to liberate the individual's own tTG from the red blood cells by hemolysing an anticoagulated whole blood sample. When tTG-specific antibodies are present in the sera they recognize and form complexes with the liberated self-tTG. The complexes can be detected by binding tTG to a solid surface coated with tTG-capturing proteins. The bound antigen-antibody complexes can be seen in a color reaction with the help of labeled anti-human IgA solution^[9]. According to the manufacturer, 5 min (not longer than 10 min) is the time till the interpretation of the results. However, a positive test result may appear within 1 to 2 min. The test result is positive when both

Table 2 Demographic data, correlation with antiendomysium antibodies, endoscopic and histological findings of duodenal biopsies for the positive individuals

	Age	Gender	IgA EmA	Biopsy	IEL	Endoscopy
Celiac at diagnosis						
CPA	28	F	Positive	III-C	> 40	Scalloped folds, nodularity
CBO	38	F	Positive	III-C	40	Mosaic pattern
NHF	41	F	Positive	III-C	> 40	Nodularity, atrophic areas
AHR	42	M	Positive	III-C	45	Erosions, edema
HB	61	M	Negative	III-C	> 40	Decreased folds
GFD transgression						
GSCS	10	M	Positive	III-C	55	Decreased folds, atrophic areas
ALM	19	F	Positive	III-A	NC	Atrophic areas
VC	23	F	Positive	III-C	> 40	Atrophic areas
FA	23	M	Positive	III-C	35	Decreased folds, atrophic areas
LGH	31	F	Positive	III-A	35	Decreased folds
RK	38	F	Positive	III-C	> 50	Atrophic areas
First-degree relatives						
LGS	21	F	Positive	I	38	Normal
RG	48	F	Positive	III-C	48	Atrophy of folds
ERA	52	F	Negative	R	R	R
Dyspepsia						
EJMS	21	F	Positive	III-A	50	Atrophy
LA	26	F	Positive	III-C	> 40	Scalloped folds, areas of atrophy

EmA: Antiendomysium; IEL: Percent of intraepithelial lymphocytes; NC: Not counted; R: Recommended.

the control line and the line in the test field can be seen; in negative cases only the control line forms^[9]. The result was interpreted immediately on site at the Endoscopy Room or in the doctor's office by the same physicians (APBR and LMSK).

The patients previously diagnosed as having CD were invited to an interview with their same physician to monitor diet, answering a questionnaire concerning the adherence to a GFD^[17]. The first-degree relatives of the patients were invited to participate in the study by phone call^[5].

Patients with clinical complaints suggestive of CD were submitted to the point-of-care test and to intestinal biopsy plus IgA EmA determination for comparison^[18].

The patients with dyspepsia, defined as persistent or recurrent pain or discomfort in the upper abdomen, were submitted to upper gastrointestinal endoscopy and biopsies by routine procedures. The mucosa of the bulb and the second portion of the duodenum was carefully studied^[12,13] and, if the previous test was positive, or if there were any changes, 5 to 7 fragments were obtained for histologic evaluation and Marsh's classification^[14]. In these cases IgA EmA test was also performed for correlation.

The diagnosis of IBS was based in Roma III criteria^[19]. Crohn's disease was diagnosed by clinical, endoscopic, imaging and histologic findings^[20]. The final diagnosis of the other cases were based on recommended tests^[3,8].

RESULTS

Table 1 shows the positivity of the Biocard Celiac-Test™ with the corresponding percentages.

The test was negative in the controls, in CD patients with strict adherence to a GFD, in the second-degree

relatives, in IBS, in Crohn's disease and diarrhea of different etiologies. Positive tests were detected in 83.3% of patients with suspicion of CD, in 100% of the patients that admitted diet transgressions, in 3.8% of the first-degree relatives and in 2.9% of patients with dyspepsia.

Considering only the female gender, the positivity of the studied test was 75.0% (3/4) for patients with CD suspicion, 66.7% (4/6) for patients with GFD transgressions, 5.3% (3/56) for first-degree relatives, and 4.3% (2/46) for patients with dyspepsia.

One female (HK) was negative for the rapid test, but positive for IgA EmA. Biopsy confirmed CD. IgA anti-tTG was determined in the sera of the patient (HB) with Biocard test positive and IgA EmA negative, with a resulting antibody level of 193 U, and CD confirmed by duodenal biopsy. IgA EmA was also determined in all the individuals with Biocard test positive ($n = 16$); 2 were negative (12.5%) for these antibodies (Table 2).

Duodenal biopsy was performed in 15 of the 16 individuals with positive Biocard test and recommended for one first-degree relative. Lesions characterized as Marsh III-C were seen in 11 cases or 73.3% (5 CD suspicion, 4 with GFD transgressions, 1 first-degree relative and 1 with and 1 without dyspepsia); Marsh III-A were detected in 2 patients with GFD transgressions and in 1 patient with dyspepsia (20.0%); Marsh type I was seen in one young first-degree relative (6.7%) (mother and aunt with CD). The number of IEL was increased in all the positive individuals (100%). Table 2 shows the demographic data of these individuals in relation to the positivity of IgA EmA and the endoscopic and histological evaluations.

DISCUSSION

CD has become more common than in the past, and can

be diagnosed at any age^[1]. However, it frequently remains undetected for long periods of time, because of failure by health care professionals to recognize the disorder, probably due to the variable clinical presentation or failure to perform appropriate diagnostic tests^[21].

In the present study, there was a young adult preponderance in all groups, indicating that it is important to exclude CD in the differential diagnosis of gastrointestinal complaints in this age group. However, we must remember that this affliction occurs also in patients of more than 60 years (as is shown here in the case of a male, 62 years, with recent-onset diarrhea)^[22].

The female preponderance, also reported by several authors in different countries, alerted us to investigate carefully women with digestive or systemic symptoms^[8,23]. When we considered only female gender, we observed that the percentage of CD case findings was higher than the total number of positive individuals in first-degree relatives and in patients with dyspepsia.

In this investigation, in the CD-suspicion group, the point-of-care test was positive in 83.3% (5/6) patients, similar to the report of Korponay-Szabó *et al*^[24]. It is possible that a patient can have only one CD-specific test positive, as was the case of the female patient with Biocard test negative and IgA EmA positive, or the male 62-year-old with this test positive, anti-tTG increased (193 U) and IgA EmA negative (Table 2). These cases demonstrate that more than one test is recommended if CD is suspected^[4].

Patients with CD should be evaluated at regular intervals by a health care team, including a physician and a dietician, but there are no clear guidelines as to the optimal means to monitor adherence to a GFD. Dietary compliance as assessed by interview is the best marker of CD due to low cost, non-invasiveness and strong correlation with serological tests and intestinal damage^[17]. Repeat serologic testing after 6 mo or more on a GFD can be helpful. The disappearance or decline of CD-specific serum antibodies during a diet is a further indication of dietary adherence and antibody testing is therefore recommended in dietary monitoring of CD (sensitivity varies from 29% to 100%)^[24]. However, antibody tests might not reveal slight dietary transgressions^[25]. In the present experience, the Biocard test recognized the disappearance of anti-tTG antibodies in all the patients with a GFD compliance and was positive in 14% (6/43) of the patients who admitted transgressions: they were young patients (median age 24 years), confirming the reports that adolescents had difficulties adhering strictly to the diet^[26-28]. The positivity of the test presumes major dietary transgressions^[25]. Notably, repeat biopsies are no longer required, but in the patients with positive tests the biopsy was performed for correlation (Table 2).

Dietary compliance varies a great deal in CD around the world. The compliance is related to the physician's recommendations but also to the collaboration of the families. It is important to know about the adherence to a GFD due to the probability of nutritional imbalances in children and adolescents^[28]. So, an easy test at

the office or at home can be very useful to detect transgressions because positivity rate correlates with dietary lapses^[24].

The prevalence of CD in first-degree relatives of patients with CD undergoing intestinal biopsy varies from 5.5%^[29] to 22.5%^[30]. In a previous study with 115 Brazilian relatives, we reported 15.6% CD prevalence^[5], with female preponderance, and in the present study there was 3.8% of test positivity (Table 2)^[31]. All were female: 1 sister and 1 daughter of the same family; 1 mother. This mother currently presents severe dyspeptic complaints. Her positivity for the point-of-care test occurred 7 years after the first IgA EmA negative determination, showing that the relatives need to be re-evaluated periodically^[32]. In some individuals, only more subtle changes of crypt lengthening with an increase in IEL, or simply an increase in IEL, are present. So, it is important that the slides be viewed by an experienced pathologist familiar with the spectrum of mucosal changes in CD. The example is the patient LGS (Table 2) with positive serologic tests, normal endoscopy and duodenal mucosa with preserved architecture, but with an increased number of IEL^[15,16].

In this investigation, there was no positivity in second-degree relatives. However, this was a small group. In a previous study with more Brazilian relatives we reported 5.9% of positivity^[32].

Although dyspepsia may be part of the clinical spectrum in CD patients, there are scarce data about its prevalence in silent CD^[33]. Duodenal biopsy undertaken during routine upper GI endoscopy in adults has been gradually incorporated into clinical practice, and is a useful tool for the diagnosis of CD in high-risk groups such as those with anemia and/or chronic diarrhea.

In this study there was 2.9% of test positivity in dyspepsia patients, both young female patients without diarrhea (Table 1). The correlations with IgA EmA and intestinal biopsy were 100% (Table 2). This result is similar to the findings of Riestra *et al*^[33] in Spain (2.2% also in women); Ozaslan *et al*^[21] in Turkey (1.5% in women). In another Brazilian region, Lima *et al*^[34] reported 1.4% positivity in women. In summary, CD should be kept in mind as a cause of dyspepsia during clinical assessments^[35]. Serological screening can be recommended for patients with refractory dyspepsia, especially females^[35]. A GFD may still bring symptomatic relief for dyspeptic symptoms in CD^[36].

Diarrhea is part of the clinical spectrum of IBS; the habitual tests are normal and the nutritional state is not compromised. There is a female prevalence probably related to hormones^[37-39]. In the present study 46 of 64 diarrhea patients were female (71.8%), median age 46.8 years, similar to previous reports^[40]. Even when CD was suspected in IBS, none of our patients were positive for anti-tTG point-of-care test (Table 1).

Patients with Crohn's disease can present chronic diarrhea as the only manifestation. At the beginning of the evaluation, differential diagnosis with CD can be pertinent, mainly in children and adolescents. In this study 62.5% (5/8) were female, mean age 35.6 years,

almost the same characteristics of the women with CD^[20,40]. None of the patients with Crohn's disease were positive for the point-of-care test (Table 1).

In the differential diagnosis of chronic diarrhea, a serological test to suggest or rule out CD is pertinent, especially in the doctor's office, to guide subsequent investigations. In 9 patients of this investigation, who were point-of-care test negative, the final diagnosis confirmed that the patients were not celiac patients^[3,41,42].

In a cost-effectiveness analysis in our geographic area, south of Brazil, the cost of IgA EmA or anti-tTG is 8 times the cost of the studied test, another reason to use this screening. The same conclusions were reported by Crovella *et al.*^[42] in another Brazilian area (Recife, Northeast).

To conclude, in this study the results of the point-of-care test showed good correlation with positivity of IgA EmA antibodies, endoscopic and histological findings (Table 2). As the test is quick, economical, easy to perform and as reliable as the conventional serological tests in CD case finding and in diet monitoring, it can be performed on site in the physician's office and in primary care^[24,43].

COMMENTS

Background

A rapid test, with a result which can be available immediately, will help the physicians to make decisions at their own offices for celiac disease.

Research frontiers

The literature only shows data in Finland populations and the authors emphasize the importance of the tissue transglutaminase antibody detection test in other geographic areas.

Applications

Due to low cost, the point-of-care test is important for celiac disease screening in developing countries.

Peer review

Overall this was a very good paper that demonstrate the value of TTG assay for diagnosis of celiac disease.

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S- Editor Tian L L- Editor Logan S E- Editor Yin DH



Sustained virological response in a predominantly hepatitis C virus genotype 4 infected population

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Received: July 12, 2009 Revised: August 8, 2009

Accepted: August 15, 2009

Published online: September 21, 2009

Abstract

AIM: To assess sustained virological response (SVR) rates in a predominantly hepatitis C virus (HCV) genotype 4 infected population.

METHODS: Between 2003-2007, 240 patients who were treated for chronic hepatitis C infection at our center were included. Epidemiological data, viral genotypes, and treatment outcomes were evaluated in all treated patients. Patients with chronic renal failure, previous non-responders, and those who relapsed after previous treatment were excluded from the study. Among all patients, 57% were treated with PEG-interferon (IFN) α -2a and 43% patients were treated with PEG-IFN α -2b; both groups received a standard dose of ribavirin.

RESULTS: 89.6% of patients completed the treatment with an overall SVR rate of 58%. The SVR rate was 54% in genotype 1, 44% in genotype 2, 73% in genotype 3, and 59% in genotype 4 patients. There was no statistical difference in the SVR rate between patients treated with PEG-IFN α -2a and PEG-IFN α -2b (61.5% vs 53%). Patients younger than 40 years had higher SVR rates than older patients (75% vs 51%, $P = 0.001$). SVR was also statistically significantly higher when the HCV RNA load (pretreatment) was below 800.000 (64% vs 50%, $P = 0.023$), in patients with a body mass index (BMI) less than 28 (65% vs 49%, $P = 0.01$), and in patients who completed the treatment duration (64% vs 8%, $P \leq 0.00001$).

CONCLUSION: The SVR rate in our study is higher than in previous studies. Compliance with the standard duration of treatment, higher ribavirin dose, younger age, lower BMI, and low pretreatment RNA levels were associated with a higher virological response.

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Key words: Hepatitis C virus infection; Sustained virological response; Genotype 4

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Dahlan Y, Ather HM, Al-ahmadi M, Batwa F, Al-hamoudi W. Sustained virological response in a predominantly hepatitis C virus genotype 4 infected population. *World J Gastroenterol* 2009; 15(35): 4429-4433 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4429.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4429>

INTRODUCTION

Chronic infection with the hepatitis C virus (HCV) affects about 170 million individuals worldwide. The natural history of chronic hepatitis C has been difficult to clearly define because of the long course of the disease; however multiple studies suggest that 20%-30% of infected patients eventually develop cirrhosis and its complications^[1]. Regardless of the mode of transmission, chronic hepatitis follows acute hepatitis C in 50%-85% of infected patients^[2]. Genotypes 1-4 account for nearly 90% of HCV-infected cases and genotype 4 is the most prevalent genotype in the Middle East, including Saudi Arabia. Genotype 1 is the next common, while genotypes 2, 3 and 5 are the least prevalent^[3-5]. Treatment of chronic HCV is aimed at slowing disease progression, preventing complications of cirrhosis, reducing the risk of hepatocellular carcinoma (HCC), and treating extrahepatic complications^[6]. The most effective therapy is the combination of PEGylated interferon (IFN) plus ribavirin. The benefit is mostly achieved in patients with HCV genotype 2 and 3 infections^[7]. Most of the published literature on management of HCV involves genotypes 1, 2 and 3. The data on treating hepatitis in the Middle East, in which genotype 4 predominates, is

limited. A recent study assessing sustained virological response (SVR) in Saudi Arabia revealed an SVR rate of around 48% in patients infected with HCV genotype 4; however, only PEG-IFN α -2a (PEGASYS) was used and they also included patients who were considered difficult to treat^[8]. Limitations of other studies include small number of patients, use of conventional interferons, and the lack of viral genotype data^[9]. Therefore, the objectives of this study were to (1) assess the SVR rates in treatment naïve patients; (2) compare the outcome of treatment using both PEG-IFN α -2b and PEG-IFN α -2a; and (3) define the predictors of SVR.

MATERIALS AND METHODS

A retrospective chart review of 400 patients treated at our center between January 2003 and January 2007 was conducted. All HCV treatment naïve patients with a positive PCR were included. Exclusion criteria included (1) pediatric patients; (2) chronic renal failure patients; (3) previous non-responders and those who had a relapse following prior treatment; (4) post renal transplant patients; and (5) patients with cirrhosis and signs of portal hypertension.

Biochemical assessments, including ALT (normal value: 0-58 IU/L), AST (normal value: 0-36 IU/L), γ -glutamyltransferase (GGT), alkaline phosphatase (ALP), bilirubin, albumin, and coagulation profile, were done according to our laboratory standards.

Serum HCV RNA was extracted using an automated extraction system. HCV detection and quantification were performed using the COBAS Ampliprep™/COBAS TaqMan™ HCV test (Roche diagnostics) utilizing different sets of primers and probes, which target a conserved region of the 5' untranslated region of the genome. The main processes of this procedure include: (1) specimen preparation to isolate HCV RNA; (2) reverse transcription of the target RNA to generate complementary DNA (cDNA) and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide detection probe specific to the target. This assay detects and quantifies HCV genotype (1-6) with a detection limit that ranges from 15 to 69 000 000 IU/mL. Prior to treatment, the HCV genotype was assayed using INNO-LiPA HCVII (Innogenetics, Ghent, Belgium).

After reviewing the patients' data, 240 patients fulfilling the inclusion criteria were selected for the study. Epidemiological data and treatment records were reviewed. Liver biopsy is not done routinely in our center unless there is suspicion of underlying cirrhosis or a concomitant liver disease. For data entry, patients were divided according to their gender, viral load (HCV RNA below *vs* above 800 000 IU/mL), age (younger *vs* older than 40), treatment (PEG-IFN α -2a *vs* PEG-IFN α -2b), liver enzyme pattern (normal *vs* abnormal), and body mass index (BMI) (below *vs* above 28) (Table 1).

Patients received either PEG-IFN α -2a (40 Kd; PEGASYS, F. Hoffmann-La Roche, Basel, Switzerland) at a dose of 180 mg/wk or PEG-IFN α -2b (12 Kd;

Table 1 Patients' characteristics

Variable	Number of patients	Percentage (%)
Age (yr)		
< 40	68	28.3
> 40	172	71.7
Sex		
Female	110	45.8
Male	130	54.2
BMI (kg/m ²)		
< 28	131	54.5
> 28	109	45.5
Liver enzymes		
Normal	115	47.9
Abnormal	125	52.1
PCR (IU/mL)		
< 800 000	138	57.5
> 800 000	102	42.5
Duration		
Completed	215	89.6
Incomplete	25	10.4
Side effects		
Present	125	52.1
Absent	115	47.9
Drug		
Peg IFN α -2A	138	57.5
Peg IFN α -2B	102	42.5
Genotype		
Genotype 1	46	19.2
Genotype 2	9	3.8
Genotype 3	15	6.3
Genotype 4	82	34.2
Genotype 5	1	0.4
Not identified	5	2.1
No records	82	34.2
ETVR		
Responded	160	66.7
No response	80	33.3
SVR		
SVR	139	58.0
No SVR	101	42.0

BMI: Body mass index; ETVR: End of treatment virological response; SVR: Sustained virological response.

PEGINTRON, Schering-Plough LTD, Singapore) at a dose of 1.5 mg/kg, and a standard dose of 1200 mg ribavirin with no weight related adjustments. While on treatment, patients were followed monthly or more frequently if required. Complete blood count (CBC) and liver enzymes were checked at each visit. To maintain the starting dose of PEGylated interferon and ribavirin, erythropoietin and granulocyte-colony stimulating factor (G-CSF) were given as early as possible to overcome any potential treatment-induced anemia or neutropenia. Patients with genotype 2 and 3 were treated for 24 wk, while patients with genotypes 1, 4 and 5 were treated for 48 wk. Viral loads were repeated at 12 wk to assess those who achieved an early virological response (EVR), at the end of treatment to assess the end of treatment virological response (ETVR), and six mo later to confirm an SVR. This study was approved by the Research Ethics Committee of our hospital.

Statistical analysis

Data were collected using a specialized data collection

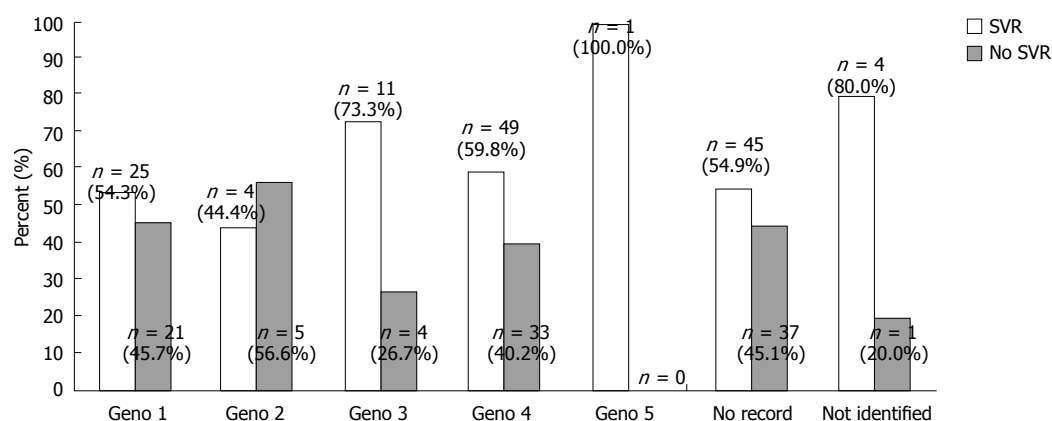


Figure 1 Response rates among the different genotypes. SVR: Sustained virological response.

Table 2 Overall treatment outcomes

Treatment outcome	Frequency (%)
SVR	139 (58)
Relapse	17 (7)
Primary non responder	61 (25)
Intolerant	23 (10)
Total	240 (100)

form, then introduced into a Microsoft Excel worksheet and finally transferred to the statistical package for social sciences version 15.0 (SPSS, Chicago, IL, USA). Analyses used included descriptive statistics as well as a Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Eighty-two (34%) patients were genotype 4, while 46 (19.2%), nine (3.8%), 15 (6.3%), and one (0.4%) had genotypes 1, 2, 3 and 5, respectively. The genotype could not be identified in five (2.1%) patients, while the genotype data was missing in 82 (34.2%) patients (Figure 1).

Two hundred and fifteen (89.6%) patients completed the treatment, while 25 (10.4%) patients could not complete the treatment because of significant side effects. In the entire cohort ($n = 240$), end of treatment Virological response (ETVR) was achieved in 160 (66.7%). 139 (58%) patients achieved SVR, while 101 (42%) did not respond or had a relapse after achieving ETVR (Table 2).

One hundred and twenty five (52.1%) had side effects, while 115 (47.9%) did not report any treatment related side effects. None of the patients required a blood transfusion for anemia. One hundred and thirty eight (57.5%) patients were treated with PEG-IFN α -2a while 102 (42.5%) patients received PEG-IFN α -2b. Among those who were treated with PEGylated IFN α -2a, the SVR was 61.5% and for those who were treated with PEG-IFN α -2b it was 53%, this did not reach statistical significance.

Younger patients achieved statistically significantly higher SVR rates compared to older patients. This observation is in agreement with other studies and we

Table 3 Characteristics of patients who achieved an SVR

Variable	Number of patients	SVR (%)	P value
Age (yr)			
< 40	51	75	0.001
> 40	88	51	
Sex			
Female	57	52	NS
Male	82	63	
BMI (kg/m ²)			
< 28	85	65	0.01
> 28	54	49	
PCR (IU/mL)			
< 800 000	88	64	0.023
> 800 000	51	50	
Drug			
Peg IFN α -2A	85	62	NS
180 μ g/wk			
Peg IFN α -2B	54	53	
1.5 μ g/kg per week			
Liver enzymes			
Normal	69	60	NS
Abnormal	70	56	
Treatment duration			
Completed	137	64	0.00001
Not completed	2	8	

NS: No significant.

believe that this is because younger patients, in addition to tolerating full treatment doses, have a less advanced fibrosis stage compared to older patients (75% *vs* 51%, $P = 0.001$).

Other statistically significant predictors of achieving a SVR in the present study include compliance to a full treatment duration (64% *vs* 8%, $P < 0.00001$), a BMI lower than 28 (65% *vs* 49%, $P = 0.01$), and a pretreatment HCV RNA load below 800 000 IU/mL (64% *vs* 50%, $P = 0.023$). SVR rates were similar in relation to patient's gender, liver enzyme pattern, and the type of drug used (Table 3).

DISCUSSION

Most of the data on HCV management originates from western populations in which genotypes 1, 2, and 3 predominate. There is less data from populations

where the prevalence of HCV is much higher and in which HCV genotype 4 predominates. Initial studies with conventional interferon and ribavirin resulted in lower SVR rates, giving the impression that genotype 4 is difficult to treat^[10]. One of the first trials on the treatment of HCV using PEGylated interferon in our region was conducted by Alfaleh *et al*^[11]. In their study, 48 wk of PEG-IFN α -2b and ribavirin combination therapy resulted in an SVR rate of around 43%. One of the possible explanations for this relatively low SVR is the use of an 800 mg fixed dose of ribavirin daily. More recently Al Ashgar *et al*^[8] published their results in which 335 patients treated with PEG-IFN α -2a and ribavirin achieved an SVR rate of around 48%. In this study, they adjusted the ribavirin dose according to the body weight. They also included renal failure patients, patients who failed previous interferon based treatment, and patients with concomitant HBV or HIV.

Other studies originating from our region, using a combination therapy of PEG-IFN α -2b and ribavirin, resulted in SVR rates of 43% to 68%^[9,11-14].

In the present study, only treatment naïve patients were included. 58% of the patients achieved an SVR (64% excluding the patients that didn't complete the treatment). The following exclusion criteria were used to achieve a more homogenous study population: previously failed interferon based treatment, renal failure patients, and patients with a concomitant HBV/HIV.

In the present study, both PEG-IFN α -2a and PEG-IFN α -2b were used, with comparable results. A fixed dose of ribavirin was also given to the whole study population with no weight-based adjustment. This approach is considered to be an effective method for improving the outcome^[9], and likely contributed to the higher SVR rate in our study compared to previous studies on genotype 4 infected patients. SVR was achieved by 64% of patients in whom the treatment duration was completed, while only 2% of patients who did not complete the treatment achieved SVR. This confirms the importance of compliance in achieving SVR, as suggested by other studies^[15].

Duration of Treatment with PEG-IFN and ribavirin is individualized according to initial treatment response, genotype, and pretreatment viral load. Patients with HCV genotype 1 and 4 require treatment for 48 wk and those with genotypes 2 or 3 seem to be adequately treated in 24 wk^[16,17]. Some investigators tried treatment durations of 16 wk for HCV genotypes 2 and 3 infected patients who had a rapid virological response at four wk, with variable results. In one study, SVR was lower in patients treated for 16 wk than in patients treated for 24 wk, and the rate of relapse was significantly greater in the 16-wk group^[18]. Other studies show that extending the treatment duration from 48 wk to 72 wk in genotype 1 patients with slow virological response to PEG-IFN and ribavirin significantly improves SVR rates^[19]. The current practice worldwide is to treat HCV genotype 4 patients for 48 wk. However, recent data suggests treating chronic HCV genotype 4 patients for 24 and 36 wk might be sufficient when viral loads are undetectable

at four and 12 wk, respectively^[14]. Whether extending the treatment to 72 wk in genotype 4 patients with a slow response will increase the SVR rate is yet to be determined.

Factors that are thought to be associated with a favorable outcome include genotype 2 and 3, mild hepatitis, minimal fibrosis, absence of obesity, and female gender^[20]. Genotype 1, serum HCV RNA concentrations over 800 000 IU/mL, advanced duration of infection, histologically advanced liver disease, increased bodyweight, relapse or no response to previous treatment, presence of cirrhosis, African-American ethnicity, and advanced age were among the factors that are thought to be associated with a less favorable outcome^[21].

In the present study, patients with genotype 4 were treated for 48 wk and achieved an SVR of 64% in the patients who completed the treatment duration. Younger patients, low pretreatment viral load, lower BMI, and compliance with the standard duration of treatment were associated with a higher SVR. This study supports the view that treatment results of genotype 4 HCV naïve patients are more favorable than has been previously suggested when higher doses of ribavirin are used.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection is a major health problem and it is the main indication for orthotopic liver transplantation among adults. PEGylated interferon (IFN) and ribavirin combination therapy is the standard treatment, but data regarding sustained virological response (SVR) in our predominantly HCV genotype 4 infected population is limited.

Research frontiers

HCV genotype 4 is the most frequent cause of chronic hepatitis C in the Middle East and North Africa. In Saudi Arabia, disease prevalence is estimated at 2.5%. Initial studies suggested that genotype 4 was difficult to treat and was associated with a low SVR.

Innovations and breakthroughs

The use of PEGylated interferon and ribavirin resulted in a higher SVR rate in patients infected with genotype 4 when higher doses of ribavirin are given. In the study, patients were given a fixed high dose of ribavirin, and it is believed that this contributed to the higher SVR rates in the study compared to previous studies. Additionally, it is shown that younger patients, patients with lower body mass index, and patients with low viral loads had a better treatment outcome.

Applications

This study supports the view that treatment results of genotype 4 HCV naïve patients are more favorable than has been previously suggested when higher doses of ribavirin are used.

Peer review

The article concerns a very topical problem-interferon therapy effectiveness in HCV-patients. The content of the article will be interesting, not only for gastroenterologists, but also for practical medicine.

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S- Editor Cheng JX L- Editor Stewart GJ E- Editor Yin DH



BRIEF ARTICLES

Paraaortic lymph node metastasis in patients with intra-abdominal malignancies: CT vs PET

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Received: May 12, 2009 Revised: August 13, 2009

Accepted: August 20, 2009

Published online: September 21, 2009

RESULTS: The sensitivity, specificity, PPV, NPV, and accuracy of CT were 61.5%, 84.9%, 50%, 90% and 80.3%, respectively. For PET, the percentages were 46.2%, 100%, 100%, 88.3%, and 89.4%. Additionally, there were 8 false positive CT cases (8/53, 15.1%) and zero false positive PET cases. Of the 13 metastatic PANs, there were 5 false negative CT scans (38.5%) and 7 (53.9%) false negative PET scans.

CONCLUSION: For detecting PAN metastasis, CT is more sensitive than PET, while PET is more specific.

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Key words: Malignancy; Paraaortic lymph node; Computed tomography; Positron emission tomography; Sensitivity; Specificity

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Lee MJ, Yun MJ, Park MS, Cha SH, Kim MJ, Lee JD, Kim KW. Paraaortic lymph node metastasis in patients with intra-abdominal malignancies: CT vs PET. *World J Gastroenterol* 2009; 15(35): 4434-4438 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4434.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4434>

Abstract

AIM: To compare the diagnostic accuracy of computed tomography (CT) and positron emission tomography (PET) for the preoperative detection of paraaortic lymph node (PAN) metastasis in patients with intra-abdominal malignancies.

METHODS: Sixty-six patients with intra-abdominal malignancies who underwent both CT and PET before lymphadenectomy were included in this study. Histopathologically, 13 patients had metastatic PAN, while 53 had non-metastatic PAN. The CT criteria for metastasis were: short diameter of > 8 mm, lobular or irregular shape, and/or combined ancillary findings, including necrosis, conglomeration, vessel encasement, and infiltration. The PET criterion was positive fluorodeoxyglucose (FDG) uptake. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of both modalities were compared with the pathologic findings, and the false positive and false negative cases with both CT and PET were analyzed.

INTRODUCTION

Paraaortic lymph node (PAN) metastasis is considered an important prognostic factor in several abdominal and pelvic malignancies, such as stomach cancer, colorectal cancer, cholangiocarcinoma, and cervical cancer^[1-4]. While it is very important to evaluate PAN metastasis in preoperative evaluations, the correct diagnosis is not always definitively determined. Because of this, sampling and pathologic confirmation of the paraaortic nodes should be carried out before starting a radical operation, which is why many surgeons, including those in our hospital, perform paraaortic node dissection before radical surgery^[5-8]. Although lymphadenectomy followed by histologic examination of the lymph nodes is still the gold standard for determining metastasis, doctors must be careful in using this technique because of the perioperative risks and postoperative complications of

PAN dissection^[3,9,10]. All of this makes preoperative, noninvasive imaging diagnosis of PAN metastasis very important^[11,12].

For preoperative evaluation of abdominal malignancies, computed tomography (CT) has usually been used as the first-line examination. However, many investigators have reported CT to have low sensitivity and low specificity when used for lymph node (LN) diagnosis. Nowadays, positron emission tomography (PET) with fluorodeoxyglucose (FDG) has been recognized as a useful diagnostic technique in clinical oncology, not only for primary tumor evaluation, but also for detection of metastasis (including nodal metastasis)^[13]. However, FDG-PET has also been reported to have low sensitivity for the detection of LN metastasis^[14], and FDG-PET cannot determine the anatomical location of small lesions, such as lymph nodes^[15]. To our knowledge, however, there has been limited study comparing CT with PET in evaluating PAN metastasis^[16,17].

The purpose of our study is to compare the diagnostic accuracy of CT and FDG-PET in the preoperative detection of PAN metastasis in patients with an intra-abdominal malignancy. We also analyze the false positive and false negative cases of both CT and PET scans and suggest clinical guidelines for using CT and PET results.

MATERIALS AND METHODS

Patients

The protocol for this study was approved by the Institutional Review Board at our institution and informed consent was not required. Our pathologic database was retrospectively searched from September 2002 to July 2006 for all patients who underwent paraaortic lymphadenectomy before or during a surgical resection operation. A total of 305 patients were selected from the database, and, among these, 113 had underlying intra-abdominal malignancies (excluding lymphoma). Finally, 66 patients (39 women and 27 men, age range 28-78 years, mean age 56 years) who underwent both CT and PET no \leq 1 mo before a lymph node biopsy were chosen for the study. The underlying malignancies were as follows: hepatobiliary cancer ($n = 11$), pancreatic cancer ($n = 10$), colorectal cancer ($n = 20$), gastric cancer ($n = 3$), cervical cancer ($n = 7$) and tubo-ovarian cancer ($n = 15$).

Imaging procedures

For CT, all patients underwent single-section spiral CT (HiSpeed CT/I, GE Medical Systems, Milwaukee, WI) or multi-detector CT scanning (four detector row; Lightspeed Plus, GE Medical Systems, Milwaukee, WI or sixteen-detector row; Sensation 16, Siemens, Erlangen, Germany) according to an established protocol. A 60% iodinated contrast material [Iopromide (Ultravist); Schering, Berlin, diatrizoate meglumine (Hypaque) or iohexol (Omnipaque 300); Nycomed Amersham, Princeton, NJ] was administered intravenously at a rate

of 2-4 mL/s by using an automatic power injector with a volume of 2 mL/kg, up to a maximum volume of 150 mL. Portal venous phase CT scans were obtained 70 s after initiating the contrast material injection, and the abdomen and pelvis, from the level of the hepatic dome to the ischial tuberosities, were scanned with a pitch of 1.0-1.5 and a reconstruction thickness of 3.0-5.0 mm. The transverse images were reconstructed with a soft-tissue algorithm.

For PET, patients fasted at least 4 h before intravenous injection of 18F-FDG, and scanning began 60 min later. Images from the neck to the proximal thigh were obtained either on an Advance PET scanner (GE advance, GE Medical Systems, Milwaukee, WI), with a spatial resolution of 5 mm in the center of the field of view, or an Allegro PET scanner (Allegro, Philips-ADAC medical systems, Cleveland, OH), with a spatial resolution of 5.3 mm in the center of the field of view. For the Advance scanner, approximately 370 MBq of 18F-FDG were intravenously injected, and the emission scan was acquired for 5 min per bed position in the 2-dimensional mode. The Allegro acquired data in the 3-dimensional mode after administration of 5.18 MBq (0.14 mCi)/kg of 18F-FDG. Transmission scans (3 min per bed position) to correct for non-uniform attenuation were obtained using point sources of 68Ge for the Advance or 137Cs for the Allegro. Transmission scans were interleaved with the multiple emission scans for the Allegro. The images were reconstructed using an iterative reconstruction algorithm: that is, either the ordered-subset expectation maximization for the Advance or the low-action maximal-likelihood algorithm for the Allegro.

Imaging analysis

All imaging analysis was performed on a picture archiving and communication system (PACS) workstation (Centricity 1.0; GE Medical Systems). Two radiologists independently evaluated preoperative CT images in the 66 patients without knowledge of the final pathologic diagnoses. They considered the following criteria as the primary findings for a metastatic PAN: short diameter > 8 mm, lobular or irregular shape, and/or combined ancillary findings including necrosis, conglomeration, vessel encasement, and infiltration (Figure 1). Reviewers characterized the PAN as metastatic or non-metastatic. When discrepancies were detected, interpretations were achieved via consensus. Two experienced nuclear medicine physicians also interpreted the preoperative PET images, where the criterion was positive FDG uptake. When discrepancies were detected, interpretations were determined via consensus. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of CT and PET for the detection of metastatic PAN were then calculated.

As a second portion of our study, a third radiologist analyzed the cases of false diagnosis for both CT and PET scans. She also evaluated the relationship between primary tumors and PAN in terms of the FDG uptake of the primary tumors on PET.

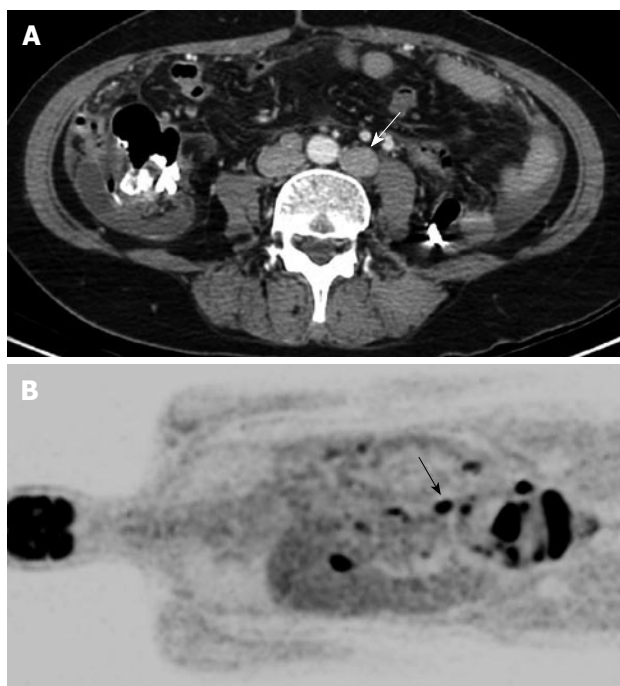


Figure 1 Typical case of metastatic paraaortic lymph node (PAN) on both computed tomography (CT) and positron emission tomography (PET). A 52-year-old woman with ovarian cancer underwent CT and PET. (A) Axial CT scan shows enlarged (short diameter was 16 mm) and conglomerated PAN (arrow) and (B) coronal view of PET image shows positive FDG uptake in this PAN (arrow).

RESULTS

In our 66 patients, we found 13 patients with metastatic paraaortic nodes and 53 patients with non-metastatic ones according to the pathologic results.

The sensitivity, specificity, PPV, NPV, and accuracy of CT for detecting PAN metastasis were 61.5% (8/13), 84.9% (45/53), 50% (8/16), 90% (45/50), and 80.3% (53/66), respectively. For PET, the results were 46.2% (6/13), 100% (53/53), 100% (6/6), 88.3% (53/60), and 89.4% (59/66), respectively. From these numbers, we can conclude that CT was more sensitive while PET was more specific, and the overall accuracy was slightly higher with PET.

With CT, 8 (15.1%) of the 53 non-metastatic nodes were false positively diagnosed (Figure 2). One of these cases showed central necrosis. There were no false positives using PET, as all 53 showed no FDG uptake.

For the 13 metastatic nodes, 5 cases (38.5%) on CT, 7 cases (53.9%) on PET, and 4 cases (30.8%) on both CT and PET were false negatively diagnosed. Among the false negative CT cases, only one (1/5, 20%) showed positive FDG uptake on PET. Among the false negative PET cases, 3 (3/7, 42.9%) were diagnosed as malignant nodes on CT (Figure 3).

In terms of the relationship between a primary tumor and PAN on FDG uptake, six of the seven false negative metastatic PAN cases showed high FDG uptake on the primary tumor. Only one patient with primary stomach cancer with ovarian metastasis showed negative FDG uptake on both the metastatic PAN and the primary



Figure 2 False positive metastatic PAN on CT. A 38-year-old man with colon cancer underwent CT and PET. (A) Coronal CT scan shows multiple enlarged (short diameter was 8 mm in the largest one) PAN (arrow), but (B) coronal view of PET image shows negative fluorodeoxyglucose uptake in these lymph nodes. This patient had underlying inflammatory bowel disease of ulcerative colitis.

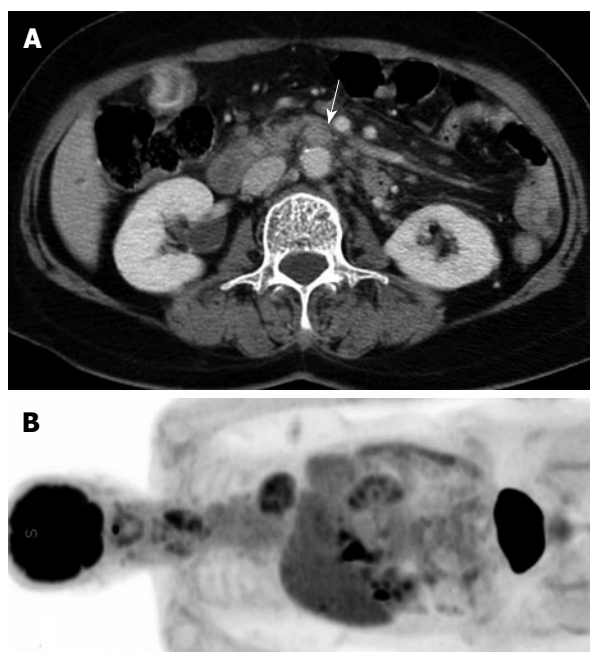


Figure 3 False negative metastatic PAN on PET. A 72-year-old woman with common bile duct cancer underwent CT and PET. (A) Axial CT scan shows enlarged (short diameter was 12 mm) PAN (arrow) with surrounding fat infiltration, but (B) coronal view of PET image shows negative FDG uptake in this lymph node. Pathology was confirmed as metastatic lymph node.

tumor. A total of 3 cases (3/66, 4.5%) showed no FDG uptake on the primary tumor, and one of these (1/3, 33%) showed negative FDG uptake on the metastatic PAN.

DISCUSSION

Our study shows that CT is more sensitive than PET, while PET is more specific than CT for the diagnosis of metastatic PAN in patients with abdominal malignancies.

PET has generally been considered a sensitive tool for detecting distant metastasis^[13,18,19]. In daily practice, PET detects some lesions that are difficult to detect by CT, such as mesenteric or pelvic nodes between bowel loops. However, when considering only PAN, CT

showed more sensitivity than PET in our study.

PET is a functional imaging modality based on the increased glucose metabolism of physiologic or pathologic conditions. Because of this, PET has a limitation associated with metabolism: the primary tumor must have a strong avidity for ^{18}F -FDG, which may or may not be the case, depending on the histopathologic type of the tumor^[14,20]. Low metabolic activity can cause a false negative PET scan^[13], while a non-tumorous hypermetabolic condition, such as a systemic inflammation or an infection, can lead to false positive uptake^[13]. The usefulness of PET imaging is also limited in patients with uncontrolled diabetes mellitus ($> 150 \text{ mg/dL}$)^[14].

In our study, PET (53.9%) showed higher false negative rates than CT (38.5%) in metastatic PAN. One false negative metastatic PAN case (14.3%) also showed no FDG uptake on the primary tumor. The FDG activity of the primary tumor may reflect that of the metastatic PAN, meaning more careful attention is required to diagnose PAN if the primary tumor shows no FDG uptake.

Among the 7 false negative PET cases, 3 (42.9%) were diagnosed as metastatic PAN using CT because they showed necrosis or conglomeration, even though the short diameter was $< 8 \text{ mm}$. This means that CT may be helpful in the characterization of false negative PET cases.

CT also showed higher false positive rates than PET in our study. Many studies discuss the criteria for the detection of metastatic lymph nodes on CT, with size as the most frequently used parameter^[14,19,21-24]. However, this places a major limitation on CT, as it will be unable to detect small, metastatic lymph nodes and might misrepresent large, reactive lymph nodes^[13,19]. All of the false positive cases in our study showed no FDG uptake, which suggests that FDG-PET may be very useful for increasing the specificity of CT.

Integrated PET-CT has been introduced into clinical practice, providing shorter total imaging time and a reduction in the number of ambiguous lesions^[25,26]. Moreover, some researchers have reported that PET-CT improves diagnostic accuracy when compared with PET alone in abdominal and pelvic cancer^[27]. Recent studies report the sensitivity, specificity, and accuracy of PET-CT for lymph node metastasis as 28.6%-51.7%, 92.9%-99.8%, and 75.0%-99.8%, respectively^[15,17,28], but this still shows the lower sensitivity and higher specificity of PET-CT. While the role of CT in PET-CT is usually limited to anatomic localization and attenuation correction^[25], the results of our study suggest that CT is useful not only for anatomic localization, but also for a reduction in the false negative rate of PET. Previous studies have also reported that dedicated CT scans do additional work in some cases, such detecting as stomach cancer, liver cancer and mucinous primary or metastatic tumors^[19,26,29]. Moreover, there are some reports about integrated PET/contrast-enhanced CT as an accurate modality for assessing colorectal cancer in recent studies^[30,31]. However, the usefulness of integrated PET/

contrast-enhanced CT in other malignancies is still being debating and additional study of this topic is needed.

There are some limitations to our study. First, the diagnoses were variable with intra-abdominal malignancy (including hepatobiliary cancer, gastrointestinal cancer and gynecologic cancer) except lymphoma. Tumor characteristics on PET and lymphatic drainage pattern can be different between these malignancies. The second limitation is that we evaluated the data not on a per-node basis, but on a per-case basis. This is a limitation of retrospective study, and further evaluation with prospective study is needed.

In conclusion, CT is more sensitive than PET for detecting PAN metastasis, but PET is more specific. PET is useful for ruling out enlarged reactive lymph nodes and reducing the false positive rate of CT scans alone. To reduce the false negative rate of PET, CT can be helpful by showing necrosis or other ancillary findings of PAN. These results all suggest that the two modalities are complementary to each other in the diagnosis of PAN metastasis.

COMMENTS

Background

Paraaortic lymph node (PAN) metastasis is considered an important prognostic factor in several abdominal and pelvic malignancies. However, the correct diagnosis is not always definitively determined. Pathologic confirmation before starting a radical operation has perioperative risks and postoperative complications. Therefore, preoperative, noninvasive imaging diagnosis of PAN metastasis is very important.

Research frontiers

For preoperative evaluation of abdominal malignancies, computed tomography (CT) has usually been used as the first-line examination. Positron emission tomography (PET) with fluorodeoxyglucose (FDG) has been recognized as a useful diagnostic technique in clinical oncology. However, there has been limited study comparing CT with PET in evaluating PAN metastasis. The aim of our study is to compare the diagnostic accuracy of CT and FDG-PET in the preoperative detection of PAN metastasis in patients with an intra-abdominal malignancy.

Innovations and breakthroughs

CT is more sensitive than PET for detecting PAN metastasis, but PET is more specific. PET is useful for ruling out enlarged reactive lymph nodes and reducing the false positive rate of CT scans alone. To reduce the false negative rate of PET, CT can be helpful by showing necrosis or other ancillary findings of PAN.

Applications

CT and PET are complementary to each other in the diagnosis of PAN metastasis in patients with an intra-abdominal malignancy.

Peer review

Preoperative evaluation of paraaortic lymph node metastasis is very important in intra-abdominal malignancy. The results of this study are interesting as a preliminary result. It may be a first step to confirm the results of a prospective study in the future.

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S- Editor Tian L L- Editor O'Neill M E- Editor Yin DH

Effects of Chai-Qin-Cheng-Qi Decoction on cefotaxime in rats with acute necrotizing pancreatitis

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Supported by National Key Technology R&D Program of China, No. 2006BAI04A15; Major Diseases Fund of Sichuan Province Administration of Traditional Chinese Medicine, No. 2007B03

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Received: July 2, 2009 Revised: August 14, 2009

Accepted: August 21, 2009

Published online: September 21, 2009

Abstract

AIM: To investigate the effect of Chai-Qin-Cheng-Qi Decoction (CQCQD) on cefotaxime (CTX) concentration in pancreas of rats with acute necrotizing pancreatitis (ANP).

METHODS: Sixty healthy male Sprague-Dawley rats were divided randomly into an ANP group (ANP model + CTX, $n = 20$), treatment group (ANP model + CTX + CQCQD, $n = 20$) and control group (normal rats + CTX, $n = 20$). ANP models were induced by retrograde intraductal injection of 3.5% sodium taurocholate (1 mL/kg), and the control group was injected intraductally with normal saline. All rats were injected intraperitoneally with 0.42 g/kg CTX (at 12-h intervals for a continuous 72 h) at 6 h after intraductal injection. Meanwhile, the treatment group received CQCQD (20 mL/kg) intragastrically at 8-h intervals, and the ANP and control group were treated intragastrically with normal saline. At 15 min after the last CTX injection, blood and pancreas samples were collected for the determination of CTX concentration using validated high-performance liquid chromatography. Pathological changes and wet-to-dry-weight (W/D) ratio of pancreatic tissue were examined.

RESULTS: Serum CTX concentrations in three groups were not significantly different. Pancreatic CTX

concentration and penetration ratio were lower in ANP group *vs* control group ($4.4 \pm 0.6 \mu\text{g/mL}$ *vs* $18.6 \pm 1.7 \mu\text{g/mL}$, $P = 0.000$; 5% *vs* 19%, $P = 0.000$), but significantly higher in treatment group *vs* ANP group ($6.4 \pm 1.7 \mu\text{g/mL}$ *vs* $4.4 \pm 0.6 \mu\text{g/mL}$, $P = 0.020$; 7% *vs* 5%, $P = 0.048$). The histological scores and W/D ratio were significantly decreased in treatment group *vs* ANP and control group.

CONCLUSION: CQCQD might have a promotive effect on CTX concentration in pancreatic tissues of rats with ANP.

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Key words: Acute necrotizing pancreatitis; Cefotaxime; Chai Qin Cheng Qi Decoction; Drug penetration; Traditional Chinese medicine

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Deng LH, Xiang DK, Xue P, Zhang HY, Huang L, Xia Q. Effects of Chai-Qin-Cheng-Qi Decoction on cefotaxime in rats with acute necrotizing pancreatitis. *World J Gastroenterol* 2009; 15(35): 4439-4443 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4439.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4439>

INTRODUCTION

Acute necrotizing pancreatitis (ANP) accounts for approximately 20% of patients with acute pancreatitis. When complicated by infection, pancreatic necrosis becomes a major contributor to late mortality^[1-3]. Early broad-spectrum antibiotics in the initial stage of disease appear to be associated with significantly decreased mortality and therefore improve outcome^[4]. The choice of the antibiotics must be driven by the flora as well as penetration in the pancreatic tissue^[5-7]. Cefotaxime (CTX), a semisynthetic broad-spectrum cephalosporin antibiotic, has an active penetrability and sustains an effective antibacterial concentration in human pancreatic tissue^[8-10].

An important criterion in determining the most appropriate antibiotic treatment for pancreatitis is antibiotic tissue concentration in the pancreas, which varies during the progression of ANP and is mainly

influenced by the changes in pancreatic tissue morphology and capillary blood flow. Previous studies have shown that the improvement of microcirculation and local infusion of the pancreas by regional arterial infusion can improve antibiotic concentrations in pancreatic tissue and reduce the pancreatic infection rate and mortality^[11,12].

Chai-Qin-Cheng-Qi Decoction (CQCQD) is modified from Da-Cheng-Qi Decoction (*Cortex Magnoliae Officinalis*, *Fructus Aurantii Immaturus*, *Radix et Rhizoma Rhei* and *Natrii Sulfas*), which is a well-known and popular traditional Chinese medicine that is used as a purgative in China and East Asia. In recent decades, CQCQD has been used widely for pancreatitis^[13]. Generally, CQCQD plays a multi-target and comprehensive role in ANP, but the exact therapeutic mechanism is still unclear.

In the present study, we performed validated high-pressure liquid chromatography (HPLC) to determinate the changes in CTX concentration and penetration ratio in pancreatic tissue of rats with ANP after oral administration of CQCQD. Our hypothesis was that CQCQD would increase CTX concentration and penetration rate in pancreatic tissue of ANP rats.

MATERIALS AND METHODS

Animals

Sixty healthy male Sprague-Dawley rats weighting 250-300 g were purchased from the Experimental Animal Center of West China Center of Medical Sciences of Sichuan University [SCXK (Chuan-11-2006)]. The animals were kept at constant room temperature with a 12-h light-dark cycle, free access to water and standard laboratory chow, and adjusted to laboratory conditions for 1 wk with a 12-h fast but free access to drinking water before the experiments. Animal experiments and performance of this study were approved by the Animal Care Committee of Sichuan University.

Preparation of CQCQD

CQCQD is composed of Chaihu (*Radix Bupleuri*) 15 g, Huangqin (*Radix Scutellariae*) 15 g, Houpo (*Cortex Magnoliae Officinalis*) 15 g, Zhishi (*Fructus Aurantii Immaturus*) 15 g, Yinchén (*Herba Artemisiae Capillaris*) 15 g, Zhizi (*Fructus Gardeniae*) 20 g, Dahuang (*Radix et Rhizoma Rhei*) 15 g, and Mangxiao (*Natrii Sulfas*) 10 g. All high-quality medicinal herbs were provided, prepared to decoction, vaporized and dried into lyophilized powder of CQCQD by Herbal Pharmacy of West China Hospital, Sichuan University, China. The lyophilized powder was stored at 4°C and freshly dissolved in drinking water (a raw herbal concentration of 2 g/mL) prior to use.

Main reagents

CTX sodium for injection (Claforan®) was a product of NCP Create Pharma Co., Ltd., China. Sodium taurocholate powder was purchased from Sigma (T-0750; St. Louis, MO, USA). All other reagents were of the highest purity available.

Animal grouping and administration

Rats were randomized divided into an ANP group (ANP model + CTX, $n = 20$), treatment group (ANP model + CTX + CQCQD, $n = 20$) and control group (normal rats + CTX, $n = 20$). After anesthesia with intraperitoneal injection of 40 mg/kg sodium pentobarbital, ANP models were induced by retrograde intraductal injection of 3.5% sodium taurocholate (1 mL/kg) using a micropump, within 5 min. Rats in the control group were administered with the same volume of normal saline. Six hours after retrograde injection, all animals were injected intraperitoneally with 0.42 g/kg CTX at 12-h intervals for a continuous 72 h; at the same time, rats in the treatment group were treated intragastrically with 20 mL/kg CQCQD at 8-h intervals, and the ANP group was given the same volume of normal saline as the positive control.

Sample collecting

Fifteen minutes after the last CTX injection, blood samples were collected *via* the inferior vena cava and centrifuged at 3600 r/min for 5 min at 4°C. The serum was kept at -70°C. Pancreatic tissues were removed quickly and stored immediately in liquid nitrogen at -70°C. Histopathological examination was also performed.

HPLC analysis

Prior to HPLC, pancreatic tissues were thawed and centrifuged at 2500 r/min for 5 min. HPLC was performed as follows: 100 μ L samples were mixed with 100 μ L water and 50 μ L internal standard, and then mixed with 500 μ L methanol. After centrifugation at 13000 r/min for 15 min at 16°C, the filtrate was evaporated under an air stream in a 40°C water bath. The residue was dissolved in 200 μ L mobile phase with methanol/0.05 mol/L phosphate buffer (18/82: v/v), and 50 μ L of the solution was injected into the HPLC system. The chromatographic separation was performed on a YMC-Pack ODS-A C18 column (5 μ m, 150 mm \times 4.6 mm). The flow rate was 0.8 mL/min. The retention time of CTX and the internal standard was 14.32 and 8.97 min, respectively. The detection was set at a wavelength of 254 nm.

Histopathological examination and wet-to-dry-weight (W/D) ratio

Pancreatic edema, inflammatory infiltration, hemorrhage and acinar cell necrosis were evaluated and scored according to the criteria shown in Table 1^[14]. For each pathological section, 10 visual fields under a high-power microscope [hematoxylin and eosin (HE) stain, $\times 400$] were selected randomly and scored by one single investigator (Xiang DK). The mean score of 10 visual fields from one pathological section was calculated as the pathological score.

We weighed the freshly collected pancreas samples in the wet state and incubated them at 210°C for 12 h, and the wet weight and dry weights were measured. The W/D ratio was used to represent water content in the pancreas.

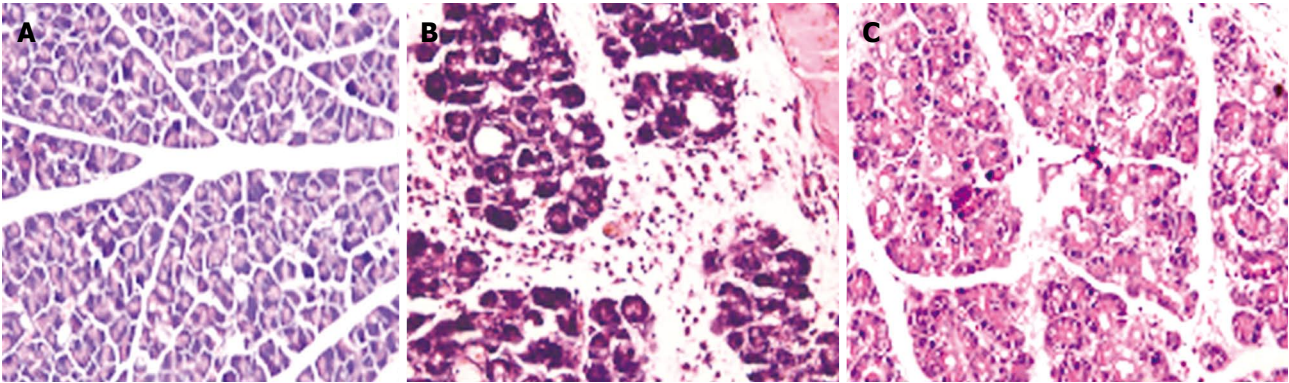


Figure 1 Histopathological changes in pancreatic tissues in three groups (HE stain). A: Control group (× 200); B: ANP rats without CQCQD treatment (× 400); C: ANP rats with CQCQD treatment (× 200).

Table 1 Pathological grading and scoring criteria for pancreatic tissue of rats with ANP	
Pathological grading	Scores
Edema	
Absent	0
Focal expansion of interlobular septae	1
Same as 1 + diffuse expansion of interlobar; septae/diffuse expansion of interlobar septae	2
Same as 2 + expansion of interacinar septae	3
Same as 3 + expansion of intercellular spaces	4
Inflammation and perivascular infiltrate (intralobular or perivascular leukocytes/HPF)	
0-1	0
2-10	1
11-20	2
21-30	3
> 30 or microabscesses	4
Acinar necrosis (necrotic cells/HPF)	
Absent	0
1-4	1
5-10	2
11-16	3
> 16	4
Hemorrhage and fat necrosis (focus)	
Absent	0
1-2	1
3-4	2
5-6	3
> 7	4

HPF: High-power field; ANP: Acute necrotizing pancreatitis.

Statistical analysis

Data were expressed as mean ± SD or percentage. *P* value was calculated using ANOVA test for multiple comparisons. *P* < 0.05 was considered statistically significant.

RESULTS

CTX concentration in serum and pancreatic tissue, and CTX penetration ratio

CTX concentration in serum (*C*_s, μg/mL) and pancreatic tissue (*C*_p, μg/g), as well as CTX penetration ratio (*C*_p/*C*_s × 100%, %) are shown in Table 2. Three groups had no significant differences in serum CTX concentration (*P* >

Table 2 CTX concentrations in serum and pancreatic tissue, and penetration ratio (<i>C</i> _p / <i>C</i> _s × 100%) in three groups (mean ± SD)				
Group	<i>n</i>	<i>C</i> _s (μg/mL)	<i>C</i> _p (μg/g)	Penetration ratio (%)
Control group	20	98.9 ± 2.4	18.6 ± 1.7	19
ANP group	20	96.8 ± 2.2	4.4 ± 0.6 ^b	5 ^b
Treatment group	20	97.4 ± 3.0	6.4 ± 1.7 ^{a,b}	7 ^{a,b}

^a*P* < 0.05 *vs* ANP group; ^b*P* < 0.001 *vs* control group. CTX: Cefotaxime.

0.05). CTX concentration in pancreatic tissue (*P* < 0.001) and CTX penetration ratio (*P* < 0.001) were significantly lower in the ANP group versus the control group. Compared with the ANP group, the treatment group had a significantly higher CTX concentration in pancreatic tissue (*P* < 0.05) and CTX penetration ratio (*P* < 0.05).

Histopathological changes and histological scores

Histopathological changes in pancreatic tissues are shown in Figure 1. Comparison between the treatment and ANP groups indicated that scores for edema and inflammatory infiltration were significantly lower in the former (*P* < 0.001), although the differences in scores for hemorrhage and necrosis were not significant. Compared with the control group, pathological scores were significantly higher in the ANP and treatment groups (*P* < 0.001) (Figure 2).

W/D ratio

W/D ratio was higher in the ANP group than the control group (7.1 ± 1.7 *vs* 1.45 ± 0.1, *P* < 0.001). Although significantly higher than that in the control group (4.5 ± 1.5 *vs* 1.45 ± 0.1, *P* < 0.001), W/D ratio in the treatment group was significantly lower than that in the ANP group (4.5 ± 1.5 *vs* 7.1 ± 1.7, *P* < 0.01) (Figure 3).

DISCUSSION

Previous studies have reported that pancreatic perfusion decreases the progression of pancreatic necrosis^[15,16]. Early antibiotic therapy may be favorable for infection of the pancreas with enteric microorganisms in the course of

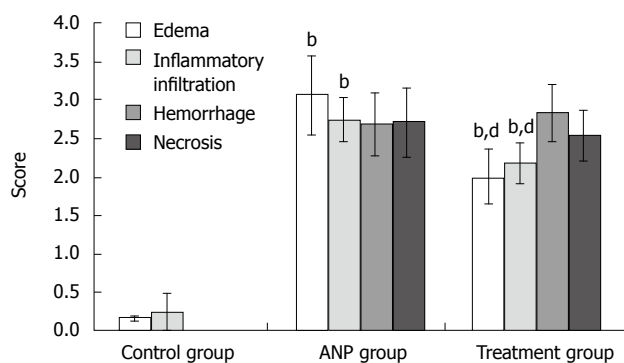


Figure 2 Histological scores of pancreatic tissues in three groups ($n = 20$, mean \pm SD). ^b $P < 0.001$ vs control group, ^d $P < 0.001$ vs ANP group.

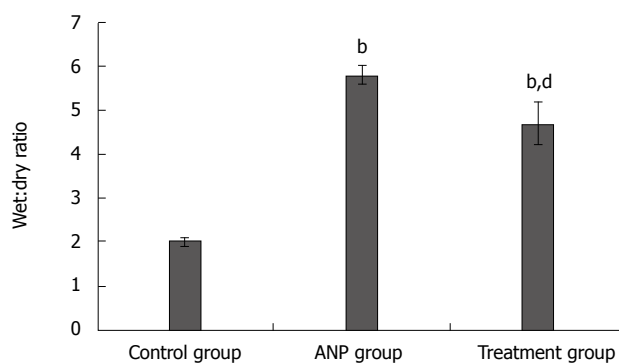


Figure 3 W/D ratio in three groups. ^b $P < 0.001$ vs control group; ^d $P < 0.001$ vs ANP group.

ANP^[17,18]. Based on pharmacokinetic and bacteriological studies of the efficacy of different antibiotics in ANP, a consensus has emerged that antibiotics in ANP must not only cover the spectrum of bacteria usually found in necrotic pancreatic tissue (including anaerobes), but also penetrate into the pancreas with a sustainable anti-bactericidal concentration. The pancreatic concentration of antibiotic is determined by the pathophysiological condition of the pancreas, alkaline pH, high ion concentration, enzymatic and hormonal regulation, pathological changes, and infection^[19]. Promoters of pancreatic perfusion and microcirculation might enhance the therapeutic effects against infection by increasing antibiotic tissue concentrations.

HPLC analysis showed that CTX concentration in healthy rats was as high as 98.9 ± 2.4 $\mu\text{g/mL}$ in serum, but reduced to 18.6 ± 1.7 $\mu\text{g/mL}$ in pancreatic tissue (CTX penetration ratio in normal rats was 19%). These results indicate that the distribution of antibiotics is restricted by the existence of a blood-pancreatic juice barrier^[20]. Furthermore, the significantly reduced CTX concentration in pancreatic tissue and penetration ratio were detected in the ANP group more than in the control group ($P < 0.001$), as found by other investigators^[11]. The results also showed significant higher pancreatic tissue concentration and penetration ratio of CTX in the treatment group, which confirmed our hypothesis that CQCQD treatment could improve antibiotic concentration and penetration ratio in pancreatic tissue of rats with ANP.

Meanwhile, pathological scoring confirmed that CQCQD improved the severity of interstitial edema, inflammatory infiltration, hemorrhage and acinar cell necrosis. The significantly reduced W/D ratio of pancreatic tissue in the treatment group confirmed that CQCQD alleviated the severity of parenchymal edema.

Although we found a promotive effect of CQCQD on CTX tissue concentration in rats with ANP, the exact mechanism remains unclear. Some previous studies have demonstrated that prescriptions composed of Dahuang, Mangxiao, Zhishi and Houpo increase the blood flow of most abdominal organs during peritonitis, reduce inflammatory exudate and lesions, inhibit the "waterfall effect" of cytokines, and therefore protect the target organ^[21-23]. We speculate that the mechanism of action of CQCQD on antibiotics pancreatic concentrations in ANP rats

may be related to promotion of pancreatic microcirculation, increasing local perfusion and blood flow of the pancreas, improving pancreatic tissue edema, and reducing anti-inflammatory responses^[24-29].

There are some methodological aspects that should be taken into consideration when interpreting the results. (1) The ANP model was induced by intraductal retrograde injection of sodium taurocholate, which could closely mimic the etiology, pathogenesis and pathology of severe human pancreatitis, and is therefore the current standard and perfect modeling method with good reproducibility and comparability^[30,31]; (2) The first CTX injection was at 6 h after induction of ANP, because it represented early antibiotic therapy in the initial stage of the disease, and the protocols for CTX administration were adopted from previous studies in small rodents^[17], as well as from our clinical protocol; (3) Seventy-two hours after modeling was chosen as the measurement time-point when pancreatic necrosis was fully developed and when stable concentrations of CTX and CQCQD would be available in the blood.

In summary, the results of the present study validate the hypothesis that CQCQD increases pancreatic tissue concentration and penetration ratio of CTX in rats with ANP, through the possible mechanism of eliminating pancreatic inflammation and edema. Thus, the study might form the basis of advanced studies on the associated therapeutic mechanisms of Chinese medicine in ANP.

COMMENTS

Background

Infection in pancreatic necrosis is still regarded as the most serious complication of acute necrotizing pancreatitis (ANP), with a morbidity of 16%-47%, which has not substantially changed during the past two decades, despite of strenuous efforts and rapid progress in treatment. Early broad-spectrum antibiotics in the initial stage of the disease appear to decrease significantly mortality and improve outcome. Based on pharmacokinetic and bacteriological studies of the efficacy of different antibiotics, antibiotics used in patient with ANP must not only cover the spectrum of bacteria usually found in necrotic pancreatic tissue (including anaerobes), but also penetrate into the pancreas with a sustainable anti-bactericidal concentration.

Research frontiers

Pancreatic perfusion and microcirculation are decreased in ANP, therefore, intravenous antibiotics might not reach an effective anti-bactericidal concentration in the pancreas. Promoters of pancreatic perfusion and microcirculation might

enhance the therapeutic effects against infection by increasing antibiotic tissue concentrations.

Innovations and breakthroughs

The therapeutic mechanisms of Chinese medicines are characterized by multiple targets and complicated networks. The innovations and breakthroughs of the present study are the linkage between antibiotic tissue concentration and Chai-Qin-Cheng-Qi Decoction (CQCQD), which may explain the therapeutic mechanisms of Chinese medicine prescribed for ANP. The study methods and results might provide the basis for advanced studies in this field.

Terminology

Acute pancreatitis is an acute inflammatory process of the pancreas that frequently involves peripancreatic tissues, and at times, remote organ systems. Pancreatic necrosis is defined as one or more areas of nonviable pancreatic parenchyma, and is associated usually with peripancreatic fat necrosis.

Peer review

In this pilot study, the authors explored the effect of CQCQD on cefotaxime concentration in pancreatic tissue of rats with ANP, using validated high-performance liquid chromatography. The results indicated that CQCQD has a promotive effect on cefotaxime concentration in pancreatic tissues of rats with ANP. The study might elucidate the associated therapeutic mechanisms of Traditional Chinese Medicine in ANP.

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CASE REPORT

Preliminary experience of helical tomotherapy for locally advanced pancreatic cancer

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Received: May 21, 2009 Revised: August 13, 2009

Accepted: August 20, 2009

Published online: September 21, 2009

15(35): 4444-4445 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4444.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4444>

INTRODUCTION

Radiotherapy for locally advanced pancreatic cancer is technically difficult and potentially deleterious, because of the proximity of adjacent vital organs. Although intensity-modulated radiotherapy recently has proven its efficacy in sparing vital organs from irradiation, digestive toxicity remains an important limiting factor^[1]. Helical tomotherapy (HT) is a new irradiation modality that combines intensity-modulated fan-beam radiotherapy with megavoltage computed tomography imaging for patient positioning^[2]. Its availability recently has opened new fields of exploration for pancreatic radiotherapy as a result of its ability to tailor very well-defined dose distributions around the target areas.

To the best of our knowledge, this report is the first to explore the use of HT and its potential for sparing vital organs in two patients with locally advanced pancreatic cancer.

CASE REPORT

Patient 1 was a 59-year-old man whose pancreatic cancer was discovered fortuitously in September 2007 at a follow-up examination for previous prostatic cancer. Clinical examination was normal. Computed tomography (CT) showed a mass in the head of the pancreas, which measured 33 mm in the greatest dimension, with portal vein thrombosis. Endosonography demonstrated infiltration of the superior mesenteric vein, which reached the superior mesenteric artery. Histological examination confirmed an adenocarcinoma. Patient 2 was a 62-year-old woman who presented in April 2008 with abdominal pain and weight loss. There was no abnormality at clinical examination. The CT scan showed diffuse dilatation of the duct of Wirsung, with an irregular mass in the pancreatic isthmus. Endosonography revealed a mass in the isthmus, which measured 25 mm in the greatest dimension, with tumor extension to posterior peripancreatic fat tissue and massive splenic artery encasement. Histological examination confirmed a papillary mucinous adenocarcinoma. For both patients, laboratory tests

Abstract

Radiotherapy for locally advanced pancreatic cancer is technically difficult and frequently associated with high-grade digestive toxicity. Helical tomotherapy (HT) is a new irradiation modality that combines megavoltage computed tomography imaging for patient positioning with intensity-modulated fan-beam radiotherapy. Its recent availability opens new fields of exploration for pancreatic radiotherapy as a result of its ability to tailor very well-defined dose distributions around the target volumes. Here, we report the use of HT in two patients with locally advanced pancreatic cancer. Doses to the bowel, kidneys and liver were reduced significantly, which allowed for excellent treatment tolerance without any high-grade adverse effects in either patient.

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Key words: Pancreatic neoplasms; Helical tomotherapy; Adverse effects; Treatment tolerance

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Chargari C, Campana F, Beuzeboc P, Zefkili S, Kirova YM. Preliminary experience of helical tomotherapy for locally advanced pancreatic cancer. *World J Gastroenterol* 2009;

revealed no biological abnormality and no elevation of tumor markers. No lymph node or distant metastases were observed at initial staging.

The patients were treated with induction chemotherapy of gemcitabine (1000 mg/m² on day 1 every week) and oxaliplatin (100 mg/m² on day 1 every 2 wk). In patient 1, assessment after six cycles showed no local or metastatic evolution. In patient 2, assessment after five cycles showed partial regression of the pancreatic mass, which measured 18 mm in the greatest dimension, and no metastatic extent. Patient 1 and 2 were referred for preoperative and exclusive chemoradiation, respectively.

In patient 1, HT delivered 45 Gy to the whole pancreas, and 50.4 Gy to the tumoral mass, using 6-MV photons, at 1.8 Gy per daily fraction, for a total duration of 39 d. In patient 2, HT delivered 45 Gy to the whole pancreas, and 66 Gy to the tumoral mass, using 6-MV photons, at 2 Gy per daily fraction, for a total duration of 64 d. For both patients, capecitabine was given at 3000 mg/m² per day in two divided doses, 5 d/wk, concurrently with radiotherapy. Chemoradiation was marked by grade 1 nausea in both patients. No other acute toxicity was observed. For patient 1, abdominal pain disappeared after completion of chemoradiation. However, he developed early hepatic metastases and was given palliative chemotherapy with gemcitabine and oxaliplatin, followed by erlotinib for progressive disease. Patient 1 maintained a complete local response that was still present at 18 mo follow-up. Patient 2 remains disease-free at 6 mo follow-up.

DISCUSSION

Conventional radiotherapy for pancreatic cancer can involve some parts of the duodenum, stomach, small intestine, and kidneys, which results in frequent severe acute gastrointestinal toxicity when chemotherapeutic agents are administered concurrently with radiotherapy. This can lead to frequent treatment disruption and limits the delivery of a sufficient radiation dose. Moreover, high-grade toxicity compromises quality of life, which is an important endpoint in patients with poor prognosis^[3]. Since gemcitabine and oxaliplatin have improved the outcome of patients with locally advanced or metastatic disease, by improving survival with clinical benefits, every effort should be made to avoid additional radiation-induced digestive toxicity that might potentially compromise the delivery of subsequent chemotherapy^[4].

For both of our patients who were at risk for high-grade toxicity, HT provided a potential tool to decrease the risk of gastrointestinal toxicity^[5]. As a result of isodose conformation, radiation doses to the bowel, right kidney and liver were reduced significantly, which allowed for excellent treatment tolerance without any high-grade adverse effects in either patient. It is reasonable to

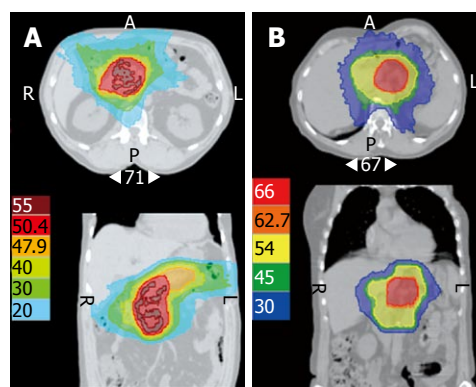


Figure 1 Distribution of isodoses with HT treatment planning in patient 1 (A) and patient 2 (B), in axial and coronal representation. The different doses are represented with different colors. Red color represents the target volume dose.

assume that HT may have spared structures that normally would not have been spared with more conventional 3D conformal radiotherapy (Figure 1). However, very low doses are distributed to larger volumes. While the problem of radiation-induced malignancy in such patients with poor prognosis should not be considered to be of primary importance, further assessment of HT remains necessary.

The present report highlights the potential of HT to reduce the dose of radiation delivered to vital organs, thus improving tolerance to irradiation.

ACKNOWLEDGMENTS

We thank TomoTherapy Europe for the sponsoring our color figures.

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S- Editor Tian L L- Editor Kerr C E- Editor Zheng XM

CASE REPORT

Free perforation of the small intestine in collagenous sprue

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Received: June 18, 2009 **Revised:** July 16, 2009

Accepted: July 23, 2009

Published online: September 21, 2009

Abstract

A 67-year-old man with celiac disease developed recurrent diarrhea, profound weakness and weight loss, with evidence of marked protein depletion. His clinical course was refractory to a strict gluten-free diet and steroid therapy. Postmortem studies led to definition of unrecognized collagenous sprue that caused ulceration and small intestinal perforation. Although PCR showed identical monoclonal T-cell populations in antemortem duodenal biopsies and postmortem jejunum, careful pathological evaluation demonstrated no frank lymphoma. Rarely, overt or even cryptic T-cell lymphoma may complicate collagenous sprue, however, small intestinal ulcers and perforation may also develop independently. The dramatic findings here may reflect an underlying or early molecular event in the eventual clinical appearance of overt T-cell lymphoma.

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Key words: Collagenous sprue; Intestinal perforation; Celiac disease; Refractory sprue; Intestinal neoplasms; Lymphoma

Peer reviewer: Dr. Daniel R Gaya, Gastrointestinal Unit, Molecular Medicine Centre, School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom

Freeman HJ, Webber DL. Free perforation of the small intestine in collagenous sprue. *World J Gastroenterol* 2009; 15(35): 4446-4448
Available from: URL: <http://www.wjgnet.com/1007-9327/15/4446.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4446>

INTRODUCTION

Collagenous sprue was initially observed to complicate the clinical course of celiac disease^[1]. The disorder is characterized by a pathologically distinctive lesion with the appearance of a patchy or diffusely thickened sub-epithelial collagen band in flattened small intestine. Most patients with extensive collagenous sprue have a progressive and deteriorating clinical disorder marked by severe pan-malabsorption of multiple nutrients, profound weight loss, and evidence of marked protein loss that fails to respond to a gluten-free diet^[2]. Etiology and pathogenesis of collagenous sprue remain obscure, although inherited and other factors may play a role^[2]. A number of treatment regimens, including steroids, have been reported anecdotally to show benefit^[3,4].

Although only limited information on the natural history, pathogenesis and clinical outcome of collagenous mucosal disorders of the small and large intestine are available, occasionally, lymphoma may complicate collagenous sprue^[5-8] and collagenous colitis^[9]. In some of these, the lymphoma was already extensive and advanced^[5-7], but in another, synchronous development of collagenous sprue and lymphoma occurred, which suggested to the authors that collagenous sprue may represent a noninvasive component of the lymphomatous process^[8]. Collagenous enterocolitis has also been noted in a patient with localized colon cancer, with complete reversion to normal following resection of the malignant lesion^[10]. It was hypothesized that intestinal sub-epithelial collagen deposition with concomitant malignancy represents a paraneoplastic morphologic marker that may be completely reversible^[10].

CASE REPORT

In November 2006, a 67-year-old man was evaluated initially in his community hospital for progressive fatigue, diarrhea and weight loss of 15 kg over 6-8 mo. Physical examination revealed bilateral lower limb edema. Initial laboratory studies were normal except for hypoalbuminemia (serum albumin, 13 g/L; normal, 35-50) and elevated tissue transglutaminase (tTG) antibody of 35 U (normal, < 20). Endoscopy of his upper and lower gastrointestinal tract appeared normal, but duodenal biopsies showed typical changes of untreated celiac disease, with crypt hyperplastic villous atrophy. A gluten-free diet was started but his

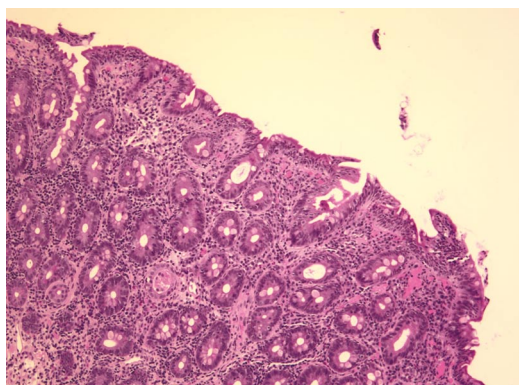


Figure 1 Antemortem biopsy from duodenum showed features of untreated celiac disease, despite a gluten-free diet. Although later postmortem PCR of this biopsy documented a monoclonal population of T cells, frank lymphoma was not detected.

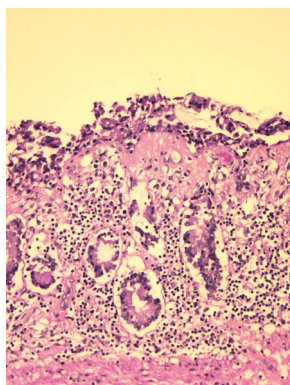


Figure 2 Postmortem small bowel section showed diffuse mucosal involvement with collagenous sprue. Note eosin-stained sub-epithelial band. Trichrome staining was also positive (HE, $\times 20$).

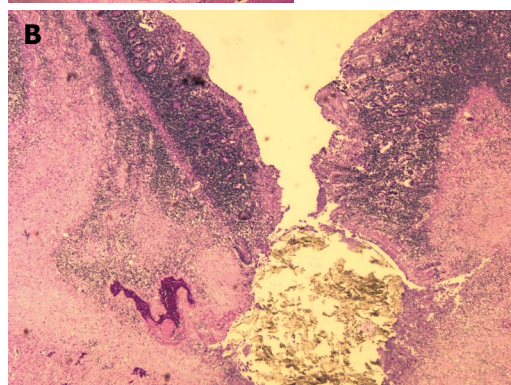
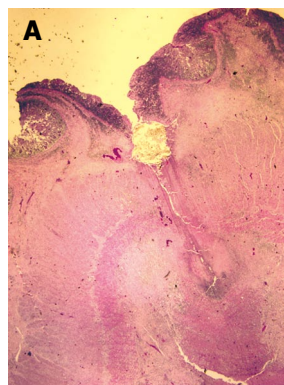


Figure 3 Autopsy showed ileum with a focal jejunal ulcer and perforation. A: Low-power photomicrograph showed jejunal ulceration with perforation in an area of collagenous sprue in postmortem material; B: High-power photomicrograph showed perforated collagenous sprue.

clinical course became complicated by progressive dyspnea that required hospital re-admission. Computed tomography (CT) scanning showed bilateral pulmonary emboli and pleural effusions with prominent mesenteric lymph nodes. He was started on anticoagulation and was maintained on coumadin for the next year. By February 2007, his fatigue resolved and exercise tolerance improved. His diarrhea resolved completely and his weight increased and normalized. Peripheral edema resolved and his serum albumin increased to 35 g/L. A repeat CT scan showed resolution of chest findings. Mesenteric lymph nodes were not enlarged.

In November 2007, diarrhea and weight loss recurred in spite of strict adherence to a gluten-free diet. Over the next month, recurrent edema also developed in his lower extremities up to his waist. Physical examination now revealed generalized wasting, brawny induration with hyperpigmentation of his lower extremities, bilateral ichthyosis, especially over the anterior tibial surfaces, and pitting edema. Laboratory investigations, including hemogram, were normal except for hypoproteinemia (35 g/L; normal, 62–82 g/L) and hypoalbuminemia (11 g/L; normal, 34–50 g/L). Serum carotene was reduced to $< 0.5 \mu\text{mol/L}$ (normal, 1.1–3.7 $\mu\text{mol/L}$), while serum tTG assay was normal (9.1 U), with a normal serum IgA (1.12 g/L; normal, 0.70–4.00 g/L). Endoscopy was normal except for scalloping of proximal duodenal mucosal folds. Biopsies showed crypt hyperplastic villous atrophy,

despite a gluten-free diet (Figure 1). Fresh biopsy material was submitted for PCR, but analysis failed. Endoscopic biopsies of stomach and colon were normal with no intra-epithelial lymphocytosis. A barium study of the small intestine showed dilation of small bowel loops but no discrete lesion, abnormal loop separation or stricture. Gluten-free diet was continued but his weight fell further by 5 kg. A contrast CT scan of the chest, abdomen and pelvis showed no new findings, although a left common femoral vein thrombus was seen. Mesenteric lymph nodes were normal. Oral budesonide 3 mg/d was prescribed^[11].

He deteriorated suddenly over 2 d with abdominal pain, worsening weakness and continued weight loss. He was readmitted to his community hospital and parenteral nutritional support was initiated. In hospital, he deteriorated rapidly with fluctuations in consciousness and died 3 d later. Autopsy showed extensive collagenous sprue (Figure 2) in the jejunum and ileum with a focal jejunal ulcer and perforation (Figure 3). Careful review failed to show lymphoma. Immunohistochemical studies (BCCA Lymphoma Referral Laboratory, Vancouver, BC, Canada) showed CD3-positive T-cells that expressed CD2 and CD7, but failed to express CD4, CD8 and CD5. CD3-positive T cells showed intracytoplasmic rather than membrane labeling. Immunostaining for CD56 was negative and cells were negative for Epstein-Barr virus by an EBER ISH stain. Stains for cytotoxic markers TIA-1, perforin and granzyme B showed that the majority of cells were negative. PCR of the previous antemortem duodenal

biopsies, along with a postmortem section of jejunum, showed an identical monoclonal T-cell band.

DISCUSSION

This report documents collagenous sprue in a patient with recurrent diarrhea and profound weight loss, with severe protein depletion despite a gluten-free diet and steroid therapy. Collagenous sprue appeared to complicate preexistent celiac disease that initially had responded clinically to administration of a gluten-free diet, similar to the original description of this disorder^[1]. Here, however, symptoms redeveloped despite a strict gluten-free diet and oral budesonide^[11]. This appeared to be related to collagenous sprue complicated by ulceration and perforation of the distal small bowel. Even though an identical monoclonal T-cell population was defined molecularly by PCR in antemortem duodenal biopsies and postmortem jejunal sections, careful pathological review failed to reveal any neoplastic lymphoid cells. It is likely that this very unusual complication of ulceration and perforation of the small intestine reflects a stage of development of cryptic T-cell lymphoma^[5], which may precede the appearance of frank pathologically defined lymphoma. Rarely, ulceration and free perforation of the small intestine may complicate celiac disease as the initial manifestation^[12]. However, the sequence of events here in collagenous sprue with ulceration and perforation has not been described previously and provides an additional dimension to our understanding of the natural history and clinical outcome of this very unusual small intestinal disorder.

While development of small intestinal ulceration and perforation clearly led to our patient's demise, it may not be an entirely surprising event in the clinical course of collagenous sprue. A number of factors may play a role. First, mucosal thickness in collagenous sprue appears to be reduced markedly so that complication of a focal ulcer with free perforation might more readily occur. Second, lymphoma has been noted previously to be responsible for free perforation of the small intestine in celiac disease^[12], and a superimposed lymphoma in collagenous sprue could occur that might present with free perforation. Finally, the collagenized layer in the small intestine may be more susceptible or more prone to perforation. Indeed, in collagenous colitis, free and spontaneous perforation of the colon has been described^[13], along with a propensity for instrument-induced colon fractures and perforation^[14]. Since there is evidence that the collagen deposited in the small and large bowel in these disorders is similar^[2], and has been hypothesized to affect the integrity of the intestinal wall^[14], this outcome with ulceration and

perforation of the small bowel during the clinical course of collagenous sprue may occur more frequently than is appreciated at present.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Louise K Coulter, Department of Pathology, Royal Columbian Hospital, New Westminster, BC, Canada, and Dr. David Good, Lymphoma Referral Laboratory, BC Cancer Agency, Vancouver, BC, Canada for their assistance in some postmortem studies.

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S- Editor Tian L L- Editor Kerr C E- Editor Ma WH



Stevens-Johnson syndrome complicating adalimumab therapy in Crohn's disease

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Received: December 26, 2008 Revised: August 25, 2009

Accepted: September 1, 2009

Published online: September 21, 2009

Abstract

The anti-tumor necrosis factor (TNF) α medications demonstrate efficacy in the induction of remission and its maintenance in numerous chronic inflammatory conditions. With the increasing number of patients receiving anti-TNF α agents, however, less common adverse reactions will occur. Cutaneous eruptions complicating treatment with an anti-TNF α agent are not uncommon, occurring in around 20% of patients. Adalimumab, a fully humanized antibody against TNF α , may be expected to cause minimal immune-mediated skin reactions compared to the chimeric monoclonal antibody, infliximab. We, however, report a case of Stevens-Johnson syndrome that required hospitalization and cessation of adalimumab in a patient with Crohn's disease (CD). In this case report, a 29-year-old male with colonic and perianal CD with associated erythema nodosum and large joint arthropathy developed severe mucositis, peripheral rash and desquamation, fevers and respiratory symptoms concomitant with a second dose of 40 mg adalimumab after a 2 mo break from adalimumab therapy. Skin biopsies of the abdominal wall confirmed erythema multiforme and the patient was on no other drugs and infective etiologies were excluded. The patient responded rapidly to IV hydrocortisone and was able to be commenced on infliximab without recurrence of the Stevens-Johnson syndrome. Desquamating skin reactions have now been described in three of the TNF α antagonists

(infliximab, etanercept and adalimumab). These reactions can be serious and prescribers need to be aware of the potential mucocutaneous side effects of these agents, especially as Stevens-Johnson syndrome is associated with significant morbidity and mortality.

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Key words: Stevens-Johnson syndrome; Crohn's disease; Adalimumab; Serious adverse effect

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Salama M, Lawrance IC. Stevens-Johnson syndrome complicating adalimumab therapy in Crohn's disease. *World J Gastroenterol* 2009; 15(35): 4449-4452 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4449.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4449>

INTRODUCTION

Managing chronic diseases is one of the greatest challenges for medicine and the community today as poor management results in increased resource utilization and financial costs. The anti-tumor necrosis factor (TNF) α medications, infliximab and adalimumab have demonstrated efficacy in the induction of remission, and the long-term maintenance of remission, in numerous chronic inflammatory conditions including rheumatoid arthritis (RA)^[1], ankylosing spondylitis^[2], psoriatic arthritis and severe chronic psoriasis^[3,4]. The use of biological anti-TNF α medications in the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) is also well accepted^[5-9]. The IBDs are life-long, chronic, conditions and many patients suffer severe attacks that require hospitalization. There are many drug combinations that can be used to manage these patients, but many patients are either resistant or intolerant to the non-biological therapies available. In view of the effectiveness of anti-TNF α medications, and the long-term nature of the diseases they treat, the number of patients receiving these

medications continues to increase.

As a fully humanized antibody against TNF α , adalimumab may potentially cause less immune-mediated skin reactions in comparison to infliximab. The risk, however, is still present and we report here the first case in the literature of the immune-mediated skin reaction Stevens-Johnson syndrome (SJS), that can be attributed to the use of adalimumab.

CASE REPORT

We report here a 29-year-old male diagnosed in 2005 with ileocolonic and fistulising perianal CD associated with the extra-intestinal complications of erythema nodosum, pustular psoriasis and large joint arthropathy. In August 2007, despite the use of azathioprine (AZA) 125 mg/d, antibiotics and 5-aminosalicylic acids, he had a Crohn's Disease Activity Index (CDAI) of greater than 300, indicating moderately severe disease activity. Endoscopy confirmed severe ulcerating inflammation of the colon and the terminal ileum. In September 2007, he was commenced on routine induction therapy with adalimumab, 160 mg at week 0, 80 mg week 2 followed by 40 mg every other week (eow) administered subcutaneously (sc). Tuberculosis had been excluded by Quantiferon gold testing and chest X-ray, while his hepatitis B and C serology were confirmed to be negative prior to commencing adalimumab treatment. Complete resolution of his colitis and healing of his perianal fistulae occurred by 12 wk. He was continued on maintenance adalimumab therapy at 40 mg (sc eow) and AZA.

In December 2007, 16 wk after commencing the adalimumab therapy, he was admitted to a district hospital for suspected cellulitis of his left leg and treated by a general physician with intravenous antibiotics. His adalimumab was withheld and AZA ceased due to concerns about infection. After 10 d on antibiotic therapy he developed a severe mucositis, peripheral rash and fever. He was transferred to the Centre for Inflammatory Bowel Diseases, Fremantle Hospital, which is a specialist IBD unit in a tertiary institution that services the southern metropolitan region of Perth, Australia. At that time his C-reactive protein was 151 mg/L (normal < 10 mg/L), Hb 90 g/L (normal 135-180 g/L), platelet $521 \times 10^9/L$ (normal $150-400 \times 10^9/L$) and WBC $7.9 \times 10^9/L$ (normal $4.00-11.00 \times 10^9/L$). His blood cultures were clear as was his CXR. The suspected cellulitis was diagnosed as erythema nodosum and the mucositis, peripheral rash and fever were considered to be an adverse reaction to the antibiotic combination he had received. He was commenced on prednisone for the drug reaction and his severe mucositis slowly improved while the erythema nodosum resolved over the following months on a weaning dose of oral prednisolone. Due to patient concerns that his symptoms may have been exacerbated by the adalimumab, this was not recommenced at that time.

In March 2008, due to the recommencement of draining from the perianal fistulae, symptoms consistent

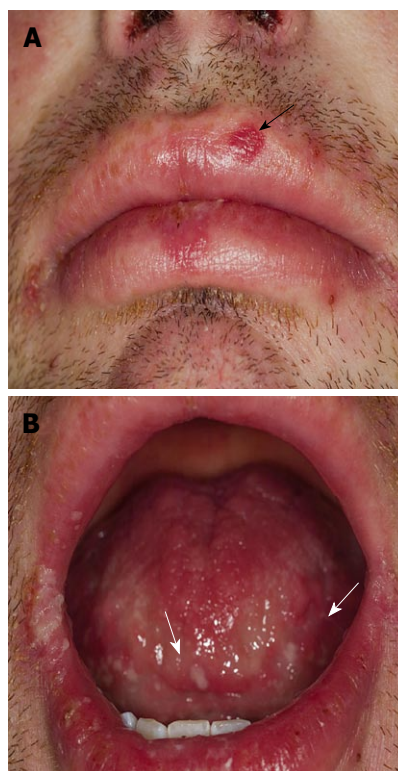


Figure 1 Mucosal lesions. A: Lip involvement with target-lesion on left upper lip progressing to a bullae (black arrow); B: Tongue desquamation demonstrated as white plaques (white arrows).

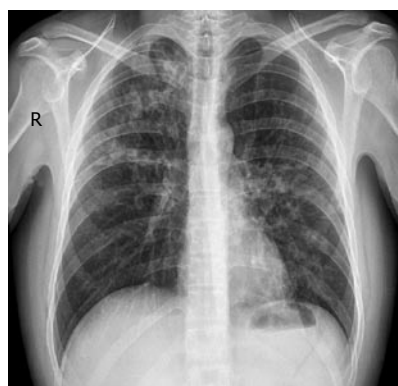


Figure 2 Chest X-Ray. Patchy bilateral alveolar infiltrates consistent with bronchial pneumonia.

with flaring of the colonic inflammation and reactivation of the erythema nodosum, the patient was recommenced on 40 mg adalimumab eow. A week after the first dose the patient felt that his abdominal symptoms had resolved quite significantly, the erythema nodosum had improved and there was no pain and minimal discharge from the perianal fistula. A day after receiving the second dose of 40 mg adalimumab, however, the patient became systemically unwell with fever. He developed a severe mucositis (Figure 1) and a peripheral rash with desquamation on his abdomen. He also suffered reactivation of the erythema nodosum on his legs that was confirmed to be erythema nodosum on skin biopsy.

The patient was also dyspnoeic and on chest X-ray patchy opacification was observed of both mid and upper

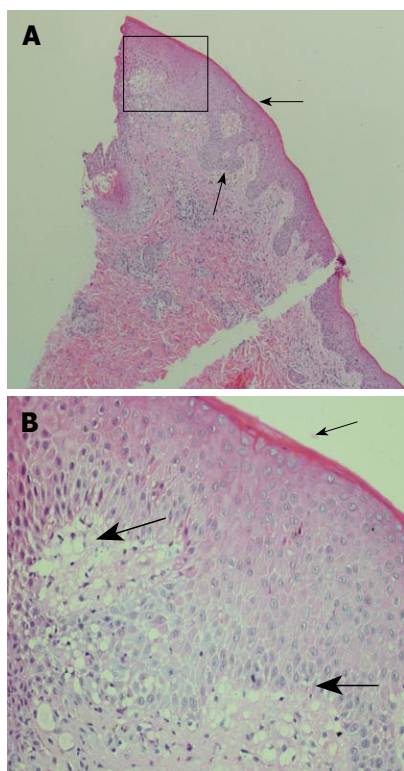


Figure 3 Histology of skin biopsies. The epidermis shows mild irregular acanthosis with a small amount of overlying compact keratin (thin arrow). There is superficial dermal oedema associated with vacuolar change of the basal layer of the epidermis (thick arrow). Within the dermis is a mixed inflammatory cell infiltrate. The findings are consistent with erythema multiforme. A: 25 × magnification; B: Insert at 100 × magnification.

zones consistent with bronchopneumonia (Figure 2). CT scan of the thorax confirmed widespread bronchopneumonia tending to confluence in the right upper lobe. The patient was taking no other medications concurrent with the adalimumab and the adalimumab was ceased. Blood and sputum culture were negative and bacterial aetiologies and other infective causes were excluded including tuberculosis, mycoplasma pneumoniae, viral infections (HSV 1 and 2, CMV, EBV, HIV, HBV, and HCV) and rickettsia. His antinuclear antibody (ANA) remained negative throughout. Skin biopsies taken from the anterior abdominal wall rash were consistent with erythema multiforme (Figure 3). Due to the combination of mucositis membrane erosions, target lesions and epidermal necrosis with skin detachment consistent with erythema multiforme, a diagnosis of SJS was made. The chest findings also supported this diagnosis, as pulmonary infiltrates are present in 1/3 of patients with SJS. Oral doxycycline was commenced in combination with intravenous hydrocortisone with a rapid improvement in his systemic symptoms and resolution of his fever and chest X-ray infiltrates. He was transferred onto oral prednisone and his mucocutaneous symptoms resolved over the course of 3-4 wk. Due to continuing symptoms from his perianal and colonic CD, the patient was commenced on an induction regime of infliximab therapy with resolution of his CD symptoms and without reactivation of the SJS.

DISCUSSION

Cutaneous reactions in patients treated with TNF α antagonists are relatively common, but make up a heterogeneous group. Recently the rate of new onset cutaneous eruptions in patients treated with infliximab for CD has been documented at 20%^[10], and in a prospectively analysed series of RA patients treated with TNF α antagonists, 35/150 (23.3%) of patients developed cutaneous reactions. Eczema and psoriasis made up the majority of these (45.7%), with infections the cause of over a third (37.1%)^[11]. There have been 3 reported cases of an interface dermatitis, with histological characteristics suggesting erythema multiforme, in patients treated with infliximab for RA. One of these subjects also developed a similar cutaneous reaction, confirmed on histology, after treatment with etanercept^[12].

With an increasing number of patients receiving an anti-TNF α agent less common adverse reactions are bound to occur, and the physician must be aware of these potential problems. There is a spectrum of severe cutaneous adverse drug reactions that may represent variants of the same disease process, and include SJS and toxic epidermal necrolysis (TEN) that are on a spectrum of disease severity. They are both characterised by varying degrees of disruption of the dermoepidermal junction. Clinically, each presents with a pattern characterised by the triad of mucous membrane erosions, target lesions and epidermal necrosis with skin detachment. A mortality rate of between 1%-5% is associated with SJS and TEN may be as high as 40%^[13]. Erythema multiforme, erythema multiforme major and atypical erythema multiforme major, are cutaneous reactions that do not involve the mucous membranes, and are primarily observed post-infection, with the majority secondary to the herpes simplex virus, rather than as a drug reaction. The distinction, however, is often difficult to make and drugs are still implicated in up to 50% of cases of erythema multiforme.

Apart from the case reported here, there has been only 1 case in the literature of adalimumab-implicated desquamating skin reactions. This occurred in a 63-year old woman with RA after her sixth injection of adalimumab. This patient developed a papulopustular rash on the palms and soles of her feet followed by desquamation. The patient remained systemically well despite the cutaneous reaction and as there was no mucous membrane involvement she was diagnosed as having erythema multiforme, but no skin biopsy was performed. Therapy with adalimumab was discontinued and the symptoms rapidly improved without specific therapy^[14]. Our case, however, was a more severe reaction with mucous membrane, skin and lung involvement that were histologically and clinically consistent with SJS. This is of particular significance due to the potentially life threatening nature of SJS. The patient was only taking one medication at the time of his second presentation and the SJS resolved on cessation of the adalimumab and treatment with hydrocortisone.

The SJS can, thus, with some confidence be attributed to the use of the TNF α antagonist, adalimumab.

Desquamating skin reactions have now been described in 3 of the TNF α antagonists (infliximab, etanercept and adalimumab). Some of these reactions can be very serious and prescribers need to be aware of the potential for the mucocutaneous adverse effects from the use of these agents, particularly due to the significant morbidity and mortality that are associated with SJS and TEN.

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S- Editor Tian L L- Editor O'Neill M E- Editor Yin DH

Hairy cell leukemia presenting as multiple discrete hepatic lesions

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Received: July 24, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: September 21, 2009

Hairy cell leukemia presenting as multiple discrete hepatic lesions. *World J Gastroenterol* 2009; 15(35): 4453-4456 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4453.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4453>

INTRODUCTION

Metastases of an unknown primary site are found in about 5% of all cancer patients^[1-3]. The recent advances in molecular technology, including gene expression profiling, raise the expectation for improved diagnosis of cancer of unknown primary (CUP). This is needed to identify subsets of patients who could potentially respond to therapy. The liver is involved in about a third of patients with CUP^[3]. In about 50% of these patients, the primary tumor is carcinoma of the lung, colon, rectum, or pancreas. Other primary tumor sites are the liver, breast, skin (melanoma), stomach *etc.* Here we describe a patient with discrete hepatic masses which proved to be hairy cell leukemia. This is in contrast to the regular liver involvement by hairy cell leukemia, which is in the form of diffuse infiltration. Since treatment of this tumor is very effective, its diagnosis should not be missed.

Abstract

The involvement of hairy cell leukemia in the liver is in the form of portal and sinusoidal cellular infiltration. Here we describe the first case of hepatic hairy cell leukemia presenting as multiple discrete lesions, which was treated successfully. We suggest that in the investigation of discrete hepatic lesions in cases of cancer of unknown primary, hairy cell leukemia should be considered. The excellent response of hairy cell leukemia to therapy highlights the need for such a consideration.

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Key words: Cancer of unknown primary; Hairy cell leukemia; Hepatic lesions

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CASE REPORT

A 61-year-old male patient presented to the outpatient clinic in 2005 with complaints of paresthesia in his hands. Abdominal imaging studies, which he underwent previously, detected focal liver lesions. In 1996, an abdominal ultrasound carried out for epigastric pain, revealed two focal hepatic lesions presumed to be hemangiomas. This was verified by a red blood cell (RBC) scan. In 2000, an abdominal computed tomography (CT) study showed ten hepatic lesions of different sizes, however, no further investigation was performed. In 2003, the patient had an inferior wall myocardial infarction. His blood count showed a hemoglobin level of 132 g/L, white blood cell count of $6.7 \times 10^9/L$ and a platelet count of $188 \times 10^9/L$. In 2005, a routine blood count showed hemoglobin of 111 g/L. A repeat ultrasound (US) detected multiple hepatic lesions, and an abdominal CT scan showed that the hepatic lesions had grown since the previous (2000) study. Another RBC scan

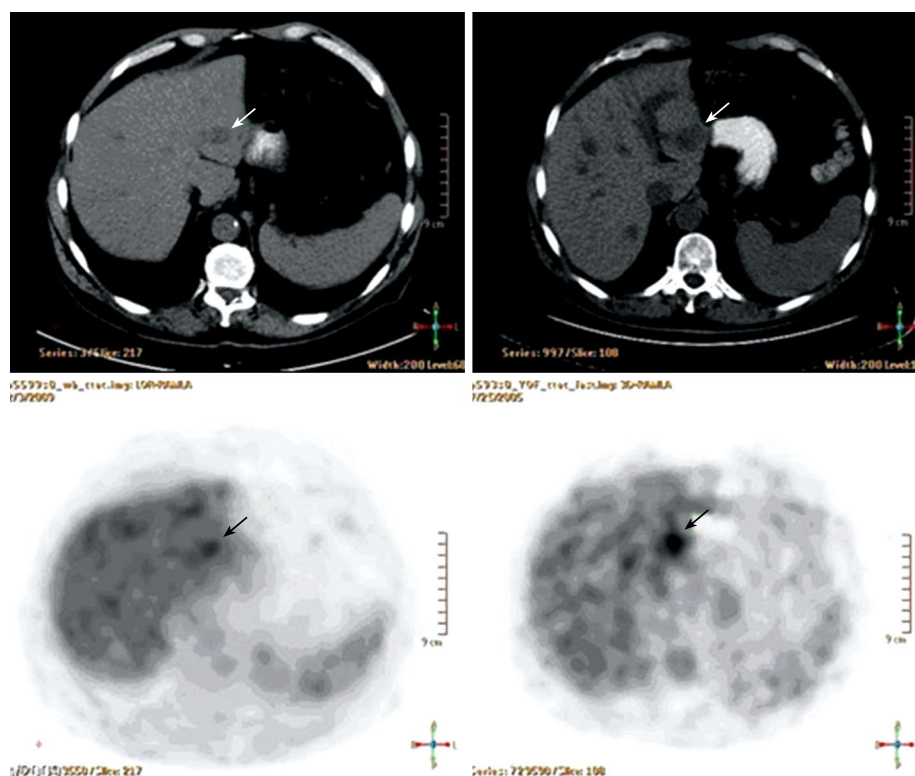


Figure 1 Axial fused PET-CT scans of the upper abdomen before (Right-2005) and after (Left-2009) treatment. Right- An FDG-avid lesion is seen in the left lobe of the liver (arrow). Left-The lesion is smaller with decreased intensity of FDG uptake (arrow).

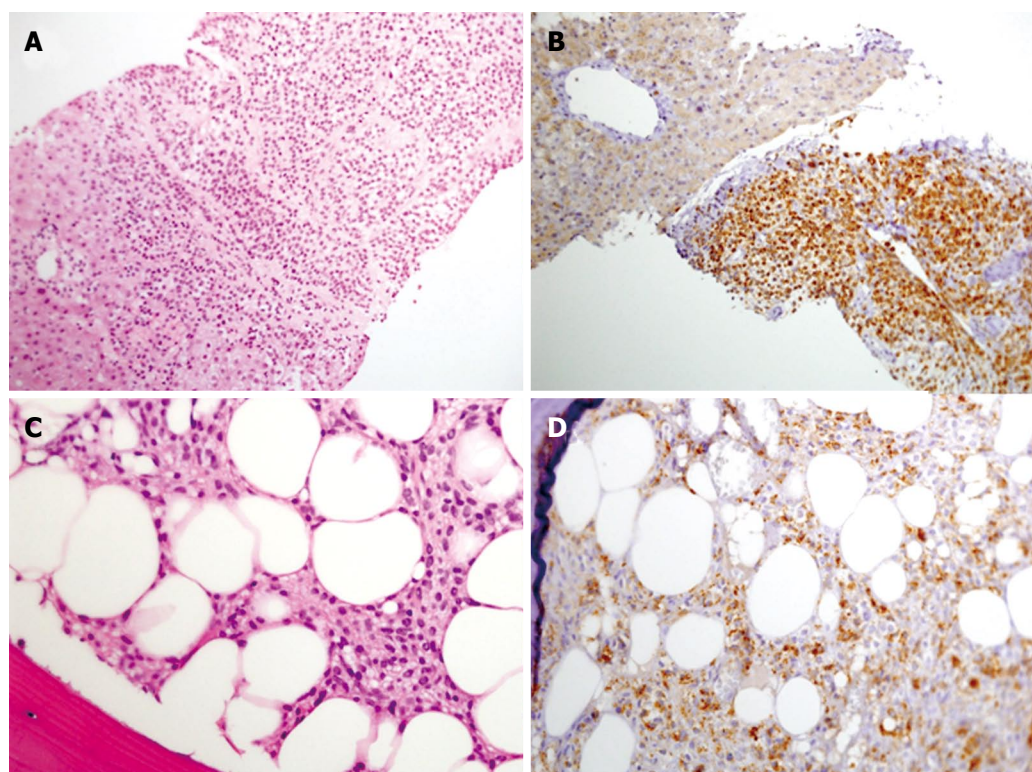


Figure 2 Histological findings. A, B: Liver core needle biopsy; C, D: Bone marrow biopsy. A: Infiltration by small to medium-sized tumor cells is seen. In the left lower part of the picture, normal residual liver parenchyma is seen (HE, $\times 200$); B: Immunohistochemical stain for TRAcP is positive in tumor cells ($\times 200$); C: Hairy leukemic cells are seen in the bone marrow (HE, $\times 400$); D: The immunohistochemical stain for TRAcP is positive in hairy cells.

again showed only two hemangiomas. Finally, a positron emission CT (PET-CT) scan showed uptake of fluorodeoxyglucose (FDG) in several hepatic lesions (Figure 1B), and a decision was made to biopsy one of the lesions. On histopathological examination, the cells of the lesion were different from the known primary and metastatic tumors of the liver, and all the immunohistochemical stains performed were negative. A neuroendocrine tumor was considered the most

likely morphological diagnosis, despite negative staining for the neuroendocrine antigens, synaptophysin and chromogranin A (Figure 2A).

The patient was referred to our clinic for further evaluation. He denied any weight loss, diarrhea or flushing. On physical examination he appeared well. No lymphadenopathy was noted, his abdomen was not tender, and no signs of an enlarged liver or spleen were found. His laboratory results showed pancytopenia

with a hemoglobin level of 110 g/L, white blood cells of 3.5×10^9 /L, and a platelet count of 120×10^9 /L. All liver function tests were normal, albumin level was 40 g/L. Blood levels of adreno-cortico-trophic hormone (ACTH) and beta-human chorionic gonadotropin (hCG) were normal, urine levels of vanilmandelic acid (VMA) and 5-hydroxyindoleacetic acid (5HIAA) were also in the normal range. An octreotide scan showed increased uptake in the liver. A bone marrow biopsy was performed, which was compatible with hairy cell leukemia with positive stains for CD-20 and Tartaric Acid Resistant Acid Phosphatase (TRAcP) (Figure 2C and D). A repeat examination of the hepatic specimen showed positive staining for CD-20 and TRAcP (Figure 2B).

The patient was started on cladribine (2-chlorodeoxyadenosine-2CDA) with a diagnosis of hairy cell leukemia with hepatic involvement. No side effects were noted. Three months after treatment, a gradual rise in his blood count was seen. A follow-up CT scan showed that the hepatic lesions had not changed in size. His paresthesia had resolved.

Two years after treatment with 2CDA his blood counts had increased, his hemoglobin level was 148 g/L, white blood cell count was 6.2×10^9 /L, and platelet count was 189×10^9 /L. Repeat CT and an abdominal magnetic resonance imaging (MRI) scan showed that the lesions were similar in size to those shown on the previous CT. A repeat PET-CT scan about three years after treatment demonstrated that only one lesion in his left hepatic lobe still showed uptake of FDG, no uptake was observed in the other lesions (Figure 1A).

PET-CT and pathological findings

PET-CT scan with F^{18} -FDG: The first PET-CT scan of 2005 (Figure 1B), shows multiple hypodense lesions of different sizes in the liver; some of the lesions show increased uptake of F^{18} -FDG. The post-treatment PET-CT scan of 2009 (Figure 1A), shows significant improvement. All the previously hypermetabolic lesions are smaller, and all foci of increased uptake, with the exception of one, disappeared. This latter lesion, although reduced in size, still shows increased F^{18} -FDG uptake but with lower intensity. These dynamic changes are consistent with involvement of the underlying disease. According to CT from the PET-CT scan of 2009, the lesions which did not show increased F^{18} -FDG uptake either in 2005 or in 2009 did not change in size. This suggests that they are of a different nature i.e. hemangioma. This was proven by a labeled red blood cell nuclide scan.

Pathological findings: The submitted liver core needle biopsy was fixed in formalin (neutral 10%, pH 7.4) and embedded in paraffin wax. Sections 4 microns thick were cut and stained with hematoxylin and eosin (HE). The histological sections showed liver tissue infiltrated by diffuse aggregates of small/medium-sized uniform tumor cells, with bland oval nuclei, clear cytoplasm with no well defined cytoplasmic borders. No cellular atypia or mitotic figures were demonstrated (Figure 2A). For immunophenotyping, we used the standard avidin-

biotin method on the paraffin sections. The slides were immunostained in the automated system ES Ventana (Ventana Medical Systems, Inc). Immunohistochemical staining showed that the cells were positive for vimentin (V9, ZYMED Laboratories, South San Francisco, CA, USA) and negative for α -smooth muscle actin/SMA (1A4, DAKO, Glostrup, Denmark), desmin (ZC 18, ZYMED Laboratories) (muscle origin), S-100 (S-100, DAKO, Glostrup, Denmark), chromogranin (Chromogranin A, DAKO, Glostrup, Denmark), synaptophysin (Synaptophysin, Z66, ZYMED Laboratories) and CD56 (T199, DAKO, Glostrup, Denmark) (neuroendocrine origin), MNF116 (MNF116, DAKO, Glostrup, Denmark), CAM 5.2 (Zym 5.2, ZYMED Laboratories), CK7 (OTVL, BioGenex, San Ramon, CA, USA), CK20 (IT-Ks20.8, BioGenex, San Ramon, CA, USA), CEA (ZC23, ZYMED Laboratories) (epithelial origin), CD31 (JC70A, DAKO, Glostrup, Denmark), CD34 (QBEnd/10, CellMarque, Rocklin, CA, USA), (vascular origin), and α -fetoprotein (α -1 DAKO, Glostrup, Denmark), and HEPA (OCH1ES, DAKO, Glostrup, Denmark), (liver origin). The Ki67 proliferation antigen was positive in about 10% of cells. Despite the fact that the tumor cells were negative for almost all the basic non-hematological immunostains, it was decided that the morphological differential diagnosis was either a neuroendocrine tumor or a glomus tumor. One month later a bone marrow trephine biopsy was performed. The bone marrow was infiltrated by sheets of tumor cells with morphological features of hairy cell leukemia (Figure 2C). The immunohistochemical stains for CD20 (L26 DAKO, Glostrup, Denmark), and TRAcP (ZY-9C5, ZYMED Laboratories), were positive and confirmed the diagnosis of hairy cell leukemia (Figure 2D). In a revision of the liver biopsy, it became clear that the tumor cells were morphologically similar to the hairy cells in the bone marrow. The immunohistochemical stains for CD20 and TRAcP were also positive and confirmed the diagnosis of hairy cell leukemia of the liver presenting as a tumoral mass (Figure 2B).

DISCUSSION

The pathological diagnosis of cancer of unknown primary requires a careful evaluation of the morphology as well as the immunohistochemistry of the affected tissue^[4]. Discrete hepatic lesions formed by infiltration of hairy cell leukemia cells were not described until last year. Hairy cell leukemia (HCL) is a rare chronic lymphoproliferative disorder usually characterized by an indolent course. The hairy cells are B cells found in the blood circulation that consist of oval nuclei and abundant cytoplasm with characteristic micro-filamentous ("hairy") projections. Patients usually present with cytopenia and splenomegaly due to infiltration of the cells into the bone marrow and spleen^[5].

Hepatomegaly can be seen in up to a third of patients^[6]. Histologically, liver involvement has been described in the form of portal and sinusoidal infiltration. Some lesions in the liver have been described as angiomatous with tumor cells and blood cells filling the sinusoids^[7].

Clinical features of liver involvement may include in addition to hepatomegaly, jaundice and increased levels of aminotransferases and alkaline phosphatase^[7]. The mechanism of production of nodular hepatic lesions in some cases of hairy cell leukemia is unknown. It is also unclear whether there is a difference in the course and prognosis of the disease in subjects with hairy cell leukemia and hepatic diffuse *vs* nodular involvement.

The introduction of purine-nucleoside analogues such as cladribine for the treatment of hairy cell leukemia has dramatically changed the prognosis in affected subjects^[8]. In a recent long-term follow-up study of 233 patients, Else *et al*^[9] found that overall, the complete response rate was 80%, and median relapse-free survival was 16 years. The outcome of patients with recurrent disease has improved with the monoclonal antibody anti-CD20, rituximab^[9,10].

Recently, Al-Za'abi *et al*^[11] have reported a case of HCL presenting as a solitary liver mass 20 years after the initial presentation with splenic involvement. These clinical features enabled correct diagnosis of the hepatic mass. No details regarding the treatment and course of the patient's disease were provided. To the best of our knowledge, our case is the first of a patient with HCL presenting with multiple discrete hepatic lesions. These lesions were present prior to the appearance of pancytopenia, which is the consequence of extensive bone marrow involvement. The patient responded to standard treatment, as seen by the disappearance of pancytopenia, and by the marked decrease in FDG uptake by the hepatic lesions.

This report highlights the need to consider the possibility of hairy cell leukemia in the investigation of discrete hepatic lesions in cases of cancer of unknown primary. The excellent response to therapy of this tumor further emphasizes the need for correct diagnosis.

ACKNOWLEDGMENTS

We thank Semyon Melnikov for his technical help. Z.F. holds the Dr. Boris (Dov) Quartin Chair in Chemical Pathology at Tel Aviv University.

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S- Editor Tian L L- Editor Webster JR E- Editor Ma WH



Muscle hematoma: A critically important complication of alcoholic liver cirrhosis

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Received: July 9, 2009 Revised: August 7, 2009

Accepted: August 14, 2009

Published online: September 21, 2009

closely related to alcoholism, and the mortality rate of the condition is extremely high. In conclusion, muscle hematoma should be recognized as an important complication of cirrhosis.

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Key words: Alcohol; Bilateral; Cirrhosis; Iliopsoas; Muscle hematoma

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Abstract

An iliopsoas hematoma can occur either spontaneously or secondary to trauma or bleeding tendency due to hemophilia and anticoagulant therapy. Although liver cirrhosis is commonly associated with coagulopathy, iliopsoas hematoma is very rare. We herein, present a case of bilateral iliopsoas hematoma in a patient with alcoholic cirrhosis, and review the literature on muscle hematoma associated with cirrhosis. A 56-year-old man with alcoholic cirrhosis was admitted in a state of shock with anemia. The cause of anemia could not be detected, and the patient was treated conservatively. The site of bleeding was not detected with either gastroduodenal endoscopy or upper abdominal computed tomography, the latter of which did not include the iliopsoas muscle. He died on the 10th day of admission and bilateral iliopsoas hematomas were found on autopsy. An iron stain was positive in the iliopsoas muscle. Eight cases of muscle hematoma associated with cirrhosis, including the present case, were found in a review of the literature. Four of these cases involved the rectus abdominis muscle, 3 involved the iliopsoas muscle and 1 involved combined muscles. Alcoholic cirrhosis accounted for 75% of the cases. One case (12.5%) was associated with virus-related cirrhosis, and another with combined virus- and alcohol-related cirrhosis. The mortality rate was 75% despite early diagnosis and low risk scores for cirrhosis. Muscle hematoma in patients with cirrhosis is

INTRODUCTION

Liver cirrhosis is commonly associated with coagulopathies, including thrombocytopenia and hypoprothrombinemia, which often cause easy bruising and bleeding^[1]. Sudden bleeding from gastrointestinal varices due to portal hypertension is also an established risk of liver cirrhosis. Muscle hematoma usually occurs either traumatically or spontaneously in patients with hemophilia^[2] and in patients receiving anticoagulant therapy^[3]. However, it is rare in cirrhosis.

We herein, report a case of spontaneous bilateral iliopsoas hematoma in a patient with alcoholic liver cirrhosis. We also include a review of the literature on muscle hematoma associated with cirrhosis. The mortality rate of this condition is extremely high in comparison to that of muscle hematomas in patients with hemophilia or under anticoagulant treatment. These findings characterize muscle hematoma as a clinically important complication of cirrhosis.

CASE REPORT

A 56-year-old man visited a clinic complaining of severe fatigue. He was found to be in a state of shock with

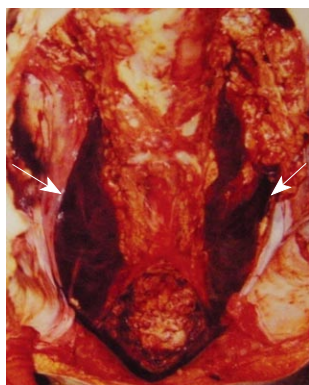


Figure 1 Bilateral iliopsoas hematoma (arrows) observed at autopsy.

severe anemia and was transferred immediately to our hospital. He had ingested 1.5 L of beer every day for 36 years and alcoholic cirrhosis had been diagnosed.

On arrival, a medical history was impossible to obtain because of his decreased level of consciousness. His body temperature was 35.0°C, his systolic blood pressure was 80 mmHg, and his pulse rate was weak but regular at 62 beats per minute. The abdomen was symmetrical and bowel sounds were weak. On palpation, there was neither rigidity nor tenderness, and no mass was palpable. The psoas position was not observed, and neurological examinations revealed only asterixis. Bilateral leg edema was noted.

The laboratory test results were as follows: white blood cell count, 3300/mm³; hemoglobin, 50 g/L; platelets, 45000/mm³; albumin, 21 g/L; total bilirubin, 6.4 mg/dL; aspartate aminotransferase/alanine aminotransferase, 58/16 U/L; alkaline phosphatase, 202 U/L; blood urea nitrogen, 32 mg/dL; serum creatinine, 2.1 mg/dL; creatine kinase, 202 U/L; prothrombin time-International Normalized Ratio, 1.48; activated partial thromboplastin time, 31.6 s. Stool samples were positive for occult blood.

A chest X-ray showed cardiomegaly and massive bilateral pleural effusion. Abdominal ultrasonography revealed a dull edge and irregular margins of the liver consistent with cirrhosis. Upper abdominal enhanced computed tomography (CT) also confirmed liver cirrhosis with splenomegaly and massive ascites but did not include the iliopsoas muscle. A gastroduodenal endoscopy did not disclose any hemorrhagic diseases. Further examinations were not performed due to the general deterioration of his condition.

Treatment, including the transfusion of red blood cell concentrate, branched-chain amino acids, and albumin as well as the administration of dopamine hydrochloride and diuretics, was initiated. On the 5th day of admission, imipenem/cilastatin sodium (1 g/d) and gamma globulin (2.5 g/d) were empirically administered for a high fever. On the 8th day, the patient received fresh frozen plasma (FFP) and antithrombin III to treat disseminated intravascular coagulation. Wide ecchymosis appeared on both sides of the patient's back on the 9th day and he died on the 10th day.

Autopsy findings revealed bilateral iliopsoas

hematoma (Figure 1) with liver cirrhosis. The source of bleeding could not be detected inside the iliopsoas muscles, and there was no evidence of either a neoplasm or an arterial anomaly. Other causes of anemia including bone marrow disorders were not detected. A histological examination with iron staining was positive in the iliopsoas muscle. The appearance of this type of staining depends on the age of hemorrhage, and indicated that the bleeding started around the day of admission in this case.

DISCUSSION

Intramuscular hematoma is classified as either spontaneous or traumatic. Causes of the former include hemorrhagic diseases, neoplasm, and arterial diseases. Iliopsoas and rectus abdominis muscle hematomas are well known as major and well documented complications of hemophilia and anticoagulant therapy^[2-5]. Although decreased levels of coagulant factors and thrombocytopenia are often observed in liver cirrhosis, intramuscular hemorrhage is very rare^[6].

Early symptoms of an iliopsoas hematoma include groin pain and femoral neuropathy. The present patient was in a drowsy state of consciousness due to shock when he was referred to our hospital. Stools were positive for blood, and therefore, only examinations for upper gastrointestinal tract were performed, and no specific findings were obtained. Unfortunately, the upper abdominal CT scan area did not involve the iliopsoas muscles.

As shown in Table 1, a review of the literature revealed 8 cases of spontaneous muscle hematoma associated with liver cirrhosis, including the present case (Table 1)^[6-11]. Of these cases, 4 involved the rectus abdominis muscle^[6-9], 3 (including the present case) involved the iliopsoas^[8,10], and one involved the gluteus maximus, the biceps femoris and the pectoralis muscles^[11]. The mean age of these patients was 55.8 years old. Five of the 8 cases were male (62.5%) and 3 were female (37.5%). Six of the 8 cases were Japanese and the others were Caucasian. Alcoholic cirrhosis accounted for 75% of the cases (6 of 8 cases), and virus-related cirrhosis (virus unknown) and a combination of hepatitis C- and alcohol-related cirrhosis each accounted for 12.5% of the cases (1 each out of 8 cases).

The mortality rate of muscle hematoma associated with cirrhosis was 75% (6 of 8 cases), which is extremely high. All 3 patients with iliopsoas hematoma and 2 of the 4 patients with rectus abdominis muscle hematoma (50%) died. Cherry and Mueller^[3] reported that rectus abdominis muscle hematoma is rarely fatal based on a large series of patients from a single institution (mortality rate 1.6%, 2 of 126 cases), most of whom were on various anti-coagulant therapies. Furthermore, the mortality rate of iliopsoas hematoma associated with hemophilia is extremely low after the induction of prophylaxis^[2]. According to a review of iliopsoas hematomas in patients receiving intravenous heparin^[5], only one of 54 patients (1.9%) died. In these

Table 1 Cases of muscle hematoma in liver cirrhosis

Case [reference]	Age (yr)/ sex	Etiology of cirrhosis	Involved muscle	Laterality	Time to diag from the onset (d)	Time to death from the onset (d)	MELD/ MELD-Na	Treatment	Course
#1 ^[7]	48/F	Alcohol	Rect. abd.	Bil		14			Died (autp)
#2 ^[6]	46/F	Alcohol	Rect. abd.	Right	< 1	> 10	29/-	Conserve., FFP	Died (autp)
#3 ^[8]	56/M	Alcohol	Rect. abd.	Right	< 1			Conserve.	Alive
#4 ^[9]	58/F	Virus	Rect. abd.	Left	< 1			Liver transplt & evac	Alive
#5 ^[10]	60/M	Alcohol	Iliopsoas	Left	6	29	24/29	Conserve.	Died (autp)
#6 ^[8]	62/M	Alcohol	Iliopsoas	Right	< 1	4		TAE	Died (autp)
#7 (present)	56/M	Alcohol	Iliopsoas	Bil		10	25/25	Conserve., FFP	Died (autp)
#8 ^[11]	60/M	Alcohol + HCV	Gluteus max & biceps femoris & pectoralis	Right	< 1	150	16/16	Conserve., FFP	Died (autp)

Autp: Autopsy; Bil: Bilateral; Conserv.: Conservative; Diag: Diagnosis; Evac: Evacuation; FFP: Fresh frozen plasma; HCV: Hepatitis C virus; Max.: Maximus; Rect. abd.: Rectus abdominis; Transplt: Transplantation.

reports, it was generally accepted that early recognition and diagnosis of rectus abdominis and/or iliopsoas hematoma could reduce mortality.

As shown in Table 1, intramuscular hematoma in patients with liver cirrhosis was diagnosed on the day of onset in 6 of the 8 cases (75%). The exceptions included case #5 (diagnosed at 6 d after the onset) and case #7 (the present case, which was diagnosed by autopsy). The Model for End-stage Liver Disease (MELD)^[12] and recently reported MELD-Na^[13] scores could be calculated in 4 (#2, #5, #7, #8) and 3 (#5, #7, #8) patients, respectively. The mean MELD and MELD-Na scores were 23.5 (range: 16–29) and 23.3 (range: 16–29), respectively, thus suggesting that the probability of death at 90 d (%) ranged from about 4% to 25%^[13]. These findings indicate that intramuscular hemorrhage associated with cirrhosis is lethal despite an early diagnosis, even in patients with low MELD or MELD-Na scores.

Huang *et al*^[14] reported interesting findings concerning spontaneous intracranial hemorrhage (SICH) in 4515 hospitalized Chinese cirrhotic patients. Among these patients, 36 experienced SICH and 78% were male. The etiology of cirrhosis included alcoholic cirrhosis in 50% of the patients, virus-related cirrhosis in 27.8%, and combined virus- and alcohol-related cirrhosis in 22.2%. The mean age at onset of SICH in cirrhosis was 53 years. They reported that the overall incidence of SICH in cirrhosis was related to the etiology of cirrhosis, and the incidence of SICH was 6 times higher in alcohol-related cirrhosis than in virus-related cirrhosis. These results are consistent with the present findings on muscle hematoma associated with cirrhosis. It seems likely that a common risk factor, namely alcohol, plays an important role in the promotion of non-variceal hemorrhage in cirrhotic patients. Several actions of alcohol may promote hemorrhage, including the inhibition of platelet adhesion to fibrinogen^[15], dose-related ethanol suppression of platelet aggregation induced by extravasation^[16], and the promotion of atherosclerosis^[17]. However, the participation of these factors in the pathogenesis is still unknown in patients with alcoholic cirrhosis. Among these cases, 6 of 8 were

reported in Japan, thus suggesting that intramuscular hematoma and/or SICH with cirrhosis may occur more frequently in Asians than in other ethnicities.

Treatment of muscle hematoma in cirrhotic patients is often challenging. Many factors, including poor general condition due to cirrhosis, lead to poor outcomes. Hematomas might improve with conservative management, as observed in case #3 in Table 1. Zissin *et al*^[18] reported in a literature review that 19 of 26 patients (73%) recovered following transcatheter arterial embolization (TAE) in anticoagulant-related rectus abdominis and iliopsoas hematoma. TAE may be useful for patients in poor condition, although it was unsuccessful in a case of cirrhosis (case #6)^[8]. Urgent liver transplantation, which was performed in case #4^[9], may be a treatment choice in the future. The bleeding site was detected by angiography in only 1 case of iliopsoas hematoma (#6). In this case, two regions of extravasation of the ilio-lumbar arterial branches were observed and TAE was performed. In the other cases, the origin of the bleeding could not be detected even at autopsy. This difficulty is one challenge associated with the treatment of this condition. Additional cases could provide further insight into effective treatment methods.

In general, intramuscular hematoma is not mentioned as one of the bleeding complications occurring in cirrhotic patients^[1]. Intramuscular hematoma may occur easily by daily motion. For example, a case of rectus abdominis hemorrhage (#3) occurred after an episode of coughing. Taking into account the extremely high mortality rate despite early diagnosis and relatively low risk scores, intramuscular hematoma should be recognized as a rare but clinically important bleeding complication of cirrhosis, especially in cases of alcoholic cirrhosis.

In conclusion, a bilateral iliopsoas hematoma was observed in a patient with alcoholic liver cirrhosis. A review of the literature on intramuscular hemorrhage in cirrhotic patients suggested that alcohol is the common risk factor. In addition, a higher incidence was observed in Asians. This literature review also indicated that muscle hematoma in cases of cirrhosis has a serious prognosis that is quite different from the prognosis

of hematomas in patients with hemophilia or under anticoagulant therapy. We should therefore always include muscle hematoma in the differential diagnosis of acute anemia in liver, especially alcoholic, cirrhosis.

ACKNOWLEDGMENTS

The authors thank Dr. Hidetoshi Matsunami, President of Matsunami General Hospital and Sosaikouseikai Clinical Foundation, for reviewing the manuscript.

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S- Editor Li LF L- Editor Webster JR E- Editor Yin DH

Delayed presentation of intrathoracic esophageal perforation after pneumatic dilation for achalasia

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Received: May 2, 2009

Revised: August 20, 2009

Accepted: August 27, 2009

Published online: September 21, 2009

Abstract

Pneumatic dilation (PD) is considered to be a safe and effective first line therapy for achalasia. The major adverse event caused by PD is esophageal perforation but an immediate gastrografen test may not always detect a perforation. It has been reported that delayed management of perforation for more than 24 h is associated with high mortality. Surgery is the treatment of choice within 24 h, but the management of delayed perforation remains controversial. Hereby, we report a delayed presentation of intrathoracic esophageal perforation following PD in a 48-year-old woman who suffered from achalasia. She completely recovered after intensive medical care. A review of the literature is also discussed.

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Key words: Intrathoracic esophageal perforation; Delayed presentation; Pneumatic dilation; Esophageal achalasia

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Lin MT, Tai WC, Chiu KW, Chou YP, Tsai MC, Hu TH, Lee CM, Changchien CS, Chuah SK. Delayed presentation of intrathoracic esophageal perforation after pneumatic dilation for achalasia. *World J Gastroenterol* 2009; 15(35): 4461-4463 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4461.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4461>

INTRODUCTION

Esophageal achalasia is an inflammatory disease characterized by esophageal aperistalsis and failure of complete LES relaxation due to loss of the inhibitory intrinsic neurons of the esophageal myenteric plexus^[1,2]. Pneumatic dilation (PD) is considered to be the first line therapy for achalasia^[3,4]. Esophageal perforation could be a hazardous event if left untreated after PD^[5]. Usually, gastrografen is ingested immediately after each PD to detect extravasations which imply perforation.

However, on rare occasions, immediate gastrografen ingestion may not always detect a perforation which could become clinically evident several hours later resulting in a delayed presentation (more than 24 h)^[6]. Hereby we report our experience in treating an achalasia patient who suffered from delayed clinical presentation of intrathoracic esophageal perforation after PD but healed after intensive medical care.

CASE REPORT

The 48-year-old female, who had a history of surgery for uterine myoma status for 2 years, was referred to our hospital due to esophageal stenosis. She suffered from progressive symptoms of dysphagia for liquid and solid foods, chest pain and food regurgitation for the previous 6 mo. She also complained of gradual body weight loss of 5 kg during this period. She had no family history of gastrointestinal cancer. Physical examination revealed an unhealthy, conscious woman with stable vital signs. The laboratory data were as follows: white blood cell count: 4.1×10^3 cells/ μ L (normal 3.5-11 cells/ μ L), hemoglobin: 10.5 g/dL (normal 12-16 g/dL), hematocrit: 32% (normal 36%-46%), mean corpuscular volume: $69.3 \mu\text{m}^3$ (normal $80-100 \mu\text{m}^3$), platelet count: $18.8 \times 10^3/\mu\text{L}$ (normal $15-40 \times 10^3/\mu\text{L}$), sodium: 142 mmol/L

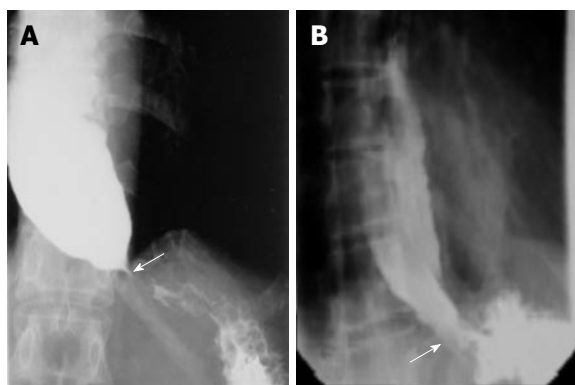


Figure 1 Barium esophagogram before and after pneumatic dilations. A: Barium esophagogram before pneumatic dilations revealed a dilated distal esophageal lumen with bird-beak signs (arrow); B: Ingestion of gastrografin revealed no obvious immediate leakage of contrast medium immediately after pneumatic dilation (arrow).

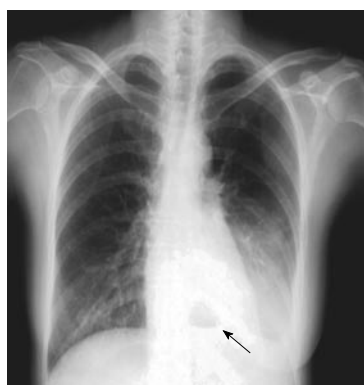


Figure 2 Chest radiograph revealed left side pleural effusion, left lower lobe consolidation and air-fluid level over retrocardiac region air fluid level over the retrosternal area (arrow).

(134-148 mmol/L), potassium: 3.7 mmol/L (normal 3.0-4.8 mmol/L). A barium esophagogram revealed a dilated distal esophageal lumen with food retention at a narrowing of the gastroesophageal junction (Figure 1A). Achalasia was confirmed using manometric findings of esophageal aperistalsis and incomplete relaxation of the lower esophageal sphincter during wet swallowing. The residual pressure was about 10 mmHg but the basal lower esophageal sphincter pressure was about 45 mmHg. Achalasia caused by tumors was excluded by computed tomography (CT). The patient received PD with a 3-cm "Regiflex" dilator. Routine gastrografin ingestion showed no sign of immediate leakage of contrast (Figure 1B). She was fine after PD with only mild chest pain and asked to be scheduled for discharge. Her vital signs remained stable in the next 24 h following PD.

Unfortunately, severe chest pain occurred 6 h later with fever up to 38°C. Chest radiograph revealed left side pleural effusion, left lower lobe consolidation and air-fluid level over retrocardiac region air fluid level over the retrosternal area (Figure 2). The chest CT revealed esophageal dilatation suspicious of rupture of the lower esophagus with prominent pleural effusion and fluid collection over the middle and lower mediastinum (Figure 3). Due to rapid progression of the dyspneic condition and high fever to 40°C, a chest drainage tube was placed immediately. Intravenous broad-spectrum antibiotics were also given immediately. She was then

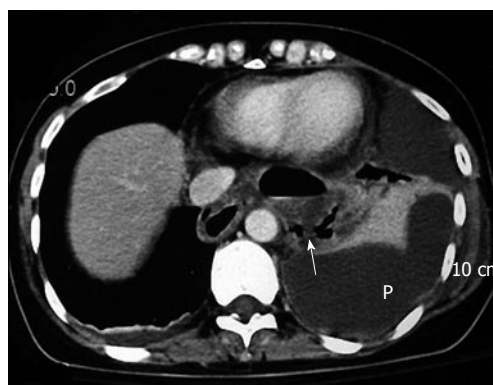


Figure 3 CT revealed esophageal dilatation and a rupture of the lower esophagus (arrow) with prominent pleural effusion (P) and fluid collection over the middle and lower mediastinum.

transferred to the cardiovascular surgery intensive care unit. She refused to receive any surgical intervention. Total parenteral nutrition was given via the venous route to ensure an appropriate nutrition supply. Fortunately, her condition improved gradually 2 wk later. A barium esophagogram revealed a dilated esophagus without any contrast leakage and absence of bird-beak signs. She was discharged safely another 2 wk later. She remained in clinical remission status 8 years after PD.

DISCUSSION

Esophageal perforation is a relatively uncommon complication even though many diagnostic and therapeutic procedures in daily practice require the passing of instruments through the esophagus, such as endoscope-guided PD for this lady who suffered from esophageal achalasia. The signs and symptoms of esophageal perforation are non-specific but may include pain, fever, dyspnea, vomiting, crepitus, and shock^[6]. Despite prompt treatment and improved management, the mortality of esophageal perforation is still significant^[7]. The esophagus lacks a serosal layer and the adventitia of the esophagus is contiguous with the connective tissue of the mediastinum. As a result, esophageal perforation allows the leakage of food residues, saliva, oropharyngeal bacteria and digestive enzymes into the mediastinum and chest cavity. Severe adverse events such as mediastinitis, pleural emphysema, empyema, or subdiaphragmatic abscess may occur rapidly in delayed diagnosed and managed patients^[8]. The impact of delayed recognition is decreasing despite the high incidence of complications. Reeder and colleagues reported a 29% mortality rate following late diagnosis^[9]. The most important predictor of treatment outcome depends on the time interval from perforation to treatment. It has been reported that delayed management of perforation for more than 24 h is associated with a 3-5 fold increase in mortality.

The diagnosis of delayed presentation of esophageal rupture remains a clinical challenge because of the non-specific findings of this rare clinical condition. Ingestion of water-soluble gastrografin immediately after each PD

is a standard examination to avoid possible extravasation of barium-containing agents that may cause serious mediastinitis^[10]. Imaging studies provide a reliable way to make a definite diagnosis of esophageal perforation. Contrast leakage on imaging studies suggests leakage of gastric juice and other substance into the mediastinum and causes the inflammatory process. Unfortunately, an immediate gastrografen test may not always detect a perforation which could become clinically evident several hours later and this must be kept in mind. Absence of contrast leakage may suggest a small or a sealed-off perforation. This may result in the tricky delayed clinical presentations following dilations. This explains the initial stable subjective and objective conditions that occurred in our patient following PD. Buecker and colleagues suggest that a negative gastrografen study should be followed-up with a barium study to increase sensitivity^[11] but then the patient may be at risk of severe mediastinitis.

The management of esophageal perforations includes fluid resuscitation, antibiotics, gastric decompression, and surgery. Broad-spectrum antibiotics that cover Gram-positive, Gram-negative, and anaerobic organisms should be started and tailored to culture results^[7]. Surgery is the treatment of choice within 24 h, but the management of delayed perforation remains controversial. Quintana and colleagues reported the mortality rate in patients with prompt surgical treatment ranged from 0% to 34%, and those without immediate treatment 68.7%-75%^[12].

However, Younes and colleagues advocated that patients with mild fever, absence of shock and sepsis might be considered for conservative management^[13]. There was no difference in mortality rate between surgery and medical treatment in these two publications^[12,13]. The other theoretical issue is whether the perforation would heal in the presence of distal obstruction, which this patient potentially had before PD as a result of her underlying achalasia; however the follow-up esophagogram revealed patency of the distal lumen. All the above favorable factors were probably the main reasons for the recovery of our patient despite her refusal of surgery. Therefore, intensive non-surgical treatment may be another option for treatment with a favorable outcome in selected patients with a good general health status.

The sizes of the balloons and the techniques of the procedure are relevant to esophageal perforation in treating achalasia under the guidance of a fluoroscope or endoscope^[3,14-16]. Graded PD with a 30 mm diameter and slower rate of balloon inflation is an effective and safe initial method of therapy for achalasia. The potential danger in increased risk of perforation is likely to interfere with the effect of dilation toward the side of the endoscope, and this can be compromising and lead to a decrease in overall efficacy of the procedure and the possibility of generating an unequal radial force on the sphincter^[17].

In summary, early identification of a delayed presentation of intrathoracic esophageal perforation requires premeditated consideration and is crucial for a favorable outcome. However, an immediate contrast study may not always detect a perforation which can

become clinically evident several hours later. Therefore, close observation of clinical symptoms and signs such as severe chest pain and fever implying potential perforations is mandatory after PD. Surgery is the treatment of choice within 24 h. Intensive non-surgical treatment could be another option for treatment with a favorable outcome in selected patients with a good general health status.

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CASE REPORT

Sorafenib induced tumor lysis syndrome in an advanced hepatocellular carcinoma patient

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Received: June 22, 2009 Revised: August 11, 2009

Accepted: August 18, 2009

Published online: September 21, 2009

Abstract

A 55-year-old male patient with hepatitis B-related liver cirrhosis was found to have advanced hepatocellular carcinoma. His AFP was initially 9828 $\mu\text{g/L}$ and rapidly dropped to 5597 $\mu\text{g/L}$ in ten days after oral sorafenib treatment. However, he developed acute renal failure, hyperkalemia, and hyperuricemia 30 d after receiving the sorafenib treatment. Tumor lysis syndrome was suspected and intensive hemodialysis was performed. Despite intensive hemodialysis and other supportive therapy, he developed multiple organ failure (liver, renal, and respiratory failure) and metabolic acidosis. The patient expired 13 d after admission.

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Key words: Sorafenib; Tumor lysis syndrome; Hepatocellular carcinoma; Hemodialysis; Hyperkalemia

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INTRODUCTION

Molecular targeted therapy is currently the new treatment modality for advanced cancer. Sorafenib is an oral multi-kinase inhibitor that blocks tumor growth and cell proliferation by targeting Raf kinase, VEGFR-2, VEGFR-3, and PDGFR- β ^[1]. Sorafenib was approved by the FDA in 2007 to treat hepatocellular carcinoma (HCC)^[2]. An uncontrolled sorafenib phase II study in 137 patients with advanced HCCs confirmed that the higher pERK patients had a longer time to progression compared to the lower pERK patients^[3,4]. Therefore, sorafenib is a specific target drug for the Raf/MEK/ERK pathway and pERK could be a useful biomarker. Sorafenib showed efficacy in prolonging patients' survival in a previous SHARP trial^[5]. The study obtained an overall 44.9% improvement and the sorafenib group had a partial response rate of 2.3% ($n = 7$) vs placebo of 0.7% ($n = 2$). Sorafenib opens a new era for advanced HCC treatment. However, that sorafenib might induce tumor lysis syndrome (TLS) had never been reported in previous studies. Here, we report sorafenib induced TLS in an advanced HCC male patient. This reported case developed multiple organ failure (liver failure, renal failure and respiratory failure) and metabolic acidosis. Despite intensive hemodialysis and other supportive therapy, he succumbed to the complication of tumor lysis syndrome.

CASE REPORT

A 55-year-old male hepatitis B carrier visited our Clinic with the symptoms of general fatigue, abdominal fullness, poor appetite and body weight loss of 5 kg over 2 mo. An abdominal sonography showed multiple mixed-hypoechoic masses (larger than 12 cm in aggregated diameter or more than 50% of whole liver capacity) involving both lobes of the liver. Additionally, there was left portal vein thrombosis and decompensated cirrhosis with ascites. Laboratory data showed that HBeAg was negative, creatinine was 106 (97-133) $\mu\text{mol/L}$. AST was 435 (0-38) U/L, ALT was 79 (0-44) U/L, total bilirubin was 74 (10-24) $\mu\text{mol/L}$, prothrombin time was 12.7 s (control was 10.7 s), albumin was 37 (35-50) g/L, and α -feto protein (AFP) was 9828.63 (1.09-8.04) $\mu\text{g/L}$. The Child-Turcotte-Pugh score was eight. His abdominal triphase computed tomography



Figure 1 Abdominal computed tomography showing bilateral multiple hepatic tumors with left side portal vein thrombosis and mild ascites.

(CT) confirmed the sonography findings, namely, advanced HCC in a background of liver cirrhosis (Figure 1). He was given oral sorafenib 400 mg twice every day and oral diuretics (furosemide 40 mg once, spironolactone 25 mg three times every day since February 28, 2009). Ten days later (March 9, 2009), his AFP level dropped to 5597.52 $\mu\text{g/L}$ and his creatinine level was 80 $\mu\text{mol/L}$. Thirty days after he received sorafenib treatment, he was found to have profound jaundice with total bilirubin of 344 (10–24) $\mu\text{mol/L}$, decreased urine output, and general weakness. Abdominal sonography showed no ascites. The laboratory data revealed a creatinine level of 177 $\mu\text{mol/L}$, BUN at 34 (3.6–7.1) mmol/L, serum sodium at 112 (135–148) mmol/L, potassium at 7.0 (3.5–5.3) mmol/L, ammonia at 92 (16–41) $\mu\text{mol/L}$, AST at 698 (0–38) U/L, and ALT at 297 (0–44) U/L. Acute renal failure with hyperkalemia was diagnosed and he was treated intravenously with calcium gluconate, sodium bicarbonate, glucose water with insulin, corticosteroid, and oral kalimate (calcium polystyrene sulfonate). The HBV DNA (Simens, VERSAN® HBV DNA 3.0, Australia) was shown to be 48 800 IU/ml (273 288 copies/mL) and antiviral therapy with entecavir (0.5 mg on day one, followed by 0.5 mg every 3 d) for the suspicion of liver failure secondary to hepatitis B exacerbation. On the next day, his serum potassium was 7.2 (3.5–5.3) mmol/L. Emergency hemodialysis began and sorafenib treatment was discontinued. On the next day, his serum potassium dropped to 4.5 mmol/L, sodium to 128 mmol/L, uric acid to 547 (238–506) $\mu\text{mol/L}$, and his creatinine level was 248 $\mu\text{mol/L}$. Half of the previous dose of sorafenib was restarted with hydration of 2000–3000 mL saline for suspicious dehydration and diuretics were discontinued. Two days later, his potassium level had increased to 5.0 mmol/L, uric acid was 851 $\mu\text{mol/L}$, phosphorus was 5.4 mmol/L (2.7–4.5) and his creatinine level was 309 $\mu\text{mol/L}$. Recurrence of TLS was suspected and sorafenib was discontinued. Allopurinol 300 mg (*po* st) and 600 mg (*po* every day) was started. Another course of emergency hemodialysis was performed after the patient failed to respond to the medication. His general

condition stabilized but multiple organ failure and metabolic acidosis persisted, despite another course of hemodialysis. He expired 13 d after admission.

DISCUSSION

Tumor lysis syndrome (TLS) is a life threatening oncological emergency. It is the result of rapid breakdown of tumor cells with release of cellular contents, such as potassium, uric acid, phosphate, purine, and proteins into the circulation^[6]. Risk factors for TLS include the following: (1) hematological malignancy; (2) high proliferation rate tumors; (3) chemo- or radio-sensitive tumors; (4) large tumor burden; and (5) dehydrated state or patients with preexisting renal impairment^[6,7]. In hematological malignancy, TLS may develop rapidly within 24–48 h or it may develop one week after effective chemotherapy. HCC is a solid tumor with slower growth rate than that of hematological malignancies. Therefore, the TLS developed one month after treatment with sorafenib in this case. His renal function was within normal limits before, and 10 d after, treatment. The occurrence of TLS in this patient could be the combination of effective therapy due to sorafenib chemosensitivity of the HCC, the use of diuretics with secondary dehydration, and advanced HCC with large tumor burden. Diuretics induced secondary dehydration could result in acute renal failure, but after effective hydration and supportive therapy, hyperkalemia and hyperuricemia persisted. Therefore, sorafenib induced TLS was the more likely cause of the symptoms described.

The degree of abnormalities in his uric acid and potassium fulfill the criteria of TLS as defined by Cairo-Bishop^[8]; however, our case had no initial calcium data due to hyperkalemia treated with calcium gluconate. The initial serum calcium was 2.32 (2.2–2.6) mmol/L and dropped to 1.65 mmol/L ten days later. The decrease of calcium level over 25% also matched the Cairo-Bishop definition of TLS^[8]. Hypophosphatemia did happen in the sorafenib treatment group (11/297) *vs* control group (2/302) ($P < 0.001$) in Llovet *et al*'s SHARP study^[5]. Phosphorus in our case was 5.4 (2.7–4.5) mmol/L and did not go over 6.5 mmol/L as Cairo-Bishop definition of TLS, which might be the result of sorafenib effect.

The prognosis of TLS is poor with a high mortality rate in solid tumor. Prevention of TLS by hydration, alkalization, or therapy to correct metabolic disturbance can be effective for some hematological malignancies^[6–9]. Based on previous reports, hemodialysis can be helpful in improving the clinical course and can even cure TLS in some patients^[6–10]. This case had a satisfactory initial course following the sorafenib therapy; however, his condition deteriorated after TLS developed. Llovet *et al*^[5] reported that sorafenib could significantly extend median survival and prolong the time of radiologically evidenced progression by approximately three months compared with a placebo in individuals with advanced hepatocellular carcinoma (HCC) with well-preserved

liver function, naive to systemic therapy. In comparison with the control group given the placebo, they reported that sorafenib was significantly associated with more diarrhea of any grade, weight loss, hand-foot skin reaction, alopecia, anorexia, and voice changes^[5]. However, in their report, TLS was not mentioned. We believed that TLS is a rare side effect of sorafenib treatment. Once it occurred, however, TLS can induce multiple organ failure with a catastrophic outcome.

In conclusion, in the therapy of HCC with sorafenib, especially in patients with high tumor burden and showing a good initial response, the possible occurrence of TLS should be kept in mind and appropriate laboratory data should be monitored. Once TLS developed, the prognosis was poor even when intensive hemodialysis was given.

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S- Editor Li LF L- Editor Stewart GJ E- Editor Yin DH



Ectopic pancreaticobiliary drainage accompanied by proximal jejunal adenoma: A case report

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Received: July 10, 2009 Revised: August 15, 2009
Accepted: August 22, 2009
Published online: September 21, 2009

Abstract

A patient with obstructive jaundice was examined by multidetector row helical computed tomography (MDCT) and magnetic resonance imaging (MRI), and his common bile duct was observed to be leading into the distal portion of the horizontal duodenum with a pancreaticobiliary union outside the duodenal wall. A mass was also found in the proximal jejunum. All the above findings were confirmed by subsequent surgery, thus contrast-enhanced MDCT and MRI with appropriate image post-processing could provide non-invasive and accurate information regarding anatomy and lesions of the pancreaticobiliary duct and duodenal union, which may improve the feasibility of surgery and reduce postoperative complications.

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Key words: Ectopic drainage; Pancreaticobiliary maljunction; Computed tomography; Magnetic resonance imaging

Peer reviewer: Norbert Senninger, Professor, Department of General Surgery, University Clinics, Westphalian-Wilhelm's -University, Waldeyerstrasse 1, D-48149 Muenster, Germany

Wu DS, Chen WX, Wang XP. Ectopic pancreaticobiliary drainage accompanied by proximal jejunal adenoma: A case report. *World J Gastroenterol* 2009; 15(35): 4467-4470 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4467.asp>
DOI: <http://dx.doi.org/10.3748/wjg.15.4467>

INTRODUCTION

Anomalous termination of the common bile duct

(CBD) is not an uncommon anatomical variation with incidence ranging from 5.6% to 23%^[1-3]. Pancreaticobiliary maljunction (PBM) is a congenital anomaly in which the pancreatic duct meets the bile duct outside of the duodenal wall. Previous studies using endoscopic retrograde cholangiopancreatography (ERCP) found some sporadic cases. As ERCP is invasive with serious complications, e.g. pancreatitis, computed tomography (CT) or magnetic resonance imaging (MRI) may be another diagnostic choice for these patients. However, few studies up till now have reported such cases by CT or MRI. This study reports findings of both multidetector row helical computed tomography (MDCT) and MRI in a patient with anomalous termination of CBD, PBM and proximal jejunal adenoma. Both MDCT and MRI provide non-invasive and accurate information about anatomy and lesions of the pancreaticobiliary duct and duodenal union (PDDU).

CASE REPORT

A 71-year-old woman presented with icteric skin and sclera for the past three months without fever, abdominal pain or distention, nausea or vomiting. Physical examination revealed no abdominal tenderness or mass. On admission, total and direct bilirubin levels were 138.5 $\mu\text{mol/L}$ and 120 $\mu\text{mol/L}$ respectively, alanine aminotransferase was 78 IU/L, aspartate aminotransferase was 184 IU/L, alkaline phosphatase was 1031 IU/L, and carcinoembryonic antigen and α -fetoprotein levels were within normal values. She had a history of cholecystectomy 18 years ago. MDCT and MRI were performed within a week. The scan parameters for the MDCT scanner (Sensation 16, Siemens Medical Solutions, Siemens, Germany) included 120 kVp, 121-124 mAs, 5-mm slice thickness, 1-mm reconstructive thickness, and a table speed of 24 mm/s (pitch, 1). Non-ionic contrast material 90 mL (Iohexol, 300 mg I/mL) was injected at a flow rate of 3 mL/s. The MR (1.5-T, Siemens Sonata, Siemens, Germany, Leonardo work station) scanning protocol included unenhanced axial T2W scan (HASTE; TR/TE, 6379 ms/83 ms), coronal T2W imaging (True-FISP; TR/TE, 3.8 ms/1.9 ms), magnetic resonance cholangiopancreatography (TSE; TR/TE, 100 ms/1.8 ms), axial T1W (GRE, TR/TE, 100 ms/1.8 ms, 70° flip angle) and Gadolinium-enhanced three-dimensional VIBE (TR/TE, 4.8 ms/2.2 ms, 70° flip angle) information. The images were retrospectively reviewed in consen-

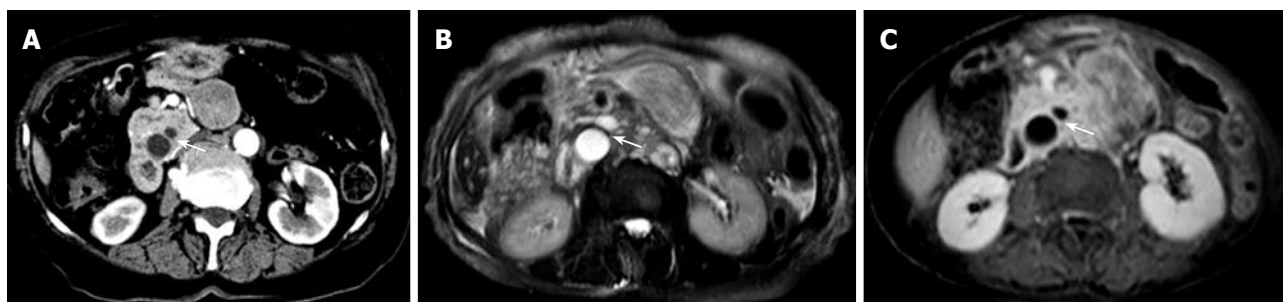


Figure 1 Anomalous junction of the common bile duct and main pancreatic duct. A: CT scan shows the junction of the common bile duct and main pancreatic duct below the level of the uncinate process (arrow); B and C: Axial MR images show the junction of the common bile duct and main pancreatic duct (arrows). (These photographs were processed by Adobe Photoshop Creative Suite 2.).

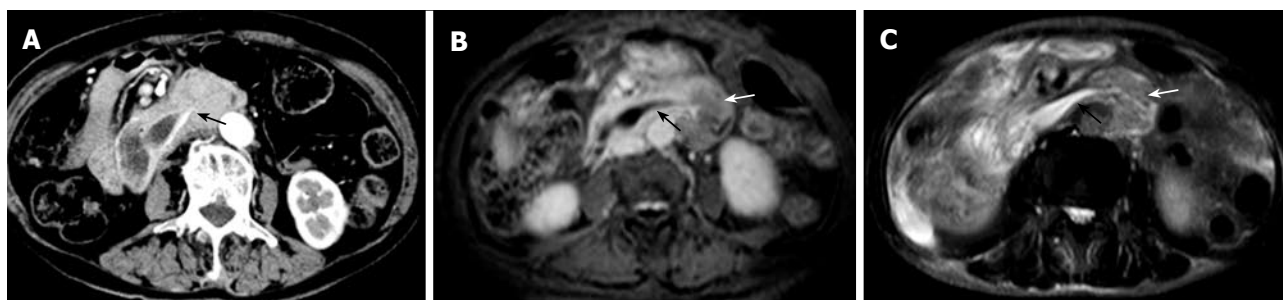


Figure 2 Anomalous termination of the common bile duct. A: Axial image from CT scan shows the termination of the common bile duct at the distal portion of the horizontal part of the duodenum (arrow); B and C: MR images demonstrate a tapered configuration of the common bile duct (black arrows) and a mass located on the proximal jejunum (white arrows). (These photographs were processed by Adobe Photoshop Creative Suite 2.).

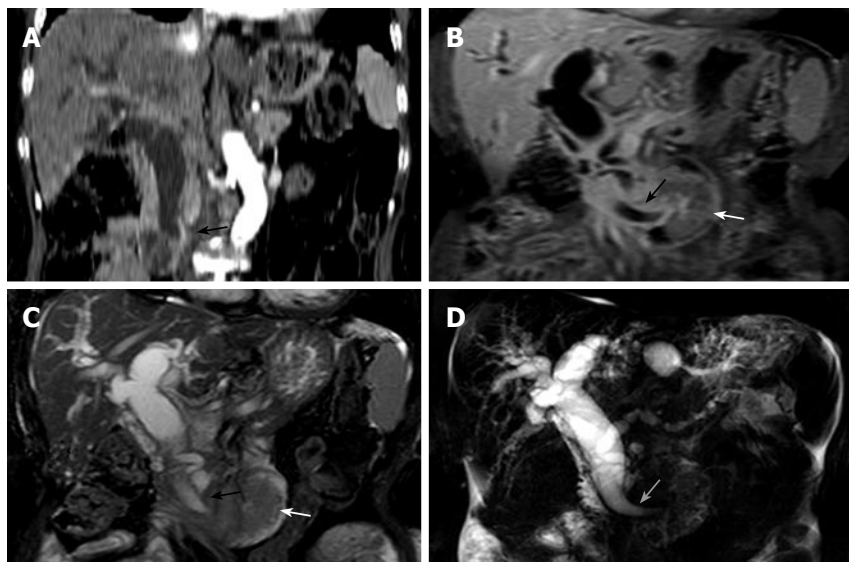


Figure 3 Dilated pancreatic and biliary ducts with ectopic drainage. A: Coronal MDCT image of CT reveals a low termination of the common bile duct (arrow); B and C: Coronal MR images show ectopic pancreaticobiliary junction, low termination of the common bile duct (black arrows), and the mass (white arrows) located on the proximal jejunum; D: MRCP shows dilated pancreatic and biliary ducts. (These photographs were processed by Adobe Photoshop Creative Suite 2.).

sus by two experienced radiologists who were unaware of other imaging and surgical findings.

On the continuous transverse images of MDCT and MR, the CBD which was joined by the main pancreatic duct (MPD) outside the duodenal wall below the level of the uncinate process (Figure 1) was found to terminate at the distal portion of the horizontal duodenum (Figure 2). The coronal images of MDCT and MR, as well as MRCP images, showed a more clear presentation than transverse images regarding the ectopic pancreaticobiliary junction and low termination of the CBD at the horizontal duodenum (Figure 3). Both MPD and extra-

and intrahepatic ducts showed extensive dilatation. The CBD was 2.4 cm at the largest diameter with a tapered configuration of the distal portion (Figure 2B and C). A discrete mass, surgically proven to be jejunal adenoma, was detected in the vicinity of the duodenojejunal flexure without dilatation of the proximal gut, but with adhesion to the proximal jejunum (Figure 2B and C). The mass was enhanced heterogeneously on contrast-enhanced CT and MR images.

During subsequent operation, the CBD was found to be dilated and it extended along the duodenum and terminated at the horizontal duodenum.

DISCUSSION

In 75% of cases, the common bile duct enters the posteromedial aspect of the descending duodenum through an intramural common channel called the hepatopancreatic ampulla (ampulla of Vater), where the CBD is joined by the main pancreatic duct and the terminal pancreatic duct is inferior and anterior to the CBD^[4]. But the CBD can terminate at anomalous sites, including the third or fourth part of the duodenum, pyloric canal, duodenal bulb, or stomach^[5,6-9]. The union of the pancreatic and biliary ducts can also locate outside the duodenal wall forming a very long common channel (usually more than 15 mm), which is representative of the appearance of a PBM.

The cause of an anomalous pancreaticobiliary system has been ascribed to unidentified abnormalities during embryogenesis. The ventral diverticulum of the foregut forms the liver, gallbladder, bile ducts and ventral pancreas. Abnormalities of the pancreaticobiliary junctions occur where these are demarcated. Boyden produced a hypothesis that ectopic bile duct drainage occurs because of the disproportional elongation and early subdivision of the primitive hepatic diverticulum which divides into the pars hepatica and pars cystica^[10,11].

Multiple modalities permit depiction of the anatomy and lesions of the PDDU, including abdominal ultrasonography, ERCP, CT and MRI. Sonographic images often do not clearly display PBM and ectopic termination of the CBD, and ERCP is invasive and can be associated with significant complications, such as a 1% to 13.5% incidence of post-ERCP pancreatitis^[12]. In contrast, MDCT and MRI are modern and non-invasive techniques revealing more anatomical details with no risk of procedure-induced complications. With appropriate image post-processing, MDCT and MRI may provide prompt identification of anatomy and abnormalities of the biliary and pancreatic ducts and the surrounding structures, which would help endoscopists and surgeons to discern the anatomy requiring special attention during ERCP operation, choledochoscopic exploration, endoscopic and choledochoscopic papillotomy and local resection of PDDU carcinomas. CT and MRI examinations can also offer valuable information in adults with underlying abnormalities of the pancreatic duct or biliary duct when the symptoms and signs are nonspecific (intermittent abdominal pain, occasional jaundice and cholangitis).

Based on the density of bile, it is easy to differentiate the CBD from surrounding soft tissue and trace its course until its termination on CT images. In high-spatial-resolution CT, the MPD and pancreaticobiliary junction can be well observed. MR imaging combined with magnetic resonance cholangiopancreatography (MRCP) has a distinctive advantage in revealing the pancreaticobiliary system. In terms of the intensity of bile, pancreatic and intestinal juice, the configuration and courses of the CBD, MPD, and enteric gut can be directly observed. At the same time, transverse images can offer additional information about the surrounding tissue and show tiny structures at the termination. In

the present case, tracing the CBD from the level below the hepatic hilum until the third part of the duodenum in continuous planar, the termination was found at the distal aspect of the horizontal duodenum on CT and MR transverse images. This was clearer on coronal images than on transverse and MRCP images. Dilated CBD and pancreatic duct made it easy and definite to determine the area of termination. The union of the pancreatic and biliary ducts was seen to be located outside the duodenal wall.

Extensive dilatation of the pancreaticobiliary ducts accompanied by a mass located on the proximal jejunum was another characteristic finding in our case. Because the gut proximal to the adenoma showed no dilatation and the CBD revealed a tapered end, it could be concluded that dilatation of the pancreaticobiliary ducts was not the result of the space-occupied mass. Miyano *et al.*^[13] reported that the majority of patients with PBM in their study showed evidence of proximal biliary dilatation. Babbitt^[14] proposed that PBM might be the etiology of choledochal dilatation and cyst formation. The resultant injury to the choledochus from enzymatic bilio-pancreatic reflux may then cause choledochal dilatation, choledochal fibrosis without dilatation, and possibly biliary atresia^[15]. PBM may result in the frequent occurrence of biliary carcinoma and pancreatitis^[16,17]. In this case, a PBM was found which may have been the cause of choledochal dilatation associated with choledochal injury and fibrosis and bile stasis, but there was no choledochal cyst formation, biliary carcinoma or pancreatitis. With bilio-pancreatic reflux, hypertension of the pancreatic duct could also result in dilatation of the MPD.

In summary, MDCT and MR imaging, with appropriate image post-processing, could provide non-invasive and accurate information about anatomy and lesions of the PDDU, which may improve the feasibility of surgery and reduce postoperative complications.

ACKNOWLEDGMENTS

We thank technicians from the Department of Radiology, West China Hospital of Sichuan University, for their kind help with this work.

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S- Editor Li LF L- Editor Logan S E- Editor Ma WH

Papers featured in the *World Journal of Gastroenterology* from 2006 to 2007

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Supported by The Education Department of Liaoning Province, No. 05W238

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Received: June 23, 2009

Revised: August 26, 2009

Accepted: September 3, 2009

Published online: September 21, 2009

Abstract

AIM: To analyze papers published in the *World Journal of Gastroenterology* (*WJG*) from 2006 to 2007. We investigated the highly cited papers for geographic distribution of the cited authors, as well as the distribution of the citing journals and year of citation.

METHODS: Papers published in *WJG* from 2006 to 2007 and their citations were retrieved from the Science Citation Index Expanded (SCIE). The papers and their citations were analyzed according to bibliometric methods, including the number of citations for a given paper, the distribution of the highly cited papers, the geographic distribution of the cited authors, and the years of citation.

RESULTS: Two thousand five hundred and six papers published in *WJG* from 2006 to 2007 were collected through SCIE, and 2335 of these were categorized as articles, reviews or proceedings. In 2006 and 2007, the average citation rate was 85.08% and 70.48%, respectively, and the average number of citations per paper was 4.33 and 2.51. Among the 2506 papers, 1963 were cited 8788 times by other articles. The mean number of citations per paper was 3.51. The papers with over three citations accounted for 54.72% of all those that were cited, and the total number of citations accounted for 85.38% of the total of 8788 citations. Thirteen papers were cited over 30 times and the highest number of citations for any one paper was 98. The cited authors came from 70 different

countries or regions, with China, Japan and the United States being the most frequent. The highest average citation rate and number of citations per paper were for authors from Canada (96.30%, 6.89), Hungary (92.31%, 5.62), Australia (88.46%, 5.46), Germany (87.04%, 5.33), and Spain (87.50%, 5.11). The impact factor was 2.081 and the self-citation rate was 9.41% in 2008. The papers published in *WJG* in 2006-2007 were cited by 1597 journals.

CONCLUSION: The papers in *WJG* have a high number of citations, and have been cited in numerous journals by authors from various countries. The results imply that *WJG* has an influential academic profile in gastroenterology around the world.

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Key words: Citation analysis; Bibliometrics; *World Journal of Gastroenterology*

Peer reviewer: Sheng-Li Ren, PhD, Department of Publication, National Natural Science Foundation of China, Beijing 100085, China

Yang H, Zhang JH, Zhang F. Papers featured in the *World Journal of Gastroenterology* from 2006 to 2007. *World J Gastroenterol* 2009; 15(35): 4471-4475 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4471.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4471>

INTRODUCTION

In the natural sciences, especially in basic and life sciences, journals have never attracted more attention. The content and academic quality of journals is emphasized, and the academic profile of a journal can be evaluated through citation analysis of a given journal's papers, as well as the journal impact factor. The pertinent indexes for evaluating a scientific journal involve the publisher of the journal, its academic background, reputation of the authors, the number of times that papers are cited, and the journal impact factor^[1]. Some of these indexes may evaluate subjectively the journal impact in a qualitative manner, and some may measure quantitatively the general profile or average level of the

journal papers^[2]. The reputation of scientific journals, types of papers in a given journal, the number of papers in a given journal, the citation rate, and the number of times each paper is cited are all essential indicators of the quality of scientific journals.

The current study analyzed several features of papers published in *World Journal of Gastroenterology* (WJG), including the citation rate of the published papers, the average number of times a given paper was cited, the geographic distribution of the cited authors, the year of citation, and the distribution of the citing journals so that we can understand the character of WJG. The results will be of interest to the editors of WJG and its readers.

MATERIALS AND METHODS

The Science Citation Index Expanded (SCIE) is a sophisticated database established by the Institute for Scientific Information^[3], and is an essential tool for evaluating papers in renowned journals. The current study analyzed features of papers published in WJG with the aid of the search function of the SCIE.

The SCIE was accessed with the Web of Science. SCIE was searched using “World Journal of Gastroenterology” for the publication title and “2006-2007” for publication year. From the papers retrieved, we selected the following information: author(s), title, source, document type, address, cited times and ISSN. The retrieved data were downloaded, saved and managed with the Microsoft Excel.

There were some limitations to the data process:

- (1) we analyzed only the address of the corresponding author, and not that of all the authors in the paper; and
- (2) all the papers from WJG and their references were retrieved on May 4, 2009.

RESULTS

Outline of papers published in WJG 2006-2007

Two thousand five hundred and six papers were published in WJG in 2006-2007, including 1311 papers from the categories of article, review and proceedings in 2006 and 1024 from these categories in 2007. Among these papers, 1963 were cited 8788 times by SCIE. The mean number of citations was 3.51 per paper. The highest number of citations for any one paper was 98. The papers with one citation accounted for 25.11% of the total number of cited papers; those with two, 20.17%; those with three, 54.72%; and those with over three, 85.38% (Table 1). Thirteen papers were cited over 30 times (Table 2).

Citation analysis of papers published in WJG 2006-2007

Among the 2506 papers, nine belonged to the “other” category, which included author’s feedback, book reviews, commentaries, editorial announcements, memorials, and news. Within the other 15 categories in WJG, viral hepatitis, reviews and editorials had high citation rates of 92.73%, 92.86% and 92.31%,

Table 1 Citation frequency of papers published in WJG 2006-2007

Cited times	No. of cited articles		No. of cited times	
	Count	Percent (%)	Count	Percent (%)
1	493	25.11	493	5.61
2	396	20.17	792	9.01
3	270	13.75	810	9.22
4	175	8.91	700	7.97
5	160	8.15	800	9.10
6	107	5.45	642	7.31
7	77	3.92	539	6.13
8	56	2.85	448	5.10
9	41	2.09	369	4.20
10	42	2.14	420	4.78
11-20	111	5.65	1720	19.57
21-30	23	1.17	546	6.21
31-40	9	0.46	316	3.60
41-100	3	0.15	193	2.20
Total	1963	100.00	8788	100.00

respectively. The highest average number of citations for the top three columns was 8.89 for reviews, 6.43 for editorials, and 6.00 for topic highlights. The citation rates for case reports and letters to the editor were low, at 64.15% and 54.55%, respectively. Their average number of citations was 1.66 and 1.43, respectively, which were far lower than the total citation rate (78.33%) and average number of citations (3.51) (Table 3). In 2006 and 2007, the total citation rate (with the “other” category excluded) was 85.08% and 70.48%, and the total average number of citations was 4.33 and 2.51.

Geographic distribution of cited authors

The cited authors came from 70 countries. Twenty-nine countries or areas had over 10 cited papers, 11 countries between 5-9 papers, and 29 countries 1-4 papers. China, Japan and the United States were ranked as the top three countries according to the highest total number of citations. The countries with the highest citation rates and average number of citations per paper included Canada (96.30% and 6.89), Hungary (92.31% and 5.62), Australia (88.46% and 5.46), Germany (87.04% and 5.33), and Spain (87.50% and 5.11) (Table 4).

Year of citation for papers published in WJG 2006-2007

The year of citation is shown in Table 5. In 2008, papers published in 2006 and 2007 were cited 3079 and 1780 times, respectively. In 2009, the figures so far are 1101 and 931, respectively. From 2006 to 2009, the self citation rate was 22.16%, 11.33%, 9.41% and 5.01%, respectively.

Distribution of citing journals

The 8788 citations from WJG 2006-2007 were in 1597 journals (Table 6). The *American Journal of Gastroenterology*, *Alimentary Pharmacology & Therapeutics*, *Gastrointestinal Endoscopy* cited papers from WJG most often. Twelve journals each had 40-49 citations from WJG, nine journals had 30-39 citations, 27 journals had 20-29 citations, 115 journals had 10-19 citations, 245 journals

Table 2 List of papers published in *WJG* 2006-2007 with over 30 citations

Rank	First author	Title	Sources	Cited times
1	Crew KD	Epidemiology of gastric cancer	12(3): 354-362, 2006	98
2	Sebastiani G	Non invasive fibrosis biomarkers reduce but not substitute the need for liver biopsy	12(23): 3682-3694, 2006	53
3	Kim KP	Diagnostic criteria for autoimmune chronic pancreatitis revisited	12(16): 2487-2496, 2006	42
4	Tamura G	Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer	12(2): 192-198, 2006	40
5	Alter MJ	Epidemiology of hepatitis C virus infection	13(17): 2436-2441, 2007	39
6	Dietrich CF	Assessment of metastatic liver disease in patients with primary extrahepatic tumors by contrast-enhanced sonography versus CT & MRI	12(11): 1699-1705, 2006	36
7	Smith MG	Cellular and molecular aspects of gastric cancer	12(19): 2979-2990, 2006	36
8	Lakatos PL	Recent trends in the epidemiology of inflammatory bowel diseases: Up or down?	12(38): 6102-6108, 2006	35
9	Engwegen JYMN	Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry	12(10): 1536-1544, 2006	34
10	Glebe D	Viral and cellular determinants involved in hepadnaviral entry	13(1): 22-38, 2007	34
11	Hocke M	Contrast-enhanced endoscopic ultrasound in discrimination between focal pancreatitis and pancreatic cancer	12(2): 246-250, 2006	31
12	Schaefer S	Hepatitis B virus taxonomy and hepatitis B virus genotypes	13(1): 14-21, 2007	31
13	Danese S	Etiopathogenesis of inflammatory bowel diseases	12(30): 4807-4812, 2006	30

Table 3 Papers and their frequency of citation, according to category

Category	2006			2007			Total			Total citation rate	Total cited times
	No. of papers	Papers cited	Cited times	No. of papers	Papers cited	Cited times	Count of papers	Papers cited	Cited times		
Basic research	178	154	682	88	63	219	266	217	901	81.58	3.39
Case reports	216	167	477	208	105	226	424	272	703	64.15	1.66
Clinical research	93	84	454	67	49	176	160	133	630	83.13	3.94
Colorectal cancer	37	36	152	22	18	56	59	54	208	91.53	3.53
Editorial	85	82	664	58	50	255	143	132	919	92.31	6.43
Esophageal cancer	10	9	53	4	3	11	14	12	64	85.71	4.57
Gastric cancer	41	39	200	23	17	48	64	56	248	87.50	3.88
<i>H pylori</i>	17	14	34	10	9	15	27	23	49	85.19	1.81
Leading article	1	1	2			0	1	1	2	100.00	2.00
Letters to the editor	18	10	41	26	14	22	44	24	63	54.55	1.43
Liver cancer (cirrhosis)	34	30	171	27	21	59	61	51	230	83.61	3.77
Rapid communications	475	383	1593	391	268	773	866	651	2366	75.17	2.73
Reviews	75	72	860	37	32	136	112	104	996	92.86	8.89
Topic highlights	70	62	482	132	120	731	202	182	1213	90.10	6.00
Viral hepatitis	37	37	134	18	14	62	55	51	196	92.73	3.56
Other	1	0	0	7	0	0	8	0	0	0.00	0.00
Total	1388	1180	5999	1118	783	2789	2506	1963	8788	78.33	3.51

had 5-9 citations, 115 journals had four citations, 172 journals had three citations, 285 journals had two citations; and 598 journals had only one citation. These citations were in a wide range of related journals, which implies that *WJG* has a widespread academic impact.

DISCUSSION

The frequency with which papers from a given journal are cited is a measure of the academic profile and quality of the journal^[4]. It is accepted widely that the academic quality of a journal is correlated positively with number of cited numbers in the journal. In citation analysis of papers published from 2006 to 2007 in *WJG*, 78.33% of all published papers were cited by a journal included in SCIE. Table 1 shows that 1074 papers were cited more than three times, with a total of 7503 citations. These accounted for 54.72% of all papers and 85.38%

of the total number of citations. The highest number of citations for one paper was 98, and papers that were cited more than four times accounted for 40.96%.

The use of subsections or categories is useful for reflecting the scope of a given journal. The editors need periodically to evaluate their choice of subsections to facilitate development of the journal, enable academic exchange and attract the attention of readers with different information needs. Among all the categories in *WJG*, reviews, editorials, topic highlights and esophageal cancer were of most interest to readers. Papers in these categories showed a high citation rate and high average number of citations. However, the categories of *H pylori*, rapid communications, case reports, letters to the editor and leading articles had fewer citations, which may have resulted from the types of papers and current research trends, and deserves further attention from the journal editors.

Table 4 Geographic distribution of cited authors

Rank	Area	Paper	No. of cited papers	Percent (%)	Cited times	Average citations per paper	Rank	Area	Paper	No. of cited papers	Percent (%)	Cited times	Average citations per paper
1	China	635	451	71.02	1499	2.36	16	Netherlands	28	24	85.71	126	4.50
2	Japan	299	230	76.92	1035	3.46	17	Canada	27	26	96.30	186	6.89
3	United States	188	156	82.98	910	4.84	18	Australia	26	23	88.46	142	5.46
4	Germany	162	141	87.04	863	5.33	19	Hungary	26	24	92.31	146	5.62
5	Italy	151	129	85.43	619	4.10	20	Brazil	22	17	77.27	60	2.73
6	Turkey	111	80	72.07	235	2.12	21	Mexico	22	17	77.27	46	2.09
7	South Korea	91	66	72.53	213	2.34	22	Poland	21	17	80.95	61	2.90
8	Greece	82	56	68.29	173	2.11	23	Sweden	18	16	88.89	83	4.61
9	India	68	54	79.41	204	3.00	24	Denmark	17	15	88.24	50	2.94
10	Taiwan, China	64	50	78.13	206	3.22	25	Serbia	17	13	76.47	26	1.53
11	Spain	56	49	87.50	286	5.11	26	Israel	13	11	84.62	62	4.77
12	England	52	39	75.00	169	3.25	27	Egypt	12	8	66.67	19	1.58
13	France	52	44	84.62	257	4.94	28	Belgium	11	8	72.73	46	4.18
14	Iran	44	34	77.27	100	2.27	29	Ireland	11	10	90.91	41	3.73
15	Thailand	29	24	82.76	72	2.48		Total	1832	2355	77.79	7935	

Table 5 Year of citation for papers published in *WJG* 2006-2007

Publication year	2006			2007			2008			2009			Total		
	Other citation	Self citation	Total	Other citation	Self citation	Total	Other citation	Self citation	Total	Other citation	Self citation	Total	Other citation	Self citation	Total
2006	130	37	167	1424	167	1591	2817	262	3079	1049	52	1101	5420	518	5938
2007				110	29	139	1585	195	1780	883	48	931	2578	272	2850
Total	130	37	167	1534	196	1730	4402	457	4859	1932	100	2032	7998	790	8788
Percent (%)	77.84	22.16	100	88.67	11.33	100	90.59	9.41	100	94.99	5.01	100	91.01	8.99	100

Table 6 Journals with a high number of citations from *WJG* 2006-2007

No.	Journals	Citations	Proportion (%)
1	World Journal of Gastroenterology	790	8.99
2	American Journal of Gastroenterology	104	1.18
3	Alimentary Pharmacology & Therapeutics	102	1.16
4	Gastrointestinal Endoscopy	101	1.15
5	Digestive diseases and Sciences	90	1.02
6	Hepatology	90	1.02
7	Journal of Gastroenterology and Hepatology	88	1.00
8	Inflammatory Bowel Diseases	82	0.93
9	Current Opinion in Gastroenterology	78	0.89
10	Gastroenterology	76	0.86
11	Gut	75	0.85
12	Liver International	67	0.76
13	Journal of Hepatology	64	0.73
14	International Journal of Cancer	55	0.63
15	Zeitschrift für Gastroenterologie	54	0.61
16	European Journal of Gastroenterology & Hepatology	53	0.60
17	Pancreas	53	0.60
18	Revista Espanola de Enfermedades Digestivas	52	0.59
19	Journal of Gastroenterology	51	0.58
	Total	2125	24.18

The authors of articles published in *WJG* were from a wide range of countries. From 2006 to 2007, China, Japan, Germany, Italy, USA and Turkey were ranked the top six countries by the number of papers and total number of citations^[5]. The authors cited in *WJG*

were also from a large range of 70 countries or regions. The papers written by authors from Canada, Hungary, Australia, Germany and Spain also had a high number of citations.

The impact factor is a routine bibliometric index to evaluate the academic profile of journals. Similar to the number of cited papers, the impact factor is correlated positively with the academic impact. According the criteria for measuring the impact factor in the Journal Citation Report, only the paper types of article, review, and proceedings are taken into account for calculations. The impact factor of *WJG* in 2008 was 2.081 $[(3079 + 1780)/(1311 + 1024) = 2.081]$. Regarding publication year, the number of times a paper was cited was low in the year of publication. In the following 2 years, the number of citations increased rapidly, which is consistent with the finding that the peak number of citations is within 2 or 3 years for most publications^[6,7].

The number of journals that cite papers from a given journal reflects the academic profile of the journal, its influence, its impact on some specialties, and its attraction to readers from different fields. The papers published in *WJG* in 2006-2007 were cited 8788 times in 1597 journals. Given the wide range of citing papers and journals, as well as the large proportion of citations in the same field, it is clear that *WJG* has a widespread influence.

In conclusion, the setting of the subsections in *WJG* is appropriate. With a high self-citation rate, and citations in papers from different countries and different journals,

it appears that *WJG* is already on the right road and its academic impact and profile are expanding gradually.

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ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Events Calendar 2009

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Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
<http://www.easl.ch/>

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
<http://www.ddw.org>

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27, 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
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Practical Solutions for Successful Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
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November 15-19, 2009
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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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Supportive foundations: The complete name and number of supportive foundations should be provided, e.g., Supported by National Natural Science Foundation of China, No. 30224801

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G,** Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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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/eid.htm>

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- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Volume 15 Number 36 September 28, 2009

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^[1]Passed away on October 20, 2007

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World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 36
September 28, 2009



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Beijing Kexin Printing House

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PUBLICATION DATE

September 28, 2009

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CSSN

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Antioxidant therapy in the management of acute, chronic and post-ERCP pancreatitis: A systematic review

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Received: June 25, 2009 Revised: August 3, 2009

Accepted: August 10, 2009

Published online: September 28, 2009

Furthermore, antioxidant therapy including allopurinol and N-acetylcysteine failed to prevent the onset of PEP in almost all trials. In conclusion, the present data do not support a benefit of antioxidant therapy alone or in combination with conventional therapy in the management of AP, CP or PEP. Further double blind, randomized, placebo-controlled clinical trials with large sample size need to be conducted.

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Key words: Antioxidant; Post-endoscopic retrograde cholangio-pancreatography pancreatitis; Oxidative stress; Therapy; Acute pancreatitis; Chronic pancreatitis

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Mohseni Salehi Monfared SS, Vahidi H, Abdolghaffari AH, Nikfar S, Abdollahi M. Antioxidant therapy in the management of acute, chronic and post-ERCP pancreatitis: A systematic review. *World J Gastroenterol* 2009; 15(36): 4481-4490 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4481.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4481>

Abstract

We systematically reviewed the clinical trials which recruited antioxidants in the therapy of pancreatitis and evaluated whether antioxidants improve the outcome of patients with pancreatitis. Electronic bibliographic databases were searched for any studies which investigated the use of antioxidants in the management of acute pancreatitis (AP) or chronic pancreatitis (CP) and in the prevention of post-endoscopic retrograde cholangio-pancreatography (post-ERCP) pancreatitis (PEP) up to February 2009. Twenty-two randomized, placebo-controlled, clinical trials met our criteria and were included in the review. Except for a cocktail of antioxidants which showed improvement in outcomes in three different clinical trials, the results of the administration of other antioxidants in both AP and CP clinical trials were incongruent and heterogeneous.

INTRODUCTION

Pancreatitis, both chronic and acute, contributes to thousands of annual hospital admissions and consecutive complications^[1]. Acute pancreatitis (AP), an acute inflammatory condition, is thought to be due to activation of enzymes in the pancreatic acinar cells, with inflammation spreading into the surrounding tissues^[2]. Patients with AP were either treated with strict bowel rest or given parenteral nutrition to allow the pancreas to rest until the serum enzyme levels returned to normal^[3]. Chronic pancreatitis (CP) is a progressive inflammatory disorder that is characterized by recurrent episodes of severe abdominal pain. Affected patients typically suffer years of disabling pain, and conventional therapeutic interventions are often unable to offer satisfactory analgesia^[4].

Oxidative stress caused by short lived intracellular reactive oxygen and nitrogen species, can oxidize lipids in the cell membrane, proteins, depolarize the mitochondrial

membrane, and induce DNA fragmentation. Active free radicals in the body can be produced during diseases or exposure to xenobiotics^[5,6].

Basic and clinical evidence suggests that the pathogenesis of both AP and CP can be associated with oxidative stress seeming independent of the etiology of pancreatitis, because oxidative stress is observed in different experimental pancreatitis models^[7,8]. Findings show that free radical activity and oxidative stress indices such as lipid peroxide levels are higher in the blood and duodenal juice of patients with AP or CP^[9,10].

Based on the mentioned findings, the idea of using antioxidant regimens in the management of both AP and CP as a supplement and complementary in combination with its traditional therapy is rational and reasonable. As a result of this hypothesis, antioxidant therapy should improve the inflammatory process that is involved in pancreatitis and therefore ameliorate the recovery rate.

In addition, pancreatitis is the most common serious complication of endoscopic retrograde cholangiopancreatography (ERCP), occurring in 1%-7% of cases^[11]. Although, the exact mechanisms involved in the pathophysiology of post-ERCP pancreatitis (PEP) are not clear, the role of oxidative stress cannot be neglected. Therefore, the use of antioxidants before, during or after this intervention has already been studied in a few clinical trials^[12,13]. Although some clinical trials have proved the benefits of using various antioxidants in AP or CP, there are still controversies^[14].

To our knowledge, there is no definite consensus on the benefits of antioxidant therapy in the management of AP or CP. Our objective was to systematically review and summarize the literature on antioxidant therapies for AP and CP as well as PEP, to provide recommendations for future research.

METHODS

PubMed, Scopus, Google Scholar, Cochrane library database, and Evidence based medicine reviews were searched for any relevant studies that investigated the use of antioxidants in the management of AP or CP and in the prevention of PEP up to February 2009. We also hand-searched references in key articles. The search terms were: AP or CP, pancreatic inflammation, antioxidant, vitamin, superoxide dismutase, manganese, glutamine, butylated hydroxyanisole, taurine, glutathione, curcumin, catalase, peroxidase, lutein, xanthophylls, zeaxanthin, selenium, riboflavin, zinc, carotenoid, cobalamin, retinol, alpha-tocopherol, ascorbic acid, beta-carotene, carotene and all MeSH terms for pharmacologically active antioxidants. Studies were limited to clinical trials and those written in the English language.

To assess the quality of clinical trials, we employed the Jadad score, a previously validated instrument that assesses trials based on appropriate randomization, blinding, and description of study withdrawals or dropouts^[15]. The description of this score is as follows: (1) whether randomized (yes = 1 point, no = 0); (2) whether

randomization was described appropriately (yes = 1 point, no = 0); (3) double-blind (yes = 1 point, no = 0); (4) was the double-blinding described appropriately (yes = 1 point, no = 0); (5) whether withdrawals and dropouts were described (yes = 1 point, no = 0). The quality score ranges from 0 to 5 points; a low-quality report score is ≤ 2 and a high-quality report score is at least 3.

Data synthesis was conducted by three reviewers who read the title and abstract of the search results separately to eliminate duplicates, reviews, case studies, and uncontrolled trials. The inclusion criteria were that the studies should be clinical trials which used an antioxidant for the treatment or prevention of pancreatitis. Outcomes of the studies were not the point of selection and all studies that analyzed the effects of an antioxidant on pancreatitis, from pain reduction^[16] to changes in plasma cytokines, were included.

Data from selected studies were extracted in the form of 2×2 tables. All included studies were weighted and pooled. The data were analyzed using Statsdirect (2.7.3). Relative risk (RR) and 95% confidence intervals (95% CI) were calculated using the Mantel-Haenszel and DerSimonian-Laird methods. The Cochran Q test was used to test heterogeneity. The event rate in the experimental (intervention) group against the event rate in the control group was calculated using L'Abbe plot as an aid to explore the heterogeneity of effect estimates. Funnel plot analysis was used as a publication bias indicator.

RESULTS AND DISCUSSION

A total of 211 potentially relevant papers were identified, of which 22 papers were eligible^[4,16-36]. Amongst the 22 papers, 19 (86%) scored 3 and only three studies^[17,25,31] scored 2 or lower according to the Jadad score. Table 1 presents controlled clinical trials of antioxidants in patients with AP or CP. Trials that used antioxidants to prevent PEP are summarized in Table 2. To perform a meta-analysis we included only four studies in which allopurinol was used in PEP.

Antioxidants in AP and CP

Glutamine: Glutamine is the most abundant amino acid both in plasma and in the intracellular free amino acid pool. It is essential for a wide variety of physiologic processes, in particular, the growth and function of immune cells including lymphocytes and macrophages^[17]. Glutamine is normally synthesized *de novo* by a number of cells and therefore is not an essential amino acid. Although glutamine is an antioxidant, in conditions of excess glutamine utilization such as sepsis, trauma, major surgery or severe AP, endogenous glutamine production may not be adequate and glutamine depletion occurs^[23].

In four studies^[17,18,22,23] glutamine was supplemented to standard total parenteral nutrition (TPN) in AP patients. In one randomized controlled study ($n = 28$), glutamine was used in AP in combination with standard TPN and demonstrated a decrease in the duration of TPN therapy and hospitalization without a change in the total

Table 1 Controlled clinical trials of antioxidants in patients with acute or chronic pancreatitis

Study/ Ref.	Drug/supplements	Study design	Jadad score	Participants	Treatment (intervention)		Outcome (results)		Adverse effects/events
					Case	Control	Clinical	Laboratory	
Bhardwaj <i>et al</i> ^[10] 2009	Combined antioxidant (organic selenium, vitamin C, β -carotene, α -tocopherol and methionine)	Randomized; double blind; placebo- controlled	5	147 patients with CP	71 patients; combined antioxidants: 600 μ g organic selenium, 0.54 g ascorbic acid, 9000 IU β -carotene, 270 IU α -tocopherol and 2 g methionine; per day; for 6 mo	76 patients; placebo	Number of painful days per month ² Numbers of oral analgesic tablets and parenteral analgesic injections per month ² Hospitalization ² Percentage of patients become pain-free ² Number of man-days lost per month ²	Lipid peroxidation (TBARS) ² Serum SOD ² Total antioxidant capacity (FRAP) ¹ Serum vitamin A ¹ Serum vitamin C ¹ Serum vitamin E ¹ Erythrocyte superoxide dismutase ²	Headache & constipation (all during the first month of treatment)
Xue <i>et al</i> ^[17] 2008	Glutamine	Randomized	1	80 patients with severe AP	38 patients; 100 mL/d of 20% AGD intravenous infusion; for 10 d; starting on the day 1 (Early treatment)	38 patients; 100 mL/d of 20% AGD intravenous infusion/for 10 d starting on the day 5 (Late treatment)	Infection rate ² Operation rate ² Mortality ² Hospitalization ² Duration of ARDS ² Renal failure ² Acute hepatitis ² Encephalopathy ² Enteroparalysis ² Duration of shock ² 15-d APACHE II core ² Infectious morbidity ² Hospital stay day ³ Mortality ³	-	-
Fuentes- Orozco <i>et al</i> ^[18] 2008	Glutamine	Randomized; double blind; controlled	4	44 patients with AP	22 patients; 0.4 g/kg per day of L-alanyl-L- Glutamine in standard TPN; 10 d	22 patients; standard TPN; 10 d		Serum IL-10 ¹ Serum IL-6 ² CRP ² Ig A ¹ Protein ¹ Albumin ¹ Leucocyte ² Total lymphocyte ¹ Nitrogen balance was (+) in treated group vs (-) in control group	-
Siriwardena <i>et al</i> ^[19] 2007	Combined antioxidant (N-acetylcysteine, selenium, vitamin C)	Randomized; double blind; placebo- controlled	5	43 patients with severe AP	22 patients; N-acetylcysteine, selenium and vitamin C; for 7 d	21 patients; placebo	Organ dysfunction ³ APACHE-II ³ Hospitalization ³ All case mortality ³ Quality of life ¹ Pain ²	Serum vitamin C ³ Serum selenium ³ GSH/GSSG ratio ³ CRP ³	-
Kirk <i>et al</i> ^[4] 2006	Combined antioxidant (selenium, β -carotene, L-methionine, vitamins C and E)	Randomized; double-blind; placebo- controlled; crossover	4	36 patients with CP	36 patients; Antiox tablet: 75 mg of selenium, 3 mg β -carotene, 47 mg vitamin E, 150 mg vitamin C, and 400 mg methionin; four times per day; for 10 wk	36 patients; placebo; four times per day; for 10 wk	Physical and social functioning ¹ Health perception ¹ Emotional functioning, energy, mental health ³ Pain ³	Plasma selenium ¹ Plasma vitamin C ¹ Plasma vitamin E ¹ Plasma β -carotene ¹	Two patients complained of nausea and one of an unpleasant taste during treatment with Antox
Durgaprasad <i>et al</i> ^[20] 2005	Curcumin	Randomized; single blind; placebo- controlled	3	20 patients of tropical pancreatitis (CP)	Eight patients; capsule: 500 mg curcumin (95%) with 5 mg of piperine; three times per day; for 6 wk	Seven patients; placebo (lactose)		Erythrocyte MDA ² GSH level ³	-

Du <i>et al</i> ^[21] 2003	Vitamin C	Randomized; controlled	3	84 patients with AP	40 patients; IV vitamin C; 10 g/d; for 5 d	44 patients; IV vitamin C; 1 g/d; for 5 d	Hospitalization ² Deterioration of disease ² Improvement of disease ¹ Cure rate ¹	Tnf- α ² IL-1 ² IL-8 ² CRP ² Serum interleukin-2 receptor ² Plasma vitamin C ¹ Plasma liperoxide ¹ Plasma vitamin E ¹ Plasma β -carotene ¹ Whole blood glutathione ¹ Activity of erythrocyte superoxide dismutase ¹ Erythrocyte catalase ¹ Cholinesterase ¹ Albumin ¹ lymphocyte count ¹ CRP ²	-
Ockenga <i>et al</i> ^[22] 2002	Glutamine	Randomized, double-blind; controlled	4	28 patients with AP	Standard TPN which contains 0.3 g/kg per day L-alanine-L-glutamine; at least 1 wk	Standard TPN	Hospitalization ² Duration of TPN ² Cost of TPN ³	-	-
de Beaux <i>et al</i> ^[23] 1998	Glutamine	Randomized; double-blind; controlled	5	14 patients with AP	Six patients; 0.22 g/kg per day of glycyl-L-glutamine in standard TPN; for 7 d	Seven patients; standard TPN	-	Lymphocytic proliferation (by DNA synthesis) ¹ TNF ³ IL-6 ³ IL-8 ² Uric acid level ²	-
Banks <i>et al</i> ^[24] 1997	Allopurinol	Randomized, double-blind, two-period crossover clinical trial	4	13 patients with CP	13 patients; 300 mg/d allopurinol; 4 wk	13 patients; placebo	Pain ³	-	-
Sharer <i>et al</i> ^[25] 1995	Glutathione precursors (S-adenosyl methionine and N-acetyl(cysteine)	Randomized	-	79 patients with AP	SAMe 43 mg/kg and N-acetyl(cysteine 300 mg/kg	-	APACHE II score reduction ³ Complication rate ³ Days in hospital ³ Mortality ³	-	-
Bilton <i>et al</i> ^[26] 1994	S-adenosyl methionine (SAMe)	Randomized; double-blind; crossover; placebo-controlled	5	20 patients with AP or CP	20 patients; SAMe 2.4g/d; 10 wk	Placebo	Attack rate and background pain ³	Free radical activity ² Serum selenium ² Serum β -carotene ² Serum vitamin E ^{2,3} Serum vitamin C ² Serum SAMe ¹ Free radical activity ² Serum selenium ² Serum β -carotene ¹ Serum vitamin E ^{1,3} Serum vitamin C ² Serum SAMe ¹	-
Salim ^[27] 1991	Selenium and β -carotene + SAMe	Randomized; double-blind; placebo-controlled	4	78 patients with CP	20 patients; SAMe 2.4 g/d, Selenium 600 μ g and β -carotene 9000 IU; 10 wk	27 patients; placebo with analgesic regimen	Pain ² Hospitalization ² Epigastric tenderness ²	WBC count ² Serum amylase ² Serum LDH ²	Allergies General malaise Headache Nausea Vomiting Dyspepsia Abdominal pain

Uden <i>et al.</i> ^[28,29] 1992, 1990	Combined antioxidant (selenium, β -carotene, vitamin C, vitamin E, methionine)	Randomized; double-blind; crossover; placebo-controlled	5	28 patients with CP	23 patients; daily doses of 600 mg organic selenium, 9000 IU β -carotene, 0.54 g vitamin C, 270 IU vitamin E and 2 g methionine; 10 wk	23 patients; placebo	Pain ²	Free radical activity ² Serum selenium ¹ Serum β -carotene ¹ Serum vitamin E ¹ Serum SAMe ²	-
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¹Significant increase as compared with control; ²Significant decrease as compared with control; ³No significant difference between groups. TBARS: Thiobarbituric acid reactive substances; FRAP: Ferric reducing antioxidant power; SOD: Superoxide dismutase; AGD: Alanine-glutamine dipeptide; CRP: C-reactive protein; MDA: Malondialdehyde; LDH: Lactate dehydrogenase; APACHE II: Acute Physiology and Chronic Health Evaluation II; GSH: Glutathione; TPN: Total parenteral nutrition; TNF- α : Tumor necrosis factor- α ; IL: Interleukin.

Table 2 Controlled clinical trials of antioxidant therapy to prevent post-ERCP pancreatitis

Ref.	Drug/supplements	Study design	Jadad score	n	Treatment (intervention)		Outcome (results)		Adverse effects/events	Other comments
					Case	Control	Primary	Other		
Romagnuolo <i>et al.</i> ^[30] 2008	Allopurinol	Randomized; double-blind; placebo-controlled	4	586	293 patients; 300 mg oral allopurinol 60 min before ERCP	293 patients; placebo	Rate of PEP ³ (5.5% vs 4.1%)	Disease-related adverse events ³ Procedure-related complications ³ Hospitalization ³	-	In the non-high-risk group ($n = 520$), the crude PEP rates were 5.4% for allopurinol and 1.5% for placebo ($P = 0.017$), favoring placebo, indicating harm associated with allopurinol, whereas in the high-risk group ($n = 66$), the PEP rates were 6.3% for allopurinol and 23.5% for placebo ($P = 0.050$), favoring allopurinol
Milewski <i>et al.</i> ^[31] 2006	N-acetylcysteine	Randomized; placebo-controlled	2	106	55 patients; 600 mg oral N-acetylcysteine 24 and 12 h before ERCP and 1200 mg IV for 2 d after the ERCP	51 patients; isotonic IV saline twice for 2 d after the ERCP	Rate of PEP ³ (7.3% vs 11.8%)	Urine amylase activity ³ Serum amylase activity ³	-	-
Katsinelos <i>et al.</i> ^[32] 2005	Allopurinol	Randomized; double-blind; placebo-controlled	4	250	125 patients; 600 mg oral allopurinol 15 and 3 h before ERCP	118 patients; placebo	Rate of PEP ² (3.2% vs 17.8%)	Hospitalization ² Severity of pancreatitis ²	-	-
Katsinelos <i>et al.</i> ^[33] 2005	N-acetylcysteine	Randomized; double-blind; placebo-controlled	3	256	124 patients; 70 mg/kg 2 h before and 35 mg/kg at 4 h intervals for a total of 24 h after the procedure	125 patients; placebo (normal saline solution)	Rate of PEP ³ Hospitalization ³	-	Nausea; skin rash; diarrhea; vomiting	-
Mosler <i>et al.</i> ^[34] 2005	Allopurinol	Randomized; double-blind; placebo-controlled	4	701	355 patients; 600 mg 4 h and 300 mg 1 h oral allopurinol before ERCP	346 patients; placebo	Rate of PEP ³ (13.0% vs 12.1%)	Severity of pancreatitis ³	-	-
Lavy <i>et al.</i> ^[35] 2004	Natural β -carotene	Randomized; double-blind; placebo-controlled	5	321	141 patients; 2 g oral β -carotene 12 h before ERCP	180 patients; placebo	Rate of PEP ³ (10% vs 9.4%)	Severe pancreatitis ²	-	-
Budzynska <i>et al.</i> ^[36] 2001	Allopurinol	Randomized; placebo-controlled	3	300	99 patients; 200 mg oral allopurinol 15 and 3 h before ERCP	101 patients; placebo	Rate of PEP ³ (12.1% vs 7.9%)	Severity of pancreatitis ³	-	-

¹Significant increase as compared with control; ²Significant decrease as compared with control; ³No significant difference between groups. PEP: Post endoscopic pancreatitis.

cost of parenteral feeding^[22]. Another similar study ($n = 44$) showed that even though TPN therapy containing glutamine reduces infectious morbidity, it has no significant effect on hospitalization and total mortality^[18]. However, both studies showed laboratory improvement in AP after administration of glutamine such as an increase in serum albumin or decrease in C-reaction protein (CRP).

Proinflammatory cytokine release was assessed in another study with a small patient number ($n = 14$). Glutamine supplementation did not significantly influence tumor necrosis factor- α or interleukin (IL)-6 release, but, in contrast, median IL-8 release was reduced by day 7 in the glutamine group while it was increased in the conventional group^[23]. Another non-blinded study examined the administration of glutamine in AP for 10 d starting either on the day of admission or 5 d after admission. Investigators reported an improvement in all clinical findings including hospitalization, infection, and mortality rate^[17]. No adverse effects were reported in these trials.

Allopurinol: Allopurinol, a xanthine oxidase inhibitor that historically has been effective in preventing attacks of acute gouty arthritis, is an effective anti-oxidant with anti-apoptotic effects. It has been shown that allopurinol is a hydroxyl radical scavenger^[37,38]. Two studies used allopurinol to reduce chronic pain in CP^[24,27]. In one clinical trial ($n = 78$), CP patients with chronic pain were admitted to hospital and received an analgesic regimen of pethidine with or without allopurinol. Their results showed that allopurinol could reduce pain and gastric tenderness. Hospitalization was also decreased in allopurinol-treated patients^[27]. Another clinical study ($n = 13$) showed that 4 wk of allopurinol administration did not reduce pain in CP when compared with placebo^[24]. Allergy, general malaise, and gastrointestinal disturbances were adverse events of allopurinol.

Vitamin C: Ascorbic acid or vitamin C is a monosaccharide antioxidant. This water-soluble vitamin is a reducing agent and can neutralize oxygen species. Vitamin C is an important antioxidant which protects the body from damage caused by inflammation, and high-dose vitamin C can improve immune function^[21]. Vitamin C alone was only investigated in one study and other studies used vitamin C in combination with other antioxidants which will be discussed later. In one randomized study ($n = 83$), 10 g/d of vitamin C was used intravenously compared to 1 g/d of vitamin C in the control group for 5 d in patients with AP. Their results indicated that 10 g vitamin C decreases hospitalization and duration of disease, and increases the cure rate. Proinflammatory cytokines and CRP were also diminished by vitamin C administration^[21].

Combined antioxidants (selenium, β -carotene, vitamin C, vitamin E and methionine): A combination of various antioxidants including selenium, β -carotene, vitamin C, vitamin E, and methionine was studied in three controlled clinical trials^[4,16,28]. In the first

clinical trial, the efficacy of antioxidant therapy in the management of pancreatitis was determined using the above combination in CP patients ($n = 28$). Their results showed that this cocktail can reduce the pain which is experienced by patients^[28]. Another study with a slightly larger sample size ($n = 36$) used the above combination at the same doses but with greater bioavailability in CP patients. In this trial, congruent with the previous trial, pain was reduced after 10 wk of the combined antioxidants. Indeed, quality of life, physical and social functioning, and health perception were also enhanced as a result of antioxidant therapy^[4]. The latest published controlled clinical trial in the field of antioxidants and pancreatitis has also used this combination at the same doses as the previous studies. In this larger clinical trial ($n = 147$), the antioxidants were administered for 6 mo, and showed that, similar to the two preceding trials, pain and hospitalization were reduced^[16]. All three studies showed that serum concentrations of the above-mentioned antioxidants were higher after a period of intake and laboratory indices of oxidative stress markers such as lipid peroxidation, free radical activity, and total antioxidant capacity improved after therapy. Another cohort study which is not presented in Table 1 examined this combination of antioxidants in 12 CP patients and showed that this combination reduces pain and hospitalization^[39]. Headache, nausea, vomiting, and constipation were some of the adverse effects of this combination. A clinical trial studied the effect of selenium, vitamin C and N-acetylcysteine (NAC) combination for 7 d in 43 AP patients. All primary endpoints including hospitalization, Acute Physiology and Chronic Health Evaluation II score, and organ dysfunction were statistically similar between the placebo and antioxidant-treated groups^[19].

Curcumin: Curcumin is a polyphenolic compound commonly found in the dietary spice turmeric^[40]. Curcumin is an inhibitor of nuclear factor- κ B and has various biological activities such as anti-inflammatory, antioxidant, antiseptic, and anticancer activity^[41]. In the one available pilot study ($n = 20$), patients with CP received 500 mg of curcumin with 5 mg of piperine or placebo for 6 wk. There was a significant reduction in erythrocyte malondialdehyde levels following curcumin therapy when compared with placebo. A significant increase in glutathione (GSH) levels was also observed. There was no corresponding improvement in pain and no adverse effects were reported^[20].

Glutathione precursors [S-adenosyl methionine (S-AdoMet)]: S-AdoMet, a highly bioactive metabolite of methionine is a precursor of glutathione, which is the key defense against reactive species. Of the two clinical trials that examined S-AdoMet in pancreatitis, in one, S-AdoMet was administered to AP patients^[25] and in the other, S-AdoMet was administered to CP patients^[26]. S-AdoMet did not enhance the clinical outcomes in either AP or CP patients. However, laboratory indices such as free radical activity were better after 10 wk of S-AdoMet administration

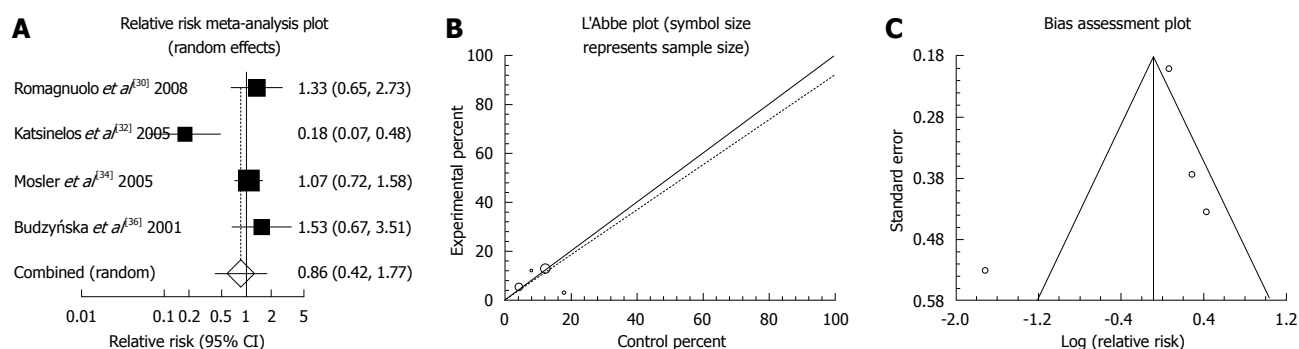


Figure 1 Individual and pooled relative risk (A), heterogeneity indicators (B), and publication bias indicators (C) for the outcome “prevention of all kinds of pancreatitis” in the studies considering allopurinol vs placebo therapy.

in CP patients. Methionine in combination with other antioxidants was discussed previously under the topic of combined antioxidants.

Antioxidants in PEP

NAC: NAC is a free radical scavenger capable of stimulating glutathione synthesis. NAC was used in two clinical trials. In one of these trial ($n = 106$), 600 mg NAC was given orally 24 h and 12 h before ERCP and 600 mg was given intravenously, twice a day for 2 d after ERCP. Their results showed that the rate of PEP was not significantly reduced. In addition, urine amylase activity, total bilirubin, alanine, aspartate aminotransferases and white blood cells showed no change^[31].

In the other double-blind, placebo-controlled trial ($n = 256$), patients received intravenous NAC at a loading dose of 70 mg/kg 2 h before and 35 mg/kg at 4-h intervals for a total of 24 h after the procedure. Similar to the previous study, there were no statistical differences in the incidence or severity of PEP grades between the groups. The mean duration of hospitalization for pancreatitis was not different in the NAC group as compared to the placebo group^[33]. The results of those studies showed the absence of any beneficial effect of NAC on the incidence and the severity of ERCP-induced pancreatitis.

Natural β -Carotene: β -carotene is a natural antioxidant which has been used as a supplement in various conditions. In a double-blind trial, 321 patients were given a single dose of natural β -carotene, 12 h prior to the procedure, and monitored for procedure complications, antioxidant levels, and plasma oxidation for 24 h post-procedure. The overall incidence of AP was not significantly different between the β -carotene and the placebo groups. The rate of severe pancreatitis was lower in the β -carotene-treated group. No reduction in the incidence of PEP was reported but there may be some protective effect of treatment with β -carotene regarding the severity of disease. Adverse events were not reported^[35].

Allopurinol: There were four randomized clinical trials which used allopurinol orally before ERCP to prevent

Table 3 Studies evaluating post-ERCP pancreatitis after allopurinol administration

Study	Allopurinol	Placebo
Romagnuolo <i>et al</i> ^[30] 2008	16/293	12/293
Katsinelos <i>et al</i> ^[32] 2005	4/121	21/118
Mosler <i>et al</i> ^[34] 2005	46/355	42/346
Budzyńska <i>et al</i> ^[36] 2001	12/99	8/101

PEP (Table 3). These studies were meta-analyzed for their primary PEP outcome. The summary RR for “prevention of all kinds of pancreatitis” in the four trials^[30,32,34,36] was 0.86 with a 95% CI of 0.42-1.77 and a non-significant RR ($P = 0.6801$, Figure 1A). The Cochrane Q test for heterogeneity indicated that the studies were heterogenous ($P = 0.0062$, Figure 1B) and could not be combined. Thus the random effect for individual and the summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among allopurinol *vs* placebo therapy was -1.961983 (95% CI: -14.671469 to 10.747502, $P = 0.5749$), and Kendall’s test on standardized effect *vs* variance indicated tau = 0, $P = 0.75$ (Figure 1C). The summary RR for “prevention of mild pancreatitis” in three trials^[30,32,36] was 1.08 with a 95% CI of 0.7-1.67, a non-significant RR ($P = 0.7238$, Figure 2A). The Cochrane Q test for heterogeneity indicated that the studies were homogenous ($P = 0.2255$, Figure 2B) and could be combined. Thus the fixed effect for individual and the summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among allopurinol *vs* placebo therapy could not be calculated because of too few strata. The summary RR for “prevention of moderate pancreatitis” in the three trials^[30,32,36] was 0.655 with a 95% CI of 0.388-1.105 and a non-significant RR ($P = 0.113$, Figure 3A). The Cochrane Q test for heterogeneity indicated that the studies were homogenous ($P = 0.0614$, Figure 3B) and could be combined. Thus the random effect for individual and the summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among allopurinol *vs* placebo therapy could not be calculated because of too few strata.

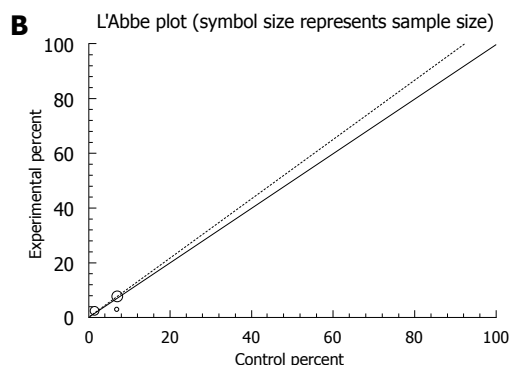
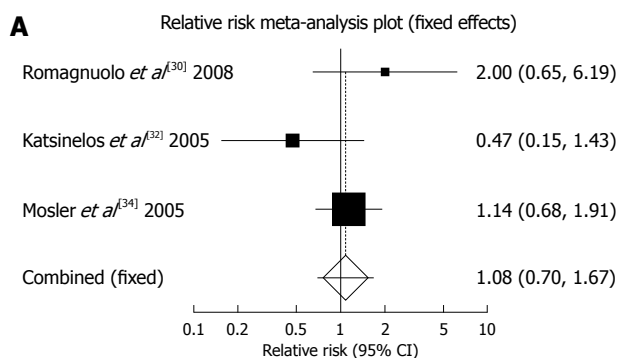


Figure 2 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome “prevention of all kinds of pancreatitis” in the studies considering allopurinol vs placebo therapy.

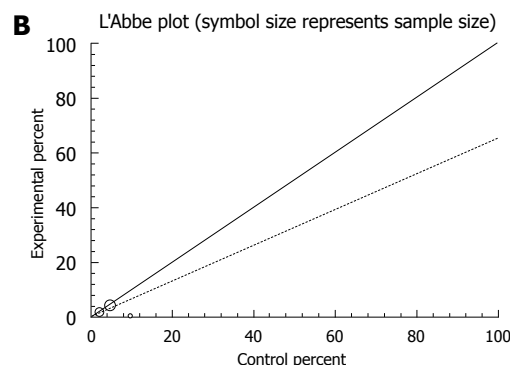
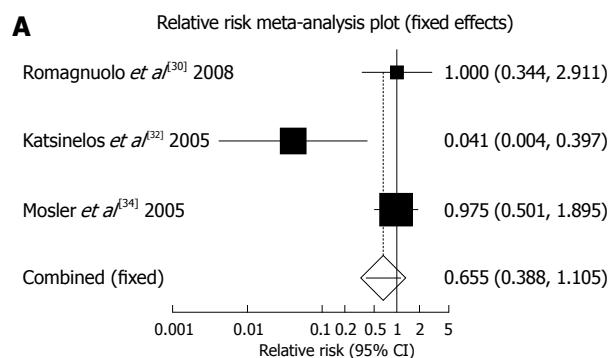


Figure 3 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome “prevention of moderate pancreatitis” in the studies considering allopurinol vs placebo therapy.

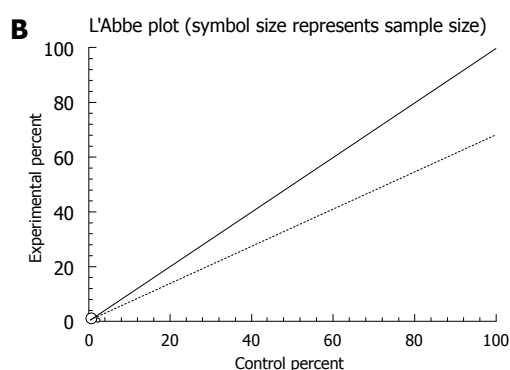
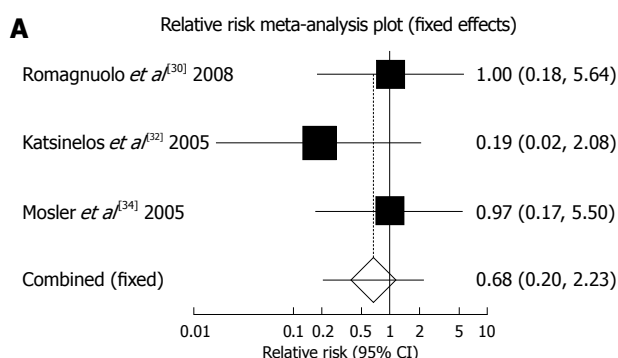


Figure 4 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome “prevention of severe pancreatitis” in the studies considering allopurinol vs placebo therapy.

The summary RR for “prevention of severe pancreatitis” of allopurinol vs placebo therapy among the three trials^[30,32,36] was 0.68 with a 95% CI of 0.2-2.23,

indicating a non-significant RR for allopurinol administration ($P = 0.5206$, Figure 4A). The Cochrane Q test for heterogeneity indicated that the studies were not significantly heterogeneous ($P = 0.6154$, Figure 4B) and the fixed effects for individual and the summary of RR was applied. Regression of normalized effect vs precision for all included studies for any adverse events among allopurinol vs placebo therapy could not be calculated because of too few strata.

Oxygen radicals play an essential role in the development of inflammation in various conditions^[42-50]. The involvement of free radicals in the pathogenesis of pancreatitis has been shown in both animal and human studies^[51]. Oxidative stress expedites mechanisms which lead to cell damage. It can directly destruct the cell membrane, accelerate lipid peroxidation, deplete cell reserves of antioxidants, and change signaling pathways inside the cells^[52,53].

Although the pathophysiology of pancreatitis has been studied before, there is no specific therapy for this disastrous disease yet. Enteral or parenteral nutrition, antibiotic therapy, surgical procedures such as removal of abscess and necrosis, and cholecystectomy have been developed to treat AP^[14]. In CP, pain management and probably surgical resection of pseudocysts are the goals of treatment. Because these treatments do not target the main problem and are recommended for symptoms and complications, investigators are still looking for new effective approaches in combination with current treatment.

Clinical studies of the evaluation of typical antioxidants on AP and CP were performed firstly at Man-

chester Royal Infirmary by Braganza and her colleagues. Two placebo controlled clinical trials^[28,29] examining combined antioxidant therapy on recurrent CP showed a significant decrease in pain and an elevation in serum antioxidant biomarkers; however, in one study in which SAME was examined as an antioxidant, alone or in combination with selenium and β -carotene, the results showed that SAME was ineffective in patients with recurrent pancreatitis. Another two recently published clinical trials^[4,16], particularly the latter study with a larger number of subjects (147), which used the same cocktail of antioxidants also showed pain reduction after administration. Therefore, the results of these studies show that such a combination of antioxidants could have a positive effect in the treatment of CP. However, we were unable to meta-analyze these three studies for pain as the primary outcome because pain reduction was assessed in a different way in each study. Except for the mentioned antioxidant cocktail, results of the administration of other antioxidants in both AP and CP clinical trials were incongruent and heterogeneous; and we cannot draw a definite conclusion on the efficacy of such therapy in the management of pancreatitis. We also evaluated the effect of etiology of pancreatitis including alcoholic, gallstone or idiopathic on the results of pain reduction and other outcomes, however, there was no relation between the cause of pancreatitis and clinical outcomes.

Furthermore, antioxidant therapy failed to prevent the onset of PEP in almost all trials (Table 2). Only one clinical trial in which 600 mg of allopurinol was administered twice before ERCP showed a significant decrease in the rate of PEP. However, our meta-analysis revealed that the RR for “prevention of mild, moderate and severe pancreatitis” of allopurinol *vs* placebo therapy was non-significant for allopurinol administration.

However, the present review indicates that there is insufficient data to support using antioxidants alone or in combination with conventional therapy in the management of AP, CP or PEP. Further double blind, randomized, placebo-controlled clinical trials with a larger sample size need to be conducted.

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S- Editor Li LF L- Editor Webster JR E- Editor Zheng XM

Adenosine: An immune modulator of inflammatory bowel diseases

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Author contributions: Ye JH and Rajendran VM both wrote the paper.

Supported by A research grant (DK-018777) from the National Institute of Diabetes and Digestive and Kidney Diseases

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Received: April 10, 2009 Revised: August 25, 2009

Accepted: September 1, 2009

Published online: September 28, 2009

Abstract

Inflammatory bowel disease (IBD) is a common and lifelong disabling gastrointestinal disease. Emerging treatments are being developed to target inflammatory cytokines which initiate and perpetuate the immune response. Adenosine is an important modulator of inflammation and its anti-inflammatory effects have been well established in humans as well as in animal models. High extracellular adenosine suppresses and resolves chronic inflammation in IBD models. High extracellular adenosine levels could be achieved by enhanced adenosine absorption and increased *de novo* synthesis. Increased adenosine concentration leads to activation of the A2a receptor on the cell surface of immune and epithelial cells that would be a potential therapeutic target for chronic intestinal inflammation. Adenosine is transported *via* concentrative nucleoside transporter and equilibrative nucleoside transporter transporters that are localized in apical and basolateral membranes of intestinal epithelial cells, respectively. Increased extracellular adenosine levels activate the A2a receptor, which would reduce cytokines responsible for chronic inflammation.

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Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Epithelial cells; Membrane transporters; Immuno-modulator

Peer reviewers: Akira Andoh, MD, Department of Internal Medicine, Shiga University of Medical Science, Seta Tukinowa, Otsu 520-2192, Japan; Elke Cario, MD, Division of Gastroenterology and Hepatology, University Hospital of Essen, Instituts group I, Virchowstr. 171, Essen D-45147, Germany

Ye JH, Rajendran VM. Adenosine: An immune modulator of inflammatory bowel diseases. *World J Gastroenterol* 2009; 15(36): 4491-4498 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4491.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4491>

INTRODUCTION

Adenosine is a purine molecule necessary for normal cell metabolism and growth. Recently, adenosine has been recognized as a potential anti-inflammatory molecule. In general, cellular adenosine is produced by both *de novo* synthesis and by absorption from the diet into the body through transporters in the gastrointestinal tract. It is thought that activation of adenosine receptors deactivates the synthesis of critical components necessary for activation of chronic inflammatory diseases, including inflammatory bowel disease (IBD). Many reviews have focused on the general aspects of adenosine activation of its receptors in inflamed tissues. This review focuses on the identification of the role of intestinal epithelial cell adenosine transporters during IBD.

CURRENT UNDERSTANDING OF INFLAMMATORY BOWEL DISEASES

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a common and lifelong disabling gastrointestinal disease^[1,2]. It has highest incidence and prevalence in the developed countries. The worldwide incidence varies greatly with that of UC ranging from 0.5-24.5/100 000 and that of CD ranging from 0.1-16/100 000 in different populations. There are more than 2 million IBD patients in the United States^[3]. The precise mechanism of IBD is still unknown. CD and UC differ in their histological presentation and cytokine profile. The accumulated data indicate that IBD results from a complex interplay of genetic, environmental, and immunologic factors. The presence of one or more genetically determined defects leads to an over-reaction of the host mucosal

immune system to normal constituents of the mucosal microflora. The genetically determined alterations of gut epithelial barrier function enhance exposure of the mucosal immune system to microflora components. The over-reaction causes either a Th1-type T cell-mediated inflammation (Crohn's disease) or a Th2-type T cell-mediated inflammation (ulcerative colitis). Multiple cytokines are released in the inflammatory process. The most important factors are tumor necrosis factor (TNF)- α , interleukin (IL)-1, interferon (INF)- γ , IL-6, 12, 13 and 17, monocyte chemotactic protein (MCP)-1 and IL-8^[4]. These cytokines attract and activate neutrophils, eosinophils, mast/plasma cells and macrophages. These inflammatory cells produce large amounts of unstable chemical species such as reactive oxygen species (ROS) or oxyradicals (i.e. superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitrite), resulting in tissue injury^[5,6].

Since the cause of IBD is still unknown, currently available treatments for the disease are non-specific and may cause side effects such as osteoporosis and suppression of the immune system. Many patients respond and maintain remission with existing therapy. Thus, at present, there is no cure for IBD. But for some patients, the available therapeutic options for IBD are still inadequate. The conventional treatments use corticosteroids, mesalamine, and immunosuppressants. These either nonspecifically block downstream inflammatory events, such as the secretion of cytokines and activation of immunocytes and neutrophils, or increase tissue adenosine levels, regardless of the nature of the underlying T cell response that generated these events. These agents have been used for treatment of mild and moderate IBD with some success for many years despite shortcomings and toxicities. The newer therapies using biologics, such as antibodies against TNF- α and α -integrin molecules, eliminate a specific major inflammatory cytokine or act by disrupting accumulation of cells at areas of inflammation. Both strategies have been successful in subsets of IBD patients but have also been associated with significant complications including fatal infections^[7-12].

Emerging treatments are being developed to target the hierarchy of the inflammatory cytokine effect including IL-12/IL-23^[13], IFN- γ ^[14], IL-6^[15], and IL-10 levels^[16]. Several antibodies currently on clinic trial include: anti-IL-12p40, an antibody against IL-12 and IL-23, the master cytokines underlying the Th1 response, for Crohn's disease^[13]; anti-IL-23p19, a potentially useful treatment for patients with resistance to anti-TNF therapy which acts by targeting IL-23 and IL-17 rather than IL-12 and IFN- γ in experimental colitis. Other approaches to the treatment of IBD currently under investigation are leukocytapheresis to eliminate effector cells^[17,18], administration of probiotics, use of GM-CSF to enhance innate immune function^[19], administration of microbe-derived agents or intestinal parasites to activate the innate immune system by inducing counter-regulatory immune responses to quell established inflammation^[20], administration of anti-CD3 antibodies^[21], autologous

hematopoietic stem cell transplant^[22], extracorporeal photophoresis to restore immunoregulation^[23], and adipose stem cell infusion^[24].

ADENOSINE MODULATES CHRONIC INFLAMMATION IN IBD

Adenosine exerts broad biologic effects, including smooth muscle contraction, neurotransmission in the peripheral and central nervous systems, platelet aggregation, pain, exocrine and endocrine secretion, lipolysis, glycogenesis, immune system development and response (e.g. severe combined immunodeficiency is due to lack of adenosine-deaminase), cardiac conduction and contractility, and anti-inflammation^[25]. It has long been reported that adenosine, a purine nucleoside that is released at injured and inflamed sites, plays a central role in the regulation of inflammatory responses and in limiting inflammatory tissue destruction^[26]. Early after the injurious or infectious signal, high concentrations of extracellular adenosine favor a transition from neutrophil infiltration to macrophage recruitment, to facilitate a highly efficient specific immune response carried out by macrophages. At later stages of immune or inflammatory processes, adenosine contributes to the resolution of inflammation, both by down-regulating macrophage activation and by advancing Th2- *vs* Th1-cell response^[26]. Some anti-inflammatory and immunomodulating drugs, such as salicylates, methotrexate and purine analogs like 6-MP and cyclosporine, exert their therapeutic actions in inflammatory diseases, at least in part, by decreasing intracellular adenosine 5'-triphosphate (ATP) concentrations and increasing extracellular adenosine levels^[27].

There are numerous reports that have demonstrated the ability of adenosine to exert anti-inflammatory actions in a variety of animal models. The anti-inflammatory effects can be achieved by increasing intracellular or extracellular adenosine levels through the mechanism of either enhanced production or inhibition of adenosine catabolism. The majority of work has been focusing on inhibition of adenosine catabolism or direct activation of adenosine receptors. A recent article by Antonioli *et al*^[28] reported that inhibition of adenosine deaminase can attenuate mucosal inflammation in experimental colitis through the mechanism of reducing mucosal myeloperoxidase activity, production of malondialdehyde and TNF- α levels as well as plasma TNF- α and interleukin-6 levels. Other studies have demonstrated that adenosine acting on the A2a receptor of T-lymphocytes can selectively suppress the expression of pro-inflammatory cytokines while sparing anti-inflammatory activity mediated by IL-10 and TGF- β ^[29]. The tissue injury and inflammation in mice with enteritis induced by *Clostridium difficile* toxin A can be alleviated by a new A2a receptor agonist, ATL 313, through the mechanism of inhibiting neutrophil infiltration, TNF- α production and adenosine deaminase activity^[30]. Adenosine can down-regulate neutrophil functions by

decreasing their adhesion, degranulation, and oxidant activities^[25]. An increase in endogenous adenosine levels by inhibition of adenosine kinase ameliorates colitis by suppression of IFN- γ in colonic tissue and CD69 expression in splenocytes, as well as maintaining tissue integrity by reducing energy demand, increasing nutrient availability, and modulating the immune system^[31]. At a molecular level, adenosine has been demonstrated to be a negative regulator of NF- κ B and MAPK signaling in human intestinal epithelial cells^[32]. Based on these findings, the adenosine system can represent a very promising target for therapies of inflammatory bowel diseases.

PHYSIOLOGY OF ADENOSINE METABOLISM BY INTESTINAL EPITHELIUM CELLS

Intracellular adenosine level is maintained by constant synthesis and degradation, as well as by trans-membrane transport through nucleoside transporters. The intracellular adenosine is produced by *de novo* synthesis from amino acids, CO₂, carbon-1-tetrahydrofolate and ribose-5-phosphate, salvage of endogenous adenine, dephosphorylation of ATP, ADP and AMP, as well as by transportation of exogenous nucleobases and nucleosides through concentrative nucleoside transporters (CNTs) or equilibrative nucleoside transporters (ENTs). Adenosine is metabolized to inosine by adenosine-deaminase, to either an end product uric acid or phosphorylated to ATP by adenosine-kinase or diffused into the extracellular space *via* ENTs^[33,34].

Most tissues and cells have *de novo* synthesis capacity to produce adenine endogenously from amino acids, CO₂ and carbon-1-tetrahydrofolate for their own use. However, some tissues and cells either lack or have very limited *de novo* synthesis capacity. These tissues and cells rely largely on exogenous nucleoside supply and salvage of endogenous nucleobases and nucleosides. Bone marrow, lymphocytes, leukocytes and intestinal epithelial cells are among them^[35-39]. The liver is the major organ supplying the nucleobases to these tissue and cells. The dietary nucleobases and nucleosides are absorbed by intestinal villous epithelial cells and degraded to end products such as uric acid. The uric acid is brought to the liver by blood and is taken up by hepatocytes and transformed into nucleobases. The synthesized nucleobases are released into the blood stream and carried to the tissues.

The intestinal epithelial cells have a very limited capacity for *de novo* synthesis; the villous cells can directly use the absorbed dietary nucleosides but the cryptal cells depend on blood supply. The impact of lack or limitation of nucleoside supply to cryptal cells on epithelial repair and barrier function during IBD has not been fully investigated^[40,41]. It is reasonable to speculate that poor absorption in the intestine or suppression of synthesis in the liver will ultimately result in disruption of epithelial barrier function in chronic bowel inflammatory diseases

like IBD, which in turn will lead to over-exposure of the innate immune system to intraluminal bacterial antigens and cause persistent inflammation or exacerbation of the diseases.

In general, the extracellular adenosine is produced by dephosphorylation of ATP by an enzymatic cascade consisting of Ntpases and ecto-5'-nucleotidase (Ecto 5'NTase), and direct diffusion of intracellular adenosine through ENTs. It is removed by enzymatic degradation by adenosine deaminase to inosine or by adenosine kinase to AMP. It can also be transported back into cells by membranous transporters like CNTs/ENTs. The extracellular adenosine level is believed to be lower than 1 μ mol/L in normal tissue but can be as high as 100 μ mol/L in inflamed or ischemic tissues^[25]. Only a high adenosine level can exert immunomodulatory and immunosuppressive effects. Luminal adenosine level is estimated to be 5 mmol/L in normal intestine, while it is 6 mmol/L in inflamed intestine due to ATP and adenosine secretion in inflammatory and other cell types^[42].

It is not entirely clear which cell types are the most important producer of extracellular adenosine, but endothelial cells, neutrophils, nerve terminal and epithelial cells have been identified in the literature^[43,44]. Extracellular adenosine binds to adenosine receptors (AR) 1, 2a, 2b and 3, all of which are expressed on the surface of immune cells. Low level expression of A1R is demonstrated in small intestine. A2bR is the only receptor expressed in epithelial cells of cecum and colon. A3R can be detected in jejunum and proximal colon^[45,46]. Adenosine receptors are members of the G protein-coupled family of receptors^[47]. A1 and A3 receptors are usually coupled with Gi proteins that inhibit adenylate cyclase, whereas the A2aR and A2bR receptors are coupled with Gs proteins that activate adenylate cyclase. Several studies have demonstrated that adenosine attenuates intestinal inflammation predominantly through the effects of the A2aR receptor of neutrophils and T-lymphocytes^[29,30,48]. However, Yang *et al.*^[49] found that activation of the A2bR can also have anti-inflammatory effects, using a gene knock-out method to delete this gene in order to show a pro-inflammatory phenotype.

MOLECULAR MECHANISM OF ADENOSINE TRANSPORT

The significance of exogenous adenosine transport by intestinal epithelial cells in the treatment of IBD and its impact on epithelial cell barrier function has not been explored. Concentrative nucleoside transporters (CNTs) have been identified as the major transporters for absorption of exogenous nucleosides from the diet. Three distinct CNTs (CNT1, CNT2 and CNT3) that exhibit different substrate specificity have been cloned and characterized from humans, rats and mice^[34]. CNT1 predominantly transports pyrimidines. CNT2 transports purine and uridine, while CNT3 transports purines and

pyrimidines. The expression of CNTs and their substrate specificity vary among species. CNT3 was not found in intestinal epithelial cells of human and rat^[34,50,51]. CNTs belong to the solute carrier family 28 (SLC-28). CNTs are expressed in the apical membranes of intestinal epithelial cells, as well as in other cell types including hepatocytes, endothelial cells, neutrophils, lymphocytes and macrophages.

CNT2 has been cloned and characterized in humans, mice, rats and rabbits. The rat CNT2 cDNA is 2.9 Kb and encodes a 659 amino acid protein with molecular weight of 72 kDa^[52]. The apparent molecular weight on western blot is usually around 60 kDa due to high hydrophobicity of membrane protein. Fourteen putative transmembrane domains were identified by hydropathy analysis. The presence of several consensus sequences for protein kinase-C (PKC) and protein kinase-A (PKA) phosphorylation sites on both N- and C-termini, and an ATP/GTP binding motif in N-terminus, suggest that CNT2 may be regulated by protein kinases and intracellular ATP and GTP. CNT2 may be a glycoprotein as there are five possible N-linked glycosylation sites. Na⁺-adenosine cotransport in brush-border membranes from rabbit ileum was identified and partially characterized in one previous study^[53]. *In vitro* expressed CNT2 in *Xenopus laevis* oocytes exhibited Na⁺-dependent adenosine uptake with an apparent *K_m* for adenosine of 6 μmol/L, with substrate selectivity to purine and uridine^[54].

REGULATION OF ADENOSINE TRANSPORT AND CNT2 EXPRESSION IN GENERAL

The CNT2 expression and adenosine uptake are highly regulated processes and are species and tissue specific^[55]. Sub-cellular trafficking (i.e. internalization of membrane transporters to sub-cellular storage vesicles) has been shown to be a regulatory mechanism for CNT2 in several cell lines including adrenal chromaffin cells, reticulocytes and cholangiocytes^[56-58]. Although CNT1 has been shown to be up-regulated in intestine and down-regulated in hepatocytes of pyrimidine-free diet fed animals, the dietary effects of adenosine on CNT2 activity and molecular expressions are not known^[59,60]. Several studies have shown that nucleoside transport functions and expressions are regulated by hormones^[61-66]. Tyrosine and glucagon have been shown to stimulate adenosine transport and CNT2 expression in both *in vitro* and *in vivo* models^[61-63]. Studies with insulin and glucose have yielded different results regarding adenosine transport and molecular expression. Insulin regulates adenosine transport through different signaling pathways that involve PI3K, MAPK, NO synthase, PKC and MAP kinase^[64-66]. One study showed that adenosine transport is up-regulated by activation of the A1 adenosine receptor through ATP-sensitive K-channels in hepatocytes, suggesting some positive feedback regulation among adenosine receptors and the

adenosine transporter^[67].

The effects of proliferative (EGF and TGF-α) and differentiating (glucocorticoid) hormones were demonstrated in IEC-6 cells (a rat intestinal cryptal cell line). A four-fold increase of adenosine transport activity and CNT2 molecular expression after treatment with dexamethasone was observed while no significant impact was noted from treatment with proliferative hormones^[68]. However, a more recent paper by the same research group found that TGF-β can transcriptionally up-regulate CNT2 gene expression in rat hepatocytes^[69]. Adenosine transport and CNT2 expression are altered during cell growth cycle and differentiation. Hepatocarcinogenesis is accompanied by loss of CNT2 expression and increased expression of ENTs. CNT2 mRNA and protein levels were increased right before the peak of incorporation of thymidine into DNA and during liver regeneration after partial hepatectomy^[70,71].

CNT2 expression was up-regulated by lipopolysaccharide (LPS), NO, INF-α and TNF-α in macrophages and B-lymphocytes in several studies^[72-74]. The LPS-induced increase of adenosine transport and molecular expression is TNF-α-dependent but not iNOs-dependent. cNOs is required for maintaining the basal transport activities of adenosine in activated B-cells. The CNT2 is recognized as an important regulator of extracellular adenosine concentrations. CNT2 expression is suppressed in inflamed tissue as a mechanism to maintain high extracellular adenosine concentration. The CNT2 expression is suppressed in neutrophils and macrophages during inflammation^[75]. There is controversy between the *in vivo* and *in vitro* studies about the functional status of adenosine transport and CNT2 expression during inflammation and the effects of inflammatory mediators.

MECHANISM OF REGULATION OF ADENOSINE TRANSPORT AND CNT2 EXPRESSION IN INTESTINAL EPITHELIAL CELLS

A systematic study for the mechanism of regulation of adenosine transport and CNT2 expression in intestinal epithelial cells is not available. Adenosine functions as a nutrient for nucleic acid metabolism, an energy carrier molecule for cell energy metabolism, and a second messenger in autocrine and paracrine hormone regulation. Adenosine transport is regulated differently from other nutrient transporters such as glucose and amino acid transporters. Luminal adenosine and ATP have been reported to regulate glucose and bile acid transport in intestine, proximal renal tubule cells and cholangiocytes^[42,76,77]. The role of the activation of purinergic receptors on adenosine uptake was also studied in vascular endothelial cells and chromaffin cells. ATP has been shown to up-regulate adenosine transport and protein expression in vascular endothelial cells and chromaffin cells^[78-80]. However, the effect of ATP on

adenosine transport in normal and inflamed intestine remains to be elucidated.

MECHANISM OF REGULATION OF Na^+ -CO-TRANSPORT IN INTESTINAL EPITHELIAL CELLS DURING CHRONIC INTESTINAL INFLAMMATION

The regulation of intestinal epithelial transporters during chronic inflammation is a very complex process, involving many cell types and immune-inflammatory mediators. The cells involved in chronic enteritis include all inflammatory cells (neutrophils, basophils, eosinophils, macrophages, lymphocytes), fibroblasts, vascular endothelial cells, nerve cells and epithelial cells. These cells produce numerous cytokines and immune-inflammatory mediators such as prostaglandins, leukotrienes, reactive oxidative metabolites (ROMs) and nitric oxide^[1-6]. So far, there are no perfect animal models for IBD though several different models exist with chemically-induced and genetically-induced enteritis. It is very difficult to fully understand the regulation of nutrient and electrolyte transport in the chronically inflamed intestine.

The mast cell has been implicated as an important player in chronic intestinal inflammation. It releases multiple inflammatory mediators including histamine, serotonin, cytokines, prostaglandins, leukotrienes and ROMs. The released mediators are very important regulators of transporters in epithelial cells. It was consistently demonstrated that blocking mast cell degranulation prevented down-regulation of a number of transporters including Na^+ -glucose and Na^+ -amino acid cotransport as well as electrolyte transporters such as $\text{Cl}^-/\text{HCO}_3^-$ exchange^[81].

Nitric oxide has been identified as an important regulating factor in the intestinal tract. Constitutive nitric oxide synthase (cNOS) plays an important role in the regulation of transporters in normal intestine. It is essential for some transporters to function properly under physiologic conditions. Inducible nitric oxide synthase (iNOS) is activated during chronic inflammation, ischemia and tissue injury. iNOS produces large amounts of nitric oxide (NO) during chronic inflammation, which is generally considered as detrimental to tissue and cells because of formation of peroxynitrous acid, though controversy continues about its role in inflammation. The mechanism of cNOS and iNOS effects on nutrient co-transporters in epithelial cells is possibly through direct and indirect effects, by stimulating the production and/or release of other inflammatory mediators such as arachidonic acid metabolites (e.g. prostaglandins and leukotrienes)^[82-84].

Prostaglandins have been shown to be very important inflammatory mediators in chronic intestinal inflammation. A large amount of prostaglandins are produced in the intestinal tissue during IBD. Prostaglandins inhibit electrolyte absorption and Na^+

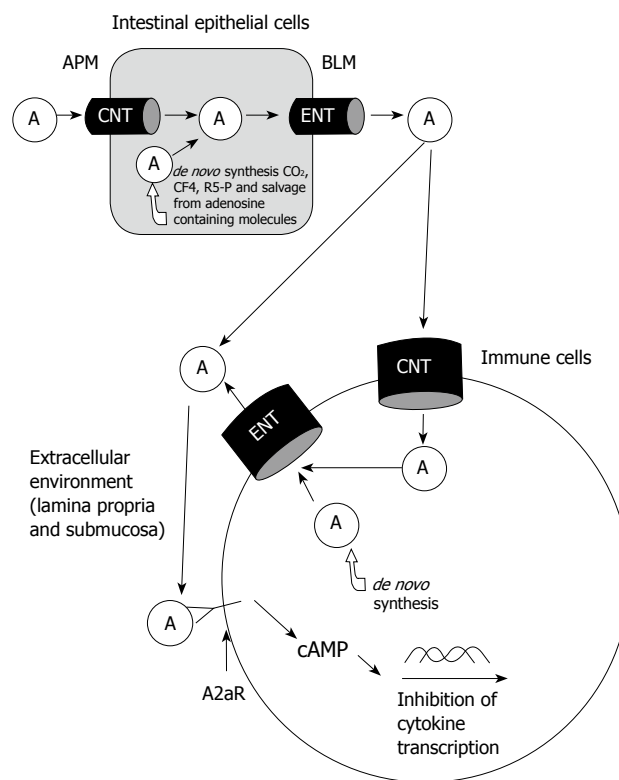


Figure 1 Pathways and roles of adenosine. Absorbed and *de novo* synthesized adenosine delivered to extracellular space. Adenosine binds to A2aR of immune cells and activates signaling pathways to inhibit the production of inflammatory mediators. A: Adenosine; APM: Apical membrane; BLM: Basolateral membrane; CNT: Concentrative nucleoside transporter; ENT: Equilibrative nucleoside transporter.

nutrient co-transporter functions^[85-89]. In addition, they also promote mucous secretion and cytoprotection in the gastrointestinal tract during IBD^[85,88]. Prostaglandin E2 (PGE2) was also reported to suppress glucose transport in the ovine intestine^[90]. Leukotrienes have been demonstrated to inhibit electrolyte transport in a similar pattern to prostaglandins^[91-98].

Corticosteroids are the most frequently used broad spectrum immunomodulators in IBD for blocking the production of most major inflammatory mediators. Corticosteroids prevent mast cell degranulation and block the phospholipase A2 (PLA2) enzyme pathway. They also down-regulate arachidonic acid release and production of prostaglandins and leukotrienes. In addition, corticosteroids suppress the production of iNOS and inducible cyclooxygenase (COX-2) during chronic inflammation^[99].

It is possible that mast cells, nitric oxide, arachidonic acid metabolites and steroids are all involved in the regulation of adenosine transport and CNT2 expression during chronic enteritis. It should be noted that adenosine itself is an inflammatory modulator. Extracellular adenosine can suppress all these above-mentioned inflammatory cells and mediators. The interplay among adenosine, inflammatory cells and mediators can be very complex and is also very interesting for further study.

CONCLUSION

The pathways of adenosine metabolism and transport are fully illustrated in Figure 1. Luminal adenosine is absorbed by intestinal epithelial cells through CNT and ENT transporters and can also be synthesized *de novo*. The increase in intracellular levels of adenosine leads to extracellular transport along with the release of adenosine from damaged cells during inflammation. Using various therapies during IBD, it may be possible to eventually increase the extracellular levels of adenosine so that the appropriate receptors can be activated to inhibit and reduce the effects of chronic inflammation on the gut. IBD is a common and lifelong disabling gastrointestinal disease. At present, there is no cure for IBD. Emerging treatments are being developed to target cytokines that perpetuate the chronic inflammatory response. Adenosine is an important modulator of inflammation and its anti-inflammatory effects have been well established in humans as well as in animal models. Therapeutic targeting of receptors such as the A2a receptor could reduce cytokine levels and thus reduce the effects of chronic inflammation during IBD.

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S- Editor Tian L L- Editor Logan S E- Editor Yin DH

Iron increases HMOX1 and decreases hepatitis C viral expression in HCV-expressing cells

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Author contributions: Hou WH, Shan Y and Rossi L each contributed to the conception, experimentation and drafting of this paper; Zheng JY performed experiments as well as contributing to the drafting of the paper; Lambrecht RW and Bonkovsky HL were instrumental in the conception of the project and the drafting of the paper.

Supported by Grant (DK RO1 38825) and contracts (DK NO1 29236 and UO1 DK 06193) from the National Institutes of Health (NIDDK)

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Received: May 26, 2009 **Revised:** September 2, 2009

Accepted: September 9, 2009

Published online: September 28, 2009

increased oxidative stress and up-regulated *HMOX1* gene expression. Iron did not affect mRNA or protein levels of Bach1, a repressor of HMOX1. Silencing the up-regulation of HMOX1 nuclear factor-erythroid 2-related factor 2 (Nrf2) by Nrf2-siRNA decreased FeNTA-mediated up-regulation of HMOX1 mRNA levels. These iron effects were completely blocked by deferoxamine (DFO). Iron also significantly decreased levels of HCV core mRNA and protein by 80%-90%, nonstructural 5A mRNA by 90% and protein by about 50% in the Con1 full length HCV replicon cells, whereas DFO increased them.

CONCLUSION: Excess iron up-regulates HMOX1 and down-regulates HCV gene expression in hepatoma cells. This probably mitigates liver injury caused by combined iron overload and HCV infection.

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Key words: Deferoxamine; Core protein of hepatitis C virus; Hepatitis C; Iron; Heme oxygenase-1; Nuclear factor-erythroid 2-related factor 2; Bach1; Oxidative stress; Nonstructural 5A protein of hepatitis C virus

Peer reviewer: Shashi Bala, PhD, Post doctoral Associate, Department of Medicine, LRB 270L, 364 Plantation street, UMass Medical School, Worcester, MA 01605, United States

Hou WH, Rossi L, Shan Y, Zheng JY, Lambrecht RW, Bonkovsky HL. Iron increases HMOX1 and decreases hepatitis C viral expression in HCV-expressing cells. *World J Gastroenterol* 2009; 15(36): 4499-4510 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4499.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4499>

Abstract

AIM: To investigate effects of iron on oxidative stress, heme oxygenase-1 (HMOX1) and hepatitis C viral (HCV) expression in human hepatoma cells stably expressing HCV proteins.

METHODS: Effects of iron on oxidative stress, HMOX1, and HCV expression were assessed in CON1 cells. Measurements included mRNA by quantitative reverse transcription-polymerase chain reaction, and protein levels by Western blots.

RESULTS: Iron, in the form of ferric nitrilotriacetate,

INTRODUCTION

Iron overload is known to be toxic to many organs, particularly to the liver. The liver is the major site of storage of excess iron. The most common form of iron overload is that related to classic hereditary hemochromatosis, in which, due to mutations in the *HFE* gene, there is excessive uptake of iron into enterocytes^[1-3]. In hemochromatosis, decreased hepatic production and secretion of hepcidin leads to increased ferroportin expression at the plasma membranes,

especially of enterocytes and macrophages. Ferroportin is the only known physiologic iron exporter from cells and its uncontrolled over expression leads to excess uptake of iron from the enterocytes into the portal blood and to increased release of iron from macrophages and other cells of the reticulo-endothelial system, including the Kupffer cells of the liver^[4-6]. The excess iron in the portal blood and/or released by Kupffer cells within the liver is taken up by hepatocytes where it is stored, chiefly in the form of holo-ferritin. Iron in ferritin is relatively non-reactive and non-toxic. However, release of tissue ferritin from damaged or dying cells leads to activation of hepatic stellate cells and a cascade of pro-inflammatory and pro-fibrogenic events. This may eventuate in the development of hepatic fibrosis, cirrhosis, and hepatocellular carcinoma, as well as all of the usual complications of advanced chronic liver disease^[7-9].

In recent years, it has become increasingly clear that only modest amounts of iron in the liver may play a role as a co-morbid factor in the development and progression of non-hemochromatotic liver diseases^[10-15]. The link between iron and non-hemochromatotic liver diseases is particularly strong for steatohepatitis, both non-alcoholic and alcoholic^[10,14,15] and viral hepatitis B and C^[16-18].

Porphyria cutanea tarda, the most common form of porphyria, is known to be triggered or exacerbated by iron and is often associated with *HFE* gene mutations, chronic hepatitis C, and alcohol use^[19-21]. The treatment of choice for porphyria cutanea tarda involves removal of iron, which leads to remission of the biochemical and clinical features of the disease. Blumberg and colleagues were among the first to stress the importance of iron status in influencing outcomes and progression of acute hepatitis B infection^[22,23]. In the case of hepatitis C infection, a number of investigators from throughout the world have noted high prevalences (35%-50%) of elevations of serum ferritin and high, albeit somewhat lower, frequencies of elevations of serum transferrin saturation in patients with chronic hepatitis C^[10,24-26]. Despite this, the occurrence of heavy iron overload in chronic hepatitis C is infrequent and is chiefly related to advanced liver disease. Increases in serum measures of iron and stainable iron in the liver have been directly correlated with more severe chronic hepatitis C and with lower likelihoods of response to currently available antiviral therapy, especially type 1 interferons^[24,27,28]. In addition, it has been shown repeatedly that reduction of body iron by therapeutic phlebotomy improves the responsiveness of chronic hepatitis C infection to interferon therapy^[29].

Heme oxygenase-1 (HMOX1) has emerged as a key cytoprotective gene and enzyme in numerous experimental and clinical contexts (For reviews, see^[30-33]). The *HMOX1* gene is under complex regulation and can be up-regulated markedly by heme, the physiologic substrate for the HMOX1 protein, by iron and other transition metallic ions, and by oxidative and heat stress and other stressful perturbations. Regulation of *HMOX1*

gene expression is related in part to alterations in levels of several transcription factors, including Bach1, and nuclear factor-erythroid 2-related factor 2 (Nrf2). Normally, Bach1 in nuclei represses *HMOX1* gene expression, whereas Nrf2, in concert with small Maf proteins, up-regulates its expression^[34-36].

The study of hepatitis C viral (HCV) infection has been difficult because of the lack of a readily available, inexpensive animal model of acute or chronic hepatitis C infection. The recent development of human hepatoma cell lines, which stably express HCV proteins, and support the replication of HCV RNA or the formation of complete infectious virions of HCV^[37,38], has facilitated studies on pathogenesis and the role of potential co-morbid factors, such as iron. We used such lines to investigate the effects of iron on oxidative stress, HMOX1 and HCV expression. Here we report that excess iron results in further increased oxidative stress and up-regulation of HMOX1 *via* Nrf2, and down-regulation of HCV protein expression in human hepatoma cells in culture (Huh-7) expressing HCV RNA and proteins. These effects are reversed by deferoxamine (DFO), the selective and potent iron chelator.

MATERIALS AND METHODS

Reagents and materials

Mouse anti-HCV nonstructural 5A (NS5A) protein was purchased from Virogen (Plantation, FL). Goat anti-human Bach1, goat anti-human GAPDH polyclonal antibodies, goat anti-mouse IgG, and donkey anti-goat IgG were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). ECL-Plus was purchased from Amersham Biosciences Corp (Piscataway, NJ). Dimethyl sulfoxide was purchased from FisherBiotech (Fair Lawn, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), zeocin, geneticin, trypsin and TRIzol were from Invitrogen Inc. (Carlsbad, CA). FeCl₃, Na₃NTA, H₂O₂, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) and its oxidation-insensitive analog 2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (Allentown, PA). DFO mesylate was from Novartis (Cambridge, MA).

Cell cultures

The human hepatoma cell line, Huh-7, was purchased from the Japan Health Research Resources Bank (Osaka, Japan). 9-13 and CNS3 cell lines derived from Huh-7 cells, which stably express HCV proteins were gifts from Dr. R Bartenschlager (University of Heidelberg, Heidelberg, Germany). Human hepatoma Huh-7 cells were maintained in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% (v/v) FBS. 9-13 and CNS3 replicon cells were cultured with additional antibiotics (1 mg/mL geneticin or 10 µg/mL zeocin), respectively. 9-13 replicon cells stably express HCV nonstructural proteins (NS3-5B), and CNS3 cells stably express subgenomic proteins from core to nonstructural protein 3 (core-NS3). The Con1 subgenomic genotype 1b HCV replicon cell line was

from Apath LLC (St, Louis, MO). The Con1 cell line is a Huh-7.5 cell population containing the full-length HCV genotype 1b replicon. The Con1 cells were maintained in DMEM supplemented with 10% (v/v) FBS and 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, and selection antibiotic 750 µg/mL geneticin. Cells were maintained in a humidified atmosphere of 95% room air and 5% CO₂ at 37°C.

siRNA transfection

A smart pool of siRNAs targeting four positions of the human Nrf2 mRNA, was purchased from Dharmacon (Lafayette, CO). Transfections of Nrf2-siRNA were performed with Lipofectamine 2000 from Invitrogen (Carlsbad, CA) as described previously^[35]. Cells were transfected for 48 h with 20–100 nmol/L Nrf2-siRNA, or an irrelevant control, and subsequently were exposed for 4 h to indicated concentrations of ferric nitrilotriacetate (FeNTA). Cells were harvested and total RNA and proteins were extracted for measurements of mRNA or protein levels by quantitative RT-PCR or Western blots.

Quantitative RT-PCR

Total RNA from treated cells was extracted and cDNA was synthesized and real time quantitative RT-PCR was performed using a MyiQ™ Single Color Real-Time PCR Detection System (BIO-RAD) and iQ™ SYBR Green Supermix Real-Time PCR kit (BIO-RAD, Hercules, CA) as described previously^[39,40]. Sequence-specific primers used for HMOX1, HCV core, NS5A and GAPDH were synthesized. We included samples without template and without reverse transcriptase as negative controls, which were expected to produce negligible signals (Ct values > 35). Standard curves of HMOX1, HCV core, NS5A and GAPDH were constructed with results of parallel PCR reactions performed on serial dilutions of a standard DNA (from one of the controls). Fold-change values were calculated by comparative Ct analysis after normalizing for the quantity of GAPDH in the same samples.

Western blotting

Protein preparations and Western blots were performed as described previously^[39,40]. In brief, total proteins (30–50 µg) were separated on 4%–15% gradient SDS-PAGE gels (Bio-Rad). After electrophoretic transfer onto immunoblot PVDF membrane (Bio-Rad), membranes were blocked for 1 h in PBS containing 5% nonfat dry milk and 0.1% Tween-20, and then incubated overnight with primary antibody at 4°C. The dilutions of the primary antibodies were as follows: 1:500 for anti-NS5A, 1:1000 for anti-Bach1, 1:2000 for anti-HCV core and anti-GAPDH antibodies. The membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (dilution 1:10 000). Finally, the bound antibodies were visualized with the ECL-Plus chemiluminescence system according to the manufacturer's protocol (Amersham, Piscataway, NJ). A Kodak 1DV3.6

computer-based imaging system (Eastman-Kodak, Rochester, NY) was used to measure the relative optical density of each specific band obtained after western blotting. Data are expressed as percentages of the controls (corresponding to the value obtained with the vehicle-treated cells), which were assigned values of one.

Cellular reactive oxygen species (ROS) production assay

Levels of cellular oxidative stress were measured using DCF assay. Briefly, cells were seeded into 24-well plates. The following day, the media were removed, and the cells were washed with PBS (PBS supplemented with 1 mmol/L CaCl₂ and 0.5 mmol/L MgCl₂), and then incubated with 100 µmol/L 2',7'-dichlorodihydrofluorescein (H₂DCF-DA) or 2',7'-dichlorofluorescein diacetate (DCF-DA) in DMEM without phenol red for 30 min at 37°C in the dark. The cells were washed twice with PBS, and then treated with selected concentrations of FeNTA for 1 h. Intracellular ROS levels were measured as an increase in fluorescence of the oxidized product of DCF-DA on a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT) at the excitation and emission wavelengths of 488 and 525 nm, respectively. The oxidation-insensitive analog of H₂DCF-DA served as a control to correct for possible changes in cellular uptake, ester cleavage, and efflux. It showed no changes in fluorescence in these studies.

Statistical analysis

Experiments were repeated at least three times with similar results. Except for Western blots, all experiments included at least triplicate samples for each treatment group. Representative results from single experiments are presented. Statistical analyses were performed with JMP 6.0.3 software (SAS Institute, Cary, NC). Initial interpretation of data showed that they were normally distributed. Therefore, appropriate parametric statistical procedures were used: Student's *t*-test for comparisons of two means and analysis-of-variance (*F* statistics) for comparisons of more than two, with pair-wise comparisons by the Kruskal-Wallis test. Values of *P* < 0.05 were considered significant.

RESULTS

Iron up-regulates HMOX1 mRNA levels in Huh-7 and cell lines expressing HCV proteins

As shown in Figure 1, *HMOX1* gene expression was significantly increased in CNS3 cells, which express HCV core to NS3, even without exposure to iron or hydrogen peroxide, compared to 9-13 cell lines, which express NS3 to NS5B, or parental Huh-7 cells. Iron, in the form of FeNTA and hydrogen peroxide (another known oxidative stressor), further up-regulated the *HMOX1* gene expression in CNS3 cells. Increase of *HMOX1* gene expression by iron in Huh-7 (6.7 fold) and 9-13 cells (5.2 fold) was greater than in CNS3 cells (1.9 fold).

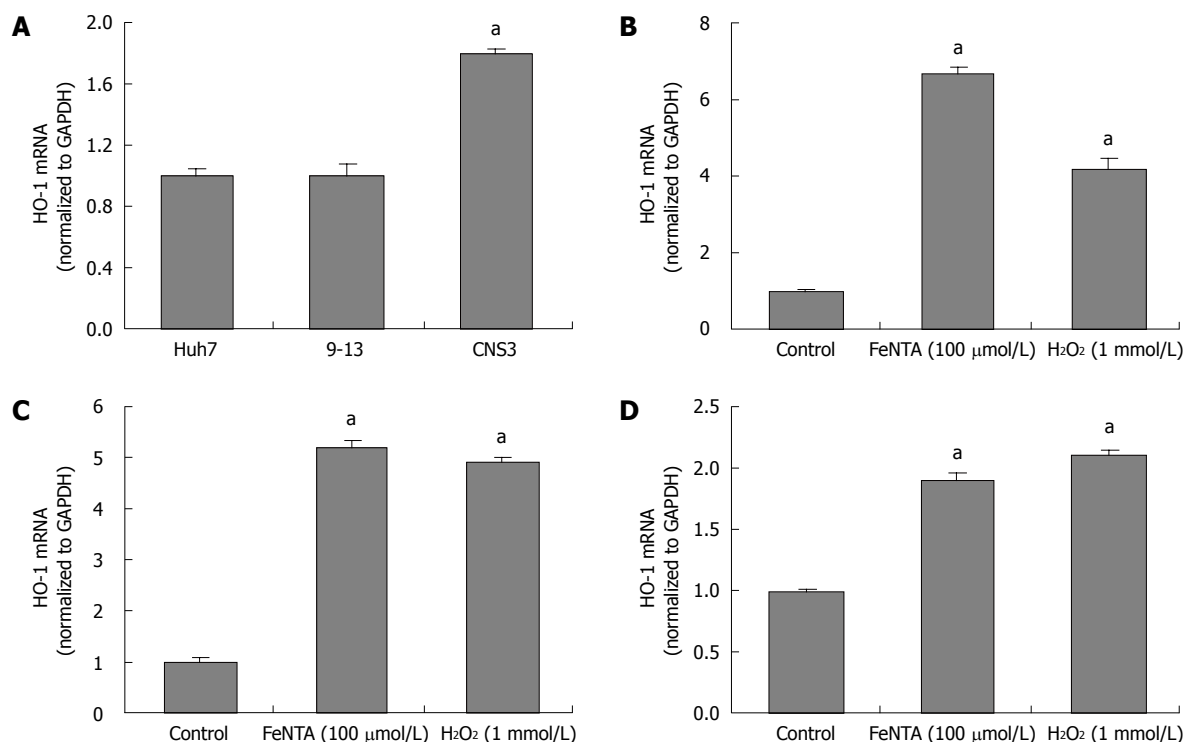


Figure 1 Iron up-regulates HMOX1 mRNA levels in Huh-7, 9-13 and CNS3 cells. A: HMOX1 mRNA levels in Huh-7, 9-13 and CNS3 cells; B: HMOX1 mRNA levels in Huh-7 cells treated with 100 μ mol/L FeNTA or 1 mmol/L H₂O₂ for 6 h; C: HMOX1 mRNA levels in 9-13 cells treated with 100 μ mol/L FeNTA or 1 mmol/L H₂O₂ for 6 h; D: HMOX1 mRNA levels in CNS3 cells treated with 100 μ mol/L FeNTA or 1 mmol/L H₂O₂ for 6 h. Values for cells without any treatment were set equal to 1. HMOX1 mRNA data are presented as means \pm SE from triplicate samples, all normalized to GAPDH in the same samples. ^a*P* < 0.05 vs control. Huh-7, 9-13 or CNS3 cells were cultured as described in Materials and Methods, and treated with 100 μ mol/L FeNTA or 1 mmol/L H₂O₂ for 6 h. Cells were harvested and total RNA was extracted. Levels of mRNA for HMOX1 and GAPDH were measured by quantitative RT-PCR.

Effects of Iron on Nrf2 and Bach1 protein levels in Huh-7 and cell lines expressing HCV proteins

Previous studies from our and other laboratories have demonstrated that Bach1 and Nrf2 act as transcriptional factors that regulate *HMOX1* gene expression in mammalian cells^[34-36], and that Huh-7 cells expressing HCV proteins show significant up-regulation of the *HMOX1* gene, and reciprocal down-regulation of the *Bach1* gene^[41]. To determine whether iron affected the *Nrf2* or *Bach1* gene expression, parental Huh-7 and cell lines (9-13 and CNS3) expressing HCV proteins were treated with FeNTA, and Nrf2 and Bach1 protein levels were measured by Western blots, as described in Materials and Methods. Cells exposed to 50 and 100 μ mol/L FeNTA showed significant accumulation of Nrf2 protein (Figure 2A-C), whereas 50 or 100 μ mol/L NaNTA did not affect Nrf2 protein levels (data not shown). In contrast, there were no detectable changes of Bach1 protein levels in either Huh-7 cells or cell lines expressing HCV proteins, suggesting that Bach1 is not involved in up-regulation of the *HMOX1* gene expression by iron (Figure 3A-C).

Nrf2-siRNA abrogates up-regulation of the HMOX1 gene expression by iron in 9-13 cells

To further establish the role of Nrf2 in up-regulation of the *HMOX1* gene expression by iron, we silenced *Nrf2* gene expression by Nrf2-siRNA as we did previously in Huh-7 cells^[35]. In comparison with control, 20 nmol/L

Nrf2-siRNA significantly reduced Nrf2 protein expression, and 100 nmol/L Nrf2-siRNA repressed Nrf2 protein expression by 92% (Figure 4A). We also successfully silenced the *Nrf2* gene expression in CNS3 cells (data not shown). HMOX1 mRNA levels were significantly induced by iron in cells without Nrf2-siRNA transfection, and this effect was blocked in cells transfected with 100 nmol/L Nrf2-siRNA, indicating that Nrf-2 siRNA plays a key role in up-regulation of the *HMOX1* gene expression by iron (Figure 4B).

Increased ROS, induced by iron, in the form of ferric nitrilotriacetate, in the cell lines expressing HCV proteins

Oxidative stress is one of the key stressors inducing the *HMOX1* gene expression^[30,31], occurring due to iron-catalyzed formation of reactive oxygen species (ROS)^[42]. We observed that the cells exposed to 50 μ mol/L FeNTA exhibited significant increases in the fluorescence intensity of H₂DCF-DA (by 1.4 fold in Huh-7, 1.7 fold in 9-13 and 1.6 fold in CNS3 cells), which are similar to the increases produced by hydrogen peroxide (1 mmol/L). 100 μ mol/L FeNTA further increased fluorescence intensity by 2.1 fold in 9-13 and 1.9 folds in CNS3 cells (Figure 5A-C), whereas 50 or 100 μ mol/L NaNTA did not affect fluorescence intensity (data not shown). The results of the same experiment done with the oxidation-insensitive analogue of the probe (DCF-DA) in CNS3 (Figure 5D), Huh-7 and 9-13 cells (data not shown) indicated no significant

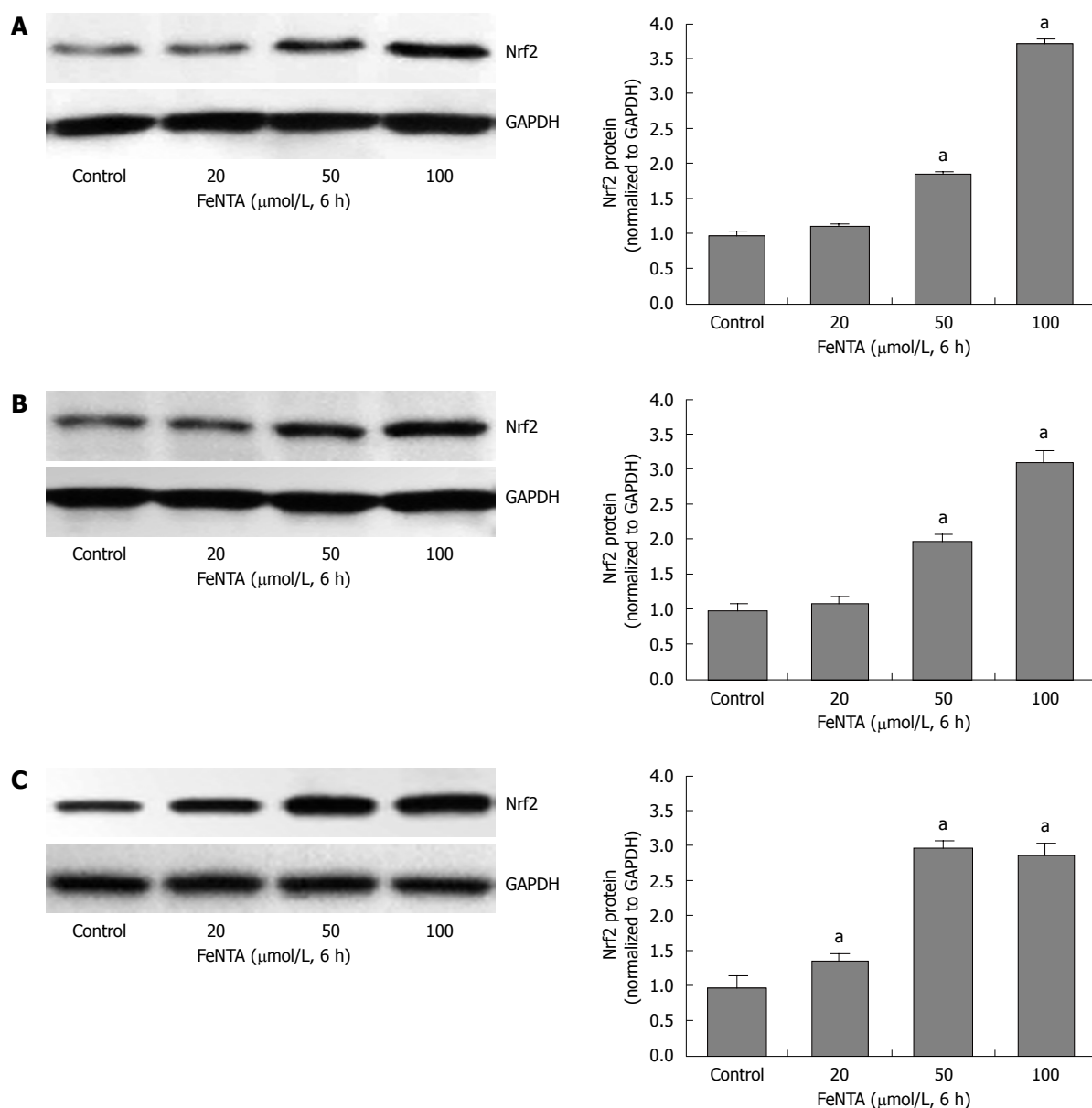


Figure 2 Effects of FeNTA on Nrf2 protein levels in Huh-7, 9-13, and CNS3 cells. A: Nrf2 protein levels in Huh-7 cells; B: Nrf2 protein levels in 9-13 cells; C: Nrf2 protein levels in CNS3 cells. ^a $P < 0.05$ vs control. Huh-7, 9-13 or CNS3 cells were treated with different concentrations of FeNTA (0, 20, 50, 100 $\mu\text{mol/L}$) or 100 $\mu\text{mol/L}$ NaNTA for 6 h, after which cells were harvested and total protein was isolated, as described in Materials and Methods. Proteins were separated on 4%-15% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-human Nrf2 and GAPDH specific antibodies. The relative amounts of Nrf2 proteins were normalized to GAPDH.

difference between control cells and cells treated with FeNTA or H_2O_2 . Therefore, the increased fluorescence intensity seen with the oxidation sensitive probe $\text{H}_2\text{DCF-DA}$ (Figure 5A-C) can be directly ascribed to changes in the oxidation of the probe in the cells. We also changed the order of adding the $\text{H}_2\text{DCF-DA}$ and FeNTA or H_2O_2 and observed the same pattern of results (data not shown).

The iron chelator DFO blocks increased ROS induced by iron in the cell lines expressing HCV proteins

DFO and deferasirox (Exjade) are widely used iron chelators to remove excess iron from the body. They act by binding iron at 1:1 (deferoxamine:iron) and 2:1 (deferasirox:iron) ratios and enhancing its elimination. By removing excess iron, these agents reduce the damage

done by iron to various organs and tissues such as the liver. In this study, DFO was used to examine whether the effects of FeNTA were blocked by DFO chelation. In comparison with 100 $\mu\text{mol/L}$ FeNTA alone, 50 $\mu\text{mol/L}$ DFO (deferoxamine:iron 1:2) significantly decreased DCF fluorescence intensity in 9-13 and CNS3 cells (Figure 6A-C). Indeed, ROS induced by iron were completely blocked by DFO in all three cell lines treated with 50 $\mu\text{mol/L}$ FeNTA and increasing concentrations of DFO (50, 100 and 200 $\mu\text{mol/L}$) (Figure 6A-C). To confirm we were truly measuring changes in $\text{H}_2\text{DCF-DA}$ oxidation and not changes in its uptake, ester cleavage, or efflux, we repeated experiments shown in Figure 6A-C with the oxidation-insensitive analogue of the probe (DCF-DA). No significant differences between control

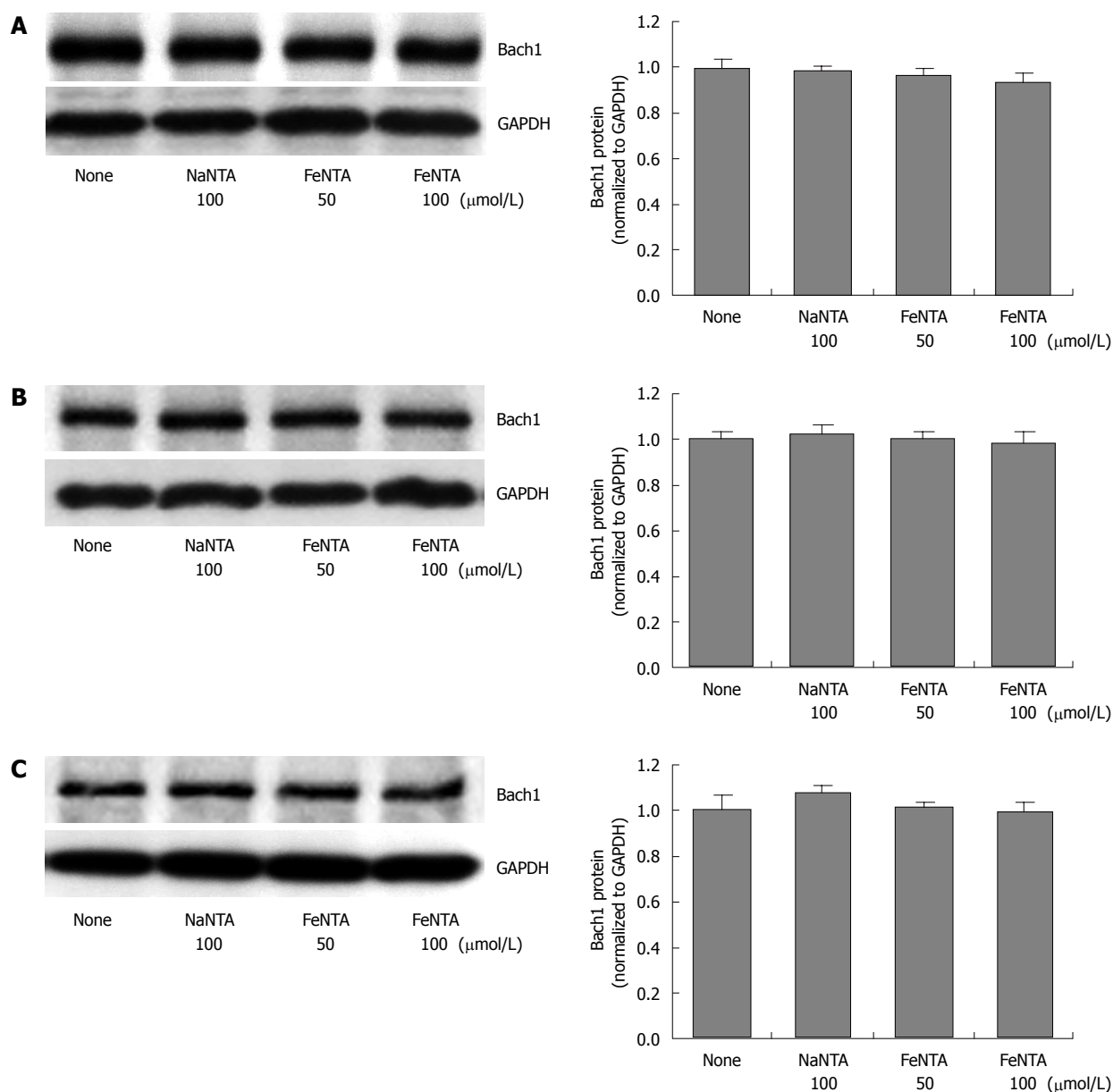


Figure 3 Effects of FeNTA on Bach1 protein levels in Huh-7, 9-13, and CNS3 cells. A: Bach1 protein levels in Huh-7 cells; B: Bach1 protein levels in 9-13 cells; C: Bach1 protein levels in CNS3 cells. Huh-7, 9-13 or CNS3 cells were treated with different concentrations of FeNTA (0, 50, 100 μmol/L) or 100 μmol/L NaNTA for 16 h, after which cells were harvested and total protein was isolated, as described in Material and Methods. Proteins were separated on 4%-15% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-human Bach1 and GAPDH specific antibodies. The relative amounts of Bach1 were normalized to GAPDH.

and treated cells were found in CNS3 (Figure 6D), Huh-7 or 9-13 cells (data not shown).

Iron decreases HCV protein expression in cell lines expressing HCV proteins

To evaluate the effect of iron in the form of FeNTA on HCV RNA and protein expression, Con1 full length HCV replicon cells were exposed to FeNTA and with or without DFO. Treatment with FeNTA resulted in a 80%-90% reduction in HCV core mRNA and protein levels (Figure 7A and B), and decreased expression of HCV NS5A mRNA by about 90% and protein by about 50% (Figure 7C and D), whereas no significant effects were produced by NaNTA, establishing that the effects are due to iron and not to the nitrilotriacetate anion

(data not shown). These down-regulatory effects were abrogated by DFO (200 μm).

DISCUSSION

The major findings of this work are as follows: (1) Iron, in the form of FeNTA, up-regulates *HMOX1* gene expression in human Huh-7, and cell lines (9-13 and CNS3) stably expressing HCV proteins (Figure 1); (2) Iron significantly increases Nrf2 protein levels in human hepatoma cells, and silencing the *Nrf2* gene with Nrf2-specific siRNA abrogates the up-regulation of *HMOX1* by iron (Figures 2 and 4); (3) Iron does not significantly change Bach1 protein levels in human hepatoma cells (Figure 3); (4) Iron increases ROS (Figures 5 and 6) but

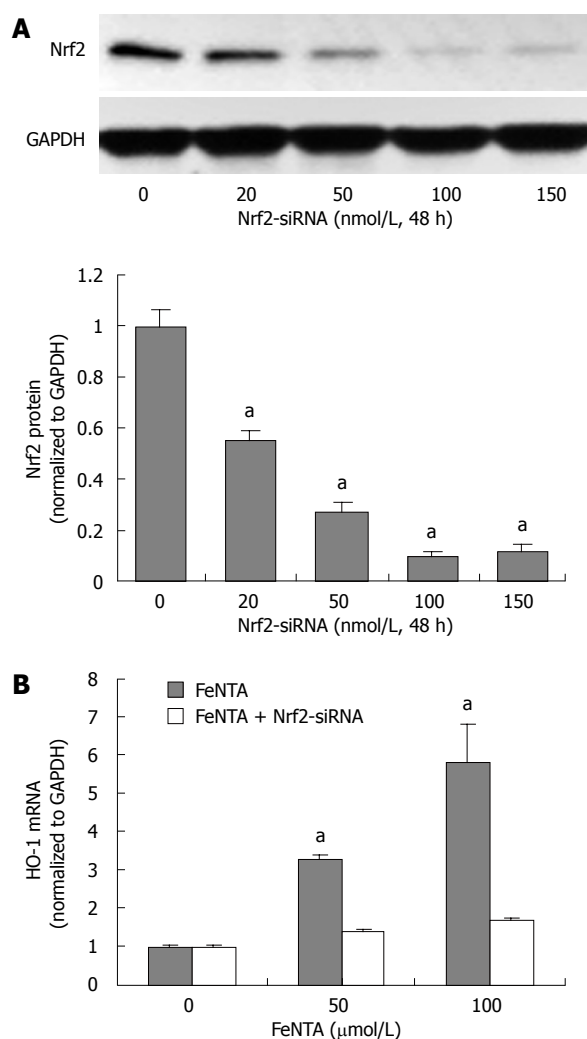


Figure 4 Silencing the Nrf2 gene abrogates up-regulation of the HMOX1 gene by iron. **A:** Dose-response effect of Nrf2-specific siRNA on Nrf2 protein levels; **B:** Effect of Nrf2-specific siRNA on FeNTA up-regulated levels of HMOX1 mRNA. ^a $P < 0.05$ vs control. 9-13 cells were transfected with selected concentrations of Nrf2-siRNA (0, 20, 50, 100, 150 nmol/L). After 48 h of transfection, cells were treated with different concentrations of FeNTA (0, 50, 100 μmol/L) for 6 h, after which cells were harvested and total RNA was isolated. The HMOX1 mRNA levels were measured by quantitative RT-PCR as described in Materials and Methods.

decreases HCV gene expression (Figure 7) in human hepatoma cells; and (5) These effects are blocked by the selective iron chelator DFO (Figures 5-7). However, none of the effects is produced by Na_3NTA , establishing that they are due to iron and not to the NTA anion. These results show clearly that iron is capable of acting directly on hepatoma cells and on HCV gene expression in hepatoma cells, without the need for mediation of effects by other tissues, organs, or cell types. Thus, it appears that iron exerts manifold effects on HCV-infected hepatocytes. On the one hand, it increases ROS and oxidative stress, acting in concert with HCV proteins, especially the core protein. On the other hand, it induces HMOX1 by increasing expression and activity of Nrf2 (Figures 1, 2 and 4), and it decreases levels of CNS3 or NS5A mRNA and protein expression (Figure 7). These latter effects are likely mediated by the recently described iron-dependent inactivation of the HCV RNA

polymerase NS5B^[43].

HMOX1 is a heat shock protein (also known as HSP 32), which can be induced to high levels, not only by heat shock, but also by a large number of physiological or pathological stressors^[30-33]. Nrf2 is a basic leucine zipper transcriptional activator^[44,45]. It protects cells against oxidative stress through antioxidant response element (ARE)-directed induction of several phase 2 detoxifying and antioxidant enzymes, including HMOX1^[35,46]. Nrf2^{-/-} mice displayed a dramatically increased mortality associated with liver failure when fed doses of ethanol that were tolerated by wild type mice, establishing a central role of Nrf2 in the natural defense against ethanol-induced liver injury^[47]. Cobalt protoporphyrin (CoPP)-mediated induction of HMOX1 involves increased Nrf2 protein stability in human hepatoma Huh-7 cells^[35]. In this study, silencing Nrf2 by Nrf2-siRNA markedly abrogated FeNTA-mediated up-regulation of HMOX1 mRNA levels. Therefore Nrf2 plays a central role in up-regulation of HMOX1 gene expression by FeNTA (Figure 4B).

Bach1, a member of the basic leucine zipper family of proteins, has been recently shown to be a transcriptional repressor of HMOX1, and to play a critical role in heme-, CoPP-, SnMP- and ZnMP-dependent up-regulation of the HMOX1 gene^[35,36,48-53]. Upon exposure to heme, heme binds to Bach1 and forms antagonizing heterodimers with proteins in the Maf-related oncogene family. These heterodimers bind to MAREs, also known as AREs, and suppress expression of genes that respond to Maf-containing heterodimers and other positive transcriptional factors. Surprisingly, ZnMP does not bind to Bach1, but it still produces profound post-transcriptional down-regulation of Bach1 protein levels by increasing proteasomal degradation and transcriptional up-regulation of HMOX1^[53]. In contrast, iron does not affect levels of Bach1 mRNA (data not shown) or protein (Figure 3), suggesting that Bach1 is not involved in up-regulation of the HMOX1 gene by iron.

Expression of HMOX1 was recently reported to be decreased in human livers from patients with chronic hepatitis C^[54,55] including some with only mild fibrosis. The reasons for this are not known currently. It is known that levels of expression of the HMOX1 gene depend in part upon genetic factors (lengths of GT repeats in the promoter^[56-59] and a functional polymorphism (A/T) at position -413 of the promoter^[60,61]). Higher expression and/or induction of HMOX1 are probably beneficial to mitigate liver cell injury in HCV infection, as well as in other liver diseases. This may be a therapeutic goal, achieved by treatment with heme or CoPP or with silymarin^[62] or other herbal products or compounds that combine anti-oxidant, iron-chelating and HMOX1-inducing effects.

Recently, we showed that HCV expression in CNS3 cells increases the levels of HMOX1 mRNA and protein^[41]. This induction is likely in response to oxidative stress. More recently, we showed that micro RNA-122, which is expressed at a high level in hepatocytes, causes down-regulation of Bach1, which, as already described,

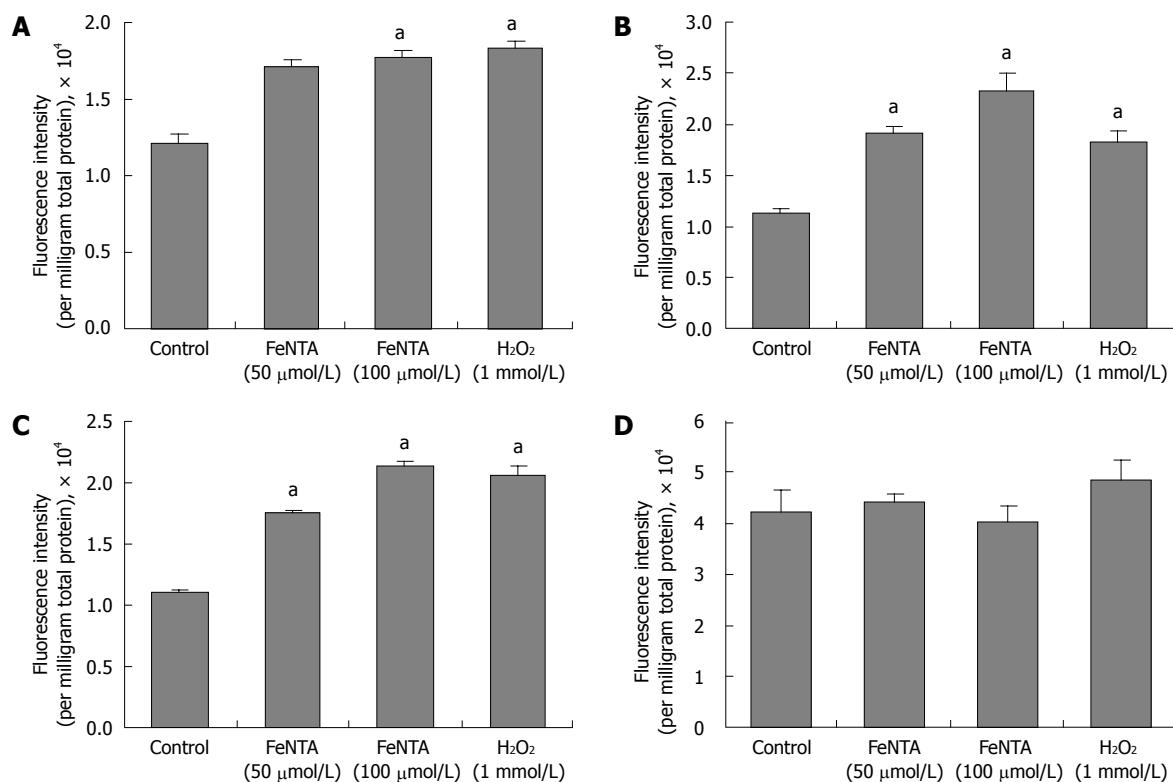


Figure 5 Effects of FeNTA on intracellular ROS in Huh-7, 9-13, and CNS3 cells. A: Fluorescence intensity with the H₂DCF-DA probe in Huh-7 cells; B: Fluorescence intensity with the H₂DCF-DA probe in 9-13 cells; C: Fluorescence intensity with the H₂DCF-DA probe in CNS3 cells; D: Fluorescence intensity with the (control) DCF-DA in CNS3 cells. Cells were preincubated with 100 μmol/L H₂DCF-DA or DCF-DA for 30 min, and then exposed to selected concentrations of FeNTA (0, 50, 100 μmol/L) for 1 h. Intracellular ROS production was measured as described in Materials and Methods. Data represent fluorescence intensity measured and expressed as relative fluorescence units per milligram total protein (mean ± SE, *n* = 3 experiments). ^a*P* < 0.05 vs control.

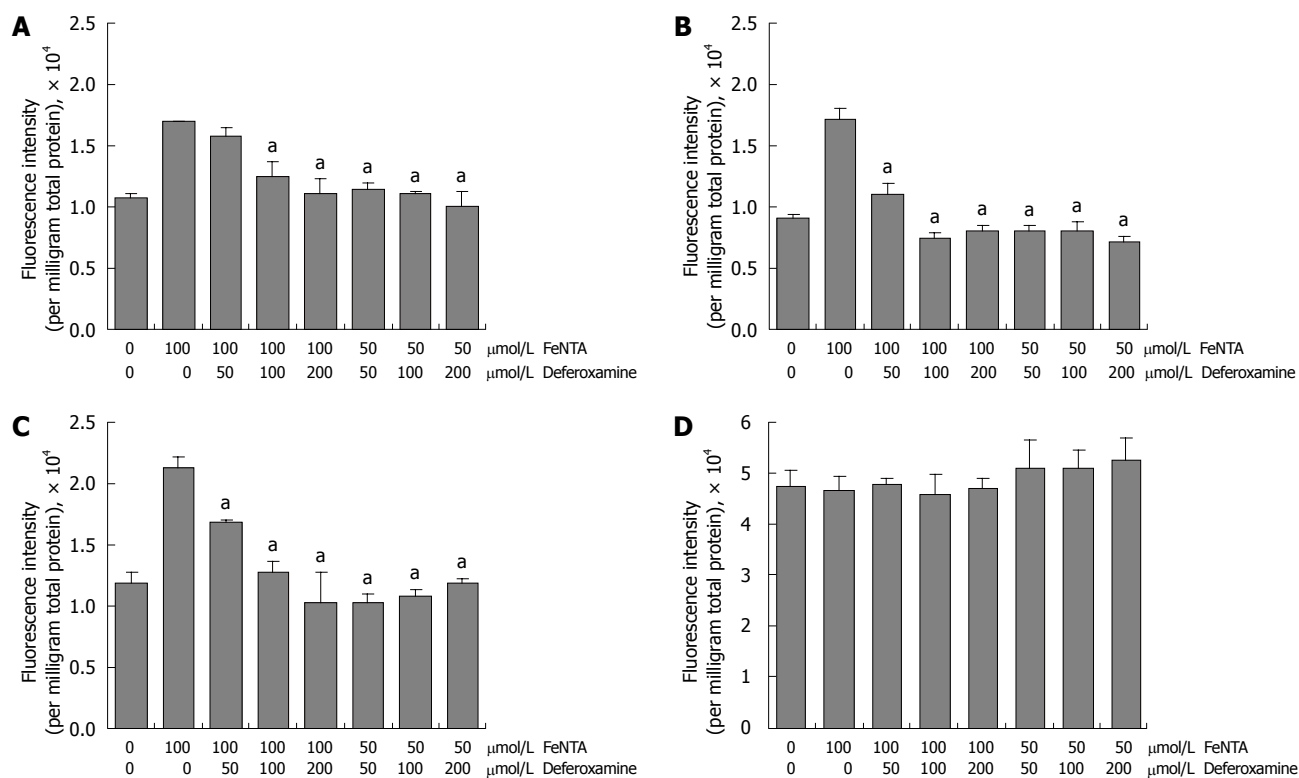


Figure 6 Effects of deferoxamine on intracellular ROS induced by FeNTA in Huh-7, 9-13, and CNS3 cells. A: Fluorescence intensity with the H₂DCF-DA probe in Huh-7 cells; B: Fluorescence intensity with the H₂DCF-DA probe in 9-13 cells; C: Fluorescence intensity with the H₂DCF-DA probe in CNS3 cells; D: Fluorescence intensity with the (control) DCF-DA in CNS3 cells. Cells were loaded with 100 μmol/L H₂DCF-DA for 30 min, treated with different concentrations of deferoxamine (50, 100, 200 μmol/L) for 30 min, and then exposed to different concentrations of FeNTA (50, 100 μmol/L) for 1 h. Intracellular ROS production was measured as described in Materials and Methods.

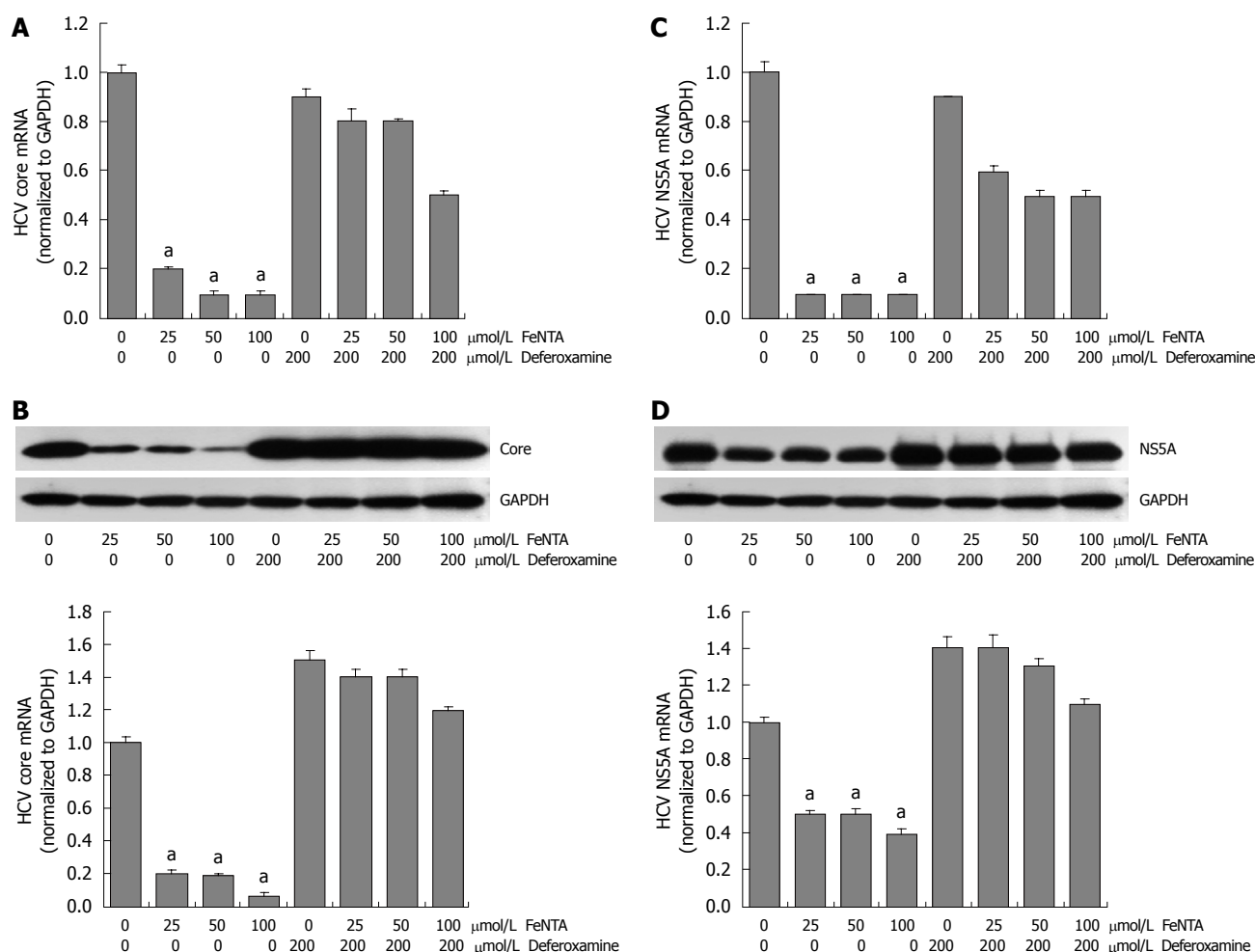


Figure 7 Effects of FeNTA on HCV core and NS5A mRNA and protein levels. A: Core mRNA levels in Con1 cells treated with FeNTA and with or without deferoxamine; B: Core protein levels in Con1 cells treated with FeNTA and with or without deferoxamine; C: NS5A mRNA levels in Con1 cells treated with FeNTA and with or without deferoxamine; D: NS5A protein levels in Con1 cells treated with FeNTA and with or without deferoxamine. Data are presented as mean \pm SE from triplicate samples, all normalized to GAPDH in the same samples. $^aP < 0.05$ vs control. The Con1 full length HCV replicon cells were treated with indicated concentrations of FeNTA and with or without deferoxamine. After 24 h, cells were harvested and total RNA and proteins were extracted. Levels of mRNA were measured by quantitative RT-PCR, and protein levels were determined by Western blots as described in Materials and Methods. Values for cells without any treatment were set equal to 1.

tonically down-regulates the *HMOX1* gene^[40]. In addition, we and others have shown that expression of micro RNA-122 is required for HCV replication in human hepatoma cells^[40,63,64]. Whether iron affects levels of micro RNA-122 has not yet been assessed, to our knowledge.

Others recently reported that iron binds to NS5B, the RNA dependent RNA polymerase of HCV, and inhibits its enzymatic activity^[43]. The HCV replicon system used in that study showed changes in the gene expression of certain genes involved in iron metabolism, including down-regulation of ceruloplasmin and transferrin receptor 1 but up-regulation of ferroportin thus producing an iron-deficient phenotype^[65]. The authors speculated that the HCV genes and proteins somehow produced these changes in order to diminish the effects of iron to inhibit NS5B RNA polymerase activity and to decrease HCV protein expression.

Regardless of these results in cell culture models, the preponderance of clinical evidence^[10-15,24-29] supports the view that iron acts as a co-morbid or synergistic factor in chronic hepatitis C infection. Because both iron and HCV infection increase oxidative stress within

hepatocytes, one attractive mechanistic explanation for the additive or synergistic affects of these two perturbations is that they act, at least in part, by increasing oxidative stress in the form of highly reactive oxygen species. These considerations provide additional rationale for the notion that reduction of iron and antioxidant therapy^[62] may be of benefit in the management of difficult to cure chronic hepatitis C^[10-15,24-29,66-68]. Iron reduction has usually been achieved with therapeutic phlebotomies. However, deferasirox (Exjade) recently has been approved in the USA and other countries as oral chelation therapy for iron overload states. Thus, studies of deferasirox for therapy of chronic hepatitis C are timely and important^[69], especially because the therapy of chronic hepatitis C currently is fraught with side effects, difficulties of adherence and rates of response that are not better than about 50%^[70-72].

In conclusion, iron can cause or exacerbate liver damage, including viral hepatitis. In the work reported in this paper we assessed effects of iron and iron chelators on liver cells, some of which also expressed genes and proteins of the HCV. Iron increased oxidative stress

and led to up-regulation of the *HMOX1* gene, a key cytoprotective gene. A mechanism for this action was to increase expression of the positive transcription factor Nrf-2. In contrast, iron did not affect expression of Bach1. Iron decreased expression of HCV genes and proteins. All the effects of iron were abrogated by DFO. The induction of HMOX1 helps to protect liver cells from the damaging effects of the HCV.

ACKNOWLEDGMENTS

We thank Ralf Bartenschlager (University of Heidelberg, Germany) for kindly supplying the HCV replicon cells used in this paper and Melanie McDermid for help in preparing the manuscript.

COMMENTS

Background

Iron overload is known to be toxic to many organs. The most common form of iron overload is hereditary hemochromatosis. In this disease, iron overload results in damage to many organs including the heart, pancreas and liver. In fact, the main site of iron deposition is in the liver. Recently it has been learned that iron plays a role in non-hemochromatotic liver disease. By insight into the mechanisms of how iron leads to this damage, novel ways to improve outcomes and success in treating these liver diseases may be achieved. One such liver disease is chronic hepatitis C.

Research frontiers

Currently chronic hepatitis C affects more than 170 million people worldwide. Standard therapy consists of a combination of pegylated alpha interferon and ribavirin. This is a difficult treatment regimen consisting of almost 1 year of therapy in many cases. Unfortunately, there is only a 50% success rate for treatment overall. There is much ongoing research seeking to improve this success rate. Until recently there were no tissue culture models for investigating hepatitis C, but cell lines have been developed which support hepatitis C viral (HCV) replication. These models allow for a unique and new way to investigate HCV replication and pathogenicity.

Innovations and breakthroughs

This article examines the role of iron in inducing heme-oxygenase 1 (HMOX1) in a tissue culture model of hepatitis C. HMOX1 is a heat shock protein that is induced by physiologic and pathologic stressors. Oxidative stress is one such stressor. The authors have shown that HMOX-1 is up regulated in cell lines that express HCV proteins. The addition of iron in the form of ferric nitrilotriacetate (FeNTA) to these cell lines further upregulates *HMOX-1* gene expression. This up regulation is independent of Bach1, a protein which functions to suppress HMOX-1. The addition of iron increased oxidative stress in these cell lines as measured by a fluorescence assay and they feel it is this oxidative stress that results in further up regulation of *HMOX1* gene expression. Conversely the expression of HCV proteins was down-regulated when HMOX1 was induced. The induction of HMOX1 likely helps to protect liver cells from the damaging effects of the HCV. The iron chelators deferoxamine (Desferal) and deferasirox (Exjade) blocked the effects of FeNTA in generating reactive oxidative stress as measured by fluorescence.

Applications

Clinical evidence supports the view that iron acts as a co-morbid factor in chronic hepatitis C infection. This may be a result of the increased oxidative stress caused by both iron and HCV infection. Therefore the use of anti-oxidant therapy and iron chelators could be of benefit in the treatment of chronic HCV infection. Recently, deferasirox (Exjade) has been approved in the USA and other countries to treat iron overload states. Studies using deferasirox as an adjunct to the treatment of hepatitis C may be an aid to advance the therapy for chronic hepatitis C.

Peer review

The manuscript is a very well written and well-designed study. In this study authors have shown the critical role of iron on HCV expression and potential use of anti-chelating agents to treat the HCV patients. The study is novel.

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S- Editor Tian L L- Editor O'Neill M E- Editor Zheng XM

EGFR and HER2 expression in advanced biliary tract cancer

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Supported by (In part) Hoffmann la Roche AG, Grenzach-Whylen, Germany

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Received: May 1, 2009 Revised: September 2, 2009

Accepted: September 9, 2009

Published online: September 28, 2009

RESULTS: 34/124 patients (27.4%) had gallbladder cancer, 47 (37.9%) had intrahepatic BTC and 43 (34.7%) had extrahepatic or perihilar BTC. EGFR expression was examined in a subset of 56 samples. EGFR expression was absent in 22/56 tumors (39.3%). Of the remaining samples expression was scored as 1+ in 12 (21.5%), 2+ in 13 (23.2%) and 3+ in 9 (16%), respectively. HER2 expression was as follows: score 0 73/124 (58.8%), score 1+ 27/124 (21.8%), score 2+ 21/124 (17%) and score 3+ 4/124 (3.2%). *HER2* gene amplification was present in 6/124, resulting in an overall amplification rate of 5%.

CONCLUSION: Our data suggest that routine testing and therapeutic targeting of HER2 does not seem to be useful in patients with BTC, while targeting EGFR may be promising.

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Key words: Cholangiocarcinoma; Gallbladder cancer; Chemotherapy; Targeted therapy

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Harder J, Waiz O, Otto F, Geissler M, Olschewski M, Weinhold B, Blum HE, Schmitt-Graeff A, Opitz OG. EGFR and HER2 expression in advanced biliary tract cancer. *World J Gastroenterol* 2009; 15(36): 4511-4517 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4511.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4511>

Abstract

AIM: To analyze the pathogenetic role and potential clinical usefulness of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) in patients with advanced biliary tract cancer (BTC).

METHODS: EGFR and HER2 expression was studied in biopsy samples from 124 patients (51% women; median age 64.8 years), with advanced BTC diagnosed between 1997 and 2004. Five micrometers sections of paraffin embedded tissue were examined by standard, FDA approved immunohistochemistry. Tumors with scores of 2+ or 3+ for *HER2* expression on immunohistochemistry were additionally tested for *HER2* gene amplification by fluorescence *in situ* hybridisation (FISH).

INTRODUCTION

Biliary tract cancer (BTC) is a heterogeneous tumor entity consisting of an intrahepatic mass forming type cholangiocarcinoma, perihilar Klatskin tumors, extrahepatic BTC, also termed intraductal growth type, and gallbladder cancer. More than 50% of tumors are diagnosed at an advanced stage with these patients having a dismal prognosis with a mean overall survival of 7-8 mo^[1,2]. Chemotherapy is widely used but of only little benefit. Therefore, new treatment options are urgently needed. Growth factor inhibitors or antibodies and small molecules such as erlotinib, gefitinib, cetuximab, panitumu-

mab, trastuzumab and lapatinib targeting the epidermal growth factor receptor (EGFR) or the human epidermal growth factor receptor 2 (HER2) have been successfully used for the treatment of colorectal, breast, lung and head and neck cancers among others^[3-7]. While there are some case reports and a phase II trial reporting promising results targeting EGFR in BTC there are no data regarding the use of trastuzumab or lapatinib also targeting HER2^[8-10]. For pancreatic carcinoma, a malignancy somewhat related to BTC, trastuzumab has been successfully used in one study and at least one further multicenter study is currently under way^[11,12].

EGFR and HER2 are receptor tyrosine kinases encoded by proto-oncogenes. Growth factors such as epidermal growth factor (EGF) or transforming growth factor (TGF) bind to these receptors at their extracellular ligand-binding domain and initiate intracellular signalling cascades, leading to tumor cell proliferation, migration, invasion, resistance to apoptosis and angiogenesis^[3,4]. In an experimental tumor model a high proportion of ErbB-2 (HER2) transgenic mice develop BTC, suggesting a role of ErbB-2 signalling in biliary carcinogenesis^[13].

Overall, overexpression of EGFR and HER2 in tumor cells has been associated with a poor prognosis, but also offers the therapeutic option of pharmacologically targeting these receptors. To date, EGFR and HER2 overexpression has been reported in up to about 80% of BTC, mostly in small patient cohorts^[14-19]. Nevertheless, there are no data regarding the correlation of immunohistochemical scores, determined by standardized methods, with clinical findings, including the overall survival of patients with advanced BTC treated by chemotherapy. The aim of this study, therefore, was to assess the clinical significance of the expression of EGFR and HER2 proteins and their potential as therapeutic targets in advanced BTC.

MATERIALS AND METHODS

Patients

Expression of EGFR and HER2 was analyzed in biopsy samples from 124 patients with advanced or relapsed, unresectable BTC (51% women, median age 64.8 years). The patients had been consecutively diagnosed at the Tumorzentrum Ludwig Heilmeyer-Comprehensive Cancer Center Freiburg between 1997 and 2004, and were followed for a median of 71 mo. All BTC cases were histologically proven adenocarcinoma. Written informed consent was obtained from all patients. Primary gastrointestinal cancers other than BTC were excluded by upper endoscopy, colonoscopy and a multislice computed tomography (CT) scan according to the consensus guidelines published in 2002^[20]. Sixty-one patients (49.2%) had been treated by chemotherapy and could be restaged for response. The chemotherapy regimens were based on 5-fluorouracil or gemcitabine, often in combination with cisplatin or oxaliplatin. The treatment response was measured by CT, magnetic resonance imaging or

Table 1 Characteristics of patients with advanced biliary tract cancer

Patients (n = 124)	n (%)
Age (yr)	
Range	32.8-84.8
Median	64.8
mean ± SD	63.4 ± 11.0
Male:female	61:63 (49:51)
Gallbladder cancer	33 (26.6)
Mass forming type	47 (37.9)
Intraductal growth type	44 (35.5)
Histological type	
Well differentiated	8 (6.5)
Moderately differentiated	80 (64.5)
Poorly differentiated	36 (29)
Stage ¹	
I	9 (7.3)
II	20 (16.1)
III	31 (25)
IV	64 (51.6)
Chemotherapy	62 (50)
Partial response	9 (14.5)
Stable disease	27 (43.5)
Progressive disease	26 (42)

¹According to AJCC/UICC classification 2002.

ultrasound according to the standard World Health Organization (WHO) criteria (WHO, 1979). The patients subgroup analyzed for EGFR was randomly selected. The different tumor types were similar between the subgroup and the whole study population. The baseline characteristics of the study population are given in Table 1.

Immunohistochemistry

Biopsy samples were fixed in 10% formalin, embedded in paraffin, cut in 5 µm sections and stained with hematoxylin/eosin for histological typing and grading. Immunohistochemistry (IHC) was performed on adjacent freshly cut deparaffinized sections using the peroxidase-labelled streptavidin-biotin technique, EGFR pharmDx™ for EGFR and Dako REAL™ detection system for HER2 staining (both Dako Glostrup, Denmark). Immunostaining was performed according to the package insert of the two FDA-approved detection systems, using only the supplied reagents and procedures.

IHC results were scored independently by two pathologists (Waiz O and Schmitt-Graeff A) blind to all clinical data. As recommended by the manufacturer additional tissue controls were performed along with the cell line controls.

The EGFR pharmDx™ kit is based on the dextran technology. First sections were treated with proteinase K at 37°C for 5 min (epitope retrieval) prior to incubation with the EGFR antibody for 1 h at room temperature. Following incubation with the primary antibody, the visualization reagent consists of both labeled polymer and HRP (Horseradish peroxidase). Subsequently added chromogen DAB+ results in a visible reaction product (brown) at the antigen site. Staining was performed using the Dako Autostainer® (Dako Glostrup, Denmark) au-

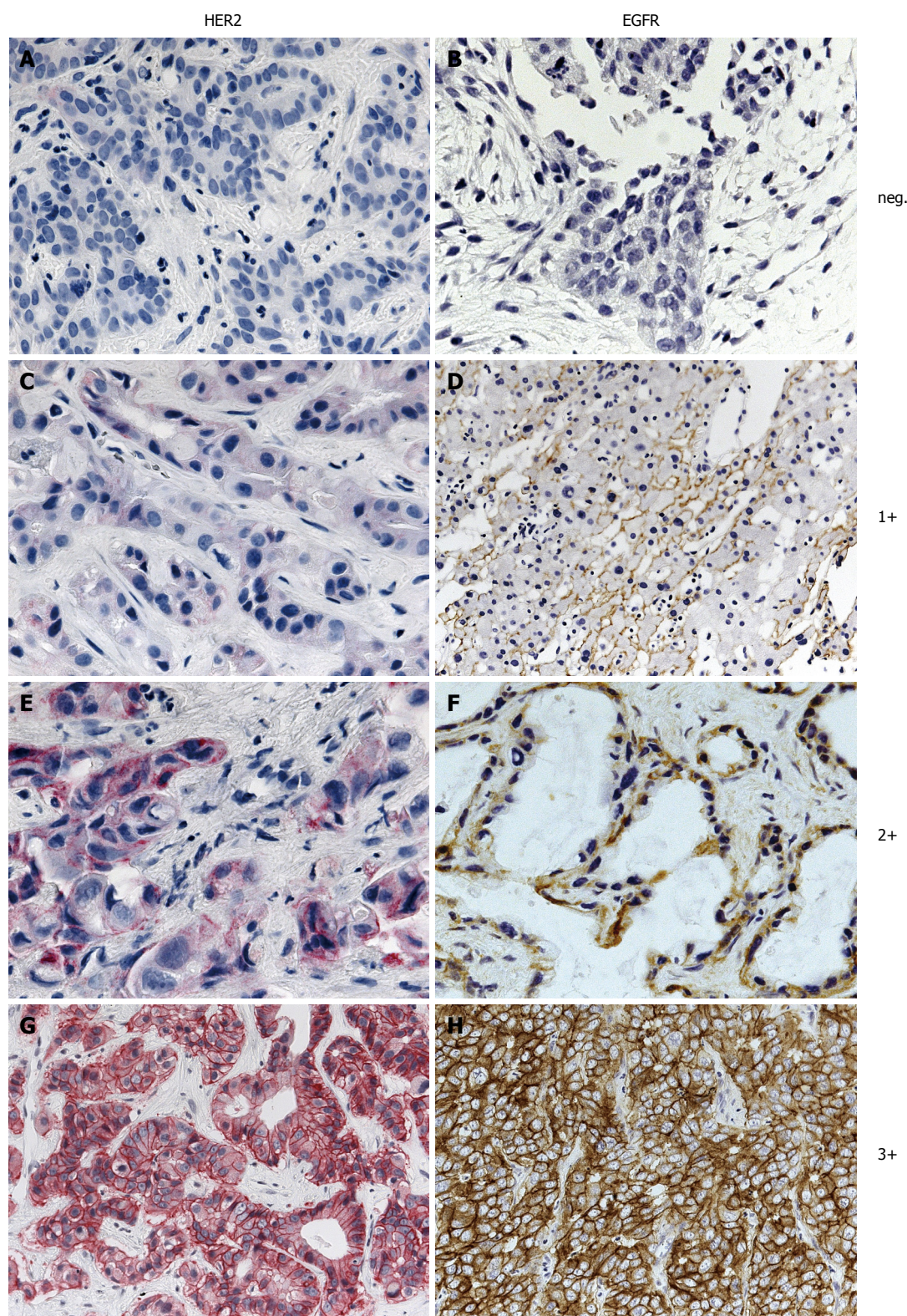


Figure 1 Immunohistochemistry of biliary tract cancer specimens for expression of HER2 (A, C, E and G) and EGFR (B, D, F and H). A: HER2 neg.; B: EGFR neg.; C: HER2 1+; D: EGFR 1+; E: HER2 2+; F: EGFR 2+; G: HER2 3+; H: EGFR 3+.

tomated system. As recommended for the interpretation of EGFR pharmDx™, both the intensity and percentage of tumor cells exhibiting membranous and/or cytoplasmic staining were assessed. If specific membrane staining was observed in less than 1% of tumor cells, the specimen was reported to be EGFR negative. The

four categories negative, weak (1+), moderate (2+) and intense (3+) were used as recommended by the manufacturer and approved by the FDA (Figure 1).

The detection of HER2 was performed with heat induced epitope retrieval (HIER) in 10 mmol/L citrate buffer (Target Retrieval Solution, pH 6.1, Dako) in a

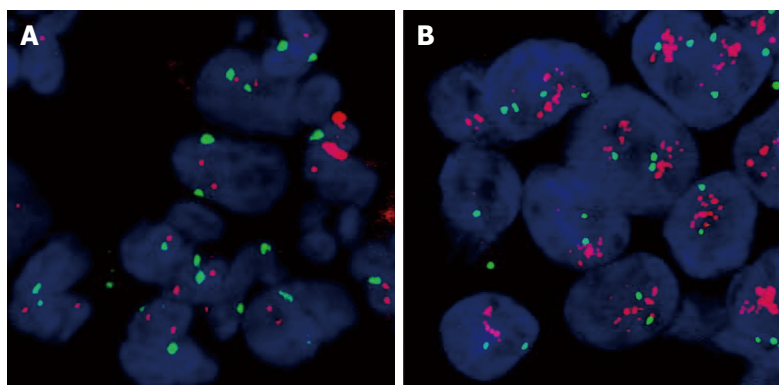


Figure 2 Dual color FISH analysis of HER2 in cholangiocarcinoma specimens. A: Nonamplified tumor with single *HER2* gene copy status. The red probe is specific for *HER2*. The green probe is specific for chromosome 17 centromere; B: Tumor with high amplification of the *HER2* gene. There are multiple red *HER2* specific signals in this tumor in relation to the green chromosome 17 signals.

water bath at 98°C for 45 min and anti-HER2 primary antibody (Polyclonal Rabbit Anti Human c-erbB-2 oncoprotein, Dako Glostrup, Denmark). Following incubation with the primary antibody, the visualization is based on the sequential application of biotinylated link antibody and streptavidin labeled with alkaline phosphatase antibody. Subsequently added chromogen Fast RED results in a visible reaction product (red) at the antigen site. HER staining was also performed using the Dako Autostainer® (Dako Glostrup, Denmark). Tumors were counted negative if less than 10% of carcinoma cells were stained, irrespective of membrane signal intensity. If 10% or more of carcinoma cells showed membrane staining, the staining intensity was classified as weak (1+), moderate (2+) or intense (3+) as recommended by the manufacturer and approved by the FDA (Figure 1). Cytoplasmic staining was discounted. Images provided by DAKO were used to define these staining categories.

Fluorescence in situ hybridization (FISH)

The PathVysion™ detection kit (Abbott, Illinois, USA) was used for FISH analysis in cases of IHC expression scores 2+ or 3+ for HER2. FISH staining was carried out according to the manufacturer's manual. The presence of gene amplification was determined using the supplied fluorescence-labelled DNA probe for chromosomal locus 17q11.2-q12 for the detection of *HER2*. FISH-stained sections were scanned at $\times 1000$ magnification and in each of three separate carcinoma areas. Twenty nuclei were analyzed. *HER2* and chromosome 17 copy number were counted for all cells and the ratio of *HER2* to chromosome 17 was calculated. A normal *HER2*:17 ratio was defined as ≤ 2 ; a ratio > 2 was interpreted as gene amplification. A normal *HER2* copy number was attested at < 4 signals per cell (Figure 2).

Statistical analysis

Data are presented as mean \pm SD or medians with range for continuous variables and as absolute and relative frequencies for categorical variables. Overall survival estimates and median survival times were calculated by the Kaplan-Meier method followed by Cox models estimating relative risks. All statistical tests were two-sided using a 5% significance level. SAS software was used for the calculations.

RESULTS

Expression of EGFR and HER2 protein

From the 124 patients examined 34 (27.4%) had gallbladder cancer, 47 (37.9%) had intrahepatic mass forming type BTC (IHCC) and 43 (34.7%) had extrahepatic or perihilar, intraductal growth type BTC (EHCC). Because EGFR expression was expected to be present two fold more frequently than HER2 expression in BTC only a subset of 56 samples was examined. EGFR expression was negative in 39.3% (22/56) of the patients. Weak (1+) positive EGFR staining was found in 21.5% (12/56), moderate (2+) in 23.2% (13/56) and intense (3+) in 16% (9/56) of the tumors. Overall 39.2% of the samples showed EGFR overexpression (scores 2+ and 3+), being significantly ($P = 0.028$) more frequent in EHCC (57.9%) than in IHCC (25%). HER2 expression was as follows: 72/124 (58%) were negative, 26 (21%) 1+, 22 (18%) 2+ and 4 (3%) 3+. Representative examples of EGFR and HER2 immunohistochemical staining and *in situ* hybridization are shown in Figures 1 and 2.

A close correlation between treatment response and gene amplification has been shown for HER2 in previous studies^[4]. However, unlike the case of trastuzumab and HER2 in breast cancer, *EGFR* gene amplification detected by FISH has not been approved as being as useful for deciding on an EGFR targeted therapy yet. Therefore, we did not study *EGFR* gene amplification in our patient cohort. Regarding HER2, based on published data and the manufacturer's recommendation, tumors with no or 1+ HER2 immunostaining were not further investigated for gene amplification. Of the 124 patients samples tested 25 were examined for *HER2* gene amplification. HER2 FISH was performed in 2+ and 3+ samples and was successfully performed in all but one tumor examined. All specimens exhibiting 3+ immunostaining (4/4) showed gene *HER2* amplification while amplification was present in 2/21 (10%) of 2+ samples. Taken together, *HER2* gene amplification could be detected in 6/124 (5%) tumors.

Correlation of EGFR and HER2 expression with clinicopathological factors

Among the 124 patients 80 (64.5%) had moderately differentiated tumors, 36 (29%) had poorly differentiated and

Table 2 EGFR and HER2 expression and clinicopathological factors (Cox's model)

Parameter	EGFR 2+ and 3+ tumors	P	HER2 2+ and 3+ tumors	P
Gallbladder cancer	5/13		8/34	
Mass forming type	6/24	0.088	7/47	0.517
Intraductal growth type	11/19		10/43	
Histological type				
Well differentiated	2/3		1/8	
Moderately differentiated	16/39	0.511	20/80	0.202
Poorly differentiated	4/14		4/36	
Stage ¹				
I	3/4		4/9	
II	2/6	0.317	4/20	0.059
III	8/17		9/31	
IV	9/29		8/64	
Chemotherapy				
Partial response	2/3		4/9	
Stable disease	3/13	0.296	4/27	0.156
Progressive disease	5/13		5/26	

¹According to AJCC/UICC classification 2002.

8 (6.5%) had well differentiated tumors. The majority of patients (64/124, 51.6%) had stage IV disease, 31 (25%) had stage III, 20 (16.1%) stage II and 9 stage I (7.3%). The patients had not undergone surgery because of unresectability, comorbidity or patients' wish. Half of the patients (62/124) had been treated with chemotherapy, resulting in tumor control in 59% (14.7% PR, 44.3% SD). Median overall survival was 13 mo with a median OS of 14 mo for patients treated with chemotherapy compared to 9 mo for patients not treated with chemotherapy. There was no statistical association between protein expression and grade, stage, overall survival and treatment response for EGFR and HER2, respectively. The frequencies of EGFR and HER2 overexpression and clinicopathological variables are summarized in Table 2. In univariate analysis EGFR and HER2 expression could not be shown to be of prognostic relevance for overall survival ($P = 0.06$ and $P = 0.49$).

DISCUSSION

Expression of the two ErbB family growth factor receptors EGFR and HER2 has been intensively studied in different tumor entities and led to the use of targeted therapy with specific inhibitors or antibodies of these receptors in colorectal, breast, lung as well as head and neck cancer^[4]. To date in other cancers monoclonal antibodies and small molecule tyrosine kinase inhibitors such as cetuximab, trastuzumab, erlotinib, gefitinib and lapatinib are under investigation. Expression of EGFR and HER2 as potential therapeutic targets has been reported in various tumors^[4,7,21,22]. For BTC, data for EGFR and HER2 overexpression have been presented in mostly small patient cohorts^[14,17,19,23]. Recently Yoshikawa *et al*^[24] described an unselected large cohort of 236 cases of resected BTC. In this study, we investigated EGFR and HER2 expression in a large cohort of patients with advanced, unresectable BTC.

In BTC the percentage of EGFR overexpressing tumors in previously reported series ranged from 8.1% to 81%. Yoshikawa *et al*^[24] showed EGFR overexpression in 26.4% of EHCC and 17.7% of IHCC. Similarly, in our study EGFR overexpression was more frequent in EHCC (57.9%) than in IHCC (25%, $P = 0.028$). In these reports by Ito *et al*^[23] and Yoshikawa *et al*^[24] EGFR overexpression was associated with biological aggressiveness of BTC. In addition, there is increasing evidence that EHCC and IHCC respond differently to chemotherapy suggesting different biological properties of these two tumor subtypes of "cholangiocarcinoma"^[25,26].

HER2 overexpression and amplification has been found in a range between 5% and 76% in BTC^[14-19,24,27]. This wide range may in part reflect the lack of standardized methodologies used within the different studies to assess HER2 status. In our cohort with advanced, unresectable BTC, HER2 overexpression was present in 20% and treatment relevant HER2 amplification in 5%. Some authors suggest that HER2 overexpression is due to gene deregulation rather than gene amplification because in some reports there is no strict correlation between protein expression and gene amplification^[19]. Overall, the highest concordance between HER2 expression and gene amplification was demonstrated for breast cancer^[7,28,29]. Our data suggest that in advanced BTC with high HER2 expression there is also a good correlation between overexpression and amplification, since all samples with a 3+ HER2 score showed gene amplification as compared to only 2/21 (10%) with a 2+ HER2 score. Similar correlations have been reported for other malignancies, including breast cancer with a tendency towards lower therapeutic relevant expression rates with improved, standardized detection methods. It has to be assumed that earlier reports overestimated HER2 expression in BTC, explaining the discrepancy between HER2 expression and gene amplification^[28,30,31]. Despite the fact that HER2 transgenic mice frequently develop BTC, HER2 does not seem to play a major role in human biliary tract malignancies. This animal tumor model may therefore be of limited value as a model for human BTC.

In recent reports in early stage, resected BTC overall HER2 amplification was about 5%-10%^[24,32]. In our cohort of patients with advanced BTC, HER2 amplification was merely 5%. Since targeted therapy with the anti-HER2 antibody trastuzumab in breast cancer is only effective when the HER2/neu receptor is overexpressed *via* gene amplification HER2 seems not to be a promising target in BTC. Therapeutic trials targeting HER2 should therefore not further be initiated.

Since EGFR expression does not predict its therapeutic usefulness, future clinical trials have to evaluate the advantage of Anti-EGFR therapy in comparison with standard treatment in patients with BTC.

In summary, our findings demonstrate that EGFR overexpression is frequent in BTC, especially in EHCC. In contrast, HER2 overexpression and gene amplification is a rare event. While therapeutic targeting

of HER2 seems to be not promising, future clinical trials in patients with BTC should focus on EGFR.

COMMENTS

Background

The epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) are involved in the carcinogenesis of many malignancies. Therapeutic molecules targeting EGFR and HER2 have been successfully used for the treatment of colorectal, breast, lung and head and neck cancers among others. It is unknown if EGFR and HER2 are overexpressed in advanced biliary tract cancer (BTC) and therefore may serve as therapeutic targets in these cancers.

Research frontiers

As the so called targeted therapies are most effective when the corresponding receptor or signalling pathway is activated, previous studies have focused on EGFR and HER2 in various tumors. There are conflicting data about overexpression of EGFR and HER2 in BTC and about the therapeutic importance of the two growth factor receptors in these tumors. Because of the low incidence, clinical trials on BTC are difficult and mostly performed in small patient cohorts. A possible selection bias might additionally explain these conflicting data.

Innovations and breakthroughs

While EGFR is significantly overexpressed in advanced BTC, HER2 overexpression and amplification is rare and therefore seems not to play a role in the carcinogenesis of BTCs.

Applications

Because of these expression data on EGFR and HER2 in BTC, and the correlation of expression and therapeutic effectiveness, especially with HER2 in other tumors, future clinical trials in BTC should focus on EGFR as a therapeutic target.

Terminology

EGFR and HER2 are receptor tyrosine kinases encoded by proto-oncogenes. Growth factors such as epidermal growth factor or transforming growth factor bind to these receptors and initiate tumor cell proliferation, migration, invasion, resistance to apoptosis and angiogenesis. BTC is a heterogeneous tumor entity with rising incidence, consisting of intrahepatic mass forming type cholangiocarcinoma, perihilar Klatskin tumors, extrahepatic bile duct tumors, also termed intraductal growth type cholangiocarcinoma, and gallbladder cancer.

Peer review

Harder *et al* describe low expression and gene amplification of HER2 in biopsy samples of advanced BTC, as assessed by immunohistochemistry and fluorescence *in situ* hybridisation, respectively. The authors suggest that deviating numbers of some earlier studies may be due to non-standardized techniques. It is convincing that in the present paper results for protein expression and gene amplification correlated well.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM

ORIGINAL ARTICLES

Protein interaction network related to *Helicobacter pylori* infection response

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Received: July 13, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: September 28, 2009

Abstract

AIM: To understand the complex reaction of gastric inflammation induced by *Helicobacter pylori* (*H. pylori*) in a systematic manner using a protein interaction network.

METHODS: The expression of genes significantly changed on microarray during *H. pylori* infection was scanned from the web literary database and translated into proteins. A network of protein interactions was constructed by searching the primary interactions of selected proteins. The constructed network was mathematically analyzed and its biological function was examined. In addition, the nodes on the network were checked to determine if they had any further functional importance or relation to other proteins by extending them.

RESULTS: The scale-free network showing the relationship between inflammation and carcinogenesis was constructed. Mathematical analysis showed hub and bottleneck proteins, and these proteins were mostly related to immune response. The network contained pathways and proteins related to *H. pylori* infection, such as the JAK-STAT pathway triggered by interleukins. Activation of nuclear factor (NF)- κ B, TLR4, and other proteins known to function as core proteins of immune response were also found. These immune-related proteins interacted on the network with pathways and proteins related to the cell cycle, cell maintenance and proliferation, and

transcription regulators such as BRCA1, FOS, REL, and zinc finger proteins. The extension of nodes showed interactions of the immune proteins with cancer-related proteins. One extended network, the core network, a summarized form of the extended network, and cell pathway model were constructed.

CONCLUSION: Immune-related proteins activated by *H. pylori* infection interact with proto-oncogene proteins. The hub and bottleneck proteins are potential drug targets for gastric inflammation and cancer.

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Key words: Gastric cancer; *Helicobacter pylori*; Inflammation; Pathway; Protein interaction network

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Kim KK, Kim HB. Protein interaction network related to *Helicobacter pylori* infection response. *World J Gastroenterol* 2009; 15(36): 4518-4528 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4518.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4518>

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram negative bacterium which infects about 50% of the world population^[1-3]. It is known to cause various gastroduodenal diseases such as chronic active gastritis in experimental animals and in humans. In human volunteers, *H. pylori* caused gastritis and hypochlorhydria^[4]. Mongolian gerbils infected by *H. pylori* also developed symptoms such as intestinal metaplasia and adenocarcinoma^[5-9]. Many scholars have demonstrated a relationship between *H. pylori* and gastric carcinoma^[3], and the World Health Organization (WHO) and the International Agency for Research on Cancer consensus group have classified *H. pylori* as a definite biological carcinogen^[10].

H. pylori colonization causes a strong systemic immune response^[11]. It induces the production of interleukins (ILs) (Korean Society for Medical Microbiology, 2004), tumor necrosis factor (TNF)^[12,13], and proinflammatory

cytokines^[14]. It also causes activation of nuclear factor κ B (NF- κ B)^[15], activator protein-1 (AP-1), c-Jun, NH₂-terminal kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase, and other cell proliferation and survival factors^[16]. Bacterial toxins, high levels of superoxides, radicals, and singlet oxygen are known to induce carcinogenesis in gastric cells. Bacterial virulence factors such as CagA and VacA^[1,17,18] induce cell hyperproliferation and the expression of oncogenes. However, the exact mechanism between *H pylori* and gastric carcinoma is unclear^[19].

Various tools have been employed to identify the relationship between *H pylori* and gastric cancer, including c-DNA microarrays^[4,20]. However, most of these methods did not consider the systematic interaction of biological components. As an alternative, a network construction and analysis of protein-protein interactions^[21] were applied to examine the inflammatory response to *H pylori* infection in a systematic manner.

MATERIALS AND METHODS

The research method used in this study mainly consisted of three steps. Step one: extraction of the genes which changed significantly during *H pylori* infection from the database and by querying web databases to gather protein-protein interactions. Step two: construction of a network and summarizing the constructed network. Step three: analysis and extension search of the network. A flow chart showing the data flow is described in Figure 1.

Searching genes related to *H pylori* infection (Step 1)

Genes related to *H pylori* infection were collected by searching PubMed. The expression of genes significantly changed ($P < 0.05$) by *H pylori* infection in the microarray^[4,11,13,20] data was examined, and genes related to the immune response were identified and collected. A total of 39 filtered genes (Table 1) were obtained.

Scanning protein interactions and construction of protein interaction networks (Step 2)

The protein interaction networks were constructed based on statistical prediction through the analysis of microarray data. Selected genes were queried to the Uniprot database to convert into proteins. The proteins were scanned by a human Protein-protein Interaction Prediction (PIPs) database (<http://www.compbio.dundee.ac.uk/www-pips/>). Protein links were then extracted from the Human Protein Reference Database reference (HPRD, <http://www.hprd.org/index.html>). Without HPRD references, any further search of the protein links was stopped. An extended network was constructed by integrating all results extracted from the PIPs server (Figure 2). Pajek (<http://vlado.fmf.uni-lj.si/pub/networks/pajek/>) was used for the construction of extended networks. Then, a core network showing simplified main pathways, major proteins, and subcellular location information was extracted from the extended network using Cytoscape (<http://www.cytoscape.org/>).

Table 1 List of proteins extracted from the literary database showing significant change after *H pylori* infection

Protein/gene name	Uniprot ID	HPRD reference
ITGB2	P05107	
LY96	Q9Y6Y9	X
TLR10	Q9BXR5	
TLR2	O60903	
TLR3	O15455	X
VCAM1	P19320	
HCK	P08631	
MAPK8	P45983	
RAC2	P15153	
SOCS2	O14508	X
STAT6	P42226	
C2	P06681	X
C3	P01024	
C4A	P0C0L4	X
CCL18	P55774	X
CCL19	Q99731	X
CCL3	P10147	X
CCL4	P13236	X
CRP	P02741	
CXCL13	O43927	
CXCL2	P19875	X
CXCL9	Q07325	X
HLA-DMA	P28067	X
HLA-DPB1	Q30154	X
HLA-DQB1	P03992	X
HLA-DRB5	Q30154	X
HSPH1	Q92598	
C11TA	P33076	
PLAT	P00750	
IFITM1	P13164	X
IRF4	Q15306	
MADCAM1	Q13477	
ALOX5	P09917	X
TLR5	O60602	X
CD53	P19397	X
TLR6	Q9Y2C9	X
SLAMF1	Q13291	
PTPRC	P08575	
FAIM3	O60667	X
CD180	Q99467	
TLR4	O00206	
TLR1	Q15399	
CXCL3	P19876	X
CD47	Q08722	
IFNGR1	P15260	
IL10RA	Q13651	X
IL18RAP	O95256	X
ITGAX	P20702	X
IL8	P10145	

Nodes with no HPRD reference were marked with x. HPRD: Human protein reference database.

Analysis of protein interaction network (Step 3)

The protein interactions of an extended network were examined whether or not the network contained known pathways related to *H pylori* infection, inflammation, and carcinogenesis. The core network was not analyzed because it was just the simplified form of the extended network.

Four factors: Shortest paths, degree (connectivity), betweenness centrality (BC), and closeness centrality (CC), were adopted to analyze general mathematical properties of the extended network and to search

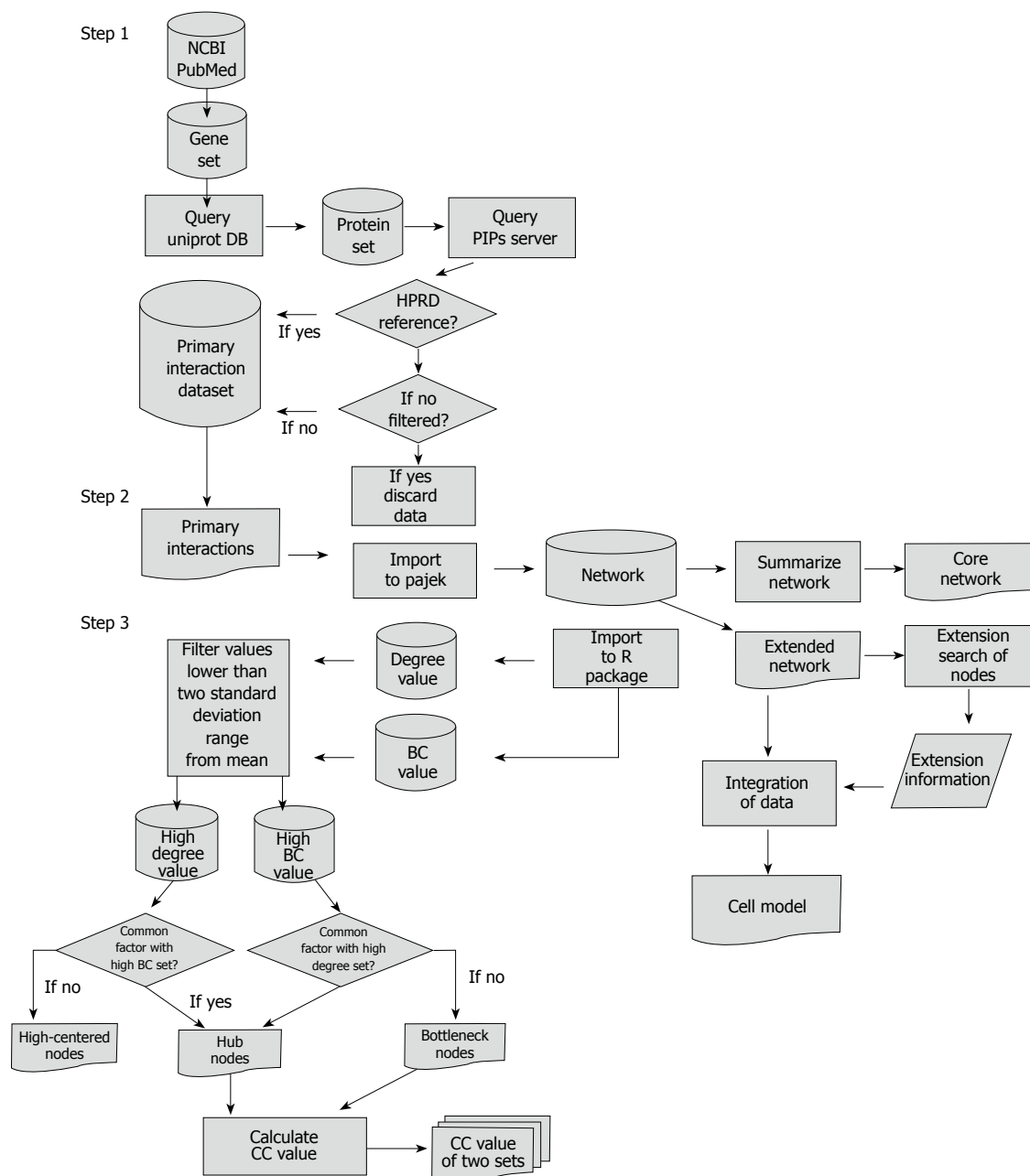


Figure 1 Flow chart showing overall methods and data flow used in this study.

topologically important proteins^[21].

Degree, the most basic characteristic of a node, is defined as the number of links the node has with other nodes. Degree distribution is obtained by counting the number of nodes with a fixed degree value, which is variable from minimum to maximum degree, and dividing it by the total number of nodes of a network^[22]. Highly concentrated nodes play a major role as a hub in a network. Degree was also used to check if an extended network was scale-free, which is frequently found in cellular networks^[22,23]. The scale-free network follows a power-law degree distribution^[22]. Power law is defined as: $P(x) = Cx^{-\alpha}$

$C = e^c$ and $P(x)$ is a probability that a selected node has exactly x links (degree value)^[23]. α is the degree exponent which determines some properties of the

network. Most of the networks found in nature are known to have degree exponent values between two and three^[22]. In this study, cumulative distribution function, a superior method of plotting data^[23], was used. The plot of log transformed probability distribution function $P(x)$ in which x has a degree value greater than or equal to x , was drawn. $P(x)$ is defined mathematically as^[23]:

$$P(x) = \int_x^{\infty} p(x') dx'$$

As the distribution follows power law,

$$P(x) = C \int_x^{\infty} x'^{-\alpha} dx' = \frac{C}{\alpha-1} x^{-(\alpha-1)}$$

A cumulative plot also follows power law, but the degree exponent of the plot is one less than the original distribution^[23]. The degree exponent was calculated by measuring the slope of the regression line and adding one to the exponent value. Other factors such as R

square, standard error, and *P*-value were also computed.

BC for node *k* is defined as:

$$b(k) = \sum_{i,j} b_{i \rightarrow j}(k) = \sum_{i,j} \frac{g_{i \rightarrow j}^k}{g_{i \rightarrow j}}$$

$g_{i \rightarrow j}$ is the number of shortest paths from node *i* to *j*, while $g_{i \rightarrow j}^k$ is the number of geodesics among $g_{i \rightarrow j}$ that passes through node *k*^[21,24]. The BC value of all nodes in the network was examined to check for bottlenecks in the network.

CC is defined as the inverse of the average length of the shortest paths to/from all the other vertices in the graph^[25]. It tells us the topological center of the network^[25]. CC was calculated by adopting the core algorithm of the R igraph package (<http://www.r-project.org/>). CC values of the protein set with either large BC value or degree were measured and compared to total CC values to check topological centrality of hubs and bottlenecks in the network.

The shortest path (geodesics) is calculated by measuring the length of all the geodesics from or to the vertices in the network. The average shortest path was measured to see how many average steps were required to link two randomly selected nodes in the network.

After computing BC and the degree of all the nodes, nodes under two standard deviation ranges from the mean were filtered out and CC values of nodes larger than two standard deviation ranges from the mean were measured. As a result, nodes with a large BC value, a large degree, both a large BC and degree, and CC value were obtained. The R package was used to calculate and analyze these values.

The network was constructed by scanning primary interactions of significantly and differentially expressed genes compared to control. Thus, it may not include hidden interactions of protein nodes between the two major nodes. For example, only the primary interaction between node A and B is available by ordinary network analysis, although the two proteins are linked *via* node C in reality. However, by extending the network, a pathway passing through node C between A and B can be found.

RESULTS

Protein interaction networks

By integrating scanned primary interactions of previously selected nodes from the PIPs server, the extended network was constructed. A core network was then derived from the extended network.

The extended network was composed of 604 nodes, connected *via* 808 edges (Figure 2). One giant network with 599 nodes and 805 edges, and two separate interactions were observed. Examining the shortest paths of the network showed that two randomly selected nodes on the network were connected *via* 4.89 links. This suggests that the nodes were very closely linked. In addition, a small world effect can be found^[26]. The distribution of the shortest paths was plotted using histograms (Figure 3A). The average value (4.89) was similar to other values of human protein networks^[21,26].

Table 2 List of proteins with a large degree value and their CC values

Protein	Degree	CC value
RELA/NF-κB3	105	0.047675522
MAPK8	68	0.046693511
NFKBIA	63	0.047164646
HCK	49	0.046599691
PTPRC	43	0.046388184
ITGB2	40	0.045643782
MAP2K1	36	0.045681818
PLAT	25	0.045451119
STAT6	24	0.046513422
HLA-DMA	24	0.04396646
TRAF4	24	0.044062843
TLR4	24	0.046527778
HLA-DRB5	23	0.04573032
TLR2	22	0.046083301
IL10RA	18	0.046206897
ALOX5	18	0.044056404

CC: Closeness centrality.

The cumulative distribution plot showed clear evidence that the extended network follows scale-free distribution (Figure 3B). By measuring the slope of the regression line of the plot drawn on the basis of log transformed cumulative data, the α value of 1.1968 in the power law distribution was determined. As the degree exponent of the cumulative plot is one less than original distribution^[23], the true degree exponent value should be 2.1968 (standard error = 0.04, coefficient of determination R square = 0.97, and *P*-value = nearly zero by the least square fit)^[27]. It is known that networks with a degree exponent larger than three do not have features that scale-free networks have^[22]. The degree exponent value of the extended network (2.1968) was lower than 3, which was similar to other networks following a scale-free distribution, rather than a random distribution.

Important nodes in the network

One of the properties of networks following scale-free distribution is the existence of a small number of highly connected nodes, called hubs which are more important than other less connected nodes^[22,28]. The hub nodes are more critical to the survival of cells (Tables 2 and 3). The scale-free networks are prone to breakdown into fragments when nodes are attacked^[29]. Other important nodes also have a large BC value. The node with a large BC functions as a bottleneck in the network, even when the node's degree is low. Nodes with a degree or BC value larger than the mean plus two standard deviations were selected. Sixteen nodes were determined to have a large degree (Table 2) and 19 nodes had a large BC (Table 4). Twelve nodes had both a large degree and a large BC (Table 3). Six nodes: NF-κB3 (Nuclear factor κB p65 subunit), MAPK8 (Mitogen-activated protein kinase 8), NFKBIA (NF-κB inhibitor α), HCK (Hemopoietic cell kinase), PTPRC (Leukocyte common antigen CD45), and ITGB2 (Integrin β-2) were the top six nodes on both degree and BC values.

Table 3 List of proteins with both a large BC and degree, and their functions

Protein name	Function
RELA/NF- κ B3	NF- κ B is a pleiotropic transcription factor which is present in almost all cell types and is involved in many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis
MAPK8	Responds to activation by environmental stress and pro-inflammatory cytokines by phosphorylating a number of transcription factors, primarily components of AP-1 such as JUN, JDP2 and ATF2 and thus regulates AP-1 transcriptional activity
NFKBIA	Inhibits the activity of dimeric NF- κ B/REL complexes by trapping REL dimers in the cytoplasm, masking their nuclear localization signals
HCK	May serve as part of a signaling pathway coupling the Fc receptor to activation of the respiratory burst. May also contribute to neutrophil migration and regulate the degranulation process of neutrophils
PTPRC	Required for T-cell activation through the antigen receptor
ITGB2	Receptor for ICAM1, ICAM2, ICAM3 and ICAM4
TLR4	Cooperates with LY96 and CD14 to mediate the innate immune response to bacterial lipopolysaccharide (LPS). Acts <i>via</i> MYD88, TIRAP and TRAF6, leading to NF- κ B activation, cytokine secretion and the inflammatory response
PLAT	Converts the abundant, but inactive, zymogen plasminogen to plasmin by hydrolyzing a single Arg-Val bond in plasminogen. By controlling plasmin-mediated proteolysis, it plays an important role in tissue remodeling and degradation, in cell migration and many other physiopathological events
TRAF4	Adapter protein and signal transducer that links members of the tumor necrosis factor receptor family to different signaling pathways by association with the receptor cytoplasmic domain and kinases
MAP2K1	Catalyzes the concomitant phosphorylation of a threonine and a tyrosine residue in a Thr-Glu-Tyr sequence located in MAP kinases. Activates ERK1 and ERK2 MAP kinases
TLR2	Cooperates with LY96 to mediate the innate immune response to bacterial lipoproteins and other microbial cell wall components
STAT6	Carries out a dual function: signal transduction and activation of transcription. Involved in interleukin-4 signaling

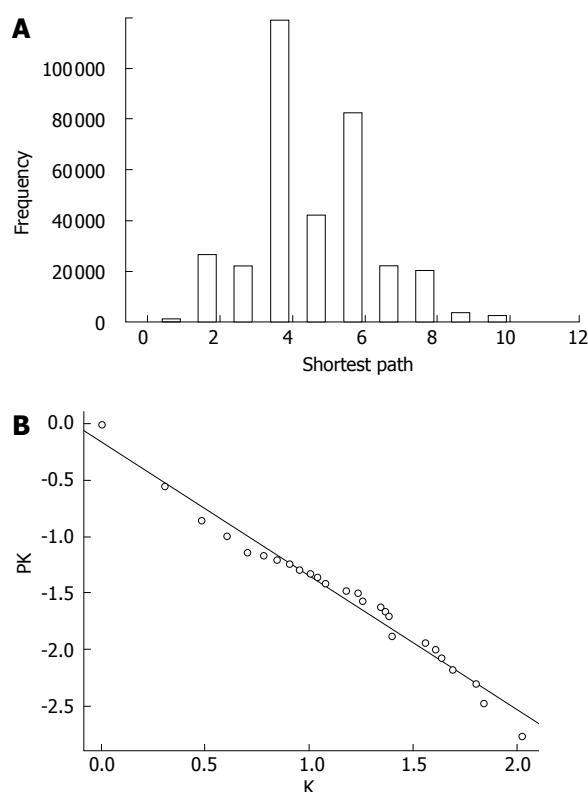


Figure 3 Properties of the extended network. A: Histogram showing distribution of the shortest path. Two randomly selected nodes were connected *via* 4.9 links; B: Cumulative degree distribution plot of the extended network showing that degree distribution follows the power law. Line indicates the degree exponent of 1.2, which is one lower than the true degree exponent of 2.2.

STAT6 (Signal transducer and activator of transcription 6), TLRs (Toll-like receptor), TRAF4 (TNF receptor-associated factor 4), and PLAT (TPA, Tissue-type plasminogen activator) also had both a large BC and degree (Table 3). NF- κ B3, NFKBIA, PTPRC, TLRs, and HLA-DRB5 are already well-known for having

Table 4 List of proteins with a large BC value and their CC values

Protein	BC	CC
RELA/NF- κ B3	62240.75685	0.047675522
MAPK8	37123.82691	0.046693511
PTPRC	34614.23746	0.046388184
HCK	33484.99898	0.046599691
NFKBIA	28771.33132	0.047164646
ITGB2	27901.91607	0.045643782
TLR4	19587.75501	0.046527778
PLAT	19339.65216	0.045451119
CCL4	18441.86414	0.043654528
HMGB1	15263.85652	0.046466826
MAP2K1	14047.72141	0.045681818
STAT6	13376.61128	0.046513422
CXCR4	11471.71166	0.044769471
CSNK2A1	11279.76894	0.046574496
HLA-DRB5	11088.56681	0.045730320
BTK	11016.20767	0.046621308
TLR2	10824.15886	0.046083301
RAC2	10692.38019	0.044872749
TRAF4	10472.80579	0.044062843

BC: Betweenness centrality.

biological functions related to immune response^[2,3,21]. STAT6 is related to the JAK-STAT pathway which sends signals from ILs directly to the nucleus^[30]. MAPK8 of the MAPK signaling pathway can be found in the signaling of other inflammatory responses in asthma, and is related to cell proliferation^[31]. TRAF4 is involved in tumor necrosis and TPA (PLAT) in plasminogen activation, respectively. Most of these nodes are related to immune response and signal transduction, suggesting that these nodes perform major functions against *H. pylori* infection.

Not only nodes with both a large degree and BC, but also nodes with a large BC and a small degree were considered important in previous research^[21], since these nodes function as bottlenecks in the network,

Table 5 List of proteins with only a large BC and their functions

Protein name	Function
HMGB1	Binds to preferentially single-stranded DNA and unwinds double-stranded DNA
BTK	Plays a crucial role in B-cell ontogeny
CSNK2A1	Casein kinases are operationally defined by their preferential utilization of acidic proteins such as caseins as substrates
CXCR4	Transduces a signal by increasing the intracellular calcium ion level
CCL4	Monokine with inflammatory and chemokinetic properties
RAC2	Plasma membrane-associated small GTPase which cycles between an active GTP-bound and inactive GDP-bound state. In active state binds to a variety of effector proteins to regulate cellular responses, such as secretory processes, phagocytosis of apoptotic cells and epithelial cell polarization. Seems to be involved in the regulation of NADPH oxidase

even without the role of hubs. Six nodes: HMGB1 (High mobility group protein B), BTK (Bruton tyrosine kinase), CSNK2A1 (CSK2A1, Casein kinase II subunit alpha), RAC2 (Ras-related C3 botulinum toxin substrate 2), CCL4 (C-C motif chemokine 4), and CXCR4 (C-X-C chemokine receptor type 4) had a large BC but a low degree. Large BC nodes such as CXCR4, CCL4, BTK, CSNK2A1, and RAC2 with the exception of HMGB1 are related to immune response and signal transduction (Table 5). HMGB1 unwinds double-stranded DNA and binds preferentially to single-stranded DNA, which may be related to the gene regulation of immune response. As expected these large BC nodes were linked to important nodes, such as hubs. HMGB1 was linked to NF- κ B3, TLR4, TLR2, and PLAT, which have a large BC degree (Figure 2). BTK interacted with NFKBIA, TLR4, HCK, and IL10RA. NFKBIA, TLR4, and HCK had a large BC and degree, while IL10RA had a large degree only. CSNK2A1 was linked to NF- κ B3, NFKBIA, PTPRC, and HSPH1. RAC2 interacted with NFKBIA, HCK, and ALOX5. Lastly, CCL4 and CXCR4 were linked to PTPRC and PLAT. Thus, it was demonstrated that the nodes with large BC play important roles in the connection and communication of nodes including hubs.

The CC values of nodes with a large degree or BC were checked to see if these proteins were near to the topological center of the network. The larger the CC value is, the closer the node is to the center of the network^[21]. NF- κ B3 was closest to the topological center, and NFKBIA was the second closest in the network (Tables 2 and 4).

Biological functions of pathways and nodes in the network

Pathways related to immune response and other biological phenomena were observed in the network (Figures 4 and 5). The network contained previously known pathways which were involved in *H pylori* infection and inflammation.

The network (Figures 2 and 4) showed interactions of IL 1, 4, 8, 10, 13, 17, and 18 receptors with JAKs and STATs that send signals from cell-surface receptors to the nucleus^[30]. IL 8 increases significantly during *H pylori* infection, thus it was used as a standard to determine the pathogenicity of different *H pylori* strains^[19]. IL 1, 10, and 18 changed significantly, which was demonstrated by microarray analysis or Western blotting data^[11,13,32].

IL 4 and 13 are proinflammatory cytokines. While IL 4 induces eosinophilic inflammation and differentiation of Th2 cells, IL 13 produces immunoglobulin E (IgE)^[33].

Interactions of Toll-like receptors (TLRs), also known to be immune-related, were observed. The TLR4 signaling pathway is associated with an immune response by interacting with MYD88 and IRAK1^[34,35] in the network (Figure 4). They were linked to proteins in the nucleus through MAPKs.

Another pathway in the network was found among the MAPKs. Interactions among MAPK 1, 3, and 8 in the network were observed. In immune-related diseases such as asthma, the activation of MAPK due to infection has also been reported^[21,36,37].

Besides full pathways, the presence of single or a few interactions having biological functions were informative. NF- κ B and AP-1 are two key regulatory factors of inflammation^[38-40]. NF- κ B1-NF- κ B3 linkage and JAK-NFKBIA-STAT linkage were found (Figure 5). The regulation of NF- κ B by AP-1(JUN) and NFKBIA was also observed (Figure 4).

Although activation of TNF α ^[13] was not found in the network, TNFSF11 (Tumor necrosis factor ligand superfamily member 11) and TRAFs (TNF receptor associated factor), related to TNF, were found. Tumor necrosis factors induce cell proliferation by activating anti-apoptosis^[16]. Cell proliferation and carcinogenesis are one of the well-known characteristics of cells infected by *H pylori*^[19]. In addition, BRCA1 (Breast cancer type 1 susceptibility protein), FOS (*c-fos*, Proto-oncogene protein)^[41], REL (C-Rel proto-oncogene protein), and VAV1 (Proto-oncogene vav), which are oncogenes, were found. The presence of TNF and the oncogenes in the network suggests that *H pylori* infection may be related to carcinogenesis.

SRC (Proto-oncogene tyrosine-protein kinase) in the network is involved in cell maintenance and communication^[21]. CDK5 (Cyclin-dependent kinase 5), RASA1 (Ras GTPase-activating protein 1) and RASA3 are related to cell growth effect^[30].

Not only protein nodes related to inflammation and carcinogenesis, but also proteins related to stress resistance were found. Infection of *H pylori* increases levels of superoxide and singlet oxygen. The stress-resistance protein, HSPH1 (Heat shock protein 105 kDa), HSPA8 (Heat shock cognate 71 kDa protein), and HSPB1 (Heat shock protein β -1) were found.

Generally, stimulation and regulation of the immune

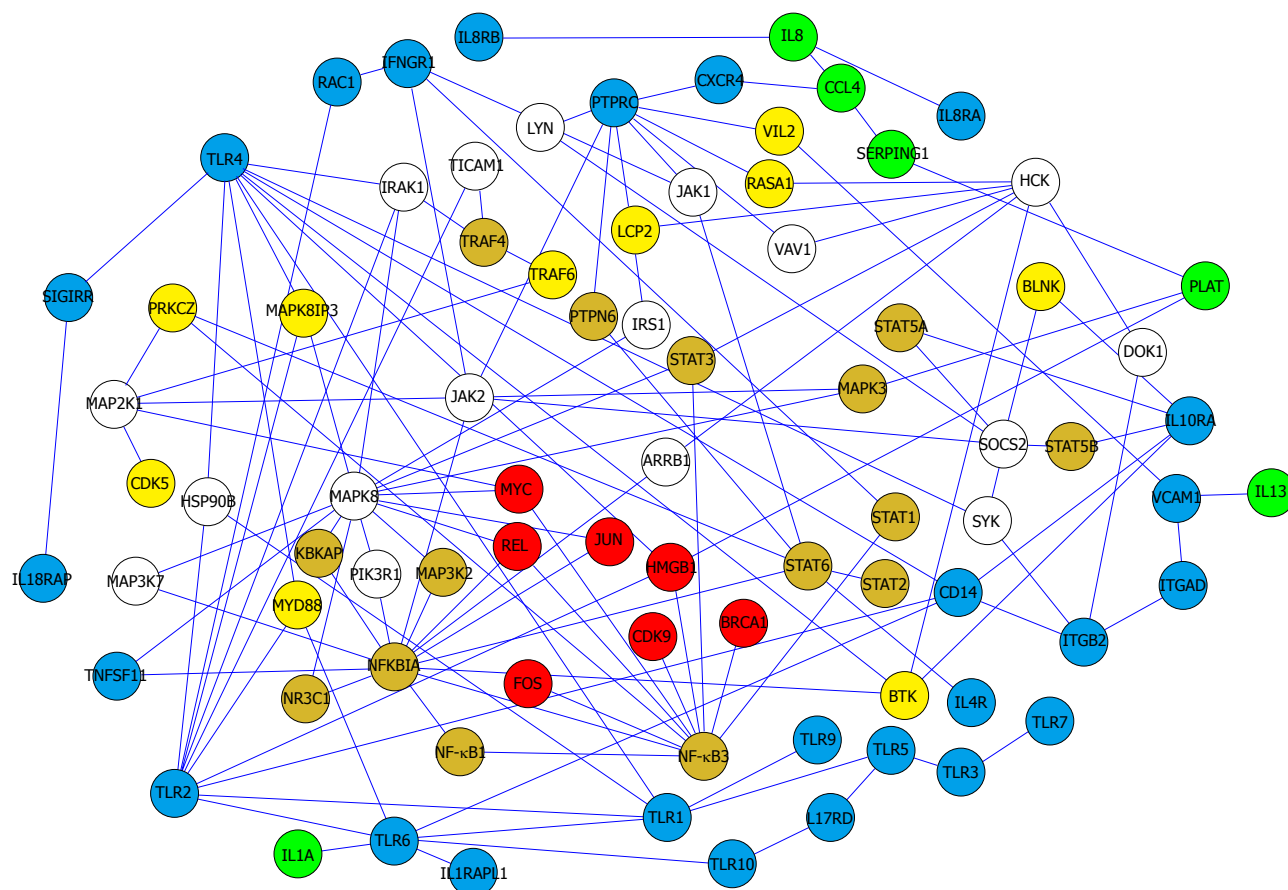


Figure 4 Core network showing simplified interactions and major pathways. Color of the nodes indicates subcellular location. Red (nucleus), orange (nucleus and cytoplasm), yellow (cytoplasm), blue (membrane), green (extracellular), and white (unknown).

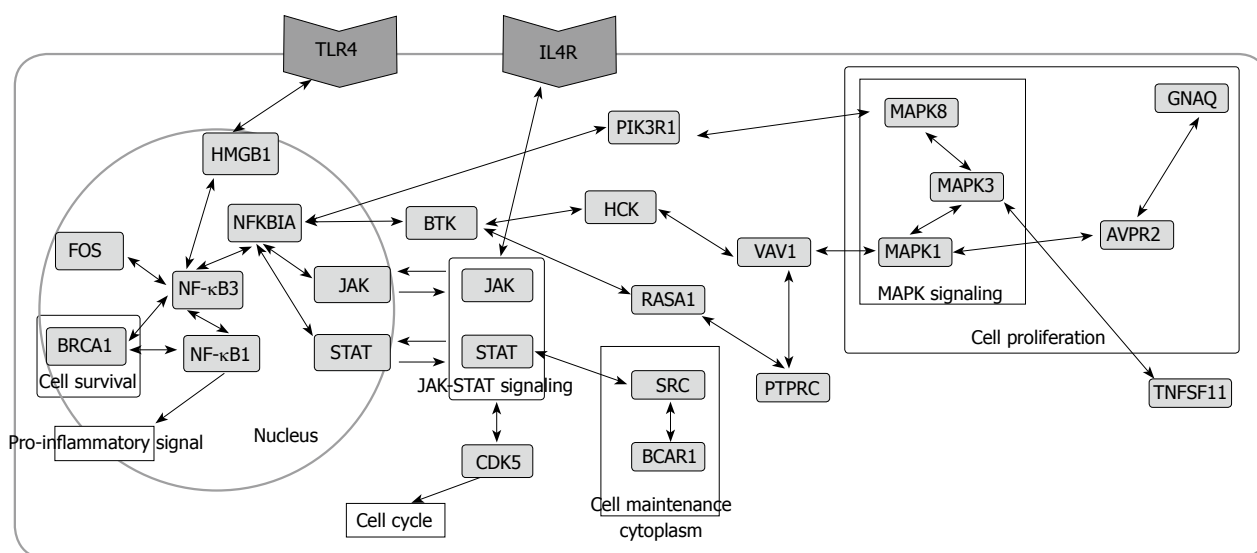


Figure 5 Cell model showing major interactions after *H. pylori* infection.

system through their receptors were found in the network. Activation of cell signaling, cell proliferation, cell survival, proto-oncogenes, and stress resistance were observed. These functions are reminiscent of the observed response of cells infected by *H. pylori*. The virtual network analysis in this study reflects the real protein-interaction-network in the cell.

Extension search of the network

A biologically important protein can be missed, as the network is constructed by searching only the primary interactions of selected genes. To overcome this problem, further interactions of nodes regardless of their degree or BC were examined. The extension of SRC led to BCAR1 (Figure 5). Thus, the role of cell maintenance^[42]

was connected with that of carcinogenesis in BCAR1. ADRB2 (β -2 adrenergic receptor) extension was linked to PRKCE, PRKACA, MAPK1, and MAPK3 (Figure 2). This pathway has not previously been reported in *H. pylori* infection, but has been found in immune-related diseases such as asthma^[21]. BRCA1 was further linked to CDK2, 4, 7, and CDC2 (Cell division control protein 2 homolog) (Figure 5). CDKs are activated proceeding to the cell cycle. The extension of BRCA1 was linked to JUND (transcription factor jun D), which binds to an AP-1 site and stimulates its promoter activity. BRCA1 extension led to ZNF467 (Zinc finger protein 467), a transcription regulator which has a possible relationship with cancer (Figure 2). The extension of MAPK1 led to GNAQ (Guanine nucleotide-binding protein G(q) subunit α) *via* GNAS (Guanine nucleotide-binding protein G(s) subunit α isoforms short) and AVPR2 (Vasopressin V2 receptor)^[21] (Figure 5). The proteins in this pathway contribute to cell proliferation, a well-known characteristic of cells infected by *H. pylori*^[19]. STAT1-CREBBP (CREB-binding protein) linkage was related to G1 arrest of a cell^[21] (Figure 5).

DISCUSSION

The correlation between inflammation caused by *H. pylori* infection and gastric cancer has been studied and supported by many researchers. It is important to understand the relationship between inflammation and the carcinogenesis mechanism. Microarray data were used to determine the global gene expression of infected cells. Microarray data showed up/down regulation of gene expression related to immune response, cell cycle, cell growth, and signal transduction, which may support the hypothesis that *H. pylori* infection causes cancer development^[4,11,13,20]. However, the data did not present a clear mechanism of carcinogenesis in a systematic manner. In this study, network analysis methods were applied to integrate previous data and construct the network model which shows the relationship between inflammation and cancer development.

The extended network showing primary interactions of significantly expressed genes (proteins in the network) was constructed. The network contained many protein nodes related to immune response and signal transduction induced by extracellular signals such as cytokines. The important nodes selected based on large BC and degree values were mostly involved in immune response and signal transduction. For example, the p65 subunit of NF- κ B (NF- κ B3), one of the most important regulatory factors of inflammation, was the node with the largest degree and BC value. Large BC nodes, the bottlenecks in the network, were linked to important nodes with a large degree, a large BC, or both. Like large BC and degree nodes, a large BC node was mostly related to immune response and signal transduction, with the exception of HMGB1. The constructed network also contained many pathways related to immune response and signal transduction. TLR4, JAK-STAT, and MAPK8 pathways are major pathways found in the network. Not

only the pathways, but important nodes such as NF- κ B and AP-1 (JUN) were also found in the network.

The network also showed many nodes related to carcinogenesis. Tumor related proteins such as BRCA1, FOS, REL, VAV1, TNFSF11, and TRAFs were found. The extension search of nodes was also linked to pathways related to cell proliferation, cell survival, and the cell cycle. The extracellular signal from ILs and TLRs goes to NF- κ B, NFKBIA, and AP-1 in the nucleus *via* the JAK-STAT and MAPK signaling pathways. The signal then goes to proteins in the cytoplasm *via* the JAK-STAT pathway and BTK, promoting cell proliferation and proceeding to the cell cycle. These activated processes are one of the characteristics of cells infected by *H. pylori*. In addition, *H. pylori* infection is known to increase levels of radicals and oxides. Radicals and oxides are widely thought to be possible mutagens. Oxidative stress may be an additional mechanism of carcinogenesis.

Another important factor of hub and bottleneck protein nodes is that they are potential drug targets. By inhibiting the functions of hubs and bottlenecks by small molecules, the function of the network can be shut down, meaning that the inflammatory and carcinogenesis processes can be stopped, theoretically. Traditionally, antibiotics have been used to treat gastric inflammation caused by *H. pylori* infection^[14]. However, this treatment has the potential problem of antibiotic resistance in the bacteria. As a potential alternative, this study presented the hub and bottleneck nodes as a drug target of gastric inflammation, cancer, and other diseases caused by *H. pylori* infection.

The analysis of protein network interactions showed immune response and carcinogenesis-related cell responses in a bigger picture. The extension search of nodes also demonstrated key signal transductions linking inflammatory response and carcinogenesis. This study showed how a systematic approach such as the network construction produces meaningful information. It also offered a relatively easy and simple framework to understand the complexity of cellular interactions having functional importance. Therefore, the application of this tool may be an alternative to find important genes and drug targets in other diseases and in complex biological systems.

ACKNOWLEDGMENTS

The authors thank Sohyun Hwang, Seung-woo Son, and Doheon Lee of Korea Advanced Institute of Science and Technology (KAIST) for their kind advice and aid.

COMMENTS

Background

The correlation between inflammation caused by *Helicobacter pylori* (*H. pylori*) infection and gastric cancer has been studied and supported by many researchers.

Research frontiers

To explain the relationship between *H. pylori* infection and cancer development,

microarray analysis was used. Microarray data showed the regulatory patterns of gene expression related to immune response, cell cycle, cell growth, and signal transduction. However, the data obtained did not show the mechanism of carcinogenesis in a systematic manner.

Innovations and breakthroughs

In this study, protein network analysis, one of the bioinformatic tools, was applied to integrate previous microarray data, and a network model was constructed showing the relationship between inflammation and cancer development. The network contained many proteins related to immune response and signal transduction induced by extracellular cytokines. Some tumor-related proteins (BRCA1, FOS, REL, VAV1, TNFSF11, TRAF) were found.

Applications

This article offered a relatively easy and simple framework to understand the complexity of cellular interactions having functional importance. This tool may be used as an alternative to find important genes and drug targets in gastric inflammation and cancer and in complex biological systems.

Peer review

This study about protein interaction network in *H pylori* infection is potentially interesting and informative.

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S- Editor Cheng JX L- Editor Webster JR E- Editor Yin DH

Attenuation of portal hypertension by natural taurine in rats with liver cirrhosis

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Author contributions: Liang J and Deng X designed research; Deng X and Zhang XL performed research; Deng X and Lin ZX analyzed data; Liang J, Lin ZX and Zhao LC wrote and revised the paper.

Supported by The National Natural Science Foundation of China, Grant, No. 30660235; Guangxi Science Foundation for Youths, Grant, No. 0728080; National "11th 5-year" Support Plan of China, Grant, No. 2006BAI0802-07

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Received: June 15, 2009 Revised: August 18, 2009

Accepted: August 25, 2009

Published online: September 28, 2009

tus of the liver tissue and reduced the expression of COL I, COL III and TGF- β 1.

CONCLUSION: NTau inhibited the LC-induced PHT by improving hyperdynamic circulation, morphology of liver and biomechanical properties of the portal vein in experimentally-induced LC rats.

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Key words: Taurine; Liver cirrhosis; Portal hypertension; Rat

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

Liang J, Deng X, Lin ZX, Zhao LC, Zhang XL. Attenuation of portal hypertension by natural taurine in rats with liver cirrhosis. *World J Gastroenterol* 2009; 15(36): 4529-4537 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4529.asp>
DOI: <http://dx.doi.org/10.3748/wjg.15.4529>

Abstract

AIM: To investigate the inhibitory effect of natural taurine (NTau) on portal hypertension (PHT) in rats with experimentally-induced liver cirrhosis (LC).

METHODS: Experimentally-induced LC Wistar rats (20 rats/group) were treated with either oral saline or oral NTau for 6 consecutive weeks. Evaluation parameters included portal venous pressure (PVP), portal venous resistance (PVR), portal venous flow (PVF), splanchnic vascular resistance (SVR) and mean arterial pressure (MAP). Vasoactive substance levels including nitric oxide (NO), nitric oxide synthase (NOS) and cyclic guanosine monophosphate (cGMP) were also measured. Histological investigation of type I and III collagen (COL I and III) and transforming growth factor- β 1 (TGF- β 1) was also performed.

RESULTS: Treatment with NTau (1) significantly decreased PVP, PVR and PVF, and increased MAP and SVP; (2) markedly increased the vascular compliance and reduced the zero-stress of the portal vein; (3) markedly decreased the amount of NO and cGMP and activity of NOS; and (4) improved the pathological sta-

INTRODUCTION

Liver cirrhosis (LC)-induced portal hypertension (PHT), also referred to as hepatocirrhotic portal hypertension, is highly susceptible to life-threatening complications such as esophageal and fundus ventriculi variceal bleeding, as well as ascites and hepatic encephalopathy (HE), resulting in high mortality among this group of patients. Currently, several treatment modalities are commonly employed for the management of LC-induced PHT, including systemic drug treatment, surgical intervention, endoscopic ligation, and liver transplantation. Although endoscopic ligation is useful in preventing initial hemorrhage in the upper digestive tract, it is less effective when dealing with recurring bleeding^[1]. Transjugular intrahepatic portosystemic stent shunt (TIPSS) can, to a certain extent, reduce various complications of PHT. It may, however, augment the incidence of HE. Therefore, TIPSS is not generally the first therapeutic option for patients with PHT owing to its inability to improve the survival rate^[2]. On the other hand, liver transplantation is regarded as the last option to combat advanced liver cirrhosis. However, the inherent risk

associated with this radical surgical procedure and the inevitable high costs of organ transplantation, together with the long-standing disturbance of the systemic hemodynamics after transplantation, practically prevents it from being a routinely used method. Presently, long-term pharmacological treatment is still the mainstay for LC-induced PHT. To date, to develop an effective therapeutic approach for the management of LC-induced PHT remains a formidable challenge to many researchers in the field.

Our previous study showed that natural taurine (NTau) markedly inhibited the contraction and collapse of hepatic stellate cells (HSCs)^[3]. It has been well established that contraction of HSCs significantly contributes to the initiation and progression of hepatocirrhotic portal hypertension^[4,5]. As a continuation of our previous efforts to evaluate the therapeutic potential of NTau for LC-induced PHT, the present study aims at investigating the inhibitory effects of NTau on PHT, specifically focusing on the perspectives of hepatic “forward” and “backward” flow theories as well as on portal venous biomechanics and hemodynamics.

MATERIALS AND METHODS

Animals and reagents

Male specific-pathogen-free (SPF) Wistar rats weighing 215 ± 18 g were provided by the Centre of Experimental Animals, Guangxi Medical University, Nanning, Guangxi Province, China. Immunohistochemical staining kits for type I collagen (COL I), type III collagen (COL III) and transforming growth factor- β 1 (TGF- β 1) were purchased from the Wuhan Boster Biological Technology Ltd (Wuhan, Hubei Province, China). Assay kits for nitric oxide (NO), nitric oxide synthase (NOS) and cyclic guanosine monophosphate (cGMP) were obtained from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, Jiangsu Province, China). The instruments used in the current project included an electromagnetic flowmeter (MFV-3200; Nihon Kohden, Japan), an 8-channel physiology recorder (RM-6000; Nihon Kohden, Japan), an ultraviolet spectrophotometer (80-2106-20; Pharmacia, UK), a UV-spectrophotometer (9100; Beijing Rayleigh Analytical Instrument Corporation, Beijing, China), and a light microscope (BX51, Olympus, Japan).

Extraction of NTau

The natural taurine (2-aminoethyl-sulfonic acid) used in the present study was extracted from black clams (*Meretrix meretrix* L.). Briefly, after the clam meat was cleaned and weighed (500 g), it was then minced in an electrical blender (4000 r/min) ten times, with each process lasting for about 10 s. Distilled water (1 L) was then added into the mince and further homogenized for 30 min. The mixture was then boiled in a water bath at 100°C for 30 min, followed by filtering the mixture through four layers of gauze. The residue on the top of the gauze was discarded and the filtrate was then centrifuged to obtain the supernatant, which was then de-acidified with HCl

(HCl:H₂O = 3). After centrifugation, the proteins were adjusted to pH 10 with NaOH (20%) aqueous solution to yield the de-alkalined proteins. After the pH value was adjusted to 5, the supernatant was further condensed. The other unwanted amino acids and pigments were removed by column chromatography using strong acid cation exchange resin as the solid phase and eluting with distilled water. The resultant NTau was quantitatively measured by high performance liquid chromatography (HPLC) and the purity of the NTau was determined to be 98.8%.

Establishment of animal model and treatment protocol

An animal model of LC was established following a previously described protocol^[6]. In brief, rats were fed with animal chow consisting of 80% corn flour, 19.5% animal fat and 0.5% cholesterol. The animals were only allowed to drink 15% aqueous alcohol. After an initial subcutaneous injection of a 40% CCl₄-olive oil solution at a dose of 5 mL/kg, the subcutaneous injections were repeated once every 3 d at a dose of 3 mL/kg for a total duration of 42 d. All rats were kept at room temperature under 12-h dark/light cycles and received humane care in accordance with the Guidelines of the Guangxi University of Chinese Medicine for the Care of Laboratory Animals.

Forty Wistar rats were randomized into 2 groups, with 20 rats in each group: a model group without NTau treatment (LC - NTau), and a model group treated with NTau (LC + NTau). During establishment of the model, rats in the LC + NTau group were concomitantly administered with 600 mg/kg NTau by gavage once daily, while LC - NTau group received only saline. Another 20 Wistar rats which received only normal animal chow and no CCl₄-olive oil solution injection were also used as the normal healthy control (NML) in the experimental design.

Influence of NTau on the “forward flow” theory of LC

Measurement of hemodynamic parameters:

Measurement of the hemodynamic parameters was conducted according to the methods described previously^[7] and was performed by an experienced technician affiliated to the first author's research group. Briefly, the animals were fasted for 8 h prior to measurement. Pentobarbital sodium (30 mg/kg) was injected through an ear vein to induce anesthesia. Then, a median epigastric incision was made in each animal which was placed in a supine position. The main portal vein (about 2 cm in length) was dissociated and exposed by blunt dissection. An incision was made followed by placement of an electromagnetic probe with an appropriate caliber connected with the electromagnetic flowmeter into the portal vein to measure the portal venous flow (PVF). Similar catheterizations were made into the portal vein and carotid artery for the measurement of the portal venous pressure (PVP) and mean arterial pressure (MAP) with the aid of a physiology recorder. All data were recorded after the hemodynamic parameters were stabilized. Portal venous resistance

(PVR) and splanchnic vascular resistance (SVR) were also calculated separately using the following equations $PVR = PVP/PVF$ and $SVR = MAP/PVF$, respectively.

Determination of NO, NOS and cGMP in portal venous blood: Heparinized portal venous blood (5 mL) was obtained and centrifuged at 3000 r/min for 10 min. The serum was extracted for determination of the concentrations of NO, NOS and cGMP using the nitrate reductase method, chemical colorimetry and radioimmunoassay respectively according to the manufacturers' instructions.

Influence of NTau on the "backward flow" theory of LC

Histological investigation of the liver tissue: Twenty-four hours after the last dosing of the experiment, the rats were anesthetized with ethyl ether, and the livers were quickly removed from the etherized animals. Tissue mass of a size measuring about 1 cm × 1 cm × 1 cm was collected from a site about 0.5 cm distant from the hepatic margin of the left lobe and then placed in a 4% paraformaldehyde solution for fixation for 24 h. The tissue mass was dehydrated in increasing concentrations of ethanol. After hydration, wax-impregnation, embedding and sectioning, HE and Masson staining were sequentially performed. Morphological changes and the degree of fiber hyperplasia of the liver tissue in rats with LC were observed under a light microscope. The grading of LC was performed according to the Knodell HAI, Scheuer, METAVIR, modified Ishak HAI and Chevallier grading systems^[8,9].

Quantitative detection of COL I, COL III and TGF- β_1 in liver tissue: Liver tissue was fixed with 10% formalin and embedded in paraffin. Then, serial sections (4 μ m) were cut. Immunohistochemical staining was performed by the Streptavidin-biotin complex (SABC) method. Briefly, the paraffin sections were baked in an oven at 60°C for 1 h and then placed into a pure xylene solution for deparaffinization twice, with each lasting for 15 min. The sections were then placed into a 3% hydrogen peroxide solution at room temperature for 30 min to inactivate endogenous peroxidase, followed by boiling in a 0.01 mol/L citrate buffer under high temperature and pressure conditions for 2 min. After this, the tissues were covered with a normal goat serum blocking buffer and placed in an incubator at 37°C for 30 min. Subsequently, anti-COL I, COL III and TGF- β_1 primary antibodies were separately added at a dilution of 1:100 to the tissues. The sections were incubated at 37°C for 30 min. After being kept at 4°C overnight, the sections were washed thoroughly in PBS (5 min × 3 times), and then biotin secondary antibody was added before incubation at 37°C for 30 min. The sections were washed again in PBS (5 min × 3 times) and in distilled water (3 min × 3 times), followed by incubation with avidin-peroxidase at 37°C for 20 min. 3,3'-diaminobenzidine (DAB) was added after smearing. The color developing was monitored under a light microscope. The staining was stopped by washing the sections in distilled water. Following stain-

ing with hematoxylin for 1 min and washing in distilled water, the sections were sequentially dehydrated in 95% and 100% ethanol for 5 min each. After air drying, the sections were sealed by neutral gum and observed under a light microscope. Mias-2000 image analysis software (Institute of Image and Graphics, Sichuan, China) was used for quantitative measurement. The ratio of the positive area of COL I, COL III and TGF- β_1 to the overall visual field area was calculated.

Determination of biomechanics of the portal vein:

After sacrifice of the rats, the main portal vein was immediately removed and connected to a three-way baroreceptor. The pressure was amplified by a dynamic electric resistor which was linked to a computer. The biomechanics of the portal vein were evaluated by measuring the corresponding pressure when the relative volume of blood vessels was changed. The vascular compliance, denoted as C, was calculated using the following formula: $C = 2\pi R \cdot \Delta R / \Delta P$, where R represents the radius of the blood vessel, ΔR and ΔP are the change of the radius and portal vein pressure respectively.

To measure the zero-stress state of the blood vessels, cross-sections were made across the portal vein and arterial rings were obtained. A cut along the ventral margin of the arterial ring was made and the expanded angle of the arteriae aorta was observed under an anatomical microscope. The photographic recording was carried out, followed by printing out on paper. The included angle, i.e. the opening angle, which was formed from the midpoint of the inner lining of the arterial ring to the two broken ends of the inner lining, was measured and used to represent changes in the zero-stress state of the blood vessels. In each rat, 5 open angles of the arterial ring were measured and their mean value was calculated.

Statistical analysis

Data were expressed as mean \pm SE. Statistical comparisons among NTau-treated animals, non-treated model controls, and healthy controls were carried out using one-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's test using the appropriate group as the control. Comparison of degrees of LC between groups was conducted by rank sum test. The analysis was performed on the SPSS for Windows (version 14.0). Differences were considered significant at $P < 0.05$ or $P < 0.01$.

RESULTS

Effect of NTau on hemodynamic alterations

Table 1 summarizes the hemodynamic data of the normal healthy animals, those having undergone LC induction and LC animals treated with NTau for six consecutive weeks. Compared with the normal healthy control group, the LC rats had very significantly higher PVP ($P < 0.01$), and the PVF and PVR were also markedly elevated in the experimentally-induced LC animals, while the MAP and SVR were considerably lower in these model animals

Table 1 Comparison of the portal hemodynamics in different groups of animals

	MAP (mmHg)	PVP (mmHg)	PVF (mL/min per kg)	SVR (mmHg/mL per min/kg)	PVR (mmHg/mL per min/kg)
NML	105.12 ± 8.56	5.26 ± 0.72	43.15 ± 1.23	2.1 ± 0.23	0.07 ± 0.03
LC - NTau	90.23 ± 4.51 ^a	7.85 ± 0.91 ^a	59.65 ± 4.32 ^a	1.61 ± 0.26 ^b	0.10 ± 0.04 ^a
LC + NTau	93.42 ± 3.25 ^c	5.38 ± 0.65 ^d	48.21 ± 2.25 ^c	1.81 ± 0.35 ^c	0.07 ± 0.05 ^c

^a*P* < 0.05, ^b*P* < 0.01 *vs* the normal healthy control group; ^c*P* < 0.05, ^d*P* < 0.01 *vs* the non-treatment model group.

Table 2 Effects of NTau on the concentrations of NO, cGMP and activity of NOS in portal venous blood

	<i>n</i>	NO (μmol/L)	cGMP (nmol/L)	NOS (U/L)
NML	20	21.41 ± 2.32	0.21 ± 0.02	2.88 ± 1.35
LC - NTau	20	48.56 ± 3.61 ^a	0.34 ± 0.04 ^a	6.45 ± 0.42 ^a
LC + NTau	20	33.14 ± 5.33 ^b	0.26 ± 0.01 ^c	3.81 ± 1.21 ^c

^a*P* < 0.05 *vs* the control group; ^b*P* < 0.01, ^c*P* < 0.05 *vs* the non-treated model group.

(*P* < 0.05). The treatment with NTau significantly attenuated PVP (*P* < 0.01), PVF and PVR (*P* < 0.05) when compared with the non-treated model group. Accordingly, the NTau treatment enhanced the MAP and SVR (*P* < 0.05). These experimental data suggest that NTau was able to improve the hemodynamic conditions in animals with LC.

Effects of NTau on the concentrations of NO and cGMP and activity of NOS in the portal venous blood

The effects of NTau on the concentrations of NO and cGMP and NOS activity are summarized in Table 2. When compared with the healthy control group, the amount of NO and cGMP and the activity of NOS were significantly increased in animals with LC. Treatment with NTau caused a significant reduction in NO (*P* < 0.01) and cGMP (*P* < 0.05) content in the blood and in the activity of NOS (*P* < 0.05) when compared with that of the non-treated LC animals. The data derived from this experiment indicate that treatment with NTau significantly mitigates the release and activation of vasoactive substances such as NO, cGMP and NOS.

Histological investigation of the liver tissue

Histological observation of normal rat liver under HE staining was characterized by intact and distinct structure of liver tissue, normal structure of hepatic lobules, and radial distribution of a cord-like arrangement of hepatocytes around the central vein (Figure 1A). In experimentally-induced LC rats, abnormal histological features were observed such as the destruction of the normal structure of hepatic lobules; extensive fibroplasia of interstitial tissue of the liver, which divided the hepatic lobules into different sizes of hepatocellular mass (i.e. formation of pseudo-lobules); extensive fatty degeneration of hepatocytes with some necrosis; and infiltration of many inflammatory cells into the portal areas and hepatic lobules. In the NTau-treated

Table 3 Degrees of liver cirrhosis of rats in different experimental groups

	<i>n</i>	Grading of LC ¹						
		S ₀	S _I	S _{II}	S _{III}	S _{IV}	S _V	S _{VI}
NML	20	0	0	0	0	0	0	0
LC - NTau ^b	20	0	0	0	0	5	12	3
LC + NTau ^d	20	0	0	2	6	7	5	0

¹ Grading criteria: S₀, normal structure of liver tissue, which is characterized by an absence of deposition of abnormal collagen fibers; S_I, occurrence of fibrous septa (FS) due to hyperplasia of collagen fibers in portal areas (P) or areas surrounding the central vein (C); extension of FS within 50% of the distance between two portal areas (P-P) or between portal and central vein areas (P-C); S_{II}, extension of fibrous septa beyond 50% of the P-P or P-C; connection of fibrous septa is either incomplete or complete; absence of completely enclosed or separated hepatic lobules; S_{III}, presence of completely enclosed hepatic lobules by FS; S_{IV}, separation of hepatic lobules by extensive hyperplasia of collagen fibers; destruction of the normal lobular structure; pseudo-lobules formed are mainly large-square-shaped (> 50%); S_V, concomitant presence of large-square- and small-round-shaped pseudo-lobules (both less than 50%); and S_{VI}, intrahepatic pseudo-lobules are mainly small-round-shaped (> 50%). ^b*P* < 0.01 *vs* the normal healthy control; ^d*P* < 0.01 *vs* the non-treated LC group.

group, the damage of the structure of hepatic lobules was still observed, but with milder fibroplasia, fewer infiltrations of inflammatory cells and only modest foamy degeneration of hepatocytes when compared with the non-treated LC rats. Under Masson staining, only a small amount of collagen was observed in the normal healthy group. In contrast, a large amount of collagen fibrils were found in the LC model rats; however, only thin collagen fibrils were observed in the NTau-treated animals.

The degree of LC in all three animal groups is presented in Table 3. It is evident that the experimental procedure involved in establishing the model, essentially the high fat diet and injection of CCl₄, succeeded in inducing LC in all rats, and the degree of LC ranged from S_{IV} to S_{VI}. The treatment with NTau succeeded in ameliorating the extent of LC.

COL I, COL III and TGF-β₁ changes in the liver tissue

Figure 2 is a bar chart presentation of the ratio of the positive area of COL I, COL III and TGF-β₁ in the different animal groups. It is evident that the induction of LC in the model rats significantly increased the amount of COL I, COL III and TGF-β₁, with COL I increasing from 9.41 ± 1.36 to 30.63 ± 8.25, COL III from 10.55 ± 2.46 to 33.65 ± 7.52, and TGF-β₁ from 15.37 ± 4.62 to 31.28 ± 7.85, respectively. The

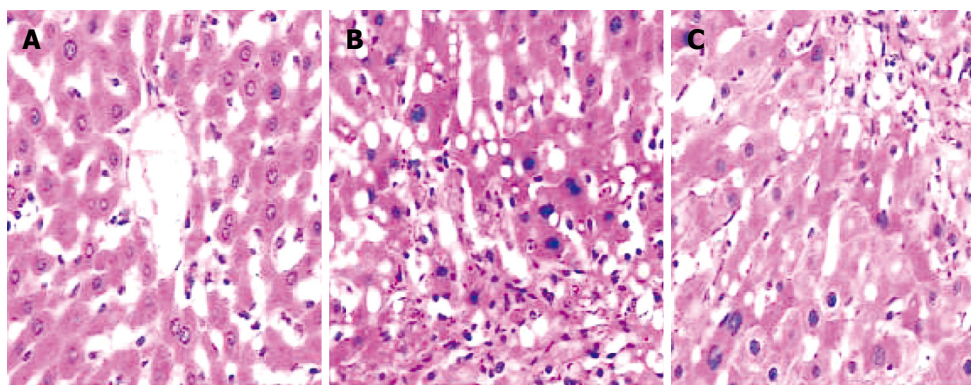


Figure 1 Histological findings of liver tissue of the rats in different experimental groups. A: Normal healthy rats; B: LC rats; C: NTau-treated groups. (magnification, 10×20).

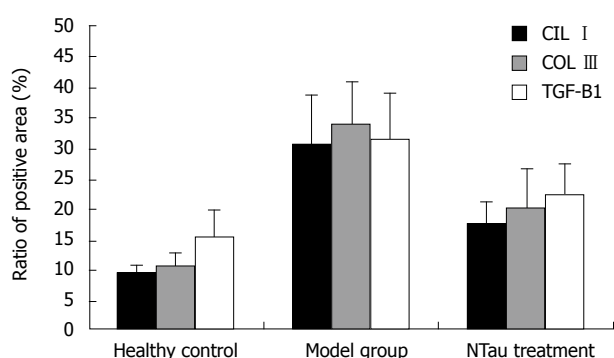


Figure 2 Bar chart presentation of the amount of COL I, COL III and TGF-β₁ in the liver tissue of rats in different experimental groups.

intervention with NTau was capable of reducing the amount of all these parameters in the LC rats, with COL I decreasing from 30.63 ± 8.25 to 17.58 ± 3.6 , COL III from 33.65 ± 7.52 to 20.34 ± 6.41 and TGF-β₁ from 31.28 ± 7.85 to 22.17 ± 5.43 , respectively. Figures 3-5 present the immunohistochemical analysis data of the expression of COL I, COL III and TGF-β₁ in the liver tissue of the different experimental groups.

COL I was only slightly expressed in the basement membrane of the central vein of the hepatic lobules in the normal healthy rats. Its expression was significantly elevated in the LC rats with thicker collagen fibrils clearly detectable in the connective tissue surrounding the hepatocytes. Compared with the non-treated model group, the rats in the NTau treatment group had conspicuously reduced expression of COL I and thinner collagen fibrils in the connective tissue surrounding the hepatocytes. There was mild expression of COL III in the interstitial connective tissue surrounding hepatocytes in the normal healthy rats. However, significantly increased and thickened COL III fibrils were observed in the interstitial connective tissue surrounding hepatocytes in the model group. Treatment with NTau was capable of markedly attenuating the expression of COL III, with thinner collagen fibrils being observed. Similarly, TGF-β₁ was only expressed mildly in the hepatocellular cytoplasm in normal rats, and there was a strongly positive expression of TGF-β₁

in hepatocellular cytoplasm in the LC rats. Similarly to expression of COL I and III, treatment with NTau showed a significant reduction in the expression of TGF-β₁ in hepatocellular cytoplasm.

Biomechanical and biodynamic changes in the portal vein

Experiments on portal vein biomechanics showed that, in general, the portal compliance decreased as the pressure in the portal vein increased (Figure 6). Among the different groups of rats, the portal compliance in the model group was significantly lower than that of the normal control group ($P < 0.05$), while the NTau treatment was able to markedly improve the portal compliance when compared with the non-treated model group ($P < 0.05$).

In addition, there was a substantial difference ($P < 0.01$) in the opening angle of the portal ring between the model group (89.23 ± 10.47 degree) and the healthy control group (76.25 ± 9.45 degree). The NTau treatment significantly reduced the opening angle of the portal ring (82.61 ± 6.31) when compared to the animals in the model group ($P < 0.05$). The direct proportion of zero-stress to opening angle indicated that the LC rats had a very significant increase in zero-stress level, and the NTau treatment was able to mitigate this abnormal elevation.

DISCUSSION

Taurine, a sulfur-containing β-amino acid [$\text{H}_2\text{N}-(\text{CH}_2)_2-\text{SO}_2\text{OH}$], is ubiquitously distributed in tissues of mammalian and marine organisms. Taurine was once thought to be a non-functional terminal metabolite of sulfur-containing amino acids in the body. However, recent studies have confirmed that taurine has a wide variety of biological functions including maintaining homeostasis and regulating physiological functions of different systems. Taurine also possesses a wide spectrum of pharmacological activities such as antipyretic, anticonvulsant, antiplatelet-aggregation, hypotensive, immunity-enhancing, liver-protecting and angiotensin-regulating effects^[10-17]. It is known that the liver is the main

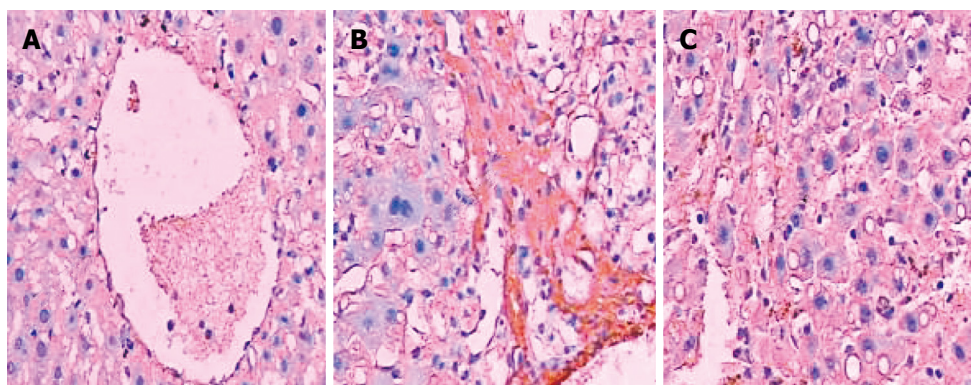


Figure 3 Immunohistochemical analysis of the expression of COL I in liver tissue of rats in different experimental groups. A: Normal healthy rats; B: LC rats. Note the expression of a large amount of COL I; C: NTau-treated rats. Note the reduction of the expression of COL I. (magnification, 10×20).

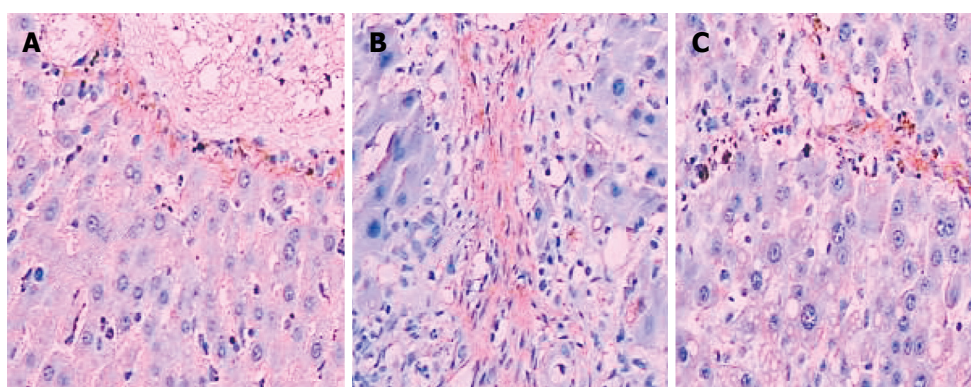


Figure 4 Immunohistochemical analysis of the expression of COL III in liver tissue of rats in different experimental groups. A: Normal healthy rats; B: LC rats. Note the abundance in the expression of COL III; C: NTau-treated rats. The expression of COL III was decreased. (magnification, 10×20).

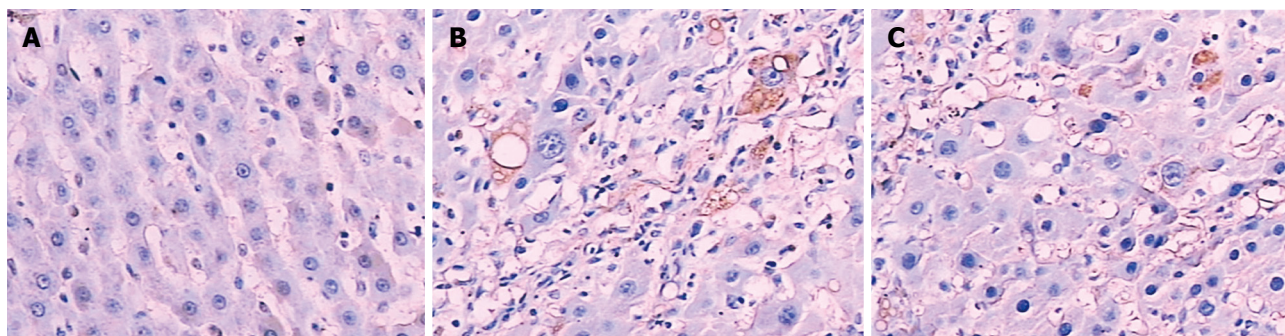


Figure 5 Immunohistochemical analysis of the expression of TGF- β_1 in liver tissue of rats in different experimental groups. A: Normal healthy rats; B: LC rats. Note the increased expression of TGF- β_1 ; C: NTau-treated rats. Note that the expression of TGF- β_1 was reduced. (magnification, 10×20).

organ for taurine biosynthesis and also an important target organ for taurine's many biological activities. Interestingly, the amount of taurine in the liver tissue of rats with chronic liver disease falls below the normal range^[18].

As a free amino acid, taurine can either be synthesized through chemical reactions or extracted from natural sources. Studies in China and other countries have demonstrated that synthesized taurine may inhibit hepatic fibrosis (HF) by inhibiting collagenation and proliferation of HSCs^[19,20]. However, the role of taurine in inhibiting PHT has hitherto not been systematically investigated. Our previous study found that NTau promoted apoptosis of HSCs in a more marked manner

than that of synthesized product^[21], and that NTau could lower PHT of LC through inhibiting contraction of HSCs^[22].

Two seemingly contradictory theories, i.e. the "backward flow" theory and the "forward flow" theory, have been put forward to explain the development of PHT in LC. In the "backward flow" theory, an increase in intrahepatic resistance is thought to be the main reason for the occurrence of PHT. The "forward flow" theory, on the other hand, considers systemic hyperdynamic circulation (SHC) as the primary cause for PHT. Recent studies have shown that both mechanisms are involved in the pathogenesis of PHT^[23]. In fact, patients with LC

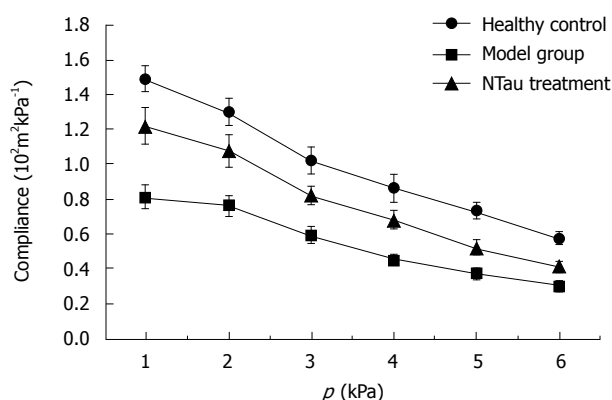


Figure 6 Comparison of the portal ring compliance among rats in different experimental groups.

may present both disturbances of blood circulation (i.e. increased resistance of “backward flow” theory) in liver tissue and abnormality in vasoactive substances (i.e. hyperdynamic circulation of “forward flow” theory).

By combining the “backward flow” and the “forward flow” theory together, this would furnish a better understanding of the pathogenesis of PHT^[24,25]. In the “forward flow” theory, the pathogenesis of PHT may be primarily attributed to initiation factors, including dilation of peripheral vessels, decrease in peripheral vascular resistance and mean arterial pressure, increase in blood volume, splanchnic blood flow and cardiac output, and development of systemic hyperdynamic circulatory syndrome (HCS)^[23,26]. HCS plays an important role in the maintenance of PHT and is also a primary cause for the development of sodium-water retention, ascites, hepato-renal and hepato-pulmonary syndromes. Our experimental study showed that NTau was capable of decreasing portal blood flow and improving systemic HCS by reducing PVF and increasing SVR and MAP. All these experimental observations indicate that NTau is able to reduce portal pressure through acting on the “forward flow” mechanism.

An increase in endogenous vasodilators is regarded as the most significant factor for peripheral arterial vasodilatation. Consequently, an increase of vasodilators may elicit a wide spectrum of pathophysiological alterations including vasodilatation of peripheral and splanchnic tertiary arterioles, decreased resistance, deficiency of effective arterial blood volume, activation of neurohumoral systems for pressure and water-sodium retention, compensatory expansion of plasma volume, increase in returned blood volume and cardiac output, and splanchnic active hyperemia. All these alterations can lead to hyperdynamic systemic and splanchnic circulation. Among the common vasodilators, NO, glicentin, prostacyclin and calcitonin gene-related peptides are the most important ones. NO is produced by L-arginine in the presence of NOS and may exert biological effects by increasing cGMP *via* activation of guanylate cyclase. It is also an important signaling molecule involved in various physiological processes such as vasodilatation. NO has also been found to be involved in the development

of a hyperdynamic circulatory state in LC^[27,28]. In our experiments, decreased activity of NOS and reduced amounts of NO and cGMP were observed after the NTau treatment, suggesting that NTau attenuates PHT of LC most probably *via* regulating the NO system.

In the “backward flow” theory, the most critical initiating determinants of portal pressure include the progression of HF, passive structural disorder in the liver, formation of pseudo-lobules and interrupted supply of blood circulation to hepatocytes, which further contribute to hepatocellular necrosis, proliferation of collagen, and formation of regenerative nodules. Consequently, compression and traction of peripheral vessels and bile duct by regenerative nodules and proliferated collagen fibrils may lead to increased resistance to blood flow of portal and hepatic veins, and subsequently result in PHT^[25,29,30]. In our study, the treatment with NTau led to the improvement of the structure of liver tissue and significantly lowered the amounts of COLI and COL III, indicating that NTau can decrease portal pressure by acting on the “backward flow” mechanism, namely by improving the pathological structure of liver tissue and inhibiting HF.

TGF- β_1 is a multifunctional peptide with a wide range of potential influences on the growth and differentiation of cells, aggregation of extracellular matrix (ECM), and immune response. It is also one of the mediators that is most closely associated with the production of fibrils. In the occurrence and development of HF, TGF- β_1 has an ability to activate HSCs and promote the gene expression of collagen as well as the synthesis and deposition of ECM^[31,32]. In our experiments, the level of TGF- β_1 in the NTau treatment group was significantly lower than that of the LC model group, suggesting that NTau inhibits the development of HF, possibly through inhibiting the expression of TGF- β_1 .

Increased resistance to PVF may elicit biomechanical changes in the portal vein by causing vascular reconstruction characterized by thickening of vessel walls, narrowing of vessel lumen and proliferation of smooth muscle cells, resulting in further maintenance and exacerbation of PHT. Such alterations in biomechanics subsequently make treatment of LC-induced PHT ever more difficult^[33,34]. Therefore, to achieve a favorable therapeutic response in the treatment of PHT of LC, terminating this vicious circle becomes particularly important, and lowering the portal pressure should not be the sole target for intervention. Vessel wall is a viscoelastic tissue with unique biomechanical properties including creep, stress-relaxation and hysteresis. Compliance and zero-stress state are usually used to describe the biomechanical properties of vessels. Indeed, reduced portal compliance and enlarged opening angle were found in rats with LC. The NTau treatment, on the other hand, was able to improve the portal compliance and decrease the opening angle, indicating that NTau could inhibit the development of LC-induced PHT by improving the biomechanical properties of the portal vein.

The pathomechanism of LC-induced PHT is complex,

for it involves SHC, structural alterations in the liver, increased resistance to PVF and biomechanical changes in the portal vein. Our present experimental data derived from the LC rat model unequivocally demonstrate that NTau can inhibit PHT of LC by improving hyperdynamic circulation, structure of liver tissue, and the biomechanical properties of the portal vein by delaying the progression of HF. Given that taurine is an important nutrient in the body with a deficiency in chronic liver diseases, the supplementation of an adequate amount of taurine may improve the functional status of the body. In view of its pharmacological and nutritional values, we believe that treatment with taurine may provide an additional dimension for the management of portal hypertension associated with liver cirrhosis.

COMMENTS

Background

Portal hypertension which acts as the main manifestation of patients in the compensatory stage of cirrhosis of the liver is the material cause of death. Treatment of portal hypertension caused by liver cirrhosis can not only enhance the prognosis of the disease but also improve the patients' quality of life. Hence, the concept of implementing treatment of portal hypertension is gradually attracting more and more attention, changing the passive state of the past which practised symptomatic treatment when complications developed.

Research frontiers

Intensive literature review shows that taurine can suppress the course of liver cirrhosis. This project investigates the function of taurine in inhibiting the course of liver cirrhosis and portal hypertension caused by liver cirrhosis from different aspects.

Innovations and breakthroughs

This study proposes the concept of the integration of the three theories for the formation of liver cirrhosis: the backward flow theory, the forward flow theory and the biomechanics of portal vein theory, then systematically explores the inhibiting effect and possible mechanism of action of taurine on portal hypertension caused by liver cirrhosis.

Applications

The treatment of portal hypertension mainly embodies medication treatment, surgical treatment, interventional treatment, liver transplant etc. At present, long-term medication treatment takes the center stage. Though there are plenty of medicines available for portal hypertension caused by liver cirrhosis, there is still a lack of effective medication. As a result, there is sound justification for investigating the therapeutic functions of taurine in portal hypertension caused by liver cirrhosis. Pure natural taurine widely exists in marine fauna. The ocean, which occupies 70.8% of the total earth's surface, is a natural medicinal resource with huge potential. It makes great sense to explore new areas of marine life's application in the search for new medicinal resources.

Terminology

Forward flow theory is one of the theories of the formation of portal hypertension caused by liver cirrhosis. The pathogenesis of PHT may be primarily attributed to initiation factors, including dilation of peripheral vessels, decrease in peripheral vascular resistance and mean arterial pressure, increase in blood volume, splanchnic blood flow and cardiac output, and development of systemic hyperdynamic circulatory syndrome. For backward flow theory, the main cause of portal hypertension caused by liver cirrhosis is the formation of diffuse fibrous septa and regenerative nodules in liver which is followed by hepatic sinus narrowing. Then intra-liver vessels constrict and the resistance of blood flow in the portal system increases which results in passive congestion of the portal system. Finally, portal hypertension develops. As vasodilatation increases, the reactivity of vessels to endogenous vascular-constriction substances falls, then functional arteriovenous fistula and portal-systemic shunting develop and result in hyperdynamic circulation over the whole body while blood flow of the portal vein increases.

Peer review

This is a small but reasonably designed experimental study to examine the impact of natural taurine in a rat model of cirrhosis. The paper particularly

concentrates on the effects of this agent on the level of portal hypertension and clearly shows that this is attenuated following repeat dosage by gavage.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Ma WH

ORIGINAL ARTICLES

Induction of apoptosis and cell cycle arrest in human HCC MHCC97H cells with *Chrysanthemum indicum* extract

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Author contributions: Li ZF and Wang ZD designed the research and contributed equally to this work; Li ZF, Wang ZD, Ji YY, Zhang S, Huang C, performed the research; Wang ZD, Ji YY, Li J, Xia XM contributed to the materials, methods and analysis; Wang ZD and Zhang S analyzed the data; Li ZF and Wang ZD wrote the paper.

Supported by Grants From the National Natural Science Foundation of China, No. 30672766 and Science and Technology Developing Foundation of Shaanxi Province, China, No. 2006 K16-G4 (1)

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Received: June 7, 2009 **Revised:** August 1, 2009

Accepted: August 8, 2009

Published online: September 28, 2009

and cell cycle proteins, including P21 and CDK4, were measured by Western blotting.

RESULTS: CIE inhibited proliferation of MHCC97H cells in a time- and dose-dependent manner without cytotoxicity in rat hepatocytes and human endothelial cells. CIE induced apoptosis of MHCC97H cells in a concentration-dependent manner, as determined by flow cytometry. The apoptosis was accompanied by a decrease in mitochondrial membrane potential, release of cytochrome C and activation of caspase-9 and caspase-3. CIE arrested the cell cycle in the S phase by increasing P21 and decreasing CDK4 protein expression.

CONCLUSION: CIE exerted a significant apoptotic effect through a mitochondrial pathway and arrested the cell cycle by regulation of cell cycle-related proteins in MHCC97H cells without an effect on normal cells. The cancer-specific selectivity shown in this study suggests that the plant extract could be a promising novel treatment for human cancer.

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Key words: Apoptosis; Cell cycle; Chinese traditional medicine; *Chrysanthemum indicum*; Hepatocellular carcinoma; Herbal medicine

Peer reviewer: Dr. Yukihiro Shimizu, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan

Abstract

AIM: To investigate the effects of *Chrysanthemum indicum* extract (CIE) on inhibition of proliferation and on apoptosis, and the underlying mechanisms, in a human hepatocellular carcinoma (HCC) MHCC97H cell line.

METHODS: Viable rat hepatocytes and human endothelial ECV304 cells were examined by trypan blue exclusion and MTT assay, respectively, as normal controls. The proliferation of MHCC97H cells was determined by MTT assay. The cellular morphology of MHCC97H cells was observed by phase contrast microscopy. Flow cytometry was performed to analyze cell apoptosis with annexin V/propidium iodide (PI), mitochondrial membrane potential with rhodamine 123 and cell cycle with PI in MHCC97H cells. Apoptotic proteins such as cytochrome C, caspase-9, caspase-3

Li ZF, Wang ZD, Ji YY, Zhang S, Huang C, Li J, Xia XM. Induction of apoptosis and cell cycle arrest in human HCC MHCC97H cells with *Chrysanthemum indicum* extract. *World J Gastroenterol* 2009; 15(36): 4538-4546 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4538.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4538>

INTRODUCTION

Hepatocellular carcinoma (HCC) is known as a common and aggressive malignant tumor worldwide. In China, HCC accounts for 90% of primary liver cancer, which is the second most common cause of death^[1]. Chemotherapy plays an important role in the treatment of cancer, but it is limited to a significant extent by its toxicities, significant

resistance to available chemotherapeutic agents and side effects, including myelosuppression, neutropenia and thrombocytopenia^[2,3]. One possible way to increase the efficacy of anticancer drugs and to decrease toxicities or side effects is to develop traditional medicines, especially from medicinal plants^[4-7].

Natural products have become increasingly important for new pharmaceutical discoveries, and among all the uses for natural products in biomedical science, traditional Chinese herbology has been a pioneering specialty^[8]. This is particularly evident in the treatment of cancers, in which more than 60% of drugs are of natural origin^[9]. Hence a new medicinal plant with anticancer activities could be a valuable substance in cancer treatment. The flowers of *Chrysanthemum indicum* (*Chrysanthemi Indici* Flos), a *Compositae* plant, is a traditional Chinese medicine and medicinal plant distributed widely in China. Oriental *Chrysanthemum indicum* traditional medicine has been used to treat vertigo, hypertensive symptoms and several infectious diseases such as pneumonia, colitis, stomatitis and carbuncles^[10]. A series of studies have demonstrated that *Chrysanthemum indicum* possesses antimicrobial^[11], antiinflammatory^[11-13], immunomodulatory^[12], and neuroprotective effects^[14]. Recently, much attention has been devoted to the anticancer activity of *Chrysanthemum indicum* on human PC3, HL 60 and HeLa cancer cells in a dose- and time-dependent manner^[15-17]. However, its anticancer mechanism of action is still not clear and needs further investigation.

Apoptosis induced by herbs has become a principal mechanism by which anticancer therapy exerts its effect^[7,18]. Upstream initiator caspases including caspase-9 activate downstream effector caspases such as caspase-3, playing a pivotal role in the induction of apoptosis. Caspase-9, triggered by chemotherapeutic drugs, is the apical caspase in the mitochondria-initiated apoptosis pathway, which requires the release of cytochrome C from the mitochondria as well as interaction with Apaf-1^[19]. This pathway, associated with changes in the permeability of the outer mitochondrial membrane and the collapse of the membrane potential ($\Delta\psi_m$), results in release of cytotoxic proteins and caspase activation^[19].

Cell cycle regulation, a fundamental mechanism determining cell proliferation, is tightly mediated through a complex network of positive factors, such as cyclin-dependent kinases (CDKs) and cyclin, and negative factors, including CDK-inhibitor (CDKI) regulatory molecules. The activated CDK4-cyclin complexes are inactivated by binding to P21, a CDKI^[20]. Plant extracts which arrest the cell cycle in cancer cells *via* regulation of CDK and CDKI proteins also can be used for therapeutic intervention^[21-23].

The aim of the present study was to examine the anticancer activities of *Chrysanthemum indicum* and related mechanisms in MHCC97H cell lines, typical human HCC cell lines, which are commonly used in the study of antitumor cells^[24]. In order to compare the actions of *Chrysanthemum indicum* on normal hepatocytes and endothelial cells, the effects of the extract were examined in rat hepatocytes and a human umbilical

vein endothelial cell ECV304 cell line. Furthermore, we investigated the effect of the extract in human MHCC97H cells and the mechanisms underlying its effect on inhibition of proliferation.

MATERIALS AND METHODS

Plant material and extraction

Fresh, ripe fruits of high quality flowers of *Chrysanthemum indicum* were procured from Xi'an traditional medicine group (Shaanxi, China) in March 2006 and the characteristics were consistent with that described in the Pharmacopeia of the People's Republic of China. Moreover, *Chrysanthemum indicum* was also authenticated by Professor Wang Jun-Xian, a taxonomist in the Department of Pharmacy in Xi'an Jiongtong University. The plant materials were air dried at room temperature and then powdered. The dried and powdered fruit of *Chrysanthemum indicum* (500 g) were extracted with 95% ethanol (EtOH) twice under refluxed temperature. After evaporation of organic solvent under reduced pressure, the resultant *Chrysanthemum indicum* EtOH extract (CIE) was concentrated under reduced pressure to give 66.5 g (13.3%) EtOH extract. The dry extract was stored in a refrigerator at -20°C until use in the experiments. CIE was dissolved in phosphate buffered solution (PBS) and diluted in cultured medium before use, and the control group was made up of medium, PBS and the cells.

Reagents, antibodies, cells, and culture medium

A human MHCC97H HCC cell line was purchased from the Liver Cancer Institute of Fudan University (Shang Hai, China). A human ECV304 cell line was obtained from the Cell Bank of Academia Sinica (Shang Hai, China). MHCC97H cells and ECV304 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO₂ in air at 37°C before use and subcultured with 0.25% trypsin-0.02% EDTA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 (Rh123), annexin V and propidium iodide (PI) were purchased from Sigma Corporation (Sigma, St.Louis, MO, USA). DMEM, FBS and trypsin were obtained from GibcoBRL, Grand Island, NY, USA. Anti-caspase-3, anti-caspase-9, anti-cytochrome C, and anti- β -actin were purchased from Santa Cruz Company. Anti-CDK4 and P21 were obtained from eBioscience Corporation.

Hepatocyte preparation, culture and viability assay

Hepatocytes were isolated by the collagenase perfusion method^[9] from 10-wk-old male Sprague-Dawley rats anesthetized with intraperitoneal administration of ketamine. The viability of the isolated hepatocytes was over 90% as determined by 0.2% trypan blue exclusion. The cells were plated in 35 mm plastic dishes at a density of 3×10^5 cells/mL in 2 mL of Williams' Medium E supplemented with 10% FBS, and were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C overnight. After overnight incubation, the culture medium was changed to fresh medium, and

cultures were incubated with varying concentrations of CIE 400, 800 and 1200 $\mu\text{g/mL}$ for 24 h. According to a previous report^[25], the cells were then trypan blue stained and a hemocytometer was used to determine the total cell count and viable cell number. Viability of cells were determined as follows: viability (%) = viable cell number/total cell count \times 100%.

Cell viability assay

ECV304 cells were seeded in a 96-well plate (5×10^4 cells/well). After 24 h seeding, cells were treated with CIE (400, 800 and 1200 $\mu\text{g/mL}$) for 24 h, in 3 parallel wells each, with untreated cells serving as a control, then the MTT assay as described by Xiao *et al*^[26] was performed. At 24 h, 20 μL MTT solution (5 mg/mL) was added to each well and incubated for a further 4 h. The medium was removed and 200 μL DMSO was added to each well. Absorbance (A) at 570 nm was measured using a microculture reader. The percentage of viable cells was calculated as follows: (A of experimental group/ A of control group) \times 100%.

MHCC97H cells were seeded into 96-well plates at a density of 5×10^4 /well and were then incubated with different concentrations of CIE (400, 800 and 1200 $\mu\text{g/mL}$) for 24, 48 and 72 h, then the MTT assay was performed. At 24, 48 and 72 h, 20 μL MTT solution (5 mg/mL) was added to each well, and the cells were further incubated at 37°C for 4 h. The MTT assay and calculation of the percentage of viable cells were the same as described above.

Apoptosis assays

In accordance with the study of Chen *et al*^[27], the apoptotic rates were analyzed by flow cytometry using an annexin V-FITC/PI kit. Staining was performed according to the manufacturer's instructions, and flow cytometry was conducted on a FACS Caliber (Becton Dickinson, Mountain View, NJ, USA). Cells that were annexin V (-) and PI (-) were considered viable cells. Cells that were annexin V (+) and PI (-) were considered early apoptotic cells. Cells that were annexin V (+) and PI (+) were considered late apoptotic cells.

Cell cycle assays

Cell cycle analyses were carried out by the method of Vinodhkumar *et al*^[28]. Briefly, cells were incubated in culture media alone or culture media containing 400-1200 $\mu\text{g/mL}$ of CIE, at 37°C for 48 h. Cells were harvested in cold PBS, fixed in 70% EtOH, and stored at 4°C. Fixed cells were washed with PBS once and suspended in 1 mL of PI staining reagent 50 mg/mL containing 100 $\mu\text{g/mL}$ Rnase, and were then incubated in the dark for 30 min. The distribution of the cell cycle was measured by a Becton Dickinson FACS analysis system and quantitation of cell cycle distribution was carried out using Multicycle Software.

Detection of mitochondrial membrane potential (MMP $\Delta\psi_m$)

Loss of MMP $\Delta\psi_m$ was assessed by flow cytometry,

using a fluorescent indicator Rh123, as described by Tang *et al*^[29] and Li *et al*^[30]. Briefly, cells were treated with different concentrations of CIE. Then, Rh123 working solution was added to the culture at a final concentration of 2 $\mu\text{g/mL}$ and then incubated in the dark at 37°C for 30 min. Cells were then washed with PBS, and fluorescence of Rh123 was detected immediately using a FACS Caliber, at an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Western blotting

Cancer cells (2.5×10^7 /well) were treated with different concentrations of CIE for 24 or 48 h. To extract cytoplasmic protein as by the method of Li *et al*^[31], cells were collected by centrifugation at 200 r/min for 10 min at 4°C. The cells were washed twice with ice-cold PBS, followed by centrifugation at 200 r/min for 5 min. The cell pellet was then suspended in ice-cold cell extraction buffer for 30 min on ice. In addition, as described by Hsu *et al*^[32], cells were then lysed in a sample buffer, followed by sonication and denaturation. Protein concentrations were measured using DC Protein Assay (Bio-Rad, Hercules, CA) and equal amounts of protein (50 μg) were subjected to SDS-PAGE on 12% gel. The proteins were then electrophoretically transferred to nitrocellulose membranes and processed for immunoblotting. Membranes were first blocked with 5% non-fat dry milk overnight at 37°C and immunolabeled using primary antibodies. Goat anti-rabbit horseradish peroxidase-conjugated antibodies (Cell Signaling Technology, Beverly, MA) were used as secondary antibodies and detected with enhanced chemiluminescence (Amersham, USA). Equal loading of each lane was evaluated by immunoblotting using the same membranes with β -actin antibodies after detachment of previous primary antibodies. The band density for the target protein in each sample was measured with image analysis software (Gene Genus, Gene Company) and normalized to β -actin expression.

Statistical analysis

All data were expressed as mean \pm SE. Statistical analysis was performed with analysis of variance (ANOVA) using the statistical software SPSS 11.0. *P*-values < 0.05 were regarded as statistically significantly.

RESULTS

Effect of CIE on numbers of viable rat hepatocytes and ECV304 cells

In order to compare the effects of CIE on rat hepatocytes and human ECV304 cells, the numbers of viable cells were measured. As shown in Figure 1A, CIE did not decrease the number of viable rat hepatocytes, used as a normal cell model. To confirm the activity of CIE in human cells, we measured the number of viable cells treated with varying concentrations of CIE in human endothelial cells (ECV304). CIE did not reduce the number of viable ECV304 cells at any dose (Figure 1B). Therefore, the effect of CIE in inhibiting

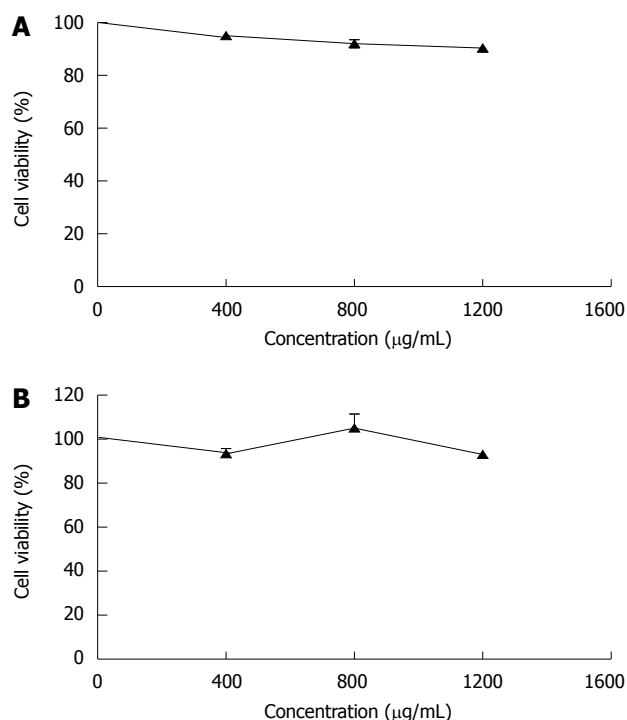


Figure 1 Effects of *Chrysanthemum indicum* extract (CIE) on cell viability of normal cells. A: Rat hepatocytes; B: Human umbilical vein endothelial cell line ECV304. Various concentrations of CIE were added, and the cells were incubated for 24 h. Cell viability was measured by 0.2% trypan blue exclusion (A) and MTT assay (B) respectively. Results presented are representative of 3 independent experiments.

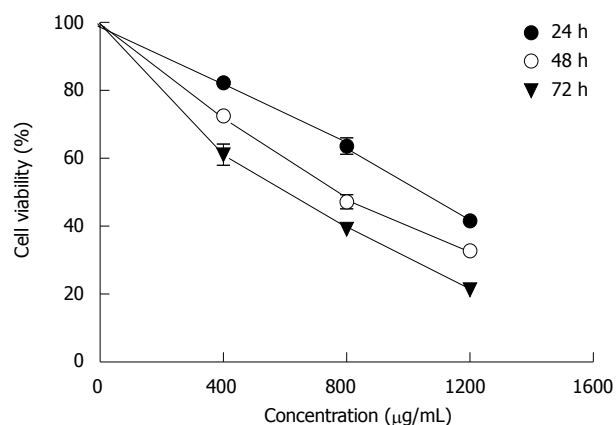


Figure 2 Dose-response curves for CIE in MHCC97H cells, following 24 h, 48 h and 72 h incubation, as assessed by MTT assay. CIE produced a concentration- and time-dependent decrease in cellular proliferation. Results presented are representative of 3 independent experiments.

proliferation of MHCC97H cells and its mechanism were examined in subsequent experiments.

Cytotoxic activities of CIE against human HCC cells

When MHCC97H cells were incubated with 400-1200 $\mu\text{g/mL}$ CIE for 24, 48, 72 h, as shown in Figure 2, there was a significant dose-dependent reduction in cell viability. The IC_{50} value at 24 h was $1009 \pm 130 \mu\text{g}$. When 1200 $\mu\text{g/mL}$ CIE was incubated with cancer cells for 72 h, viable cells amounted to only 25% of control. These findings indicated that CIE significantly decreased

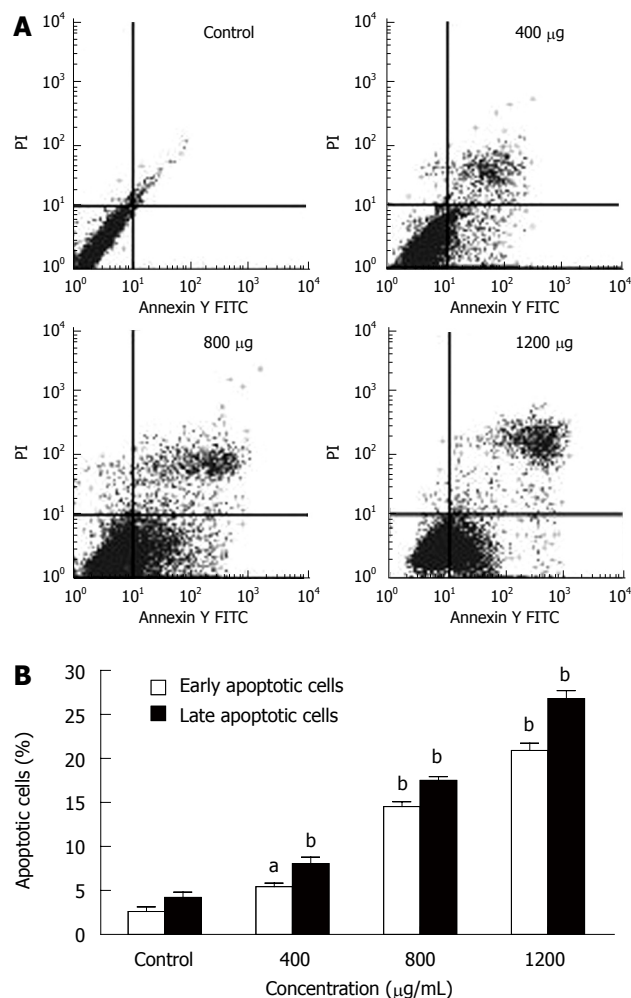


Figure 3 Effect of CIE on the induction of MHCC97H cell apoptosis. A: The apoptosis of MHCC97H cells induced by CIE were determined by flow cytometry at 24 h; B: CIE induced a concentration-dependent increase in early and late cellular apoptosis. Results presented are representative of 3 independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

proliferation of MHCC97H cells in a dose- and time-dependent manner. Hence, the proliferation inhibitory effect of CIE on MHCC97H and its mechanisms were tested in the following experiments.

CIE induces MHCC97H cell apoptosis

MHCC97H cells were incubated with different CIE concentrations (400, 800 and 1200 $\mu\text{g/mL}$) for 24 h and were analyzed by flow cytometry. Pretreatment of MHCC97H cells with various concentrations of CIE induced significant apoptosis (Figure 3A). The numbers of early and late apoptotic cells were significantly increased compared with the control group (Figure 3B). The proportion of early and late apoptotic cells in the 1200 $\mu\text{g/mL}$ treatment group was more than 10 times higher than in the drug-free cells.

Effects of CIE on cell morphology

After incubation with CIE at different concentrations (400, 800, 1200 $\mu\text{g/mL}$), the cells were examined by phase contrast microscopy for evidence of morphological apoptosis induced by CIE (Figure 4). The control cells

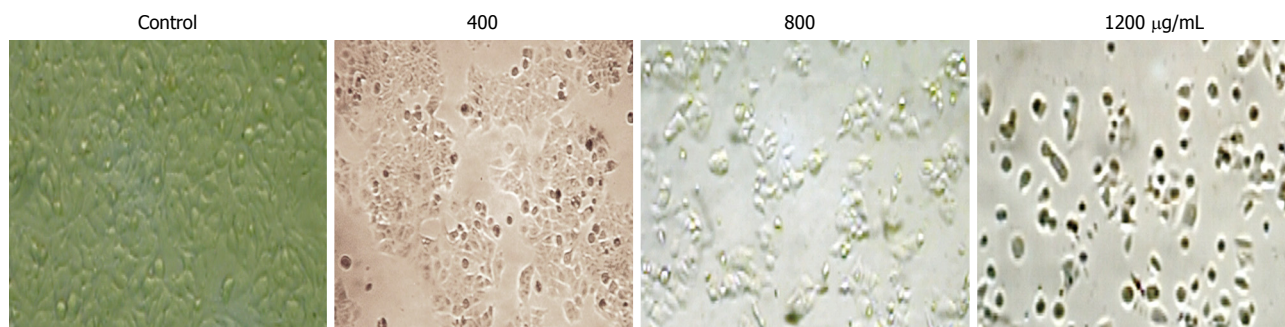


Figure 4 Morphological changes in MHCC97H cells with CIE. Cells were observed by phase contrast microscopy in controls and after treatment with 400, 800, 1200 µg/mL CIE ($\times 200$).

showed a typical polygonal and intact appearance (control), whereas the CIE-treated cells displayed morphological changes with preapoptotic characteristics, such as cellular shrinkage (400, 800 µg/mL), rounding (800 µg/mL), and poor adherence (1200 µg/mL), as well as round floating shapes (1200 µg/mL).

CIE causes loss of $MMP\Delta\psi_m$

To explore whether CIE-induced apoptosis involved the $MMP\Delta\psi_m$, we used a fluorescent indicator, Rh123 to detect the $MMP\Delta\psi_m$ when MHCC97H cells were treated with 400-1200 µg/mL of CIE for 24 h. As shown in Figure 5A and B, after exposure to different CIE doses, cells exhibited much lower Rh123 staining (236.7 ± 9.3 , 170.7 ± 13.9 , 105 ± 10.5) than controls (275 ± 14.5 ; $P < 0.05$ or $P < 0.01$), indicating that CIE can significantly decrease $MMP\Delta\psi_m$ associated with cancer cell apoptosis.

CIE-induced apoptosis is caspase-dependent

To determine whether apoptosis induced by CIE was a mitochondrial-dependent caspase pathway, we further tested whether cytochrome C could be released from the mitochondria into the cytoplasm. As shown in Figure 6A, although there was no detectable cytochrome C in the cytosolic fraction of continuously growing MHCC97H cells, the level of cytochrome C released from the mitochondria increased dose-dependently in the presence of CIE concentrations ranging from 400 to 1200 µg/mL. In accordance with mitochondrial cytochrome C release into the cytoplasm, caspase-9 protein expression was increasingly detected. Accordingly, caspase-3 protein expression was also increased dose-dependently on exposure to CIE (Figure 6A and B). Taken together, these findings suggest that CIE exerted a significant apoptotic effect on MHCC97H cells in a concentration-dependent manner through the mitochondrial pathway, and was accompanied by a decrease in $MMP\Delta\psi_m$, release of cytochrome C, and activation of caspase-9 and caspase-3.

Cell cycle analysis

The cell cycle of cancer cells was also determined by flow cytometry. MHCC97H cells treated with CIE 400, 800, 1200 µg/mL for 48 h showed an accumulation of cells in the S phase of the cell cycle. In contrast, the population of cells in G0-G1 and G2/M phases was

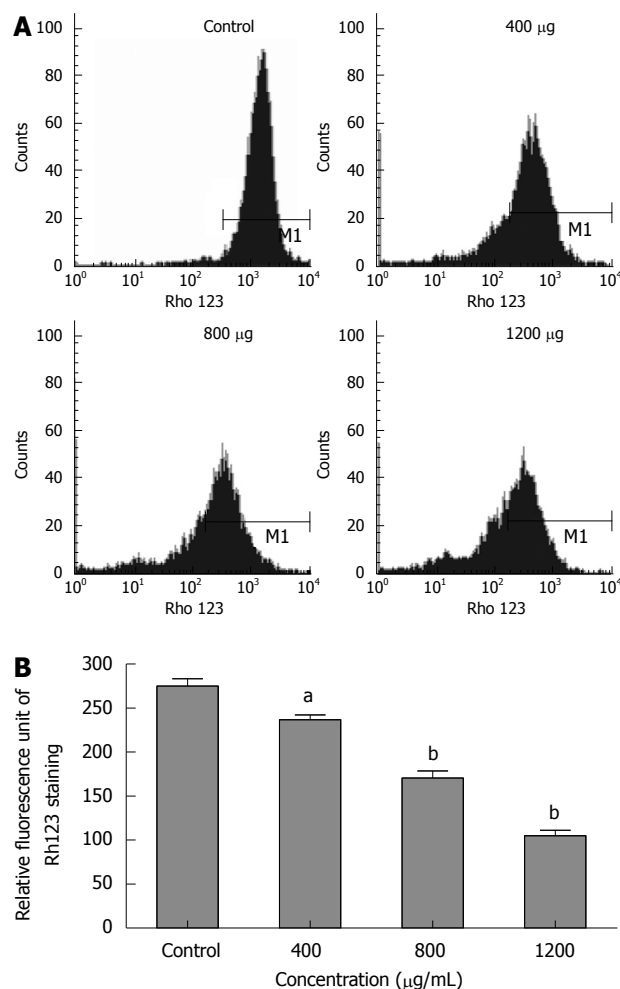


Figure 5 Effect of CIE on MHCC97H cellular mitochondrial membrane potential ($MMP\Delta\psi_m$). A: The $MMP\Delta\psi_m$ of MHCC97H cells were determined by flow cytometry at 24 h after CIE; B: Results presented are representative of 3 independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

significantly decreased, especially at 400 µg/mL CIE (Figure 7A-C). In addition, as shown in Figure 7A, cancer cells incubated with higher doses of CIE for 48 h also showed a sub-G1 peak indicating apoptosis. These observations suggest that a small number of cancer cells escape from the S phase and undergo apoptosis, particularly at the 1200 µg/mL CIE concentration. Therefore, with the dose-dependent increase in cancer cell apoptosis, the proportion of cells arrested in the

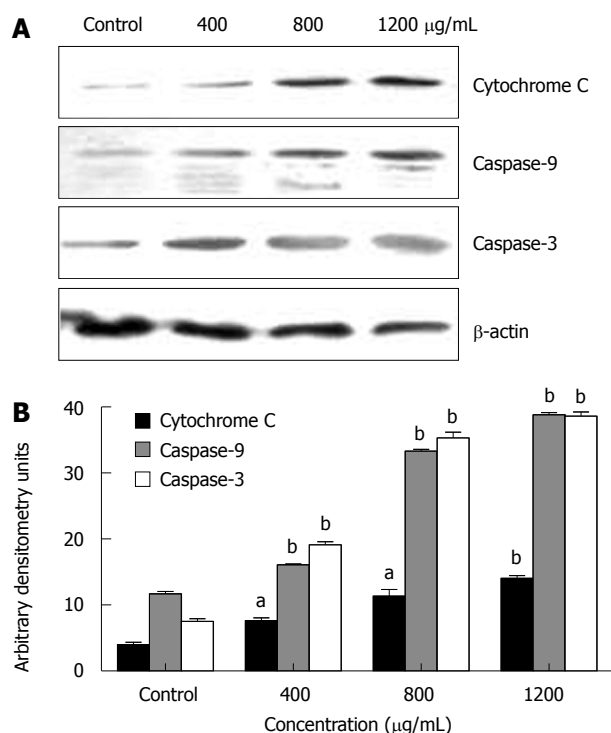


Figure 6 Effect of CIE on apoptosis-related protein expression. A: The expressions of cytochrome C, caspase-9 and caspase-3 were assessed by Western blotting; B: Results presented are representative of 3 independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

S phase by CIE decreased as the CIE concentration increased from 400 to 1200 μg/mL.

The mechanism of action of CIE on the cell cycle

To determine the mechanism by which CIE arrested the cell cycle in the S phase, Western blotting was used to determine the expression levels of cell cycle-regulating proteins including P21 and CDK4. P21 protein expression was markedly higher than that of the control group at all CIE doses tested. In contrast, CDK4 levels were significantly lower than that of the control group, as shown in Figure 8A and B. The results suggested that CIE could arrest the cell cycle *via* upregulation of P21 and downregulation of CDK4.

DISCUSSION

So far, the underlying mechanisms of the pharmacological effect of *Chrysanthemum indicum* in cancer therapy have been unclear, and this study examined the effect of CIE and its underlying mechanisms on inhibition of tumor cell proliferation. In the present study, we have demonstrated that CIE potently inhibits the proliferation of MHCC97H cells by inducing apoptosis (Figures 3 and 4) and arresting the cell cycle (Figure 7) but has no cytotoxicity in rat hepatocytes and human endothelial cells (ECV304) that were used as representatives of normal cells (Figure 1).

Morphological changes in apoptotic characteristics, such as cellular shrinkage, rounding, poor adherence, and round floating shapes in CIE-treated cells were also observed by phase-contrast microscopy (Figure 4). The

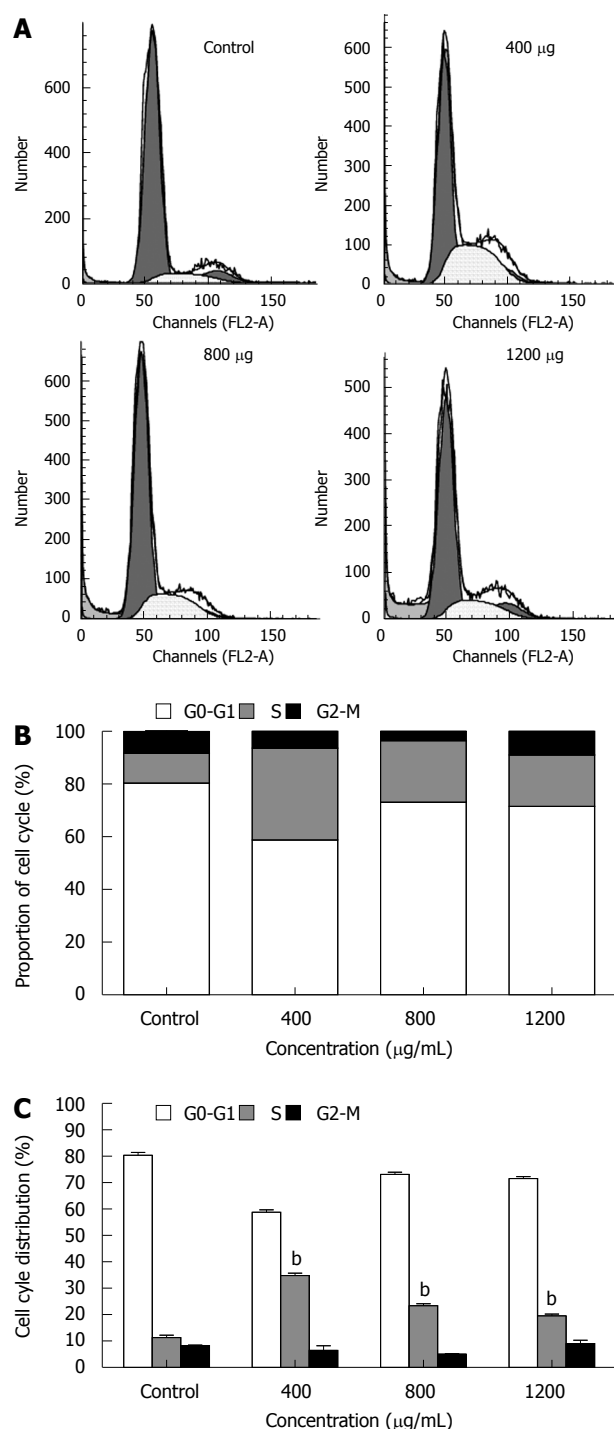


Figure 7 Effect of CIE on MHCC97H cell cycle. A: The cell cycle of MHCC97H cells in the presence of CIE was determined by flow cytometry at 48 h; B and C: Results presented are representative of 3 independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

induction of cancer cell apoptosis without side effects is recognized as an important target in cancer therapy. Apoptosis triggered by activation of the mitochondrial-dependent caspase pathway represents the main programmed cell death mechanism^[19]. The mitochondrial-dependent apoptosis pathway is activated by various intracellular stresses that induce permeabilization of the mitochondrial membrane, leading to cytochrome C release^[33]. Flow cytometry with Rh123 staining showed

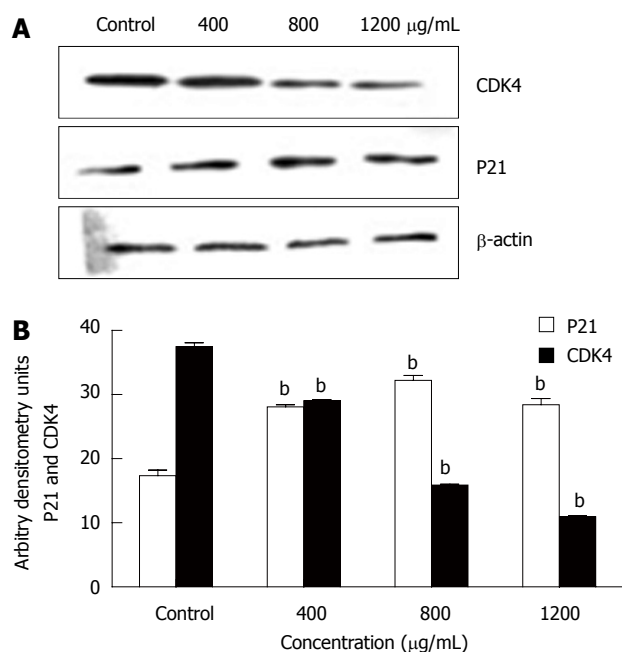


Figure 8 Effect of CIE on cell cycle-related protein expression. A: The expression of CDK4 and P21 was assessed by Western blotting; B: Results presented are representative of 3 independent experiments. ^b*P* < 0.01 vs control group.

disruption of $\text{MMP}\Delta\psi_m$ in the CIE-treated cells (Figure 5), indicating that the mitochondrial apoptotic pathway played a pivotal role in CIE-induced apoptosis of MHCC97H cells. Furthermore, cytosolic cytochrome C activates pro-caspase-9 by binding to Apaf-1 and in the presence of dATP, and contributes to activation of caspase-9 and caspase-3, thus triggering apoptosis^[34,35]. In this study, caspase-9 and caspase-3 levels reached a maximum at about 24 h in the cells after exposure to CIE 1200 μg/mL (Figure 6). These results indicated that CIE-induced apoptosis of MHCC97H cells was mediated by loss of $\text{MMP}\Delta\psi_m$, increased cytosolic translocation of cytochrome C, and activation of caspase-9 and caspase-3.

The inhibition of tumor cell growth without toxicity in normal cells has attracted attention as an important target in cancer therapy. Dysregulation of the cell cycle mechanism has also been shown to play an important role in various cancer cell growths, including HCC. In this study, CIE inhibited MHCC97H cell proliferation partly as a result of accumulation of cells in the S phase of the cell cycle. The present study, to the best of our knowledge, is also the first to demonstrate that CIE induced arrest of the cell cycle in the S phase in HCC cells (Figure 7). The S phase is associated with DNA synthesis and plays a crucial role in cell cycle progression. Recently, a series of S phase chemotherapeutic agents such as *Smilax glabra* Roxb^[36], baicalein from *Scutellariae radix* roots^[37] and others^[38] have been found to inhibit cancer cells, including HCC. Furthermore, in accordance with these results, CIE upregulated P21 and downregulated CDK4 (Figure 8), indicating that cell cycle-related proteins were involved in the CIE-induced cell cycle arrest in MHCC97H cells. One of the CDKI proteins, P21 can perform a key function in controlling cell cycle progression by negatively regulating

CDK4 activity^[38]. Inappropriate expression of cell cycle-related proteins, such as CDK4 and P21, could be one of the major factors contributing to HCC development^[39]. Moreover, CDK4 and P21 play important roles in regulation of the S phase of the cell cycle^[37,38,40,41]. These findings, taken together with the present study, suggest that upregulation of P21 and downregulation of CDK4 are likely to be involved in the S phase arrest induced by CIE in HCC cells.

Additionally, in clinical studies, *Chrysanthemum indicum* can be used in combination with other chemotherapeutic agents or traditional Chinese medicines in treatment of other cancers. Xiang *et al*^[42] found that patients with metastatic breast cancer postoperatively receiving *Chrysanthemum indicum* as one of the main components, in combination with other traditional Chinese medicines, had a 5-year overall survival rate of 70% and a complete response rate of 60%, and in combination with chemotherapeutic agents, had a 5-year overall survival rate of 77% and a complete remission rate of 80%, without adverse effects. Bi *et al*^[43] demonstrated that *Chrysanthemum indicum*, in combination with traditional Chinese medicines, achieved a response rate of 67% in advanced stage esophageal carcinoma patients, without myelosuppression or toxicities of the liver and kidney.

In conclusion, different effects of CIE treatment were observed in cancer and normal cells. CIE exerted a significant apoptotic effect on MHCC97H cells through the mitochondrial-dependent caspase-3 pathway. It arrested the cell cycle of cancer cells in the S phase by upregulation of P21 and downregulation of CDK4. In addition, the cancer-specific selectivity shown in this study suggests that the herb could be a promising novel plant with potential in the treatment of human cancer without side effects.

COMMENTS

Background

Ethnopharmacology used in folk medicine continues to be an important source of discovery and development of novel therapeutic agents in cancer. The flowers of *Chrysanthemum indicum*, a *Compositae* plant, is common in ethnopharmacology, and has long had wide spread use in the treatment of hypertension, colitis, pneumonia and carbuncles by traditional Chinese practitioners. Recently, much attention has been devoted to the anticancer activity of *Chrysanthemum indicum*, especially in hepatocellular carcinoma (HCC). However, the underlying mechanisms of the pharmacological effect of the plant extract in cancer therapy have been largely undetermined.

Research frontiers

Induction of apoptosis and arrest of the cell cycle by plant extracts has become a principal mechanism by which anticancer therapy is effective. Apoptosis triggered by the activation of the mitochondrial-dependent caspase pathway represents the main programmed cell death mechanism. Permeabilization of the outside mitochondrial membrane plays a vital role in cell apoptosis, during which loss of the mitochondrial membrane potential and release of cytochrome C into the cytosol, followed by caspase-9-dependent activation of caspase-3 occurs, resulting in apoptosis. Dysregulation of the cell cycle mechanism has also been shown to perform an important function in growth of various cancer cells, including HCC. The S phase is associated with DNA synthesis and plays a crucial role in cell cycle progression. One of the CDKs, P21, can influence key functions in the control of the cell cycle by negatively regulating CDK4 activity, and plays an important role in regulation of the S phase of the cell cycle.

Innovations and breakthroughs

So far, there has been no evidence found to show that the mitochondrial

pathway is involved in induction of apoptosis and cell cycle arrest by *Chrysanthemum indicum* extract (CIE) in human HCC cells. Therefore, the present study examined the anticancer activities of CIE and related mechanisms in MHCC97H cell lines. The data showed that CIE could induce apoptosis and arrest the cell cycle of MHCC97H cells. CIE exerted a significant apoptotic effect on MHCC97H cells through the mitochondrial-dependent caspase-3 pathway, and arrested the cell cycle in the S phase in cancer cells by upregulation of P21 and downregulation of CDK4.

Applications

This study suggests that *Chrysanthemum indicum* could be a promising plant with potential in the novel treatment of human cancer, particularly HCC.

Peer review

The manuscript written by Li ZF *et al* describes that *Chrysanthemum indicum* extract can induce apoptosis and cell cycle arrest in a hepatoma cell line. Many patients with HCC still die each year, and novel therapeutic strategies are needed. The data are encouraging and promising.

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S- Editor Li LF L- Editor Cant MR E- Editor Ma WH

Effects of lysophosphatidic acid on human colon cancer cells and its mechanisms of action

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Received: June 26, 2009 Revised: August 1, 2009

Accepted: August 8, 2009

Published online: September 28, 2009

migration of SW480 cells, and protected from apoptosis. The Ras/Raf-MAPK, G12/13-Rho-RhoA and PI3K-AKT/PKB signal pathways may be involved.

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Key words: Lysophosphatidic acid; Colon cancer; Proliferation; Apoptosis; Adhesion; Migration; Signal pathway

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Sun H, Ren J, Zhu Q, Kong FZ, Wu L, Pan BR. Effects of lysophosphatidic acid on human colon cancer cells and its mechanisms of action. *World J Gastroenterol* 2009; 15(36): 4547-4555 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4547.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4547>

Abstract

AIM: To study the effects of lysophosphatidic acid (LPA) on proliferation, adhesion, migration, and apoptosis in the human colon cancer cell line, SW480, and its mechanisms of action.

METHODS: Methyl tetrazolium assay was used to assess cell proliferation. Flow cytometry was employed to detect cell apoptosis. Cell migration was measured by using a Boyden transwell migration chamber. Cell adhesion assay was performed in 96-well plates according to protocol.

RESULTS: LPA significantly stimulated SW480 cell proliferation in a dose-dependent and time-dependent manner compared with the control group ($P < 0.05$) while the mitogen-activated protein kinase (MAPK) inhibitor, PD98059, significantly blocked the LPA stimulation effect on proliferation. LPA also significantly stimulated adhesion and migration of SW480 cells in a dose-dependent manner ($P < 0.05$). Rho kinase inhibitor, Y-27632, significantly inhibited the up-regulatory effect of LPA on adhesion and migration ($P < 0.05$). LPA significantly protected cells from apoptosis induced by the chemotherapeutic drugs, cisplatin and 5-FU ($P < 0.05$), but the phosphoinositide 3-kinase (PI3K) inhibitor, LY294002, significantly blocked the protective effect of LPA on apoptosis.

CONCLUSION: LPA stimulated proliferation, adhesion,

INTRODUCTION

Colorectal cancer (CRC) is a common form of cancer and a major cause of cancer death. The incidence of CRC has been rapidly increasing in recent years. Although the incidence of CRC was substantially lower in Asia than in the USA in the mid-twentieth century, the incidence in Japan and China has been rapidly increasing^[1,2]. Thus, CRC is now a leading cancer killer worldwide.

Lysophosphatidic acid (LPA) was first found in the ascitic fluid from ovarian cancer patients. It is a bioactive glycerophospholipid generated and released by platelets, macrophages, epithelial cells, and some tumor cells. Studies have shown the presence of high levels of LPA in the ascitic fluid of patients with ovarian cancer^[3] and LPA is known to be an "ovarian cancer activating factor", which exerts growth factor-like effects through four specific G protein-coupled receptors (LPA₁₋₄).

LPA is a potent mediator with a broad range of cellular responses, including regulation of cell proliferation, protection from apoptosis, modulation of chemotaxis and transcellular migration^[4,5], which mediates survival of ovarian cancer cells, macrophages, fibroblasts, and neonatal cardiac myocytes. Some of these cellular responses indicate that LPA is a mediator of tumor progression.

In a recent study we found that plasma levels of several LPAs, including 18:1-LPA and 18:2-LPA, were significantly increased in CRC patients compared with controls^[6]. This is the first report of high levels of LPA in plasma of CRC patients. It implies that LPA may play roles in CRC development. In order to clarify these roles of LPA in CRC development, the LPA effect on the CRC cell line, SW480, was studied *in vitro*.

LPA was found firstly to be increased in the body fluids of ovarian cancer patients, so the roles of LPA in ovarian cancer have been widely studied. A few preliminary studies of LPA in CRC have been reported, but not in the cell line SW480. Our previous study revealed a high expression of LPA receptors on SW480 cells, especially LPA receptor 2^[7,8]. This study aimed to investigate the effect of LPA on proliferation, migration, adhesion, and apoptosis in the CRC cell line, SW480.

MATERIALS AND METHODS

1-Oleoyl LPA (18:1 LPA) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Inhibitor of phosphoinositide 3-kinase (PI3K), LY290042, and inhibitor of mitogen-activated protein kinase (MAPK), PD98059, are from Cell Signaling (Beverly, MA, USA). Rho kinase inhibitor, Y-27632, was from Biomol (Beverly, MA, USA). Boyden transwell migration chambers and 24-well plates were from Corning Costar Corporation (Cambridge, MA).

SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mL/L fetal bovine serum, streptomycin (100 mg/L), and penicillin (100 kU/L) at 37°C in 50 mL/L CO₂ incubator. Cells were serum starved for 12 h before LPA treatment.

Cell proliferation assay

Methyl tetrazolium (MTT) colorimetry assay was employed to measure cell proliferation. SW480 cells (2×10^3 /well) were seeded in 96-well plates. After cells were starved for 12 h, DMEM containing LPA supplemented with 1 g/L bovine serum albumin was put into the wells. After 24, 48, 72 and 96 h of culture, 20 μ L of MTT solution (5 g/L) was added to each well. Four hours later, the medium was removed and 150 μ L of dimethyl sulfoxide was added to each well. Absorbance value was measured at 490 nm on a Microplate Reader (EXL800). Each assay was performed in quintuplicate.

Annexin V staining

After treating with cisplatin or 5-FU, LPA and/or inhibitors, the cells were resuspended in binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂). Then the cells were stained with 5 μ L of annexin-FITC and 5 mg/L propidium iodide (PI), and then analyzed by flow cytometry (FACSCalibur cytometer, BD Biosciences), and CellQuest (BD Biosciences) was used to quantify the apoptotic cells. Experiments were performed in triplicate.

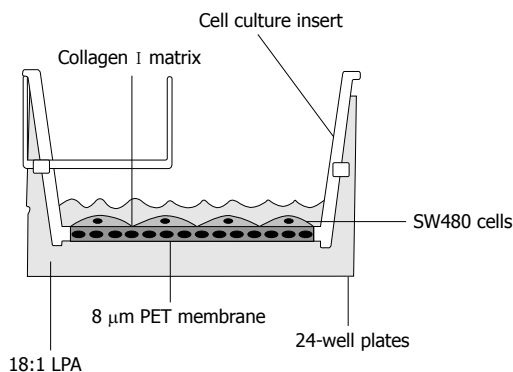


Figure 1 Costar transwell cell culture chamber inserts.

Cell adhesion assay

Flat-bottom 96-well plates were coated with 2 μ g of collagen I (0.04 g/L) (Sigma Chemical Co.) in phosphate-buffered saline overnight at 4°C. Plates were blocked with 2 g/L BSA for 2 h at room temperature followed by washing three times with DMEM. SW480 cells (4×10^4 /well) were added to each well. Four hours later, unbound cells were removed, washing twice with DMEM. Bound cells were fixed by methanol and stained with crystal blue. Stained cells were counted with a phase contrast microscope. Experiments were performed in triplicate.

Cell migration assay

Migration assays were performed in Costar transwell cell culture chamber inserts (coated with collagen I; Corning Costar Corporation, Cambridge, MA) with an 8 μ m pore size as described as Figure 1. Briefly, SW480 cells (5×10^4 cells in 100 μ L of starvation medium) were used for cell migration, which was conducted for 4 h at 37°C. Migrated cells were fixed, stained and counted in five randomly ($\times 200$) selected fields with a phase contrast microscope, and the average numbers of cells per field were counted.

Administration of LPA and inhibitors

SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA at different doses, and then reconstituted in DMEM containing 10 g/L BSA. All inhibitors including LY294002 (50 μ mol/L), PD98059 (10 μ mol/L), and Y-27632 (10 μ mol/L) were applied to cells 30 min before the action of LPA.

Statistical analysis

Statistical significance was assessed by one-way ANOVA using SPSS software. Data are presented as the mean \pm SE.

RESULTS

LPA stimulation of proliferation of SW480 cells

SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA at different doses. After different time periods, MTT assay was performed to evaluate the

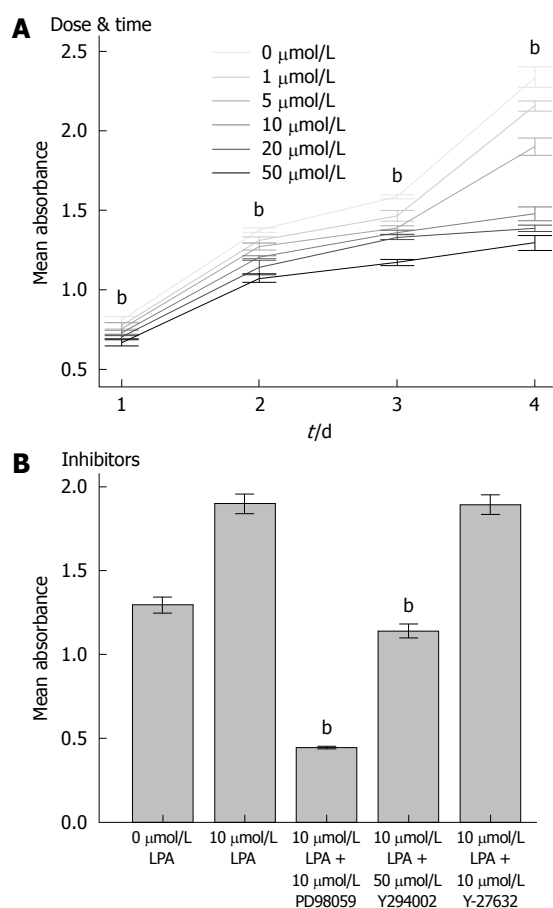


Figure 2 LPA effect on SW480 cell proliferation. Results presented as mean \pm SE, $n = 5$. A: Dose and time effect of LPA on the proliferation of SW480 cells. SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA at different doses. At different time points, MTT assay was performed to evaluate cell numbers. $^bP < 0.001$ vs 0 $\mu\text{mol/L}$ LPA; B: Inhibitors blocked LPA-induced cell proliferation. SW480 cells were starved in serum-free DMEM for 12 h and treated with LPA (10 $\mu\text{mol/L}$). Inhibitors including LY294002 (50 $\mu\text{mol/L}$), PD98059 (10 $\mu\text{mol/L}$), and Y-27632 (10 $\mu\text{mol/L}$) were applied to cells 30 min before the action of LPA. ninety-six hours later, MTT assay was performed to evaluate cell growth. $^bP < 0.001$ vs 10 $\mu\text{mol/L}$ LPA.

activity of cell growth. It was found that LPA significantly stimulated the proliferation of SW480 cells in a dose- and time-dependent manner ($P < 0.001$, Figure 2A). LPA, especially when the concentration was ≥ 10 $\mu\text{mol/L}$, remarkably stimulated cell growth compared with the control group.

In order to investigate the signal pathways which mediated the stimulation effect of LPA on SW480 cells, inhibitors against key molecules of several signal transduction pathways were applied to the LPA-treated group. Three inhibitors were employed including PI3K inhibitor (LY290042), MAPK inhibitor (PD98059), and Rho kinase inhibitor (Y-27632). It was found that after applying the inhibitors, the stimulation effect of LPA on cell growth was significantly blocked by PD98059 and LY290042 ($P < 0.001$, Figure 2B); especially PD98059. This indicated that the Ras/Raf-MAPK signal pathway and the PI3K-AKT/PKB signal pathway may be involved in the LPA stimulation effect on proliferation of SW480 cells.

LPA induction of migration of SW480 cells

SW480 cells (1×10^5 cells in 100 μL of starvation medium) were seeded on the transwell inserts with an 8 μm pore size. Different doses of LPA in DMEM were added to the lower chamber of the transwell. Cells were then incubated at 37°C for 4 h. Cells migrated to the lower surface of inserts were fixed, stained, and quantified. It was found that LPA significantly enhanced SW480 cell migration toward the lower chamber of the transwell in a dose-dependent manner compared with the control ($P < 0.001$, Figure 3A and B). This indicates that LPA has a significant chemotactic effect on SW480 cells.

In order to investigate the signal pathways which mediated the chemotactic effect of LPA on SW480 cells, some inhibitors against key molecules of signal transduction pathways were employed. It was demonstrated that Rho kinase inhibitor (Y-27632 at 10 $\mu\text{mol/L}$) dramatically blocked the chemotactic effect of LPA on SW480 cells ($P < 0.001$, Figure 3C). This indicated that Rho kinase and G12/13-Rho-RhoA signal pathways may mediate the LPA effect on SW480 cell migration.

LPA induction of adhesion of SW480 cells

SW480 cells were seeded in 96-well plates. After the cells had undergone 12 h of starvation, LPA at different doses was added to the cells. SW480 cells were allowed to adhere to the plates for 4 h at 37°C in the incubator. Unbound cells were washed away twice. Adhered cells were fixed, stained, and quantified. Images of adhered cells under different doses of LPA were taken (Figure 4A). It was demonstrated that LPA significantly increased SW480 cell adhesion to extracellular matrix (ECM) in a dose-dependent manner compared with controls ($P < 0.001$, Figure 4B).

Some inhibitors were used to determine the mechanisms which mediated the LPA effect on adhesion. It was found that Rho kinase inhibitor (Y-27632) and LY294002 dramatically inhibited LPA upregulation of adhesion; especially Y-27632 ($P < 0.001$, Figure 4C and D). This indicated that the G12/13-Rho-RhoA signal pathway and the PI3K-AKT/PKB signal pathway may participate in the LPA effect on the adhesion of SW480 cells.

LPA protected SW480 cells from apoptosis

SW480 cells (1×10^5 /well) seeded in 24-well culture plates were starved for 24 h and then treated with cisplatin (10 mg/L) or 5-FU (8 mg/L) for 24 h in the absence or presence of LPA (20 $\mu\text{mol/L}$). Inhibitors including LY294002 (50 $\mu\text{mol/L}$) and PD98059 (10 $\mu\text{mol/L}$) were added to the LPA-treated group.

Apoptotic cells were detected by flow cytometry after Annexin V and PI staining. Apoptotic cells were defined as Annexin-positive, PI-negative (Figure 5). After cells were exposed to cisplatin and 5-FU, the apoptotic population was $20.2\% \pm 2.3\%$ and $14.2\% \pm 2.6\%$, respectively. However, after the action of LPA, the apoptotic population dropped to $14.6\% \pm 2.1\%$ in the cisplatin-treated group and $10\% \pm 2.8\%$ in the 5-FU-treated group. LPA protected 27.7% of cells from cisplatin-induced apoptosis and protected 29.6% of cells

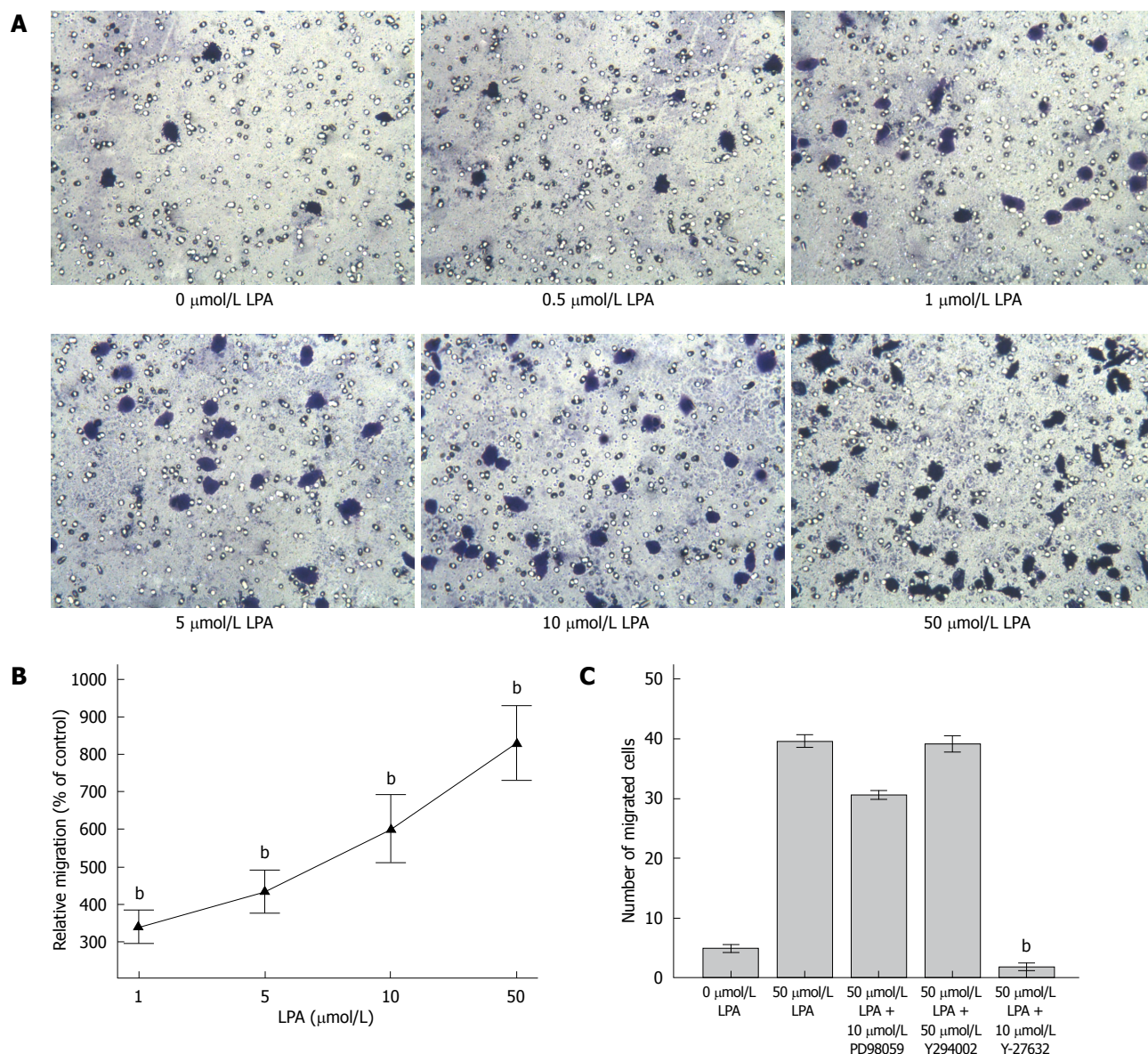


Figure 3 LPA stimulated migration of SW480 cells. A: LPA stimulated migration of SW480 cells ($\times 200$). SW480 cells ($1 \times 10^5/100 \mu\text{L}$) were seeded into the inserts of transwell chambers after starvation for 8 h. Cells were incubated at 37°C for 4 h. Cells on the outside surface of inserts were fixed and stained. Typical images are presented; B: Migrated cells were quantified and relative migration rates (mean \pm SE) are presented. $^bP < 0.001$ vs $0 \mu\text{mol/L}$ LPA; C: Effect of inhibitors on LPA-induced cell migration. $50 \mu\text{mol/L}$ LPA in $600 \mu\text{L}$ medium was added to the lower chamber of transwell. Inhibitors including $10 \mu\text{mol/L}$ PD98059, $50 \mu\text{mol/L}$ LY294002 and $10 \mu\text{mol/L}$ Y-27632 were added to cells in the upper chamber. Cells on the outside surface of inserts were fixed, stained, and quantified. Data were analyzed using one-way ANOVA with post-hoc *t*-tests. $^bP < 0.001$ vs $50 \mu\text{mol/L}$ LPA.

from 5-FU-induced apoptosis. This suggests that LPA effectively protected SW480 cells from apoptotic death induced by the chemotherapeutic agents.

In order to elucidate the mechanisms of LPA protection from apoptosis, LY294002 and PD98059 were added to the LPA-treated group. Apoptotic population increased to $50.2\% \pm 3.2\%$ and $32.5\% \pm 3.6\%$ respectively after exposure to LY294002 (PI3K inhibitor) and PD98059 (MAPK inhibitor). This indicated that the PI3K, MAPK, PI3K-AKT/PKB signal pathways and the Ras/Raf-MAPK signal pathway may be involved in the LPA apoptotic-protection effect.

DISCUSSION

LPA, the simplest glycerophospholipid, was initially

found in the ascites of ovarian cancer patients at significant levels ($2\text{--}80 \mu\text{mol/L}$), and plays an important role in the development of ovarian cancer. LPA exerts growth factor-like effects through four specific G protein-coupled receptors (LPA₁₋₄). The effects include mitogenesis, secretion of proteolytic enzymes, and migration activity, which are accompanied by stress fiber formation and focal adhesion assembly in ovarian cancer cells.

We found recently that the level of LPA increases not only in the body fluid of ovarian cancer patients, but also in the plasma of patients with CRC. There are many studies focused on the LPA role in ovarian cancer cells. It has been found that LPA plays important roles in the progression of ovarian cancer and acts as an ovarian cancer promoter. Since LPA also increases in the plasma of CRC patients, what are the roles of LPA in CRC?

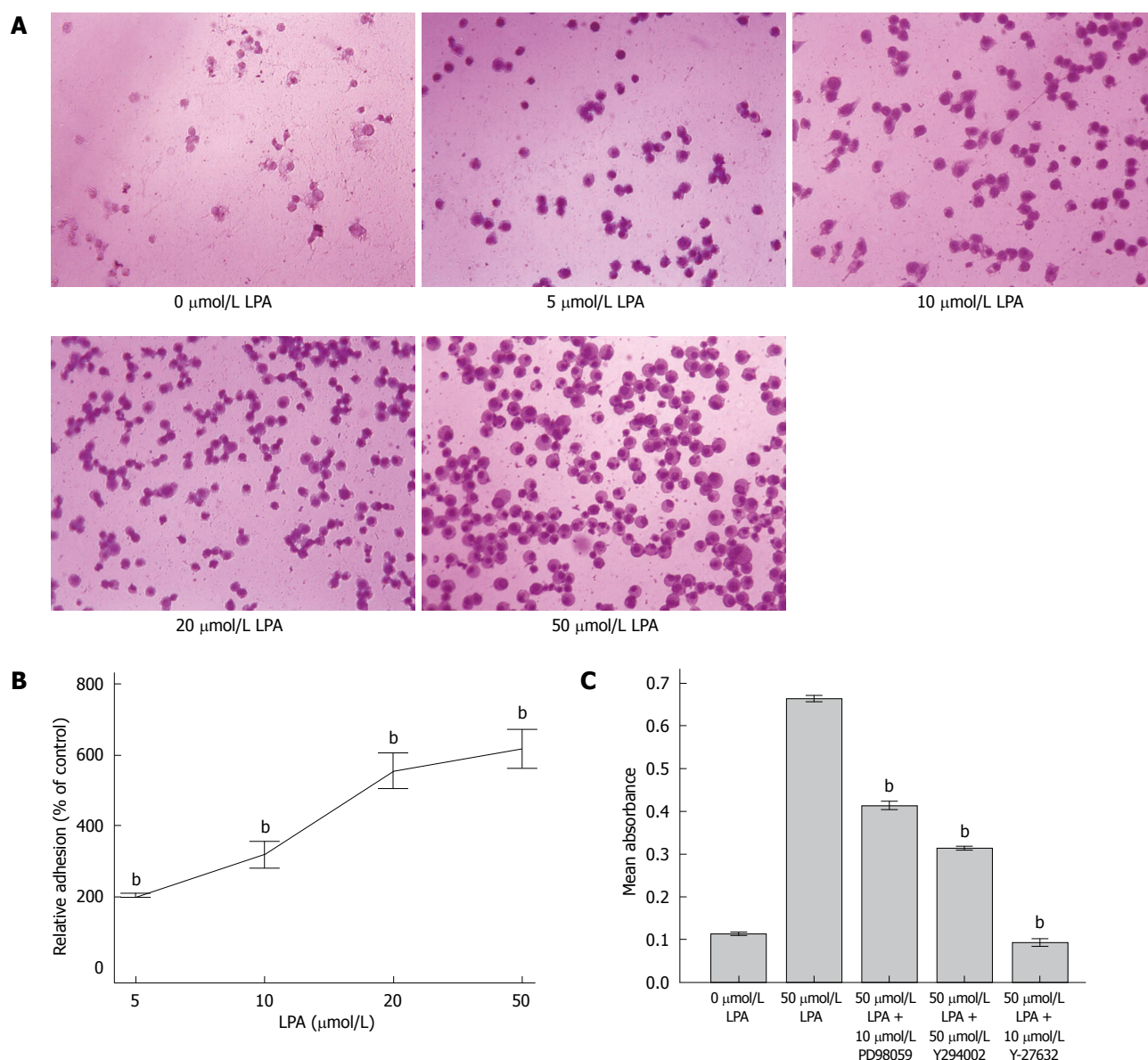


Figure 4 LPA stimulated adhesion of SW480 cells. A: Typical image of stained adhered cells ($\times 200$); B: Adhered cells were quantified and relative adhesion rates (mean \pm SE) are presented. ^b $P < 0.001$ vs 0 $\mu\text{mol/L}$ LPA; C: Effect of inhibitors on LPA induced adhesion. 50 $\mu\text{mol/L}$ LPA or LPA plus inhibitors (including 10 $\mu\text{mol/L}$ PD98059, 50 $\mu\text{mol/L}$ LY294002 and 10 $\mu\text{mol/L}$ Y-27632) were added to cells in 96 well plate. Adhered cells were quantified and presented. Results presented as mean \pm SE, $n = 5$. ^b $P < 0.001$ vs 50 $\mu\text{mol/L}$ LPA.

There are few studies regarding LPA effects on CRC. Furthermore, our previous study has shown that LPA receptors (LPA_{2,4}) are highly expressed in SW480 cells. In the present study, we preliminarily investigated the roles of LPA in the proliferation, migration, adhesion and apoptosis of SW480 cells and its mechanisms of action. We found that LPA significantly stimulated the proliferation of SW480 cells in a dose-dependent and time-dependent manner. This is consistent with the reports in ovarian cancer, in which LPA promotes growth of ovarian cancer similar to growth factor^[9].

We found that the MEK1 inhibitor, PD98059, significantly inhibited the LPA effect on the proliferation of SW480 cells. MEK1, a MAPK, is a key molecule of the Ras/Raf1/MEK/ERK signal pathway^[10]. We also found that the PI3K inhibitor, LY290042, partially inhibited the effect of LPA on the cell proliferation. This

indicated that LPA stimulates the proliferation of SW480 cells through the Ras/Raf1/MEK/ERK pathway, and that the PI3K-AKT/PKB signal pathway may also be partially involved in the LPA effect on proliferation.

MAPK transfers major cell proliferation signals from the cell surface to the nucleus. There are three major subfamilies of MAPK, including the extracellular-signal-regulated kinase (Ras/Raf1/MEK/ERK or ERK MAPK), the c-Jun N-terminal or stress-activated protein kinase (JNK or SAPK), and MAPK14^[11]. The Ras/Raf1/MEK/ERK pathway is one of the most important pathways for cell proliferation. Several lines of evidence suggest that, in CRC, the Ras/Raf1/MEK/ERK pathway, but not the JNK pathway or the p38 MAPK pathway, is the major regulator of cell proliferation. There is growing evidence that activation of the Ras/Raf1/MEK/ERK pathway is involved in the

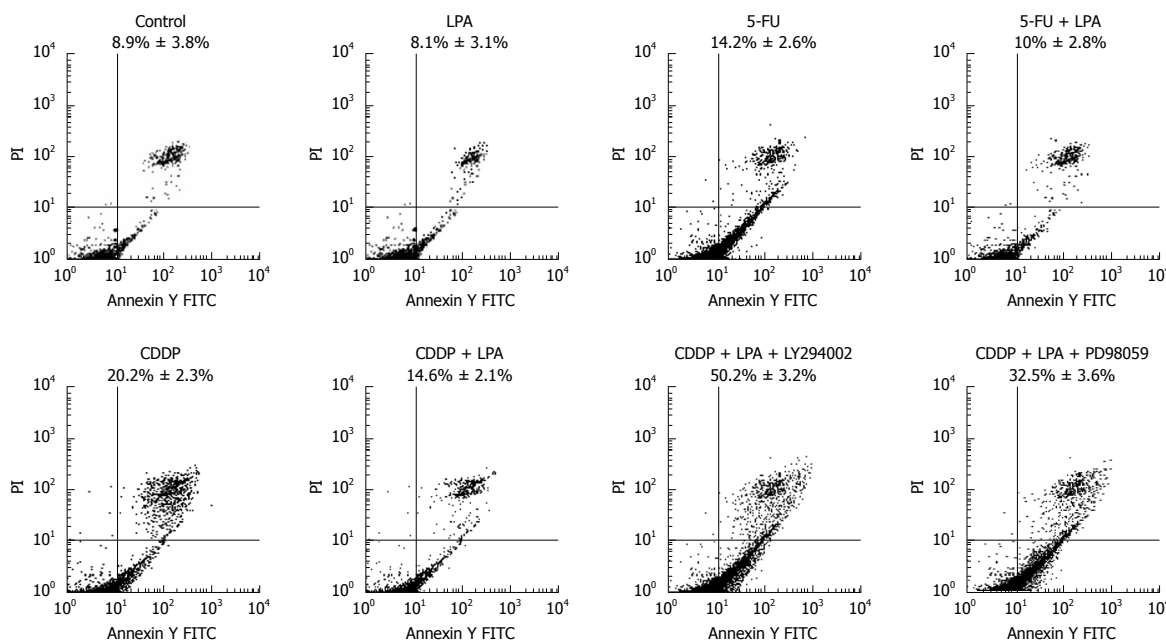


Figure 5 LPA protected cells from cisplatin- & 5-FU-induced apoptosis. SW480 cells were serum-starved for 24 h and treated for 24 h with 10 μ g/mL cisplatin (CDDP); 10 μ g/L cisplatin + 20 μ mol/L LPA; 8 μ g/mL 5-FU; 8 μ g/mL 5-FU + 20 μ mol/L LPA; 10 μ g/mL cisplatin + 20 μ mol/L LPA + 50 μ mol/L LY294002; 10 μ g/mL cisplatin + 20 μ mol/L LPA + 10 μ mol/L PD98059, respectively. Cells were harvested, stained with Annexin V/PI, and analyzed by FACS analysis. The Annexin V-positive and PI-negative population was defined as apoptotic cells. Representative results from three separate experiments are presented.

pathogenesis, progression, and oncogenic behavior of human CRC^[12]. This supports our observations that LPA stimulated growth of CRC cell line SW480 through the Ras/Raf1/MEK/ERK pathway.

There are several studies reporting LPA's effect on the proliferation of CRC cells. Zhang *et al*^[13] has found that LPA facilitates proliferation of colon cancer cells *via* induction of Krüppel-like Factor 5 (KLF5). KLF5 is a transcriptional factor highly expressed in the crypt compartment of the intestinal epithelium. LPA stimulated the KLF5 expression in colon cancer cells, SW480 and HCT116. Moreover, LPA-mediated KLF5 induction was partially blocked by inhibition of MAPK kinase and protein kinase C (PKC). This also indirectly indicated that the MAPK signaling pathway is involved in the proliferation of SW480 cells.

Yang *et al*^[14] reported that LPA-induced colon cancer cell proliferation requires the β -catenin signaling pathway. LPA activated the main signaling events in the β -catenin pathway, but inhibition of PKC blocked the effects, suggesting PKC involvement in LPA-induced activation of the β -catenin pathway. This also indirectly indicated that the PKC signaling pathway is involved in the proliferation of SW480 cells.

Balavenkatraman *et al*^[15] reported that DEP-1 protein tyrosine phosphatase inhibits proliferation and migration of colon carcinoma cells and is upregulated by protective nutrients. Upregulation of DEP-1 expression, and in turn inhibition of cell growth and migration, may present a previously unrecognized mechanism of chemoprevention by nutrients. This result contradicts other reports.

In our study, it was found that LPA significantly enhanced the migration and adhesion of SW480 cells

in a dose-dependent manner. The stimulation effect of LPA on cell adhesion, invasion and migration has been reported in other cancer types, including ovarian cancer^[16-19], pancreatic cancer^[18], and breast cancer^[20,21]. Enhanced migration activity and increased adherence to ECM are two major factors which contribute to tumor metastasis. Cell-ECM adhesions can alter the cell's capacity to attach and migrate through surrounding tissues. Changes of the expression and activities of the components of such adhesions could make an important contribution to preventing cancer invasion. Our study showed that LPA stimulated both migration and adhesion to ECM of colon cancer SW480 cells. This means that LPA significantly promotes the metastatic potential of SW480 cells. In our study, Rho kinase inhibitor, Y-27632, significantly inhibited cell migration and adhesion induced by LPA. LY294002 partially inhibited the LPA effect on adhesion. This indicated that the G12/13-Rho-GEFs-RhoA signal pathway may mediate the effect of LPA on both migration and adhesion, and that the PI3K-AKT/PKB pathway may partially mediate the LPA effect on adhesion.

Rho GTPase family proteins, including Rho, Rac1, and Cdc42, control a wide variety of cellular processes, such as cell adhesion, motility, proliferation, differentiation, and apoptosis^[22]. One of the best effectors of Rho is Rho-associated kinase (ROCK). ROCK is a target effect molecule downstream of RhoA. Rho activates ROCK by phosphorylation of Ser854 and Thr697, and induces a series of actions downstream to stimulate adhesion and migration. Y-27632 is a novel and specific inhibitor of ROCK, which is cell permeable and inhibits ROCK- I and ROCK- II by competing with ATP.

It has been reported in other tumors that LPA

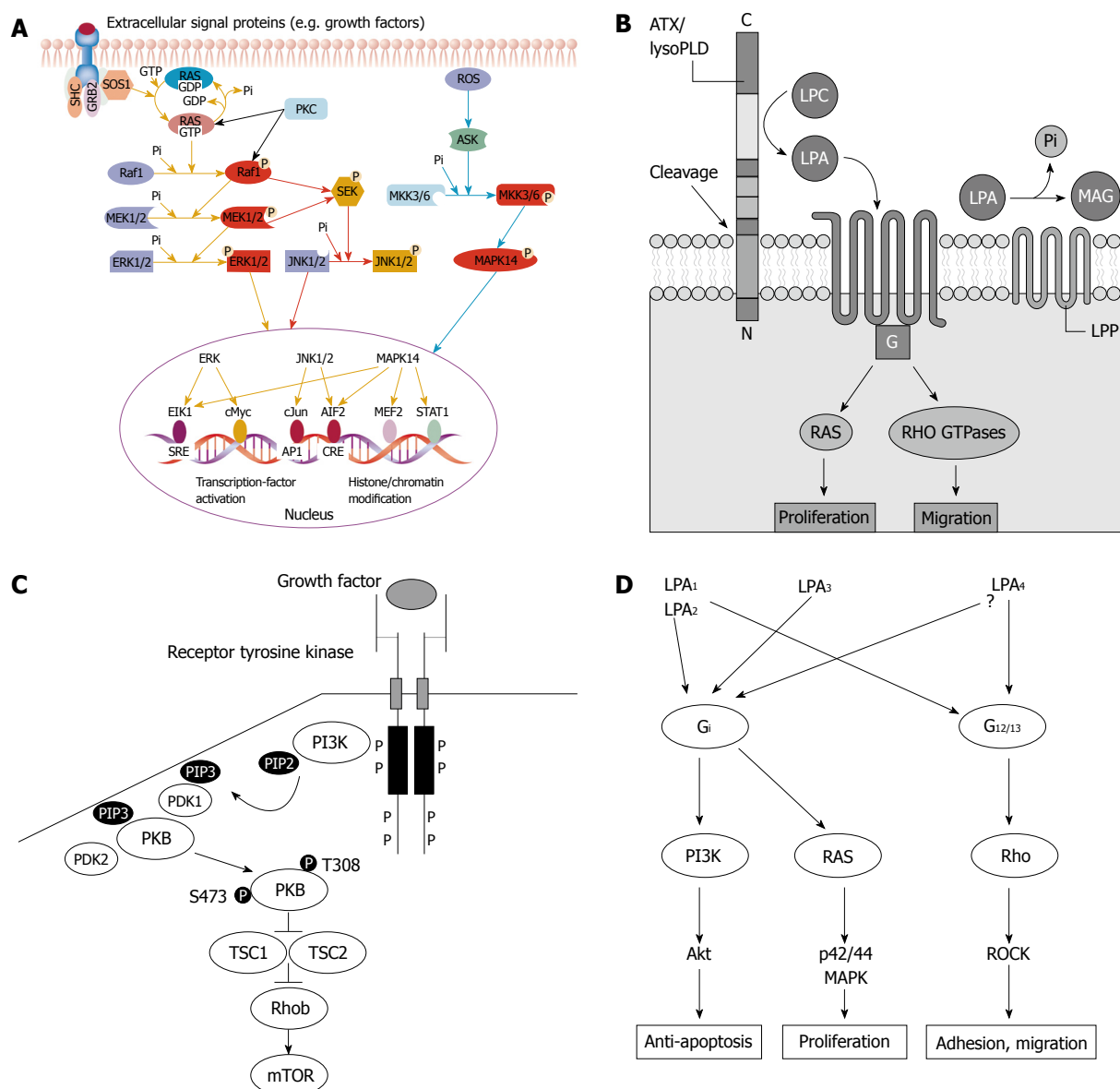


Figure 6 LPA effect and signal transduction pathway. A: Ras/Raf1/MEK/ERK signal pathway^[27]; B: G12/13-Rho-GEFs-RhoA signal pathway; C: PI3K-AKT/PKB signal pathway; D: Possible signal pathways mediated by LPA effect on SW480 cells.

drives the formation of focal adhesions, the tyrosine phosphorylation of focal adhesion proteins, and ROCK. Our results suggested that in colon cancer SW480 cells, the G12/13-Rho-GEFs-RhoA pathway may mediate migration and adhesion induced by LPA.

Several studies have also found that LPA promotes the metastatic potential of CRC.

Tatsuta *et al*^[23] found that LPA significantly increased the peritoneal and pleural metastases of intestinal adenocarcinomas induced by azoxymethane through RhoA activation. This is consistent with our study, in which the Rho signaling pathway was found to be involved in the LPA effect on metastasis. Other mechanisms have also been found.

Shiada *et al*^[24] found that cross-talk between LPA₁ and epidermal growth factor receptors mediates up-regulation of sphingosine kinase 1 to promote gastric cancer cell motility and invasion. Down-regulation of SphK1 attenuated LPA-stimulated migration and invasion of

MNK1 cells. Shiada *et al*^[25] also reported that LPA acts as a potent stimulator of colon cancer progression, although the binding to LPA₁ and LPA₂ induced slightly different responses. Komuro *et al*^[26] found that LPA₁ expression was increased in the early stage of adenoma.

We also found that LPA significantly protected SW480 cells from apoptosis induced by chemotherapeutic drugs, while LY294002 and PD98059 effectively blocked the LPA effect on apoptosis, indicating that the PI3K-AKT/PKB and the Ras/Raf-MAPK signal pathways may mediate the LPA effect on apoptosis; especially the PI3K-AKT/PKB pathway. The apoptosis protection roles of LPA have been reported in ovarian cancer cells.

PI3K can be divided into three classes. Class I PI3K is the most studied class of PI3K, consisting of an 110 kDa catalytic subunit and a regulatory subunit of 85 kDa. The activity of PI3K protein family is associated with cytoskeletal organization, cell division, inhibition of apoptosis and glucose uptake. The phospholipid

products of PI3K activate downstream targets, including PDK, Akt and PKC. LY294002 blocks PI3 kinase-dependent Akt phosphorylation and kinase activity. In SW480 cells we observed that the PI3K-AKT/PKB pathway may partially mediate the effect of LPA on proliferation.

In this study, it has been found that LPA significantly stimulated the proliferation, adhesion, and migration of human colon cells, SW480, and protected their apoptosis. The Ras/Raf-MAPK signal pathway may be involved in the LPA effect on proliferation. The G12/13-Rho-RhoA signal pathway may be associated with the LPA effect on adhesion and migration. The PI3K-AKT/PKB signal pathway may participate in the anti-apoptotic effect of LPA. This indicates that LPA probably acts as a promoter of the development of CRC. To decrease the LPA level in CRC patients and to block the LPA action (Figure 6) could be the aim of new strategies of treatment and prevention of CRC. The pathways involved in the LPA effects which we have discovered in this study could be new treatment targets of CRC.

COMMENTS

Background

Colorectal cancer (CRC) is a major cause of cancer death worldwide. The incidence has been rapidly increasing in recent years. Lysophosphatidic acid (LPA) was initially found in the ascites of ovarian cancer patients. Recently the authors group found that LPA levels increase not only in the body fluid of ovarian cancer patients, but also in the plasma of patients with CRC. There are many studies of LPA roles in ovarian cancer cells which have found that LPA stimulates the progression of ovarian cancer. Since LPA increases in the plasma of CRC patients as well, what are the roles of LPA in CRC? This study preliminarily investigated the roles of LPA in the proliferation, migration, adhesion and apoptosis of SW480 cells and its mechanisms of action.

Research frontiers

LPA was firstly found in the ascitic fluid from ovarian cancer patients. It is a bioactive glycerophospholipid generated and released by platelets, macrophages, epithelial cells, and some tumor cells. Studies have shown the presence of high levels of LPA in the ascitic fluid of patients with ovarian cancer and LPA is known to be an "ovarian cancer activating factor", which exerts growth factor-like effects through four specific G protein-coupled receptors (LPA₁₋₄). LPA is a potent mediator with a broad range of cellular responses, including regulation of cell proliferation, protection from apoptosis, modulation of chemotaxis and transcellular migration, which also mediates survival of ovarian cancer cells, macrophages, fibroblasts, and neonatal cardiac myocytes. Some of these cellular responses indicate that LPA is a mediator of tumor progression.

Innovations and breakthroughs

Since LPA was found in the ascites of ovarian cancer patients, there have been many studies on LPA, but most of the studies have focused on ovarian cancer. There are some studies of LPA in colon cancer, but this present study has some different findings from other studies. Firstly, because the expression level of LPA receptor varies in different colon cancer cell lines, and different LPA receptors mediate different responses to LPA, so LPA effects on SW480 cells are different from other colon cancer cell lines. Secondly, some mechanisms found in this study are not completely the same as findings in other studies. They found some pathways which mediate the LPA effect on proliferation, migration, and adhesion which are different from other studies.

Applications

In order to completely block the growth, metastasis and progression of CRC, the mechanisms for its development need to be clarified. The provided some information about the LPA effects on colon cancer cells and some mechanisms of action. These results will help to design targeted strategies to block LPA's stimulation effect on colon cancer.

Peer review

LPA is associated with inflammation and has been thought to be one of the

mediators of inflammation-induced promotion of cancer. LPA is one of the possible keys for inflammation-induced carcinogenesis in GI tract. Actually there are at least 511 papers on LPA and cell proliferation, among which there are nine papers on LPA and colon cancer. In this particular manuscript, the study was conducted carefully on many aspects including proliferation, apoptosis, cell adhesion, migration *etc.*, of colon cancer cells. Title, abstract, methods and results were carefully written.

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S- Editor Tian L **L- Editor** Logan S **E- Editor** Zheng XM



BRIEF ARTICLES

Early graft dysfunction following adult-to-adult living-related liver transplantation: Predictive factors and outcomes

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Received: June 28, 2009 Revised: August 13, 2009

Accepted: August 20, 2009

Published online: September 28, 2009

Abstract

AIM: To describe a condition that we define as early graft dysfunction (EGD) which can be identified preoperatively.

METHODS: Small-for-size graft dysfunction following living-related liver transplantation (LRLT) is characterized by EGD when the graft-to-recipient body weight ratio (GRBWR) is below 0.8%. However, patients transplanted with GRBWR above 0.8% can develop dysfunction of the graft. In 73 recipients of LRLT (GRBWR > 0.8%), we identified 10 patients who developed EGD. The main measures of outcomes analyzed were overall mortality, number of re-transplants and length of stay in days (LOS). Furthermore we analyzed other clinical pre-transplant variables, intra-operative parameters and post transplant data.

RESULTS: A trend in favor of the non-EGD group (3-mo actuarial survival 98% vs 88%, $P = 0.09$; 3-mo graft mortality 4.7% vs 20%, $P = 0.07$) was observed as well as shorter LOS (13 d vs 41.5 d; $P = 0.001$) and smaller requirement of peri-operative Units of Plasma (4 vs 14; $P = 0.036$). Univariate analysis of pre-transplant variables identified platelet count, serum bilirubin, INR and Meld-Na score as predictors of EGD. In the multivariate analysis transplant Meld-Na score ($P = 0.025$, OR: 1.175) and pre-transplant platelet count ($P = 0.043$, OR: 0.956) were independently associated with EGD.

CONCLUSION: EGD can be identified preoperatively and is associated with increased morbidity after LRLT. A prompt recognition of EGD can trigger a timely treatment.

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Key words: Small-for-size graft dysfunction; Living-related liver transplantation; Graft-to-recipient body weight ratio; Partial liver transplantation; Allograft dysfunction

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Gruttadauria S, di Francesco F, Vizzini GB, Luca A, Spada M, Cintonino D, Li Petri S, Pietrosi G, Pagano D, Gridelli B. Early graft dysfunction following adult-to-adult living-related liver transplantation: Predictive factors and outcomes. *World J Gastroenterol* 2009; 15(36): 4556-4560 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4556.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4556>

INTRODUCTION

Small-for-size graft dysfunction (SFGSD) is one of the greatest limiting factors for the expansion of segmental liver transplantation from living donors^[1], and is characterized by: (1) onset within 2 wk after living-

related liver transplantation (LRLT); (2) a graft-to-recipient body weight ratio (GRBWR) below 0.8%; (3) total bilirubin higher than 5 mg/dL, and/or output of ascites through abdominal drainages of more than 1 L/d; and (4) exclusion of technical (e.g. arterial or portal occlusion, outflow congestion, bile leak), infective (e.g. sepsis) and immunological (e.g. acute cellular rejection) complications.

By definition, SFGSD can be diagnosed only in the presence of a GRBWR of less than 0.8%, or a ratio of graft volume (GV) relative to the standard liver volume (SLV) of the recipient (GV/SLV) of less than 30%^[1-4]. However, despite a GRBWR above 0.8%, some recipients of LRLT may have a worse clinical course.

The aim of this study was to analyze a group of LRLT recipients in order to identify those who developed a clinical picture of SFGSD in the absence of a GBWR of < 0.8% and with a GV/SLV ratio highest than 30%. Those patients were defined as affected by early graft dysfunction (EGD).

MATERIALS AND METHODS

We evaluated the rate of EGD in 73 consecutive recipients of adult-to-adult LRLT performed at our institute between July 2004 and September 2008, and whose GRBWR was > 0.8% and with a GV/SLV ratio higher than 30%. Follow-up in months was 27.34 ± 13.77 .

There were 43 males and 30 females, with a median age of 57 years (range 18-68 years). The etiology of the liver disease was related to hepatitis C virus infection in 47 cases, to hepatitis B virus infection in nine patients, to both B and C virus infection in three patients, and to non-viral causes in 14 patients. Twenty-two patients had hepatocellular carcinoma (HCC). Donor liver resection resulted in 73 right hepatectomies (liver segments 5-8). Graft implantation was performed with the piggy back technique and, in all cases, with the use of veno-venous bypass. Details of surgical procedures are reported elsewhere^[5,6]. Volumetric computed tomography (CT) scan was used to calculate liver and spleen volumes.

The main measures of outcomes analyzed were overall mortality, number of re-transplants and length of stay in days (LOS).

In order to identify predictors of EGD, epidemiologic pre-transplant variables such as age of the recipient and donor, sex of the recipient and donor, recently reported as markers of graft function^[7], were evaluated (Table 1).

Furthermore, we analyzed other clinical pre-transplant variables such as: serum bilirubin, serum albumin, serum sodium, INR, platelets count, WBC count, Child-Pugh score, MELD score, Meld-NA score, recently described^[8-10], percentage of donor liver steatosis, liver volume and spleen volume evaluated using CT, spleen/liver volume ratio (S/LVR), GBWR and GV/SLV (Table 2).

Then we observed the following intra-operative parameters: mean arterial pressure, systemic vascular resistance, cardiac output, cardiac index, units of

Table 1 Univariate analysis of epidemiologic data in the group with EGD *vs* the group without EGD, median (range)

	With EGD (10 pts)	Without EGD (63 pts)	P value
Age recipient	52.72 (38-61)	57.6 (18-68)	NS
Age donors	29.5 (26-54)	30 (18-53)	NS
Sex recipient (M/F)	5/5	38/25	NS
Sex donors (M/F)	5/5	39/24	NS

EGD: Early graft dysfunction.

transfused packed red blood cells, units of transfused platelets, and units of transfused fresh frozen plasma (Table 3).

Finally as post transplant data we looked at the LOS.

Statistical analysis

Survival analysis was performed using the Kaplan-Meier analysis with SPSS (SPSS Inc., Chicago, Ill, United States), and a descriptive analysis was used for the outcome. Normality was tested with the Wilk-Shapiro test. Differences between the two groups were tested using the unpaired Student's *t*-test, Mann-Whitney test, χ^2 test; $P < 0.05$ were considered significant. Multivariate analysis was performed to identify independent determinants for occurrence of EGD (logistic regression stepwise backward procedure).

RESULTS

Ten out of 73 patients (13.7%) fit our criteria for EGD. No statistically significant differences were found between EGD and non-EGD recipients in terms of 3-mo patient and graft mortality [one patient out of ten (10%) *vs* one patient out of 63 (1.6%), $P = 0.13$; two patients out of ten (20%) *vs* three patients out of 63 (4.7%), $P = 0.07$], number of re-transplants during the first 3 mo after LRLT [one patient out of ten (10%) *vs* two patients out of 63 (3.2%), $P = 0.33$] and 3-mo and 1-year actuarial patient survival (88% *vs* 98%; $P = 0.09$ by the log-rank test; 80% *vs* 94%, $P = 0.12$ by the log-rank test).

The 4-year actuarial patient survival was 77.78% *vs* 88.01%, ($P = 0.201$ by the log-rank test) (Figure 1). Although the statistical analysis doesn't indicate any statistical significance, probably due to the small size of the sample examined, the survival analysis points out a lower survival rate (77.78%) on the EGD patient *vs* non-EGD patient (88.01%); this is clinically relevant.

In the EGD patients, we observed two deaths: one because of sepsis and the second one due to multiorgan failure. In the non-EGD group, we observed six deaths: three because of neoplastic recurrence of HCC and three due to multiorgan failure. HCC recurrence could be explained by the advanced stage of the tumor at the pathologic examination, although the patients were classified within Milan criteria.

We did observe a significant difference between the two groups in terms of LOS, with the EGD group having

Table 2 Univariate analysis of pre-transplant clinical data in the two groups: EGD *vs* non-EGD

	EGD (10 pts)	Non-EGD (63 pts)	P value
Pre-transplant serum bilirubin (mg/dL)	8.71 (1.27-29.21)	2.01 (0.28-24.82)	0.013
¹ Pre-transplant serum albumin (g/dL)	2.6 (2.2-3.3)	2.8 (1.31-4)	NS
Pre-transplant serum sodium (mEq/L)	133 (122-145)	138 (126-144)	NS
Pre-transplant INR	1.38 (1.27-2.55)	1.22 (0.81-2.55)	0.001
Pre-transplant platelets (mmc)	48 000 (22 000-60 000)	71 000 (24-400)	0.007
Pre-transplant WBC (mmc)	4575 (1700-7200)	4200 (1500-15 500)	NS
Child-Pugh score, points	10.00 (8-12)	8.0 (5-12)	NS
MELD score	20.50 (12-40)	15.0 (6-28)	NS
Meld-Na score	24.25 ± 7.9	18.13 ± 5.8	0.004
Steatosis (No/Macro 1%-2%/Macro 10%-20%/Macro 25%-30%)	(6/2/2/0)	(35/15/9/4)	NS
Liver volume (mL)	780 (590-1186)	1016 (557-1482)	NS
Spleen volume (mL)	983 (648-1382)	709 (161-2711)	NS
S/LVR	0.96 (0.55-2.34)	0.78 (0.13-2.95)	NS
GRDWR	1.26 (0.79-1.59)	1.48 (0.81-2.96)	NS
GS/SLV	59.52 (37.34-70.19)	68.5 (38.7-132.6)	NS

¹Neither group of patients received albumin supplementation before transplant. S/LVR: Spleen/liver volume ratio; GRDWR: Graft-to-recipient body weight ratio; GS/SLV: Graft-to-recipient standard liver volume. Data are expressed as mean (range), or mean ± SD.

Table 3 Univariate analysis of intraoperative parameters in the two groups: EGD *vs* non-EGD

	EGD (10 pts)	Non-EGD (63 pts)	P value
MAP, mmHg	73.8 ± 13	76.7 ± 12	NS
SVR dyn s cm ⁻⁵	676 (350-1429)	704 (308-1249)	NS
C/O, L/min	6.9 (3.7-12)	9.4 (6-11.8)	NS
C/I, L/min per meter ²	4.5 (3.6-7.5)	4.2 (2.2-6.4)	NS
PRBC	12 (0-47)	3 (0-34)	NS
Units plasma transfused	14 (0-47)	4 (0-34)	0.036

MAP: Mean arterial pressure; SVR: Systemic vascular resistance; C/O: Cardiac output; C/I: Cardiac index; PRBC: Units of transfused packed red blood cells. Data are expressed as mean (range), or mean ± SD.

a longer median LOS (13 d *vs* 41 d, $P = 0.001$) and greater median number of units of plasma transfused during surgery (4 *vs* 14, $P = 0.036$).

At univariate analysis of the variables collected, INR, platelet count, serum bilirubin and Meld-Na score, were identified as predictors of EGD (Table 3).

In the multivariate analysis (logistic regression, backward stepwise procedure), we analyzed INR, platelet count, serum bilirubin and Meld-Na score. Meld-Na score ($P = 0.025$, OR: 1.175) and pre-transplant platelet count ($P = 0.043$, OR: 0.956) were the variables independently associated with occurrence of EGD (Table 4).

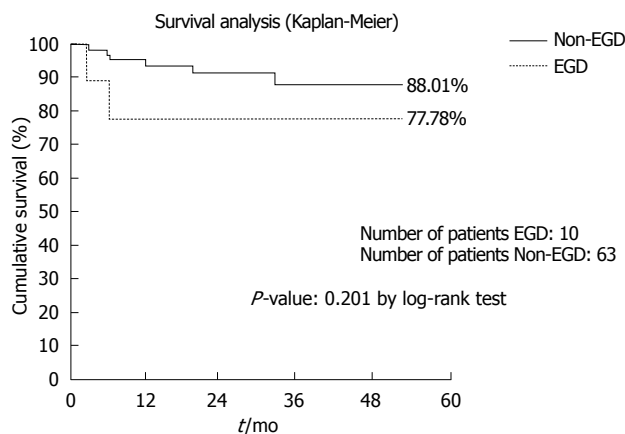
In conclusion, the main clinical outcomes of the two groups were not statistically significant in terms of both early and late patient survival, probably because of the small size of the sample. In fact, as the survival rate was 77.78% *vs* 88.01% for EGD and non-EGD patients, we can hypothesize that survival rate acquires a statistically significant difference by enrolling a larger number of patients.

DISCUSSION

A GRBWR below 0.8% is considered mandatory for

Table 4 Multivariate analysis of pre-transplant epidemiologic and clinical data in the two groups: EGD *vs* non-EGD (logistic regression, backward stepwise procedure)

	P value	OR	95% CI per OR
Meld-Na score	0.025	1.175	1.021; 1.352
Pre-transplant platelets, mmc	0.043	0.956	0.915; 0.999

**Figure 1** Survival analysis.

the diagnosis of SFSGD. Despite these findings in the literature, there are few patients who fully develop SFSGD by classic definition.

On the other hand, there are many patients who do not do well immediately after LRLT. We observed a clinical picture similar to that of SFSGD in patients who received partial livers that could not be described as small (GRBWR > 0.8).

In this study, the relevant clinical impact of EGD is suggested by the reduced 3-mo and 1-year patient survival and the increased graft-loss rate in the group of patients with this condition, even though there was no statistically significant difference, which is probably

due to an insufficient sample size (and a small number of events). The increased LOS in the EGD group reflects the increased time of recovery. Those patients who developed EGD were in fact those with worse INR, platelet count and total bilirubin. In addition, as previously reported by Yoshizumi *et al*^[11], we noted that patients with a higher MELD score, higher Child Pugh score and hyponatremia, tended to have a worse outcome.

In fact, in the EGD group (Table 3), these parameters were higher than in the non-EGD patient.

Our data, although not significant in accordance to others^[10], are clinically relevant especially at the time of selection of donors and recipient.

Our study was also aimed at finding objective criteria for identifying those patients who had a worse clinical course in the 2 wk after LRLT, and with a GRBWR above 0.8%. Our data support the hypothesis that SFGSD and EGD have a multi-factorial genesis in which the combination of the donor's factors (GV and quality of the graft) and the recipient's factors (portal hypertension and stage of liver disease) lead to allograft dysfunction after partial liver transplantation^[3,9,10,12].

The clinical variables identified at the univariate analysis as predictors of EGD confirmed the relevant roles of liver disease and portal hypertension in graft dysfunction.

Serum bilirubin, INR, and Meld-Na score are markers of liver function and platelet count is a marker of portal hypertension. However, at the multivariate analysis, the only variables independently associated with occurrence of EGD were Meld-Na score and pre-transplant platelet count.

The transplant community is now focused on the possibility of detecting predictive factors based on simple biochemical and imaging assessments which could allow physicians to treat those patients at risk of EGD immediately after surgery.

It has been demonstrated that in patients with cirrhosis and severe portal hypertension, the occlusion of the splenic artery causes a significant reduction in portal pressure, which is directly related to the spleen volume and indirectly related to the liver volume^[13]. This concept is at the center of our strategy for performing early splenic artery embolization for the treatment of SFGSD following LRLT^[14].

EGD can be identified preoperatively and is associated with increased morbidity after LRLT. Obviously, a prompt recognition of EGD can trigger a timely and appropriate treatment.

COMMENTS

Background

Small-for-size graft dysfunction (SFGSD) following living-related liver transplantation (LRLT) is characterized by early graft dysfunction (EGD) when the graft-to-recipient body weight ratio (GRBWR) is below 0.8%. However, patients transplanted with GRBWR above 0.8% can develop dysfunction of the graft.

Research frontiers

The study was aimed at finding objective criteria for identifying those patients who had a worse clinical course in the 2 wk after LRLT and had a GRBWR above 0.8%. They describe a condition that they define as EGD which can be identified preoperatively and seems to be associated with increased morbidity after LRLT.

Innovations and breakthroughs

A GRBWR below 0.8% is considered mandatory for the diagnosis of SFGSD. Despite the findings in the literature, there are few patients who fully develop SFGSD by classic definition. The authors observed a clinical picture similar to that of SFGSD in patients who received partial livers that could not be described as small (GRBWR > 0.8).

Applications

A prompt recognition of EGD can trigger a timely and appropriate treatment.

Terminology

The authors describe a condition that they define as EGD which can be identified preoperatively and seems to be associated with increased morbidity after LRLT.

Peer review

This is an important study which impacts on the field. Gruttadauria and colleagues report herein the parameters which allow preoperative identification of a condition defined as EGD i.e. the transplant Meld-Na score and pre-transplant platelet count. The study is original, well designed and performed.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM



COX-2 polymorphisms -765G→C and -1195A→G and colorectal cancer risk

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Received: April 21, 2009 Revised: August 26, 2009

Accepted: September 2, 2009

Published online: September 28, 2009

with an increased risk of developing CRC and the GG/AC haplotype seems to protect against CRC. These findings suggest a modulating role for the COX-2 polymorphisms -765G→C and -1195A→G in the development of CRC in a Dutch population.

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Key words: Colorectal carcinoma; Cyclooxygenase-2; Genetic polymorphism

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Hoff JH, te Morsche RHM, Roelofs HMJ, van der Logt EMJ, Nagengast FM, Peters WHM. COX-2 polymorphisms -765G→C and -1195A→G and colorectal cancer risk. *World J Gastroenterol* 2009; 15(36): 4561-4565 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4561.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4561>

Abstract

AIM: To determine the possible modulating effect of the COX-2 polymorphisms, -765G→C and -1195A→G, on the risk of colorectal cancer (CRC) in a Dutch population.

METHODS: This case-control study includes 326 patients with CRC and 369 age- and gender-matched controls. Genotypes of the COX-2 polymorphisms -765G→C and -1195A→G were determined by polymerase chain reaction-based restriction fragment length polymorphism. COX-2 genotypes and haplotypes were analyzed and odds ratios with 95% confidence intervals were estimated by logistic regression.

RESULTS: The -765GG genotype was associated with an increased risk of developing CRC (OR, 1.45; 95% CI, 1.03-2.04). No significant difference was observed in the genotype distribution of the -1195A→G polymorphism between patients and controls. The GG/AC haplotype was present significantly less often in patients than in controls (OR 0.44; 95% CI, 0.22-0.85). When the AC, AG and GG haplotypes were investigated separately, the AC haplotype showed a tendency to be less frequent in patients than in controls (OR_(AG/AC) 0.78; 95% CI, 0.57-1.06).

CONCLUSION: The -765GG genotype is associated

INTRODUCTION

Colorectal cancer (CRC) is a common disease in both men and women. CRC includes cancerous growths in the cecum, colon, sigmoid and rectum. In Western countries, 5% of the population ultimately develop CRC, thus this disease is an important public health issue^[1]. CRC is ranked the third most common form of cancer worldwide in terms of incidence^[2]. In the Netherlands, CRC is the second most common form of cancer affecting women and the third most common form of cancer affecting men. In 2003 in the Netherlands 9898 new cases of CRC were diagnosed^[3].

CRC is usually observed in one of three specific patterns: sporadic, inherited or familial. The sporadic form accounts for approximately 70% in the population and is most common in individuals older than 50 years of age, probably as a result of interactions between low penetrance genes and environmental factors. Fewer than 10% of the population has an inherited predisposition to colon cancer. Inherited colon cancer is usually the result of a single germ line mutation. The third pattern, familial colon cancer, includes those families in which CRC develops too frequently to be considered as sporadic colon

cancer and which are not in a pattern consistent with an inherited syndrome. Up to 25% of all cases of CRC are estimated to fall into this category^[1].

Cyclooxygenase (COX), also known as prostaglandin endoperoxidase H synthase, is a modifier gene and key enzyme in the conversion of arachidonic acid into prostaglandins.

The COX family consists of two isozymes: COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is involved in the homeostasis of various physiological functions. COX-1 is well known as the housekeeping gene. COX-2 is an inducible form and its expression can be induced by mitogenic and proinflammatory stimuli. Increased expression of COX-2 is observed in many types of cancers. COX-2 is also associated with many stages of cancer development, e.g. invasion, metastasis, hyperproliferation, transformation and tumor growth^[4,5].

Recent studies suggest that single nucleotide polymorphisms (SNPs) in the COX-2 promoter may alter the enzyme function of COX-2 by differential regulation of COX-2 expression. A differential COX-2 expression may influence the risk of the development of gastrointestinal adenocarcinomas, including CRC^[6-9].

In a study of African-Americans, an inverse association was found between the Val511Ala polymorphism and the risk of CRC^[8]. In two studies the promoter polymorphisms -765G→C and -1195A→G were associated with an increased risk of CRC^[9,10], whereas Ulrich *et al*^[11] reported a reduced risk of CRC associated with the -765G→C polymorphism. The inconsistent results may indicate that the COX-2 polymorphisms -765G→C and -1195A→G may play a role in carcinogenic processes in combination with specific life-style conditions or dependent on the racial composition of a particular population.

The purpose of our study was to determine the possible modulating effect of the COX-2 polymorphisms -765G→C and -1195A→G on the risk of sporadic CRC in a Dutch population. The results of this research will lead to a better understanding on the role of SNPs in the COX-2 promoter in colon cancer carcinogenesis. Such knowledge in future may eventually lead to better preventive measures for CRC.

MATERIALS AND METHODS

Patients and controls

This case-control study included 326 patients with CRC (59.8% men, 40.2% women) and 369 cancer-free controls (59.1% men, 40.9% women). In the patient group, 31.0% had a proximal tumor and 68.1% had a distal tumor, whereas in 0.9% of cases localization of the tumor was unknown (see legend of Table 1). All subjects were of Caucasian origin with a mean age of 63.7 years and were recruited at Radboud University Nijmegen Medical Center, the Netherlands. The patient and control groups were matched for gender and age. The characteristics of patients and controls are summarized in Table 1.

Table 1 Characteristics of patients with colorectal cancer (CRC) and controls (mean ± SD)

	Patients with CRC (n = 326)	Controls (n = 369)
Age (yr)	62.7 ± 11.7	64.5 ± 10.7
Male gender	195 (59.8%)	218 (59.1%)
Female gender	131 (40.2%)	151 (40.9%)
Localization of tumor ¹		
Proximal ²	101 (31.0%)	
Distal ³	222 (68.1%)	

¹Note that the localization of the tumor was unknown in 3 patients;

²Proximal tumor: cecum, ascending and transverse colon; ³Distal tumor: descending colon, sigmoid, rectosigmoid junction and rectum.

Genotyping

DNA from patients and controls was isolated from whole blood using the Pure Gene DNA isolation kit (Gentra Systems, Minneapolis, MN) and stored at 4°C. Genotypes of the COX-2 -765G→C and -1195A→G polymorphisms were determined by polymerase chain reaction (PCR)-based restriction fragment length polymorphism, according to the method of Zhang *et al*^[5].

First, PCR was used to amplify the COX-2 promoter region containing the polymorphism -765G→C and -1195A→G. The primers used to amplify the COX-2 promoter region were 765F5'-TATTATGAGGAGAATTTACCTTTCGC-3'/765R5'GCTAAGTTGCTTTCACAGAAGAAT-3', and 1195F5'CCCTGAGCACTACCCATGAT-3'/1195R5'GCCCTTCATAGGAGATACTGG-3'. PCR was performed using a 25 µL reaction mixture containing 100 ng of DNA, 10 mmol/L of Tris/HCl (pH 9.0), 50 mmol/L of KCl, 0.1% of Triton X-100, 2 mmol/L of MgCl₂, 200 nmol/L of each primer, 250 µmol/L of deoxyribonucleotide triphosphates and 2.5 U Taq DNA polymerase. The PCR profile for the -1195A→G polymorphism consisted of an initial melting step of 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 72°C and a final elongation step of 7 min at 72°C. Cycle conditions for the -765G→C polymorphism were 4 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 54°C, 30 s at 72°C and finally the same elongation step as for the -1195A→G PCR assay. The samples were then analyzed by agarose gel electrophoresis for control of the PCR products.

The PCR products (10 µL) were incubated with 10 U of restriction enzymes *Pvu*II and *Hba*I at 37°C for determination of the -1195A→G and -765G→C genotypes, respectively. Finally, the samples were analyzed by agarose gel electrophoresis. The -765G→C and -1195A→G genotypes that could be detected were: 765CC (100 bp fragment), 765GC (100 + 74 + 26 bp fragments), 765GG (74 + 26 bp fragments), 1195AA (273 bp fragment), 1195GA (273 + 220 + 53 bp fragments) and 1195GG (220 + 53 bp fragments), respectively.

Statistical analysis

The data analysis was performed using SPSS software

Table 2 Genotype distribution and OR of the *COX-2* -1195A→G and -765G→C polymorphisms in patients with CRC and controls

Genotype	Patients with CRC (n = 326)	Controls (n = 369)	OR (95% CI)
-1195AA	213 (65.3%)	232 (62.9%)	Reference
-1195GA	101 (31.0%)	124 (33.6%)	0.90 (0.66-1.23)
-1195GG	12 (3.7%)	13 (3.5%)	1.01 (0.45-2.25)
-765GG	241 (73.9%)	249 (67.5%)	Reference
-765GC	75 (23.0%)	112 (30.4%)	0.69 (0.49-0.97)
-765CC	10 (3.1%)	8 (2.2%)	1.29 (0.50-3.33)

OR: Odds ratio; CI: Confidence interval.

(Version 14.0, SPSS, Chicago, IL, USA). Logistic regression was used to assess the association between the genotypes and the risk of CRC. The statistical significance of the -1195A→G and -765G→C genotype distributions between the patient and control groups was determined by Chi-square analysis. A *P*-value of < 0.05 was used as the criterion of statistical significance and all analyses were adjusted for age and sex. A test for deviation from the Hardy-Weinberg equilibrium, by comparing the expected to observed genotype frequencies, was used. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated. Based on the two polymorphisms tested, a haplotype analysis was performed. In the two populations studied, seven different haplotypes could be distinguished: AC/AC, AG/AC, AG/AG, GC/AC, GG/AC, GG/AG and GG/GG. The localization of the tumor, distal or proximal, was also included in the database analyses.

RESULTS

Using cancer-free controls as a reference we tested for an association of the two *COX-2* polymorphisms with CRC. The genotype distributions in patients and controls of the two *COX-2* polymorphisms investigated are summarized in Table 2. The observed genotype distributions for the -765G→C and -1195A→G polymorphisms in patients with CRC and controls were in accordance with the Hardy-Weinberg equilibrium, with *P*-values of 0.19 and 0.99 for patients with CRC and 0.24 and 0.46 for controls, respectively. When both polymorphisms were investigated separately, there was no significant difference in the -765G→C or -1195A→G allele frequency between the patient and control group. However, the -765GG genotype was more frequent in patients than in controls (OR, 1.45; 95% CI, 1.03-2.04). There was no significant difference in the genotype distribution of the -1195A→G polymorphism among patients and controls.

Next, the potential association of genotype distribution of the two *COX-2* polymorphisms with tumor localization was investigated. We distinguished proximal and distal tumor localization. Proximal included the cecum, colon ascendens and colon transversum and distal included the rectum, sigmoid, colon descendens and flexura lienalis. No association between the -765G→C and -1195A→G polymorphisms and tumor

Table 3 *COX-2* haplotypes in patients with CRC and controls

Haplotype	Patients with CRC (n = 326)	Controls (n = 369)	OR (95% CI)
AC/AC	9 (2.8%)	8 (2.2%)	1.16 (0.40-3.42)
AG/AC	59 (18.1%)	74 (20.1%)	0.83 (0.54-1.27)
AG/AG	145 (44.5%)	150 (40.7%)	Reference
GC/AC	1 (0.3%)	-	-
GG/AC	16 (4.9%)	38 (10.3%)	0.44 (0.22-0.85)
GG/AG	84 (25.8%)	86 (23.3%)	1.01 (0.68-1.50)
GG/GG	12 (3.7%)	13 (3.5%)	0.92 (0.38-2.24)

localization was detected.

Also no association of the genotype distribution of the -765G→C and -1195A→G polymorphisms in the patient group was found with gender and age.

Based on the two polymorphisms tested, a haplotype analysis was performed in the two populations studied and seven haplotypes could be distinguished (Table 3). A significant difference between the *COX-2* haplotypes was observed. The GG/AC haplotype was less frequent in patients (OR, 0.44; 95% CI, 0.22-0.85). When the AC, AG and GG haplotypes were investigated separately; the AC haplotype tended to occur less frequently in patients than in controls (OR_(AG/AC) 0.78; 95% CI, 0.57-1.06).

DISCUSSION

The *COX-2* protein was detected in 70% of all colorectal cancer tissues. In adjacent normal colorectal tissue in the same slide the *COX-2* protein was not observed. These results suggest that increased expression of *COX-2* is associated with CRC^[12]. SNPs in the *COX-2* promoter may alter the enzyme activity of *COX-2* by differential regulation of *COX-2* expression, which may influence the risk of the development of CRC^[7-9]. It has been recently demonstrated that the polymorphisms -765G→C and -1195A→G may have a functional effect on *COX-2* expression and enzyme activity^[7-9]. Both the -765G→C and -1195A→G polymorphisms were shown to display a lower *COX-2* promoter activity, which may result in a lower expression of the *COX-2* enzyme^[5,13].

We investigated the potential association of the *COX-2* polymorphisms -765G→C and -1195A→G and the risk of developing CRC, and found that the -765GG genotype was present more often in patients than in controls. As demonstrated by Zhang *et al*^[5] the reporter gene expression driven by the -765G-containing *COX-2* promoter was higher as compared to the -765C-containing counterpart. This indeed could mean a higher *COX-2* expression in -765GG individuals.

A study in American Caucasians reported a reduced risk of colorectal adenomas in individuals bearing the -765GG genotype, but this lower risk was found only among users of non-steroidal antiinflammatory drugs (NSAIDs). In addition, a lower risk of adenoma among -765CC genotypes was found only in non-users of NSAIDs^[11]. Zhang *et al*^[5] and Tan *et al*^[9] reported that the -765GC genotype was associated with an increased

risk of esophageal squamous cell carcinoma (ESCC) and CRC, in Chinese populations. The findings of Tan *et al*^[9] and Zhang *et al*^[5] seem in contrast with our results, since we found a reduced risk of CRC with the -765G/C genotype. However, racial differences in the study populations may explain these apparent contradictory results, since the distribution of the COX-2 polymorphisms studied here differs considerably between the Chinese and Dutch study populations. The genotype frequencies found in our Dutch patients with CRC for the -765G → C and -1195A → G polymorphisms were: 73.9% GG, 23.0% GC, 3.1% CC and 65.3% AA, 31.0% GA, 3.7% GG, respectively. Zhang *et al*^[5] in a Chinese population reported genotype frequencies of 90.6% GG, 9.4% GC, 0% CC and 30.5% AA, 52.9% GA and 16.6% GG. Tan *et al*^[9] in Chinese patients with CRC recently reported approximately the same genotype frequencies as Zhang *et al*^[5]: 91.6% GG, 8.4% GC, 0% CC and 34.5% AA, 49.4% GA and 16.1% GG. These findings suggest that ethnic differences in genotype frequencies of COX-2 polymorphisms may have a significantly different modulating effect on disease phenotypes in different ethnic populations.

According to Zhang *et al*^[5] and Tan *et al*^[9] in a Chinese population, the -1195GA and -1195AA genotypes were associated with an increased risk of ESCC and CRC, respectively. This again is not in line with our findings, since we could not demonstrate a significant difference in the allele distribution of the -1195A → G polymorphism between our Dutch patients with CRC and controls.

We also investigated the potential association of the genotype distributions of the -1195A → G and -765G → C polymorphisms with tumor localization. No association between the two polymorphisms and tumor localization was found, which is in accordance with the results of Tan *et al*^[9] who found a very similar distribution of both COX-2 genotypes in patients with colon ($n = 403$) or rectal ($n = 597$) cancer.

The COX-2 GG/AC haplotype (-1195G-765G/-1195A-765C) was found to be present less frequently in patients. When the AC, AG and GG haplotypes were investigated separately, the AC haplotype tended to be less frequently present in patients with CRC than in controls (OR_(AG/AC) 0.78; 95% CI, 0.57-1.06). This is in line with the findings of Zhang *et al*^[5] who demonstrated that the luciferase expression of the AG constructs was higher than the expression of the AC constructs, suggesting that the AC haplotype was associated with a lower COX-2 expression and a decreased risk of CRC.

However, Zhang *et al*^[5], Tan *et al*^[9] and Moons *et al*^[14] found an association of the AC haplotype with an increased risk of ESCC, CRC and esophageal adenocarcinoma (EAC). These findings are in contrast with our results, as described above. In addition, the predicted expression levels of the COX-2 protein are higher in AG versus AC haplotype individuals, according to Zhang *et al*^[5], which is not in agreement with the hypothesis that high expression of COX-2 is a risk factor for colorectal or esophageal carcinoma. It should be noted however

that haplotype frequencies of AC are very low in the patient and control populations studied by Zhang *et al*^[5] and Tan *et al*^[9], being 4.5% *vs* 1.6% and 3.8% *vs* 1.8%, respectively, compared to 21.2% *vs* 32.6% in our study. In the study of Moons *et al*^[14] the AC haplotype occurred in 25.0% of the total study population, who were patients with esophageal adenocarcinoma, Barrett's esophagus and reflux esophagitis, a proportion which is very close to our data. In the study of Moons *et al*^[14] unfortunately no cancer-free controls were included, but patients with Barrett's esophagus or reflux esophagitis were used as controls, both of which would confer a risk of esophageal adenocarcinoma.

In summary, we found a significant difference in the -765G → C polymorphism distribution between the patients with CRC and the control group; the -765GG genotype was associated with an increased risk for CRC. The GG/AC haplotype was found less frequently in patients with CRC and may be associated with a reduced risk of CRC. These findings suggest a modulating role for the COX-2 polymorphisms -1195A → G and -765G → C in the development of CRC in a Dutch population.

COMMENTS

Background

Cyclooxygenase-2 (COX-2) is a key enzyme in the development and progression of neoplasms. COX-2 was found to be over-expressed in gastrointestinal tumors, including those of the colon/rectum. The corresponding COX-2 gene is polymorphic and two single nucleotide polymorphisms (SNPs; -1195A → G and -765G → C) were demonstrated to influence the expression of COX-2. Therefore, these polymorphisms may modulate the risk for gastrointestinal cancers, such as cancers of the colon/rectum.

Research frontiers

In this study, the -765GG genotype was associated with an increased risk of developing CRC and the GG/AC haplotype seemed to protect against CRC.

Innovations and breakthroughs

The COX-2 polymorphisms -765G → C and -1195A → G may modulate the development of CRC in a Dutch population.

Applications

Screening for the COX-2 -765GG genotype in a population at risk of colorectal cancer may be valuable in future in order to select the high risk patients. Information and prevention programs can then be focused on these patients.

Terminology

COX-2 is an enzyme that catalyzes the conversion of arachidonic acid in prostaglandin H₂, the precursor of other prostaglandins, prostacyclin and thromboxanes. These regulatory compounds play a role in many biological processes such as cell proliferation, angiogenesis, immune function and inflammation, which are all crucial in the development and progression of neoplasms.

Peer review

The manuscript, reported by Hoff *et al*, analyzes COX-2 polymorphisms -765G → C and -1195A → G and colorectal cancer (CRC) risk in a Dutch population. This manuscript contributes in the effort to characterize some molecular signatures as risk factors for colon cancer in different ethnic populations.

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S- Editor Tian L L- Editor Cant MR E- Editor Ma WH

BRIEF ARTICLES

Lack of correlation between *p53* codon 72 polymorphism and anal cancer risk

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Supported by The National Council for Scientific and Technological Development (CNPq), No. 142678/2007-4, Brazilian Government

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Received: June 5, 2009 **Revised:** August 27, 2009

Accepted: September 3, 2009

Published online: September 28, 2009

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Key words: Anus neoplasms; Arginine; Genetic polymorphism; Polymerase chain reaction; Proline; Tumor suppressor protein *p53*

Contu SS, Agnes G, Damin AP, Contu PC, Rosito MA, Alexandre CO, Damin DC. Lack of correlation between *p53* codon 72 polymorphism and anal cancer risk. *World J Gastroenterol* 2009; 15(36): 4566-4570 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4566.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4566>

INTRODUCTION

Squamous cell carcinoma (SCC) of the anus is a relatively uncommon malignancy, affecting approximately 4600 patients per year in the United States^[1]. Globally, annual incidence rates of invasive anal cancer range from 0.1 to 2.8 cases per 100 000 among men and 0.0-2.2 cases per 100 000 among women^[2]. In particular, anal cancer rates among men who have sex with men are notably higher^[3]. Invasive anal cancer, like invasive cervical cancer, has been causally linked to high-risk human papillomavirus (HPV) infection^[4,5]. According to a recent review, HPV is detected in 71% of invasive anal cancers, with approximately 72% of the HPV-positive cases being associated with HPV 16 and/or 18 infection^[6]. This estimate of HPV 16 and 18 prevalence is similar to that found in invasive cervical cancer^[7].

Although many risk factors for the development of anal cancer have been identified, such as the practice of receptive anal intercourse and immunodeficiency, the molecular mechanisms related to anal carcinogenesis remain unclear. Mutations in the *p53* gene are the most common genetic alterations in human cancer and they can be found in up to 80% of anal carcinomas^[8]. In addition to gene mutations, some polymorphisms in the *p53* gene have been suggested to play a role in different malignancies^[9-11]. Recent studies have focused on a common single-base-pair polymorphism at codon

Abstract

AIM: To investigate the potential role of *p53* codon 72 polymorphism as a risk factor for development of anal cancer.

METHODS: Thirty-two patients with invasive anal carcinoma and 103 healthy blood donors were included in the study. *p53* codon 72 polymorphism was analyzed in blood samples through polymerase chain reaction-restriction fragment length polymorphism and DNA sequencing.

RESULTS: The relative frequency of each allele was 0.60 for Arg and 0.40 for Pro in patients with anal cancer, and 0.61 for Arg and 0.39 for Pro in normal controls. No significant differences in distribution of the codon 72 genotypes between patients and controls were found.

CONCLUSION: These results do not support a role for the *p53* codon 72 polymorphism in anal carcinogenesis.

72, which results in a Pro (CCC) or Arg (CGC) residue at this position. The two polymorphic variants have been shown to have not only structural differences, as reflected by distinct electrophoresis patterns of migration, but also different biological properties^[12-14]. The Arg variant has been demonstrated to be more susceptible to degradation by the HPV E6 protein than the Pro variant, with individuals who are homozygous for Arg having a higher risk of being affected by HPV-associated malignant tumors^[15].

In this article, we present the results of what is believed to be the first study to investigate the potential association of *p53* codon 72 polymorphism with invasive carcinoma of the anal canal.

MATERIALS AND METHODS

Cases and controls

Thirty-two patients with histologically confirmed primary SCC of the anal canal (mean age 60.3 years, range 30-81 years) were enrolled prospectively in the study. As a non-malignant control group, we studied 103 consecutive healthy blood donors with no previous history of cancer (mean age 47.7 years, range 40-72 years). Demographic characteristics of cases and controls are shown in Table 1. After pretreatment assessment, including a complete medical history and physical examination, colonoscopic examination, computed tomography of the abdomen and pelvis, and chest radiography, the patient's AJCC (American Joint Committee on Cancer) tumor stage was determined^[16]. The distribution was as follows: 6.2% stage I ($n = 2$), 53.1% stage II ($n = 17$), 34.3% stage III ($n = 11$) and 6.2% stage IV ($n = 2$).

The study was approved by the Ethics and Scientific Committee of the Santa Casa Hospital Complex and Hospital de Clínicas de Porto Alegre. Informed consent was obtained from all patients and controls before being enrolled in the study.

DNA extraction and genotyping

p53 codon 72 polymorphism was studied in blood samples collected by venous puncture. Genomic DNA was extracted from peripheral lymphocytes using Ultra Clean DNA Bloodstain Kit (MoBioLabs, Solana Beach, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of codon 72, modified from the technique described by Ara *et al.*^[17], was used to identify *p53* genotypes. The forward primer used was 5'-TTGCCGTCCCAAAGCAATGGATGA-3', and the reverse primer was 5'-TCTGGGAAGGGACA GAAGATGAC-3'. Each PCR reaction mixture (50 mL) contained 10 pmol each primer, 1.5 mmol/L MgCl₂, 200 mmol/L each dNTP, 1 U Platinum®*Taq* DNA polymerase (Invitrogen, Sao Paulo, Brazil), and 100-300 ng genomic DNA. Reaction mixtures were preincubated for 5 min at 94°C. PCR conditions were 94°C for 1 min and 55°C for 1 min, followed by 72°C for 1 min for 35 cycles. The final extension was at 72°C for 10 min.

Table 1 Demographic characteristics of cancer patients and controls n (%)

Characteristics	Cases	Controls	P
Gender			
Male	7 (22)	22 (21)	NS
Female	25 (78)	81 (79)	
Race			
White	28 (87)	96 (93)	NS
Non-white	4 (13)	7 (7)	
Age (yr); mean (range)	60.3 (30-81)	47.7 (40-72)	< 0.001
Total	32 (100)	103 (100)	

NS: Not significant.

After confirmation of an amplified fragment of the expected size (199 bp) on an agarose gel, 10 µL PCR product was digested with 6 U restriction enzyme *Bst*UI (New England Biolabs, Ipswich, MA, USA) at 60°C for at least 4 h. DNA fragments were electrophoresed through a 2.5% agarose gel and stained with ethidium bromide. RFLP results were confirmed by sequencing the PCR fragments from nine randomly selected samples (three of each genotype) using an automated sequencing system (ABI Prism 310 Genetic Analyzer; Applied BioSystems, Foster City, CA, USA). Sequencing reactions were performed using the BigDye® Terminator V3.1 cycle sequencing reaction kit (Applied BioSystems) according to the manufacturer's instructions. Forward and reverse primers were utilized as sequencing primers.

Statistical analysis

Univariate statistics were used first to compare cases and controls for demographic variables and genotype prevalence. The χ^2 test was used to analyze categorical variables and ANOVA was used to compare the continuous variable age. The association between the *p53* polymorphism and anal cancer was determined using the logistic regression method to assess ORs and 95% CI. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of *p53* codon 72 polymorphism by PCR-RFLP was performed in all cases and controls. The Arg allele was cleaved by *Bst*UI, which yielded two small fragments (113 and 86 bp). The Pro allele was not cleaved by *Bst*UI, which had a single 199-bp band. Heterozygotes contained three bands, which corresponded to 199, 113 and 86 bp. The PCR results were confirmed by DNA sequencing.

The distribution of the codon 72 genotypes in patients and controls did not deviate from the Hardy-Weinberg equilibrium. The genotype frequencies in cases and controls are presented in Table 2, with no association with anal cancer risk being observed. The relative frequency of each allele was 0.60 for Arg and 0.40 for Pro in patients with anal cancer, and 0.61 for Arg and 0.39 for Pro in normal controls.

We also analyzed the codon 72 polymorphism of the healthy controls according to their age. The genotype

Table 2 Distribution of *p53* codon 72 polymorphism in cancer patients and controls *n* (%)

	Total	Arg/Arg	Arg/Pro	Pro/Pro	OR ¹	CI	P
Anal cancer	32	10 (31.2)	19 (59.4)	3 (9.4)	1.6	0.6-4.9	0.325
Controls	103	31 (30.1)	62 (60.2)	10 (9.7)			

¹Adjusted for age. Arg/Arg vs Arg/Pro and Pro/Pro.

distribution in the 78 controls under 50 years old was as follows: 25 Arg/Arg (32.1%), 6 Pro/Pro (7.7%), and 47 Arg/Pro (60.3%). The distribution in the 25 controls over 50 years old was: 6 Arg/Arg (24.0%), 4 Pro/Pro (16.0%), and 15 Arg/Pro (60.0%). No significant difference in the genotype distribution was found between these two age groups ($P = 0.407$).

DISCUSSION

High-risk HPV infection has been implicated in the pathogenesis of different malignancies^[17-21]. Several biochemical and genetic studies have shown that HPV E6 and E7 proteins exert a cooperative effect on cellular transformation and immortality by interfering with the function of cellular tumor suppressor proteins^[22-24].

A common polymorphism has been known in codon 72 of the *p53* gene, with two alleles encoding either Arg (*p53*Arg) or Pro (*p53*Pro)^[13,14]. Storey *et al*^[15] have investigated the effect of this polymorphism on the susceptibility to E6-mediated degradation and found that individuals homozygous for Arg are seven times more susceptible to HPV-associated cervical carcinogenesis than heterozygotes are. Since then, the effect of codon-72 polymorphism of *p53* on cervical cancer has been studied, with contradictory results being reported. Overall, as demonstrated in a recent meta-analysis, compared with the heterozygous genotype (Pro/Arg), the homozygous genotype (Arg/Arg) of codon-72 of *p53* is associated with an approximately 20% increased risk of cervical cancer^[25].

Invasive anal cancer, like invasive cervical cancer, has well-documented precursors, known as anal intraepithelial neoplasia 2-3 (histology) or high-grade squamous intraepithelial lesions (cytology)^[6]. Anal cancer also has been causally linked to high-risk HPV infection, therefore, we decided to evaluate, perhaps for the first time, the potential role of codon 72 polymorphism as a risk factor for development of this type cancer.

In order to minimize sources of bias and avoid misinterpretation of the results, standard safeguards were adopted. Patients and controls were matched ethnically and derived from a population living in the same geographic region (Southern Brazil), and were enrolled consecutively in a single institution. All PCR results were confirmed by DNA sequencing.

We investigated the allele and genotype frequencies at *p53* codon 72 in 32 patients with anal cancer and 103 healthy individuals from southern Brazil. No significant differences in the relative allele frequency and in the distribution of genotypes were found between patients

and controls. These results are in line with several studies that failed to demonstrate a correlation of the *p53* codon 72 polymorphism with development of non-cervical HPV-associated epithelial malignant tumors, such as head and neck and oral SCCs^[26,27].

The association between codon 72 polymorphism and risk of cancer has been reported in different populations^[28]. Studies have been conducted to evaluate this polymorphism as a risk factor for different types of cancer, such as gastric^[29], lung^[9] and breast carcinomas^[11]. So far, the published data have been inconclusive. The conflicting results found in the literature might be attributed to variations in protocols among different laboratories, or to poor selection of control groups^[29]. They also might have been caused by the inherent characteristics of the population being analyzed, as there are considerable variations in the distribution of the codon 72 genotypes in various populations. This polymorphism seems to be maintained by natural selection influenced by environmental factors, such as the degree of exposure to the UV-B component of sunlight^[30]. The resulting North-South Arg/Pro gradient has been reported in different geographical regions. Population-based studies have indicated that the Arg allele is most prevalent in individuals with light complexion and least prevalent in those with darker complexion, with a clear and consistent decline in the prevalence of the Pro allele, with increasing northern latitude^[30-32].

The population from Southern Brazil, in contrast with other regions of the country, is composed mainly of Caucasian individuals who are descended from European immigrants^[33]. Although most of these immigrants came from Portugal, Germany and Italy^[34], the genotype distribution found in our healthy controls was notably different from the genotype distribution observed in those countries^[35-37]. This can be explained partially by the process of miscegenation among different ethnic groups (Caucasians, Amerindians and Afro-Brazilians) that took place during Brazilian colonization^[38]. Each specific population seems to have its own characteristic genotype distribution that can differ markedly from the polymorphic frequencies found in other populations, even when neighboring countries are compared.

We believe, however, that the lack of correlation between the codon 72 genotype distribution and anal cancer risk observed in our study cannot be interpreted solely as a result of population ethnicity. In a previous study of cancer patients and normal individuals from Southern Brazil, we were able to detect a significant

association of *p53* codon 72 polymorphism with breast cancer risk^[39]. We analyzed blood samples collected from 118 women with primary breast carcinoma and from 202 female blood donors (healthy controls) through PCR-RFLP and DNA sequencing. The Arg/Arg genotype was significantly associated with an increased risk for breast cancer (OR 2.9; 95% CI: 1.43-3.6; $P < 0.002$). The relative frequency of each allele was 0.75 for Arg and 0.25 for Pro in patients with cancer, and 0.62 for Arg and 0.38 for Pro in normal controls ($P < 0.001$). In the present study, the relative frequency of each allele observed within the control group (0.61 for Arg and 0.39 for Pro) was therefore very similar to our previous observation in normal controls derived from the same population.

In summary, we did not detect significant differences in the allele distribution at codon 72 of *p53* between patients with invasive anal cancer and healthy controls. Our results do not support the hypothesis that *p53* codon 72 polymorphism is associated with anal cancer susceptibility. The role of the genetic susceptibility to high-risk HPV infection and anal cancer, however, merits further investigation.

COMMENTS

Background

A common Arg/Pro polymorphism at codon 72 of the *p53* gene has been studied as a risk factor for human papilloma virus (HPV)-associated malignancies. Although anal cancer has been associated repeatedly with high-risk HPV infection, this polymorphism has not been investigated in this type of cancer up until now.

Research frontiers

Although several risk factors for the development of anal cancer have been determined, the molecular mechanisms involved in anal carcinogenesis remain unclear. In this context, the identification of new factors involved in progression of the anal carcinoma represents a critical step towards development of new anticancer strategies in this malignancy.

Innovations and breakthroughs

This is believed to be the first study to investigate *p53* codon 72 polymorphism in patients with anal cancer. The authors did not detect differences in the allele distribution at codon 72 of *p53* between patients with invasive anal cancer and healthy controls. In contrast to previous observations with cervical cancer, this polymorphism does not seem to be associated with anal cancer susceptibility. The results, however, are in line with several studies that failed to demonstrate a correlation of the *p53* codon 72 polymorphism with development of non-cervical HPV-associated epithelial malignant tumors, such as head and neck and oral squamous cell carcinomas.

Applications

In the process of identifying genetic causes of cancer, it is important to determine precisely which elements of a biological pathway are responsible for affecting tumor suppression or development. Then, treatments and preventive measures can be directed to those individuals who would benefit most. Previous studies have identified *p53* codon 72 polymorphism as a potential contributing factor in HPV-associated carcinogenesis. This study found that *p53* codon 72 polymorphism is not a likely risk factor for anal cancer, and future research should focus on other parts of the *p53* gene pathway to understand its role in development of this type of cancer.

Terminology

Mutations in the *p53* gene are the most common genetic alterations in human cancer. In addition to gene mutations, some polymorphisms in the *p53* gene have been suggested to play a role in different malignancies. A polymorphism is known in codon 72 of the *p53* gene, which results in a Pro or Arg residue at this position. These two polymorphic variants have been shown to have different biological properties, including differences in cancer susceptibility.

Peer review

Contu *et al* Have described the lack of a functional role of a specific *p53* polymorphism in the pathogenesis of anal cancer. The major point of this paper and the spectrum of methods used are rather confined, but the point is clear and the paper is well written. Although this paper presents negative data, I feel that the attempt to perform genetic analysis on a relatively uncommon malignancy (anal cancer) may be viewed favorably clinically.

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S- Editor Tian L L- Editor Kerr C E- Editor Zheng XM

Hemoperfusion with polymyxin B-immobilized fiber column improves liver function after ischemia-reperfusion injury

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Received: July 3, 2009 Revised: August 3, 2009

Accepted: August 10, 2009

Published online: September 28, 2009

reperfusion in both groups, but the AST was significantly ($P < 0.05$) lower in the DHP-PMX group 360 min after reperfusion.

CONCLUSION: DHP-PMX therapy reduced the hepatic warm I/R injury caused by THVE in a porcine model.

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Key words: Ischemia-reperfusion injury; Total hepatic vascular exclusion; Polymyxin B-immobilized fiber column

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Sato H, Oshima K, Kobayashi K, Yamazaki H, Suto Y, Takeyoshi I. Hemoperfusion with polymyxin B-immobilized fiber column improves liver function after ischemia-reperfusion injury. *World J Gastroenterol* 2009; 15(36): 4571-4575
Available from: URL: <http://www.wjgnet.com/1007-9327/15/4571.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4571>

Abstract

AIM: To investigate the usefulness of direct hemoperfusion with a polymyxin B-immobilized fiber column (DHP-PMX therapy) for warm hepatic ischemia-reperfusion (I/R) injury after total hepatic vascular exclusion (THVE) using a porcine model.

METHODS: Eleven Mexican hairless pigs weighing 22-38 kg were subjected to THVE for 120 min and then observed for 360 min. The animals were divided into two groups randomly: the DHP-PMX group ($n = 5$) underwent DHP-PMX at a flow rate of 80 mL/min for 120 min (beginning 10 min before reperfusion), while the control group did not ($n = 6$). The rate pressure product (RPP): heart rate \times end-systolic arterial blood pressure, hepatic tissue blood flow (HTBF), portal vein blood flow (PVBF), and serum aspartate aminotransferase (AST) levels were compared between the two groups.

RESULTS: RPP and HTBF were significantly ($P < 0.05$) higher in the DHP-PMX group than in the control group 240 and 360 min after reperfusion. PVBF in the DHP-PMX group was maintained at about 70% of the flow before ischemia and differed significantly ($P < 0.05$) compared to the control group 360 min after reperfusion. The serum AST increased gradually after

INTRODUCTION

In ischemia-reperfusion (I/R), the generation of reactive oxygen species on reoxygenation inflicts tissue damage and initiates a cascade of deleterious cellular responses leading to inflammation, cell death, and ultimately organ failure^[1]. Hepatic I/R injury occurs in various clinical settings, such as transplantation, trauma, liver or bowel resection, and hemorrhagic shock^[2]. Severe hepatic I/R injury can lead to liver or multiple organ failure and is associated with increased morbidity and mortality^[3]. Total hepatic vascular exclusion (THVE), which involves the total occlusion of the liver vasculature at the hepatoduodenal ligament (i.e. Pringle's maneuver) and the occlusion of the inferior vena cava below and above the liver, is used during the resection of large and posterior portions of the liver clinically^[4,5]. As this technique induces hepatic I/R injury, inhibition of this injury caused by THVE is necessary to obtain a better postoperative course.

The Toraymyxin polymyxin B-immobilized fiber column (PMX cartridge; Toray Industries, Tokyo, Japan) was developed in Japan in 1994 as an extracorporeal

hemoperfusion device that uses polymyxin-B fixed to α -chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxin and reduce various cytokines, even in patients with high plasma cytokine levels^[6]. This method has been used to treat endotoxemia^[7] and was reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 (PAI-1) levels immediately^[8]. DHP-PMX therapy has also been found effective for severe sepsis secondary to intra-abdominal infection^[9] and acute lung injury or acute respiratory distress syndrome caused by sepsis^[10]. Recently, we reported the efficacy of DHP-PMX therapy in normothermic cardiopulmonary bypass^[11], a pulmonary warm I/R injury model^[12], and a small intestine warm I/R injury model^[13].

In this study, we evaluated the usefulness of DHP-PMX therapy in warm hepatic I/R injury with a porcine THVE model.

MATERIALS AND METHODS

Animals

All animals were cared for in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the guidelines set forth in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication 85-23, revised 1985). The study was performed under the supervision of the Animal Care and Experimental Committee of Gunma University, Showa campus, Japan.

Operative procedure

Eleven Mexican hairless pigs (both sexes, weighing 22-38 kg) were used in this study. They were not allowed access to food for 24 h before the experiment. After administering ketamine hydrochloride (250 mg) and atropine (0.5 mg) intramuscularly, the pigs were intubated endotracheally and ventilated mechanically at a tidal volume of 25 mL/kg and a rate of 12 breaths/min. During the experiment, general anesthesia was maintained with a mixture of 1%-2% isoflurane and 100% oxygen. Lactated Ringer's solution (20 mL/kg per hour) was infused *via* a catheter inserted into the right subclavian vein. A laparotomy was performed *via* a midline incision. The liver was skeletonized completely by dividing all of the suspensory ligaments and dissecting the retrohepatic vena cava from the posterior abdominal wall. The portal vein, hepatic artery, and common bile duct were isolated and their collaterals were occluded separately. THVE was achieved by clamping the infrahepatic and suprahepatic vena cava after clamping the portal vein and hepatic artery. An active venovenous (v-v) bypass system was started as a portosystemic shunt just before THVE to prevent congestion of the portal vein and lower body. This system consisted of a centrifugal pump system (Lifestream; St. Jude Medical, Chelmsford, MA) and venous cannulas. The blood-contact surfaces of these

components were heparin-coated. The v-v bypass system was established with drains (12 Fr) inserted into the splenic and right external iliac veins for blood removal, with another drain inserted into the right external jugular vein (12 Fr) for blood return. Blood from the portal vein and infrahepatic vena cava was bypassed into the right external jugular vein *via* a Y-shaped shunt. The bypass blood flow was maintained at more than 20 mL/kg per minute with systemic heparinization (200 U/kg). Liver ischemia was induced by total exclusion of hepatic inflow for 120 min. After releasing the clamps to end the ischemia, the bypass system was removed. The splenic, right external iliac, and right external jugular veins were ligated after removing the cannulas. The parameters described below were measured and the animals were observed for 360 min after reperfusion.

Experimental groups

The experimental study involved two groups: the DHP-PMX ($n = 5$) and control ($n = 6$) groups. The animals were assigned randomly to either group. In the DHP-PMX group, a double-lumen catheter was positioned in the right atrium through the left subclavian vein and DHP-PMX was performed through the catheter at a flow rate of 80 mL/min for 120 min (beginning 10 min before reperfusion). Direct hemoperfusion was not performed in the control group.

Monitoring and sampling

The external iliac artery was cannulated for monitoring the arterial blood pressure and collecting blood samples. Arterial blood pressure and heart rate (HR) were monitored directly through a catheter connected to a transducer (Spectramed TA 1017; San-ei, Tokyo, Japan). The rate pressure product (RPP: $\text{HR} \times \text{end-systolic arterial blood pressure}$) was also calculated. Blood samples were collected from the same catheter before and after the procedure [before ischemia and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion]. All samples were centrifuged at $900 \times g$ for 15 min at 4°C, and the serum or plasma was frozen at -80°C for later measurement.

Hepatic tissue blood flow (HTBF)

HTBF was measured with a laser Doppler flowmeter (Laser Blood Flow Monitor MBF 3; Moor Instruments, Devon, UK) before ischemia and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. The laser probe was always placed on the right median lobe of the liver. HTBF is expressed as a percentage of the flow before ischemia.

Portal vein blood flow (PVBF)

PVBF was measured before ischemia and 30, 60, 120, 240 and 360 min after reperfusion using an electromagnetic blood flowmeter (Model MFV-3100; Nihon Kohden, Tokyo, Japan). PVBF is expressed as a percentage of the flow before ischemia.

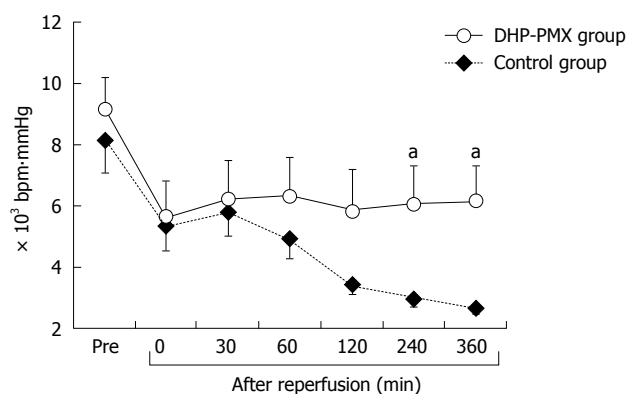


Figure 1 The rate pressure product (RPP: heart rate \times systolic arterial blood pressure) before ischemia (pre) and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. Data are expressed as the mean \pm SE. ^a $P < 0.05$ vs the control group.

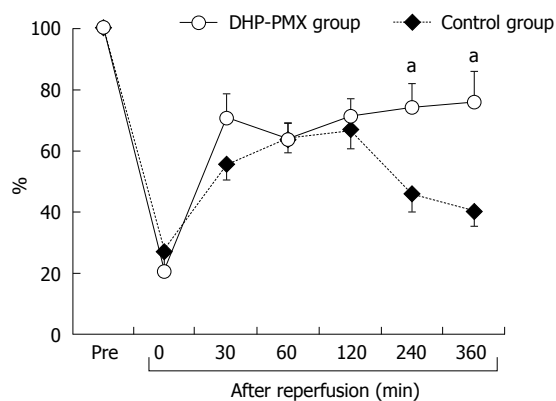


Figure 2 The hepatic tissue blood flow (HTBF) before ischemia (pre) and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. The HTBF was evaluated as a percentage of the flow before ischemia. Data are expressed as the mean \pm SE. ^a $P < 0.05$ vs the control group.

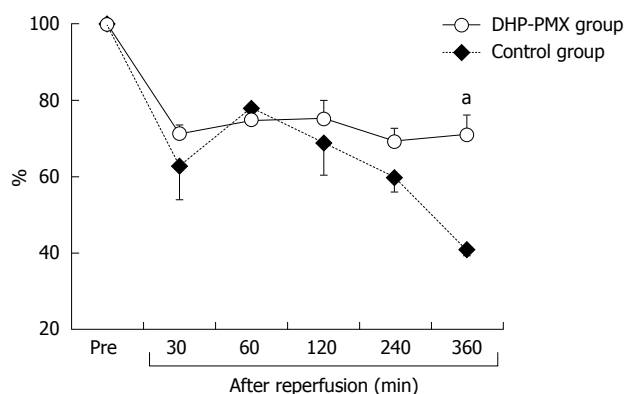


Figure 3 The portal vein blood flow (PVBF) before ischemia (pre) and 30, 60, 120, 240 and 360 min after reperfusion. The PVBF was evaluated as a percentage of the flow before ischemia. Data are expressed as the mean \pm SE. ^a $P < 0.05$ vs the control group.

Serum aspartate aminotransferase (AST) assays

Serum AST levels were measured at 37°C using an ultraviolet rate assay on an autoanalyzer (Hitachi 736-60; Hitachi, Tokyo, Japan) with blood samples collected and preserved using the method described above.

Statistical analysis

The results are expressed as the mean \pm SE. StatView ver. 5.0 (Abacus, Berkeley, CA) was used for the statistical analyses. Statistical comparisons were made using repeated measure analysis of variance followed by Fisher's protected least significant difference. $P < 0.05$ were considered to be statistically significant.

RESULTS

All animals survived until the endpoint of the study (360 min after reperfusion).

The changes in RPP

The changes in RPP were similar in both groups until reperfusion. The RPP in the control group decreased gradually until 360 min after reperfusion, while that in

the DHP-PMX group was maintained at about 6000 bpm·mmHg and differed significantly ($P < 0.05$) from the RPP in the control group 240 and 360 min after reperfusion (Figure 1).

The changes in HTBF

The HTBF decreased to about 20% of the baseline immediately after reperfusion in both groups (Figure 2). After reperfusion, the HTBF in the DHP-PMX group was maintained above 60% of the baseline, while no improvement was seen in the control group; the HTBF in the control group was significantly ($P < 0.05$) lower than that in the DHP-PMX group 240 and 360 min after reperfusion (Figure 2).

The changes in PVBF

The PVBF decreased after reperfusion in both groups (Figure 3). The PVBF was similar in both groups 30 and 60 min after reperfusion. Subsequently, the PVBF in the DHP-PMX group was maintained at about 70% of the flow before ischemia, while that in the control group decreased gradually beginning 120 min after reperfusion; a significant ($P < 0.05$) difference was observed between the two groups 360 min after reperfusion (Figure 3).

The changes in serum AST

As shown in Figure 4, the serum AST before ischemia did not differ significantly between the two groups. The serum AST increased gradually after reperfusion in both groups, although the increment in the DHP-PMX group was smaller than in the control group and differed significantly 360 min after reperfusion (Figure 4).

DISCUSSION

Polymyxin B binds to endotoxin, which is an outer membrane component of Gram-negative bacteria and is thought to be an important pathogenic trigger for the production of inflammatory mediators. Several preclinical studies have demonstrated that hemoperfusion or plasmapheresis over immobilized polymyxin B removes

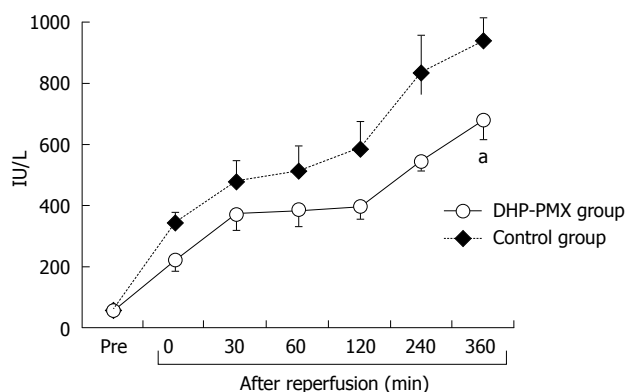


Figure 4 The serum AST before ischemia (pre) and immediately (0 min) and 30, 60, 120, 240 and 360 min after reperfusion. Data are expressed as the mean \pm SE. ^a $P < 0.05$ vs the control group.

endotoxin from blood^[14-16]. Recently, some studies have shown improved hemodynamic status^[17] and survival^[18] in patients with sepsis who were treated with PMX. Moreover, DHP-PMX therapy was found to be effective for patients with septic shock infected with either Gram-negative or Gram-positive bacteria, which do not release endotoxins^[19]. Therefore, DHP-PMX therapy is used in patients with severe sepsis or septic shock and its clinical effect has been described. In addition, studies have reported on the mechanism of DHP-PMX action. Kushi *et al*^[20] showed that the adsorption of pathogenic bacteria prevented the release of inflammatory cytokines and reduced the stimulation of vascular endothelial cells to lower the PAI-1 level, rather than direct inhibition of PAI-1 production by DHP-PMX therapy. Tani *et al*^[8] postulated that the reduction in plasma endotoxins by endotoxin adsorption contributed to the cessation of cytokine gene expression and the excretion of cytokines. In addition, DHP-PMX therapy improved the PaO₂/FiO₂ ratio in patients with acute lung injury or acute respiratory distress syndrome caused by sepsis, and this appeared to be related to decreases in the blood neutrophil elastase and IL-8 levels^[10]. We hypothesized that DHP-PMX therapy might be effective in various inflammatory situations and have evaluated the utility of DHP-PMX therapy in normothermic cardiopulmonary bypass in a pig model^[11], pulmonary warm I/R injury in a canine model^[12], and small intestine warm I/R injury in a canine model^[13] with satisfactory results. In this study, we evaluated whether DHP-PMX therapy could reduce hepatic I/R injury in a THVE model.

We found that RPP, PVBF, and HTBF in the DHP-PMX group were preserved significantly ($P < 0.05$) better than in the control group after reperfusion. In addition, the increase in serum AST was significantly ($P < 0.05$) lower in the DHP-PMX group. These results demonstrate that DHP-PMX therapy reduces the hepatic warm I/R injury caused by the THVE technique.

The natural ligands of cannabinoid receptors are lipid-like substances called endocannabinoids, and include arachidonoyl ethanolamine or anandamide and 2-arachidonoylglycerol. Cannabinoid-2 receptor agonists

have been reported to have a protective effect against I/R injury in the liver and other organs by reducing endothelial cell activation, the expression of adhesion molecules such as intercellular adhesion molecule and vascular cell adhesion molecule, the levels of tumor necrosis factor- α and chemokines, neutrophil infiltration, lipid peroxidation, and apoptosis^[21,22]. In addition, Wang *et al*^[23] demonstrated that the absorption of anandamide during DHP-PMX therapy eliminated the diverse negative effects of anandamide, such as hypotension, immunosuppression, and cytotoxicity. Considering these results, DHP-PMX treatment appears not only to remove endotoxins, but also to reduce the inflammatory reaction by inhibiting various inflammatory cascades and to have an effective role in I/R injury. Further studies are required, including the suitable timing and duration and the detailed mechanisms of DHP-PMX therapy in I/R injury.

In conclusion, DHP-PMX therapy reduced the hepatic warm I/R injury caused by the THVE method using a porcine model.

COMMENTS

Background

Total hepatic vascular exclusion (THVE) is used during the resection of large and posterior portions of the liver clinically. This technique induces hepatic ischemia-reperfusion (I/R) injury, and severe hepatic I/R injury is associated with increased morbidity and mortality. The inhibition of the hepatic I/R injury caused by THVE is necessary to obtain a better postoperative course.

Research frontiers

The Toraymyxin polymyxin B-immobilized fiber column (PMX cartridge) was developed as an extracorporeal hemoperfusion device. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxin. The authors had already demonstrated the efficacy of DHP-PMX therapy in a pulmonary warm I/R injury model and a small intestine warm I/R injury model. In this study, the authors investigated whether DHP-PMX therapy reduced the hepatic warm I/R injury caused by THVE in a porcine model.

Innovations and breakthroughs

The authors found that systemic hemodynamics, blood flow for liver and liver function were preserved significantly better in the group treated with DHP-PMX therapy than in the group with no treatment after reperfusion.

Applications

Their results demonstrate that DHP-PMX therapy reduces the hepatic warm I/R injury caused by the THVE technique.

Terminology

The PMX cartridge (Toray Industries, Tokyo, Japan) was developed in Japan in 1994 as an extracorporeal hemoperfusion device that uses polymyxin-B fixed to α -chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. DHP-PMX therapy can remove circulating endotoxin and reduce various cytokines, even in patients with high plasma cytokine levels. This method has been used to treat endotoxemia and was reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 levels immediately. DHP-PMX therapy has also been found effective for severe sepsis secondary to intra-abdominal infection and acute lung injury or acute respiratory distress syndrome caused by sepsis.

Peer review

This is an interesting study that investigates the utility of direct hemoperfusion with a polymyxin B-immobilized fiber column (DHP-PMX therapy) on warm hepatic I/R injury with THVE using a porcine model. The title accurately reflects the major topic and contents of the study. The abstract gives a clear delineation of the research background, objectives, materials and methods, results and conclusions. Materials and Methods are very well described. The results are clearly presented and the conclusions are scientifically reliable and valuable.

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S- Editor Tian L L- Editor Logan S E- Editor Zheng XM

BRIEF ARTICLES

Epithelioid angiomylipoma of the liver: Cross-sectional imaging findings of 10 immunohistochemically-verified cases

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Received: July 1, 2009

Revised: August 18, 2009

Accepted: August 25, 2009

Published online: September 28, 2009

Abstract

AIM: To retrospectively evaluate the computed tomography (CT)/magnetic resonance imaging (MRI) imaging features of epithelioid angiomylipoma of the liver (Epi-HAML), with pathology as a reference.

METHODS: The CT/MRI findings (number, diameter, lobar location, and appearance of lesions) in a series of 10 patients with 12 pathologically proven epithelioid angiomylipomas of the liver were retrospectively analyzed. The imaging features, including attenuation/signal intensity characteristics, presence of fat, hypervascular, outer rim, and vessels within lesion, were evaluated and compared with that of non-Epi-HAML in 11 patients (13 lesions). The Fisher exact test was used to compare difference in probability of imaging features between the two types.

RESULTS: For 21 patients, CT images of 15 patients and MR images of six patients were available. No patient underwent two examinations. For the 15 patients with a CT scan, all HAML lesions in the two groups (10 Epi-HAML and seven non-Epi-HAML) manifested as hypoattenuation. For the six patients with MRI, all lesions (two Epi-HAML and six non-Epi-HAML) were hypointense on T1WI (fat suppression) and hyperintense on T2WI. There were 10 non-Epi-HAML, but only two Epi-HAML lesions showed the presence of

fat, which significantly different between the two types ($P = 0.005$). On the dynamic contrast enhancement (DCE) imaging, eight Epi-HAML, and 13 non-Epi lesions manifested as hypervascular. Punctate or curved vessels were displayed in 10 Epi-HAML as well as in nine non-Epi lesions and outer rim enhancement could be found with eight Epi-HAML as well as six non-Epi lesions.

CONCLUSION: Little or no presence of adipose tissue was found to be an imaging feature of Epi-HAML, compared with the non-Epi type. In addition, hypervascularity with opacification of central punctiform or filiform vessels on DCE would be a characteristic enhancement pattern for Epi-HAML.

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Key words: Epithelioid angiomylipoma; Liver; Immunohistochemical staining; Magnetic resonance imaging; Computed X-ray tomography

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Xu PJ, Shan Y, Yan FH, Ji Y, Ding Y, Zhou ML. Epithelioid angiomylipoma of the liver: Cross-sectional imaging findings of 10 immunohistochemically-verified cases. *World J Gastroenterol* 2009; 15(36): 4576-4581 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4576.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4576>

INTRODUCTION

Hepatic Angiomylipoma (HAML) is a rare benign tumor and the etiology is unclear. Some cases have been associated with the tuberous sclerosis complex (TSC). HAML belongs to a family of tumors that have collectively been called "PEComa"^[1-3]. Histologically, HAML is composed of a heterogeneous mixture of blood vessels, smooth muscle, and adipose cells, of varying proportions and distributions, not only among different tumors, but from area to area within the same tumor. Thus, according to the line of differentiation and the predominance of tissue components, HAML is usually subcategorized into mixed,

lipomatous ($\geq 70\%$ fat), myomatous ($\leq 10\%$ fat), and angiomatous types. The smooth muscle cell component is the most specific for the diagnosis. Depending on the dominant cell type, HAML can be subcategorized into epithelioid, spindle, and intermediate forms^[1]. Epithelioid AML (Epi-AML) was first described in the liver in 2000^[4], diagnosis can be difficult, for example the differentiation of Epi-AML from hepatocellular carcinoma and the metastatic sarcomatoid variant of renal cell carcinoma. Only one case report of Epi-AML has been mentioned or reviewed in the literature^[4-7]. In this paper, 10 immunohistochemically verified cases were retrospectively analyzed and the CT/MR imaging findings were summarized and compared with those of non-epi-HAML.

MATERIALS AND METHODS

Patients

The imaging examinations of 21 cases (10 Epi-HAML and 11 non-Epi-HAML) with pathologically proven HAML were included in this study. Proof of diagnosis was based on findings at liver resection and pathological manifestations, including immunohistochemical staining. Cases were collected from one university hospital over a seven-year period and were identified by reviewing pathology databases. In our patients, AML were incidentally detected on cross-sectional imaging performed for various reasons, such as abdominal pain ($n = 15$), suspected gallbladder stone ($n = 3$), or urinary ($n = 3$) diseases. Institutional review board approval and patient consent were not required for this retrospective study because patient privacy was maintained and patient care was not impacted.

Imaging protocols and methods

Helical multiphasic CT was performed in 15 patients using a Siemens Sensation 16 (Siemens Medical Solutions) or a PHILIPS Marconi MX8000 4 slice CT unit with 5 to 7 mm contiguous sections. After non-enhanced acquisitions of the liver, patients underwent helical multiphase CT that included both hepatic arterial phase and portal venous phase imaging (30-35 s and 80-85 s, respectively), after *i.v.* infusion of 90-100 mL nonionic contrast material (iopromide, Ultravist 300, Bayer Schering Pharma). Contrast material was injected at a rate of 3 mL/s with a power injector (Envision CT, MEDRAD).

MRI was performed in six patients using 1.5-T MR units (Magnetom avanto, Siemens Medical Solutions) with the combination of a phased-array body coil and spine array coil for signal reception. Baseline MR images, including a respiratory-navigated T2-weighted turbo spin-echo sequence [TR/TE, 2000/104 ms; slice thickness, 7 mm; flip angle, 150°; matrix, 207 (phase) \times 384 (read); FOV, 33-38 cm] and a breath-hold T1-weighted fast low angle shot (FLASH) sequence [TR/TE, 112/4.76 ms; slice thickness, 7 mm flip angle, 70°; matrix, 114 (phase) \times 256 (read); FOV, 33-38 cm]. Dynamic imaging, breath-hold T1-weighted FLASH sequence was performed using the following parameters: TR/TE, 230/2.47 ms; flip angle, 70°; matrix, 135 (phase) \times 256 (read); effective slice thickness, 7 mm; and FOV, 33-38 cm. Dynamic imaging was

performed before and after administration of gadopentate dimeglumine (Magnevist; Bayer Schering Pharma), consisting of late arterial (delay time 20-25 s), portal (70-90 s), and equilibrium (180 s) phases. The contrast-enhanced imaging was acquired after a bolus injection of 30-35 mL of contrast with a fixed delay. The contrast material was injected into the antecubital vein at a rate of 2.5 mL/s *via* a power injector (Spectris, Medrad, Indianola, PA, USA). Three dynamic phases were repeated for 18-21 s during a single breath-hold.

Imaging analysis

Imaging studies were evaluated on film by two abdominal radiologists (with experience ranging from 5 to 10 years) in consensus. Readers were not blinded to the pathology results.

The following imaging criteria were analyzed: number of lesions; lesion diameter; lesion location according to the hepatic segment numbering system of Couinaud; attenuation at non-enhanced CT, classified as hypoattenuating, isoattenuating, or hyperattenuating to the adjacent liver parenchyma; signal intensity characteristics of the lesions at non-enhanced T1-weighted (including T1WI and T1WI with fat suppression) and T2-weighted MRI; presence of fat tissue, hypoattenuating foci (-20 to -120 Hu) on non-enhanced CT images or hyperintense on T1WI but hypointense on T1WI with fat suppression; enhancement pattern at contrast-enhanced CT or MRI with regard to three-phase dynamic enhancement; presence of the central vessels in lesion at contrast-enhanced imaging; and presence of outer rim enhancement.

Pathology

All tissues were reviewed independently by one pathologist. Histopathological diagnosis was made according to the World Health Organization's classification of tumors of the liver and intrahepatic bile ducts^[8]. The most important diagnostic criterion was the presence of HMB-45-positive myoid cells. All tumor tissues had been fixed in neutral buffered formalin and were routinely embedded in paraffin. Hematoxylin-eosin stained sections were evaluated and immunohistochemical studies were performed on representative blocks by the EnVision Plus system (DAKO, Glostrup, Denmark) with a panel of antibodies (HMB-45, SMA, S-100, CD34, A103, MSA, Vimentin, CD68, CD117, HepPar-1, AFP, AE1/AE3, and CK8).

Statistical analysis

Statistical analysis was performed by using software (Intercooled Stata, version 9.0 for Windows, 2005; Stata Corp, College Station, TX, USA). We used the Fisher exact test to compare probability of these imaging features for Epi-HAML and non-Epi lesion. A *P* value less than 0.05 was considered statistically significant.

RESULTS

General data

The data are summarized in Tables 1 and 2. Age at diagnosis of hepatic angiomyolipoma varied between

Table 1 Synopsis of demographics and imaging findings in 10 patients with Epi-HAML

No.	Age (yr)/sex	Size (cm), segment	Unenhanced CT/MR	Fat	Vascularity	Outer rim	Vessels in lesion
1	51/F	6.5, 4.0 VI/VII, IV	Hypo	No	Hypovascular	Yes	Yes
2	42/F	4.2, I	Hypo	No	Hypovascular	No	No
3	35/F	7.5, VI	Hypo	No	Hypervascular	No	Yes
4	36/F	1.5, IV	Hypo	No	Hypervascular	Yes	Yes
5	17/F	10.0, V/VII/VIII	Hypo	No	Hypervascular	Yes	Yes
6	55/F	5.0, II/III	Hypo	No	Hypervascular	Yes	Yes
7 ¹	33/F	6.0, 1.0, II/III/IV, VII	Hypo	Yes	Hypervascular	No	Yes
8	36/F	3.0, IV	Hypo	No	Hypervascular	Yes	Yes
9	46/F	4.0, II/III	T1 hypo, T2 hyper	No	Hypervascular	Yes	Yes
10	47/F	2.5, VI	T1 hypo, T2 hyper	No	Hypervascular	Yes	Yes

¹The demonstration of hypervascularity and vessels with lesion were only for one lesion (large) of No. 7 patient. Hyper: Hyperattenuation; Hypo: Hypoattenuation; T1 hypo: Hypointense on T1WI (fat suppression); T2 hyper: Hyperintense on T2WI.

Table 2 Synopsis of demographics and imaging findings in 11 patients with non-Epi-HAML

No.	Age (yr)/sex	Size (cm), segment	Unenhanced CT/MR	Fat	Vascularity	Outer rim	Vessels in lesion
1	45/F	6.0, VI/VII	Hypo	Yes	Hypervascular	No	No
2	34/F	4.5, II/III	Hypo	No	Hypervascular	No	Yes
3	37/F	5.5, VIII/IV	T1 hypo, T2 hyper	Yes	Hypervascular	Yes	Yes
4	40/F	12.0, VII/VIII/IV 2.0, 1.0, II/III	T1 hypo, T2 hyper	Yes	Hypervascular	Yes	Yes
5	50/F	6.5, IV	T1 hypo, T2 hyper	Yes	Hypervascular	Yes	Yes
6	43/M	2.0, VI	Hypo	Yes	Hypervascular	No	No
7	47/M	2.0, IV	Hypo	Yes	Hypervascular	No	No
8	46/M	3.0, VIII	Hypo	Yes	Hypervascular	No	Yes
9	44/F	4.0, II	Hypo	No	Hypervascular	No	No
10	50/F	3.0, VI	Hypo	Yes	Hypervascular	No	Yes
11	21/F	12.0, VI/VI	T1 hypo, T2 hyper	No	Hypervascular	Yes	Yes

Table 3 Comparison of imaging features between Epi-HAML and non-Epi-HAML¹

	Epi-HAML, <i>n</i> = 12 (10)	Non-Epi-HAML, <i>n</i> = 13 (11)	<i>P</i> value
Fat	2 (1)	10 (8)	0.005
Hypervascular	8 (8)	13 (11)	0.082
Vessels in lesions	10 (9)	9 (7)	0.645
Outer rim	8 (7)	6 (4)	0.428

¹Data without parentheses are the number of lesions and data in parentheses are the number of patients. The Fisher exact test was used to analyze difference in per-lesion probability of imaging features between two types.

17 and 55 years (mean 40.7 years). In the Epi-HAML group, there was history of TSC for one patient, and a combination of left kidney AML for another one. Among 10 patients, eight had solitary lesions and two had two lesions. Lesions had a mean diameter of 4.6 (range, 1.5-10.0). In the non-Epi-HAML group, there was right kidney AML in one patient, 10 had solitary lesions, and one had three lesions. Lesions had a mean diameter of 4.9 (range, 1.0-12.0).

Comparison of CT/MRI findings between Epi-HAML and non-Epi-HAML

The CT/or MR images of 21 patients (CT images for 15 patients and MR for six) were available for retrospective analysis. The results are shown in Tables 1-3.

At non-enhanced CT, 10 Epi-HAML and seven non-Epi HAML were hypoattenuating to the surrounding liver. Two Epi-HAML and six non-epi lesions were hypointense on T1WI (fat suppression) and hyperintense on T2WI. The presence of fat was detected in 10 non-Epi HAML but only two Epi type lesions (Figure 1) and there were significant differences between the two lesion types ($P < 0.01$, Table 3).

On arterial phase, eight Epi-HAML and 13 non-Epi-HAML showed obvious enhancement (Figures 1-3). Punctate or curved vessels could be seen within 10 Epi-HAML as well as nine non-Epi type lesions (Figures 1-4) on arterial or/and portal phase. Outer rim enhancement could be found in eight Epi-HAML as well as in six non-Epi-HAML on enhanced imaging (Figures 3 and 4).

Pathology

Immunohistochemical studies showed that tumor cells were positive for HMB-45 and A103, but negative for cytokeratin (HepPar-1, AFP, AE1/AE3, CK8.) in all cases. Smooth muscle actin (SMA) and desmin staining were weak to moderate in epithelioid cells. MSA staining was similar to desmin but was more weak when positive. The endothelial cells lining the blood vessels were positive for CD34. CD117 and S-100 were negative in all cases.

Follow-up

Imaging and clinical follow-up was available in 10 patients with Epi-HAML and ranged from one to six years.

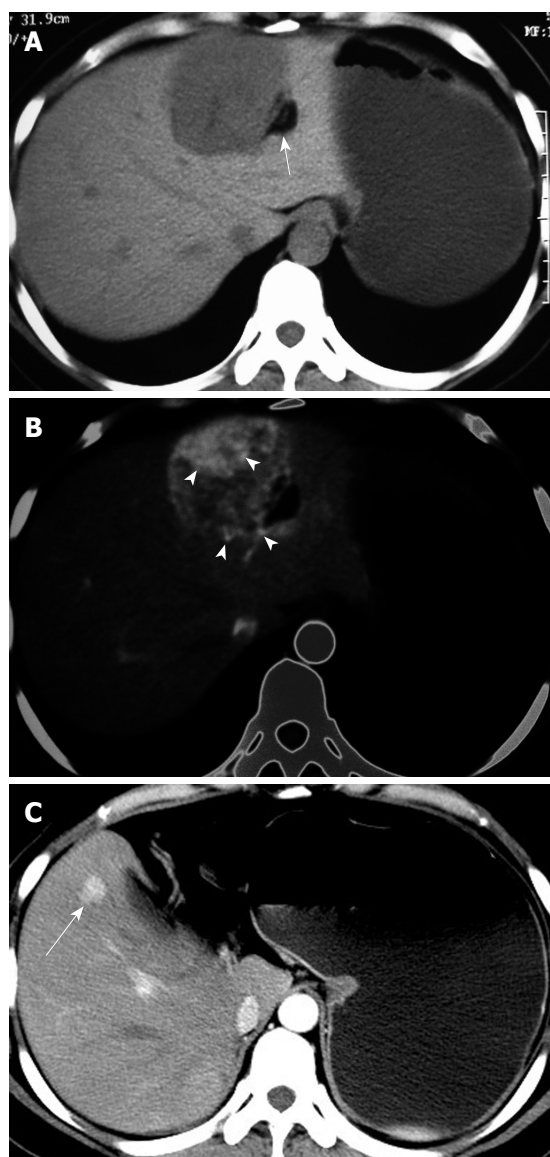


Figure 1 33-year-old woman with epithelioid angiomyolipoma in left lobe of liver (patient 7). A: Non-enhanced CT scan shows hypoattenuating lesion with fat components (arrow, CT value mean-35 Hu) in segments II/III/IV; B: Contrast-enhanced CT scan shows inhomogeneous enhancement lesion with punctiform or filiform enhanced vessels (arrowheads, the window width and level was adjusted) on arterial phase; C: Contrast-enhanced CT scan at 1-year follow-up shows enhanced recurrent nodule (arrow) on arterial phase image after the left lobe surgery.

Recurrent hepatic lesions were found in one patient (Figure 1), pubic bone destruction and metastatic nodule in body soft tissue were proved with biopsy in another.

DISCUSSION

Previous literatures showed variable imaging appearances for HAML^[9-12]. The imaging characteristics of HAML are correlated with its histological components, and demonstration of blood vessels and mature adipose tissue are the most important radiographic features. Color Doppler sonography shows a punctiform or filiform vascular distribution pattern. Contrast-enhanced CT shows marked enhancement of the soft tissue components in the arterial phase. MRI is the most

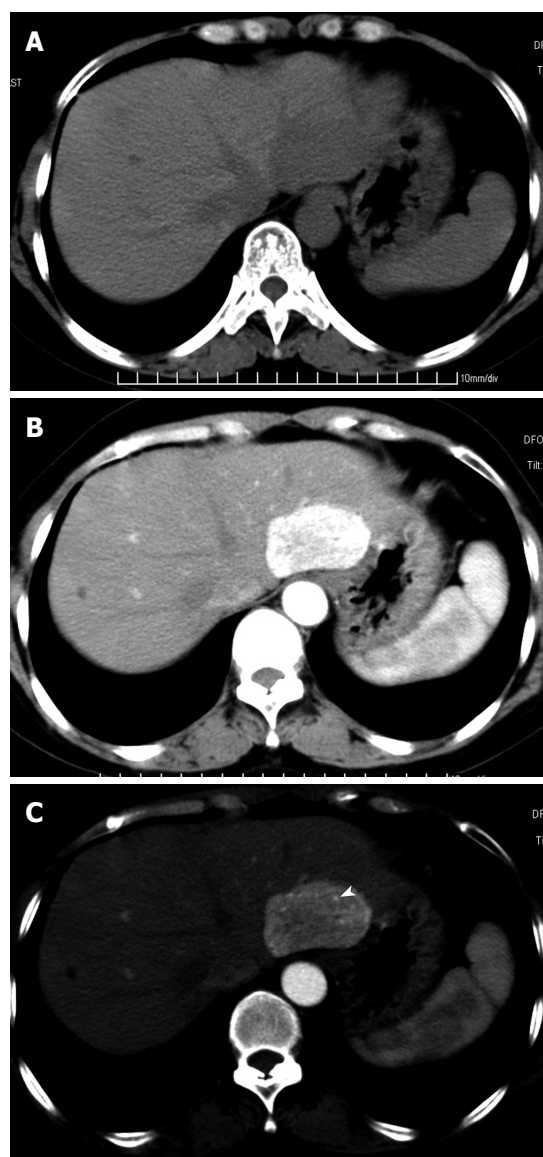


Figure 2 55-year-old woman with epithelioid angiomyolipoma in left lobe of liver (patient 6). A: Non-enhanced CT scan shows hypoattenuating lesion in segment II; B: contrast-enhanced CT scan shows obviously enhanced lesion on arterial phase; C: the punctate enhanced vessels (arrowhead) within the lesion is show on an arterial phase image by adjusting the window width and level.

specific imaging entity for the detection of lipomatous components; however, because hepatic AML can have such variation in the amount of adipose tissue present, detection varies based on the percentage of the lesion that is composed of adipose tissue. MRI findings include hypointensity or hyperintensity on T1WI, slight hyperintensity on T2WI, dense enhancement in the arterial phase, and hypointensity in the delayed phase.

In our study, most of the Epi-AML tumors (10/12) were completely devoid of adipose tissue, which is the characteristic finding compared with that of non-Epi type. The result was consistent with a previous report^[1]. After *i.v.* contrast administration, there was obvious enhancement on the arterial phase for most of these lesions, suggesting that Epi-AML was a hypervascular tumor. Most lesions (seven) manifested as hypoattenuation or hypointensity on portal/equilibrium phase. By adjusting the window

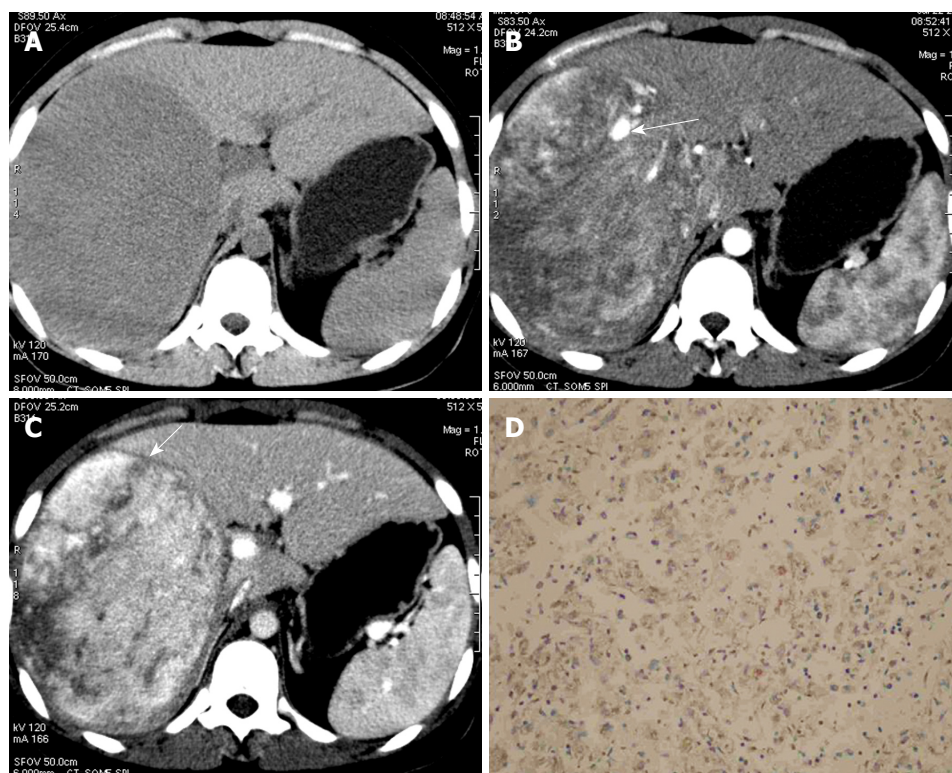


Figure 3 17-year-old girl with epithelioid angiomyolipoma in right lobe of liver (the patient had a history of tuberous sclerosis complex, patient 5). A: Non-enhanced CT scan shows homogeneous hypoattenuating lesion in segment V/VII/VIII; B: Contrast-enhanced CT scan shows inhomogeneous enhanced lesion with opacification of central vessels (arrow) on the arterial phase; C: The slight enhanced outer rim (arrow) around the inhomogeneous enhancement lesions is shown on portal phase contrast-enhanced CT; D: Immunohistochemical staining for HMB-45 shows diffusely positive staining in tumor cells within the cytoplasm (EnVision, $\times 100$).

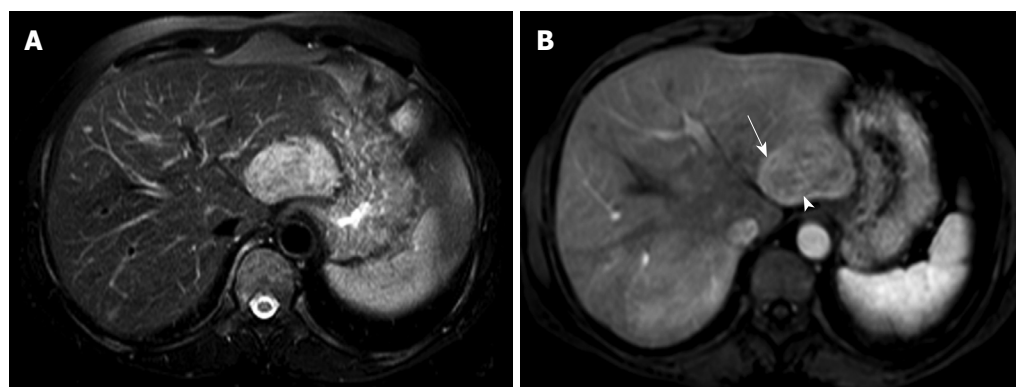


Figure 4 46-year-old woman with epithelioid angiomyolipoma in left lobe of liver (patient 9). A: Non-enhanced MRI T2WI shows hyperintense lesion in segment II/III; B: Contrast-enhanced MRI shows obvious enhanced lesions with outer rim enhancement (arrow) and opacification of punctate or curved vessels (arrowhead) on arterial phase.

width and level, punctate or curved vessels could be seen within 10 lesions on enhanced scanning. In addition, intact or discrete outer rim enhancement within 8 lesions was observed. According to the pathology specimens and literature report, it is not true capsular but a pseudocapsule, which is composed of the compressed liver parenchyma and sparse fibrosis tissues with small vessels, resulting in delayed enhancement on late phase^[13]. A similar manifestation can be found in other hypervascular lesion, such as hepatocellular carcinoma (HCC) and focal nodular hyperplasia (FNH). The distinction between these lesions can be difficult. However, the enhancement patterns were somewhat different among Epi-AML, HCC, and FNH. Most of the HCC enhanced markedly in the arterial phase

and decreased rapidly on the portal venous/equilibrium phase. Additionally, capsule could be found in most of the HCC, so the margins of HCC were more clear than those of AML in the portal venous phase, a suggestive finding for correct diagnosis^[14]. The enhancement pattern of FNH was similar to AML, in both of them prolonged enhancement could be shown in the portal venous phase. However, most of FNH enhanced homogeneously on the arterial phase except the central scar, which was characteristic of FNH and could be enhanced on the portal venous phase/delayed phase^[15].

Five cases of hepatic malignant angiomyolipoma have been reported^[5,16-19]. Strict histology criteria for defining hepatic angiomyolipoma as malignant have not been put

forward, but it should be suspected, especially in tumors with many mitoses. Those with necrosis and significant cellular pleomorphism might show aggressive behavior. There is clinical evidence of aggressive behavior such as recurrence and metastases beyond the liver, for two cases in these 10 Epi-AML cases. However, there are no characteristic findings for malignant HAML on cross-sectional imaging. Therefore, although most HAMLs are biologically benign, this tumor should be considered to have malignant potential, especially for Epi-AML. So resection and careful follow-up are recommended.

It is important to recognize the limitations of our study. Firstly, the study is retrospective; secondly, the numbers reported are limited and no one patient had both CT and MRI. A further limitation is that not every patient underwent regular imaging follow-up and documentation was incomplete.

In summary, little or no adipose tissue was found to be an imaging feature of Epi-HAML, *vs* non-Epi-HAML. In addition, hypervascularity with opacification of central punctiform or filiform vessels on DCE would be a characteristic enhancement pattern for Epi-HAML.

COMMENTS

Background

Hepatic Angiomyolipoma (HAML) is a rare benign tumor and belongs to a family of tumors that have collectively been called "PEComa". The immense variability of the morphological appearance is due to the different heterogeneous mixtures of vessels, epithelioid cells, and lipocytes. As a specific form, the epithelioid HAML (Epi-HAML) has characteristics in pathology and diagnosis that can be difficult, e.g. differentiation of Epi-AML from hepatocellular carcinoma and the metastatic sarcomatoid variant of renal cell carcinoma.

Research frontiers

Previous studies showed variable imaging appearances for HAML. The imaging characteristics of HAML are correlated with its histological components. Both CT and MRI, for the most part, do not allow the definitive differentiation of HAML from hepatocellular carcinoma, adenoma, liposarcoma, lipoma, hamartoma, and sometimes even from focal nodular hyperplasia, especially if the fat content is low. The Epi-HAML has morphological characteristics of tumor cells. In addition, the tumors are devoid of fat or only scattered fat cells distribution in pathology. However, the imaging features of Epi-HAML have not been unequivocally described and evaluated. In this study, the authors summarized CT/MRI imaging features of Epi-HAML and compared with those of non-Epi-HAML.

Innovations and breakthroughs

Previous reports have showed demonstration of mature adipose tissue is the most important radiographic features and highlighted the importance in the diagnosis of HAML, in particular for mixed, lipomatous types of HAML. This is the first study to report that blood vessels are also an imaging feature in Epi-HAML. Furthermore, the study would suggest that little or no adipose tissue might be imaging characteristics of Epi-HAML, *versus* non-Epi-HAML.

Applications

By understanding the imaging characteristics, this study could help radiologists familiarize themselves with the appearance of Epi-HAML, and improve the confidence in the diagnosis of incidental liver tumor, especially in cases where the fat content is low and the interpretation of histological findings is difficult.

Terminology

Immunohistochemistry is a method of analyzing and identifying cell types based on the binding of antibodies to specific components of the cell. The most important diagnostic criterion for HAML is the presence of HMB-45-positive myoid cells.

Peer review

The manuscript reported the imaging characteristics of Epi-HAML, only a few reports had been published about the lesion before. It will be helpful for radiologists to obtain knowledge of the lesion.

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BRIEF ARTICLES

Effect of preoperative transcatheter arterial chemoembolization on angiogenesis of hepatocellular carcinoma cells

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Author contributions: Xiao EH designed the research; Xiao EH, Guo D and Bian DJ performed the research; Guo D and Bian DJ contributed new reagents/analytic tools; Xiao EH, Guo D and Bian DJ analyzed the data; Xiao EH and Guo D wrote the paper. Supported by The National Science and Technology Projects of 11th Five-Year Plan, No. 2007BAI05B06; the National Council of Natural Sciences of China, No. 30070235, 30470508, 30870695, the Provincial Council of Natural Sciences of Hunan, No. 06JJ20081

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Received: May 7, 2009 Revised: July 30, 2009

Accepted: August 6, 2009

Published online: September 28, 2009

Abstract

AIM: To evaluate the effects of four types of preoperative transcatheter arterial chemoembolization (TACE) on angiogenesis of hepatocellular carcinoma (HCC) cells.

METHODS: A total of 136 patients with HCC underwent liver resection. One to five courses of TACE prior to liver resection were performed in 79 patients (TACE group), in which one to four courses of chemotherapy alone were performed in 11 patients (group A); one to five courses of chemotherapy combined with iodized oil were performed in 33 patients (group B); one to three courses of chemotherapy combined with iodized oil and gelatin sponge were performed in 23 patients (group C); one to three courses of chemotherapy combined with iodized oil, ethanol and gelatin sponge were performed in 12 patients (group D). The other 57 patients only received liver resection (non-TACE group). The microvessels were marked by CD31. The expression of CD31 and vascular endothelial growth factor (VEGF) protein were detected by immunohistochemical methods.

RESULTS: The mean microvessel density (MVD) in HCC cells was significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). The expression of VEGF protein in HCC cells were significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). MVD and the expression of VEGF

protein were positively correlated. Mean MVD and the expression of VEGF protein were closely related to the number of courses of TACE and the interval of TACE.

CONCLUSION: Four different types of preoperative TACE regimens enhanced angiogenesis in HCC cells by up-regulating the expression of VEGF protein. It is necessary to repress angiogenesis of liver cancer after TACE.

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Key words: Angiogenesis; Hepatocellular carcinoma; Immunohistochemistry; Transcatheter arterial chemoembolization; Vascular endothelial growth factor

Peer reviewer: Dr. Paolo Del Poggio, Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

Xiao EH, Guo D, Bian DJ. Effect of preoperative transcatheter arterial chemoembolization on angiogenesis of hepatocellular carcinoma cells. *World J Gastroenterol* 2009; 15(36): 4582-4586 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4582.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4582>

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asian countries. It is responsible for more than 250 000 deaths worldwide each year, 40% of which occur in China ranking HCC second after gastric carcinoma^[1-3]. Surgical resection is recognized as the most effective treatment method for patients with HCC^[4]. Although recent advances in treatment have helped prolong the survival of patients with HCC, they consequently increase the risk of intrahepatic recurrence and extrahepatic metastasis. Only a minority of patients currently diagnosed with HCC may benefit from this radical option^[5].

Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with HCC^[6-11]. Various mixtures of anticancer drugs, lipiodol and gelatin sponge have been used as TACE agents. There have been few reports comparing the efficacy of different TACE regimens in patients with HCC^[12-14].

There is ample evidence that tumor angiogenesis is the pathological basis and a necessary condition for solid tumor growth and metastasis^[15]. Vascular endothelial growth factor (VEGF) is a strong angiogenesis factor in HCC^[16], and plays an important role in the development and prognosis of liver cancer. In the present study, we examined the effects of the four main types of TACE used clinically (pure intra-arterial chemotherapy, chemotherapy plus lipiodol, chemotherapy plus lipiodol plus gelatin sponge, and chemotherapy plus lipiodol plus alcohol plus gelatin sponge) on angiogenesis of HCC cells *in vivo*.

MATERIALS AND METHODS

Patients

From February 1992 to February 2001, a total of 136 patients with HCC were referred to our hospital for surgery, of which, 122 were men and 14 were women with a mean age of 45 years (range: 20 to 70 years). A diagnosis of HCC was obtained for all patients by preoperative ultrasound (US) and/or computed tomography (CT) and/or magnetic resonance imaging (MRI) and/or plasma AFP levels, and was then confirmed by biopsy.

Surgical procedure

The patients were randomly divided into two groups. In the TACE group, 79 patients underwent 1-5 courses of chemoembolization prior to liver resection, in which one to four courses of chemotherapy alone were performed in 11 patients (group A), one to five courses of chemotherapy combined with iodized oil were performed in 33 patients (group B), one to three courses of chemotherapy combined with iodized oil and gelatin sponge were performed in 23 patients (group C), and one to three courses of chemotherapy combined with iodized oil, ethanol and gelatin sponge were performed in 12 patients (group D). Fifty patients underwent one course of TACE, 19 patients underwent two courses of TACE and 10 patients underwent three or more courses of TACE during an interval of 52.8 ± 12.2 d (mean \pm SD). Twenty-five patients had an interval of one month or less, 29 patients had an interval of two months or less, 16 patients had an interval of three months or less and 9 patients had an interval of more than three months. In the non-TACE group, 57 patients received initial liver resection without preoperative TACE. The extent of liver resection carried out was based on the location of the tumor, the severity of concomitant liver cirrhosis and preoperative liver reserve function.

Immunohistochemical methods

The formalin-fixed, paraffin-embedded specimens were examined immunohistochemically using anti-CD31 antibody and anti-VEGF antibody (LSAB Kit, Dako). Breast cancers were used as positive controls. Negative controls were generated by substituting the primary antibody with phosphate-buffered saline (PBS). CD31 and VEGF immunostained cells showed a brownish-yellow color in the cytoplasm (Figure 1A and B). Microvessel

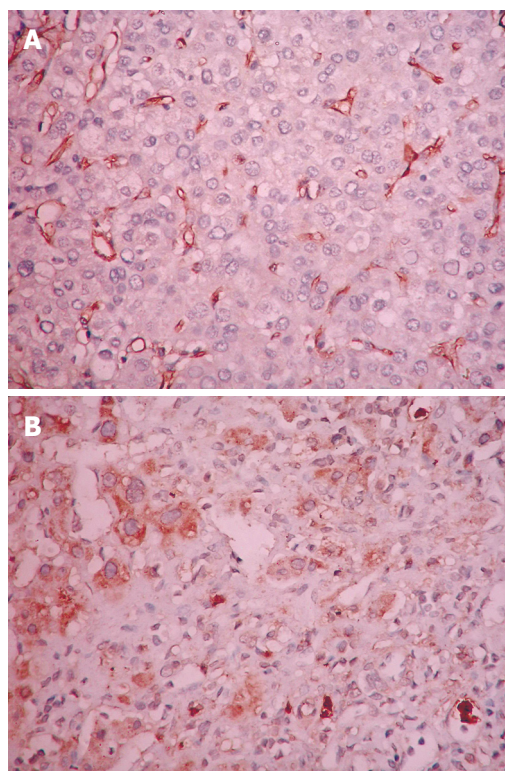


Figure 1 Expression of CD31 (A) and VEGF (B) protein detected by immunohistochemical methods after chemotherapy combined with ethanol, iodized oil and gelatin sponge (Dako Envision, peroxidase method $\times 400$).

density (MVD) counting followed the method of Weidner *et al*^[17], i.e. first, the regions with the highest density of CD31-positive cells were chosen and counted under a low-power microscope ($\times 40$). Then microvessel numbers were counted under a high-power microscope ($\times 400$). Each isolated brown vascular endothelial cell or cluster of endothelial cells was counted as a vascularization. VEGF counting followed the method of Park *et al*^[18], i.e. negative (-) was $< 5\%$ of positively stained cells, weakly positive (+) was 5% to 15% of positively stained cells, moderately positive (++) was 15% to 50% of positively stained cells and strongly positive (+++) was $> 50\%$ of positively stained cells. All slides were reviewed and scored in a blind test by two observers without knowledge of the corresponding clinical data. A few cases with discrepant scoring were jointly re-evaluated until agreement was reached.

Statistical analysis

MVD was expressed as mean \pm SD and analyzed using the two-sample *t*-test for the two groups, and by analysis of variance for multiple comparisons. VEGF was analyzed using table χ^2 test. The correlation between MVD and VEGF was analyzed using Pearson correlation analysis. *P*-value < 0.05 was considered statistically significant.

RESULTS

Correlation between methods of TACE and expression of CD31 and VEGF protein

The mean MVD was (47.71 ± 23.33), (56.05 ± 22.45),

Table 1 Expression of VEGF protein in groups A, B, C, D, and the non-TACE group

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
A	11	2 (18.2)	4 (36.4)	3 (27.3)	2 (18.2)
B	33	5 (15.2)	6 (18.2)	9 (27.3)	13 (39.4)
C	23	2 (8.7)	6 (26.1)	4 (17.4)	11 (47.8)
D	12	1 (8.3)	2 (16.7)	4 (33.3)	5 (41.7)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

Table 2 Correlation between expression of CD31 and VEGF protein

Group	Cases	MVD
VEGF (-)	27	35.47 ± 17.35
VEGF (+)	34	44.12 ± 15.84
VEGF (++)	33	52.56 ± 17.29
VEGF (+++)	42	60.72 ± 23.46

(54.36 ± 24.46), (51.90 ± 19.41) and (44.36 ± 17.67) in groups A, B, C, D and the non-TACE group, respectively. The mean MVD was significantly higher in groups A, B, C and D than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was significantly higher in groups B, C and D than in group A or the non-TACE group ($\chi^2 = 12.63$, $P < 0.05$) (Table 1). When the expression of VEGF protein was increased, MVD increased significantly, and both were positively correlated (Pearson correlation, $r = 0.445$, $P < 0.05$) (Table 2).

Correlation between courses of TACE and expression of CD31 and VEGF protein

The mean MVD was (44.36 ± 17.67), (54.01 ± 23.83), (53.38 ± 22.64) and (51.94 ± 22.64) in the non-TACE group, the one-course TACE group, the two-course TACE group and the three-, four- and five-course TACE group, respectively. The mean MVD was significantly higher in the one-course TACE group than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was higher in the TACE groups than in the non-TACE group ($\chi^2 = 16.786$, $P > 0.05$) and decreased as the courses of TACE increased (Pearson correlation, $r = 0.331$) (Table 3).

Correlation between interval of TACE and expression of CD31 and VEGF protein

The mean MVD was (44.36 ± 17.67), (49.20 ± 19.84), (55.30 ± 23.31), (61.48 ± 26.63) and (44.25 ± 17.52) in the non-TACE group, the 1 mo-interval TACE group, the 1-2 mo interval TACE group, the 2-3 mo interval TACE group, and the > 3 mo interval TACE group, respectively. A comparison between the groups showed that the mean MVD was higher in the 1 mo-interval TACE group, the 1-2 mo interval TACE group and the 2-3 mo interval TACE group than in the non-TACE group ($P < 0.05$). The expression of VEGF protein was higher in the TACE interval groups than in the non-

Table 3 Correlation between courses of TACE and expression of VEGF protein

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
One-course	50	5 (10.0)	14 (28.0)	12 (24.0)	19 (38.0)
Two-course	19	3 (15.8)	4 (21.1)	3 (15.8)	9 (47.4)
Three-, four- or five-course	10	2 (20.0)	0 (0.0)	5 (50.0)	3 (30.0)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

Table 4 Correlation between interval of TACE and expression of VEGF protein

Group	Cases	Expression of VEGF (%)			
		-	+	++	+++
1 mo interval	27	4 (14.8)	7 (25.9)	7 (25.9)	9 (33.3)
1-2 mo interval	28	2 (7.1)	7 (25.0)	6 (21.4)	13 (46.4)
2-3 mo interval	16	3 (18.8)	2 (12.5)	5 (31.3)	6 (37.5)
> 3 mo interval	8	1 (12.5)	2 (25.0)	2 (25.0)	3 (37.5)
Non-TACE group	57	17 (29.8)	16 (28.1)	13 (22.8)	11 (19.3)

TACE group ($\chi^2 = 12.488$, $P = 0.407$) and was highest in the 1-2 mo interval TACE group (Table 4).

DISCUSSION

TACE is one of the most common and effective palliative treatments. The prognosis of patients treated with TACE depends not only on the use of an effective TACE regimen but also on tumor factors^[12]. According to the literature, very limited data are currently available regarding the molecular mechanism of TACE treatment in patients with HCC^[13-15]. We believe that the current study is the first to detail the correlations between the expression of CD31 and VEGF protein and different TACE regimens, courses of TACE and interval of TACE.

There is ample evidence that tumor angiogenesis is the pathological basis and a necessary condition for the growth and metastasis of solid tumors^[15]. VEGF is a strong factor in the angiogenesis of HCC^[16], and plays an important role in the development and prognosis of liver cancer. It also has a biological effect by combining its specific VEGF receptor (vascular endothelial growth factor receptor, VEGFR), of which VEGFR-1 and VEGFR-2 are mainly distributed in vascular endothelial cells. Binding of VEGFR-1 and VEGF allows vascular endothelial cell migration, maintains tubular structure and regulates vascular permeability. Binding of VEGFR-2 and VEGF also promotes vascular endothelial cell proliferation and maturation^[19]. This study showed that MVD of HCC was positively correlated with the expression of VEGF protein.

Whether angiogenesis of liver cancer after TACE is enhanced is still controversial. Some reports have suggested that the mean MVD of HCC specimens before and after TACE were not statistically significantly

different^[20-23]. However, there are other reports which show that the mean MVD was significantly higher in the TACE group than in the non-TACE group^[24-26]. The current study showed that the mean MVD of HCC was significantly higher in the TACE groups than in the non-TACE group. The expression of VEGF protein was significantly higher in the TACE groups than in the non-TACE group. After chemoembolization, the expression of VEGF protein increased, and the change in expression and MVD showed a significant positive correlation. It has also been reported that serum VEGF in patients with liver cancers was significantly increased after TACE^[27,28]. Tumor tissue ischemia and hypoxia after TACE are important in promoting increased VEGF expression^[29]. Therefore, it is necessary to repress angiogenesis in liver cancer after TACE.

The study on the effects of the interval between surgical resection of the tumor and the end of embolization on MVD, revealed that MVD was not significantly different in the group with an interval of less than 30 d or in the group with an interval of more than 90 d, but was significantly increased in the group with an interval of 31-90 d compared with the control group. This showed that after chemoembolization, tumor hypoxia-ischemia results in a series of biochemical changes which cause increased angiogenesis and a gradual increase in MVD, reaching a peak in 1-3 mo. More than three months later, the blood supply to the residual tumor improves, the formation of tumor angiogenesis slows, the residual cancer cells grow, infiltration occurs and damage to generated tumor angiogenesis occurs.

The mean MVD and expression of VEGF protein in liver cancer has a tendency to decrease as the time from TACE therapy increases, although this difference was not significant. It was reported that survival after a number of pre-operative TACE treatments was significantly better than that after a single TACE treatment^[30]. Therefore, multiple pre-operative TACEs should be carried out in suitable patients.

In conclusion, the present study demonstrated that angiogenesis of residual HCC cells following treatment with four types of TACE is significantly increased and is positively correlated with the expression of VEGF protein. The effect of TACE on angiogenesis of HCC cells has a close correlation with the number of courses of TACE and the interval of TACE.

COMMENTS

Background

Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with hepatocellular carcinoma (HCC). Various mixtures of anticancer drugs, lipiodol and gelatin sponge have been used as TACE agents. However, there have been few reports comparing the effects of different TACE regimens on angiogenesis of HCC cells.

Applications

According to the results of this study, it is necessary to repress angiogenesis of liver cancer after TACE.

Terminology

TACE indicates transcatheter arterial chemoembolization; MVD indicates

microvessel density.

Peer review

TACE stimulates angiogenesis. It would be very useful from a clinical standpoint to identify which type of TACE stimulates more angiogenesis and give appropriate "prophylactic" anti-angiogenic therapy. The authors have the merit of confirming that TACE is an angiogenesis stimulating procedure and also give new information on this process -- e.g. angiogenesis peaks about 1 mo after TACE and then seems to wane off. The number of observations and the histological specimen (surgical) are adequate.

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S- Editor Tian L L- Editor Webster JR E- Editor Ma WH

Pseudolymphoma of the liver associated with primary biliary cirrhosis: A case report and review of literature

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Received: April 10, 2009 Revised: August 14, 2009

Accepted: August 21, 2009

Published online: September 28, 2009

pseudolymphoma into lymphoma in the liver, the exact nature of development from benign pseudolymphoma to malignant lymphoma is still not fully understood and cases of hepatic lymphoma need to be followed carefully.

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Key words: Liver diseases; Pseudolymphoma; Primary biliary cirrhosis

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Okada T, Mibayashi H, Hasatani K, Hayashi Y, Tsuji S, Kaneko Y, Yoshimitsu M, Tani T, Zen Y, Yamagishi M. Pseudolymphoma of the liver associated with primary biliary cirrhosis: A case report and review of literature. *World J Gastroenterol* 2009; 15(36): 4587-4592
Available from: URL: <http://www.wjgnet.com/1007-9327/15/4587.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4587>

Abstract

We report a case of two pseudolymphomas of the liver in a 63-year-old Japanese woman with primary biliary cirrhosis. One of the lesions was found incidentally during a medical examination, presenting as a 10 mm hypodense nodule that revealed hyperdensity in the early phase and hypodensity in the late phase in computed tomography (CT) after injection of contrast medium. Retrospectively, the 10 mm nodule had first been discovered as a 4 mm nodule during CT 4 years previously. Superparamagnetic iron oxide-enhanced MRI revealed another 4 mm hyperintense nodule in segment 6 in addition to the 10 mm hyperintense nodule in segment 7. CT during arterial portography revealed two hypointense nodules. Findings with other imaging modalities such as ultrasonography, magnetic resonance imaging, and hepatic angiography were consistent with hepatocellular carcinoma. A right posterior segmentectomy was performed, and the lesions were microscopically diagnosed as pseudolymphoma. To the best of our knowledge, only 31 other cases of this disease have ever been reported, with a highly asymmetrical male:female ratio of 1:9.7. Although we could find only one case of transformation of hepatic

INTRODUCTION

Pseudolymphoma in the liver is an extremely rare disease. Although the exact etiology remains unknown, it is speculated that the disorder is a reactive immunological response to a chronic infection or inflammation^[1]; hepatic pseudolymphoma can develop in patients with autoimmune diseases^[2,3], malignancy^[4], or hepatitis^[5] or who are administered interferon therapy^[6]. It has been reported that diagnosis of pseudolymphoma is difficult without histopathological examination, since image findings are quite similar to hepatocellular carcinoma (HCC)^[2]. Although pseudolymphoma is generally thought to be benign, the risk of malignant transformation into lymphoma remains controversial.

We report a case of hepatic pseudolymphoma in a female Japanese patient with primary biliary cirrhosis (PBC) and discuss the literature.

CASE REPORT

A 63-year-old female with a history of PBC and resection of the left adrenal gland for primary aldosteronism, was admitted to our hospital for further evaluation of

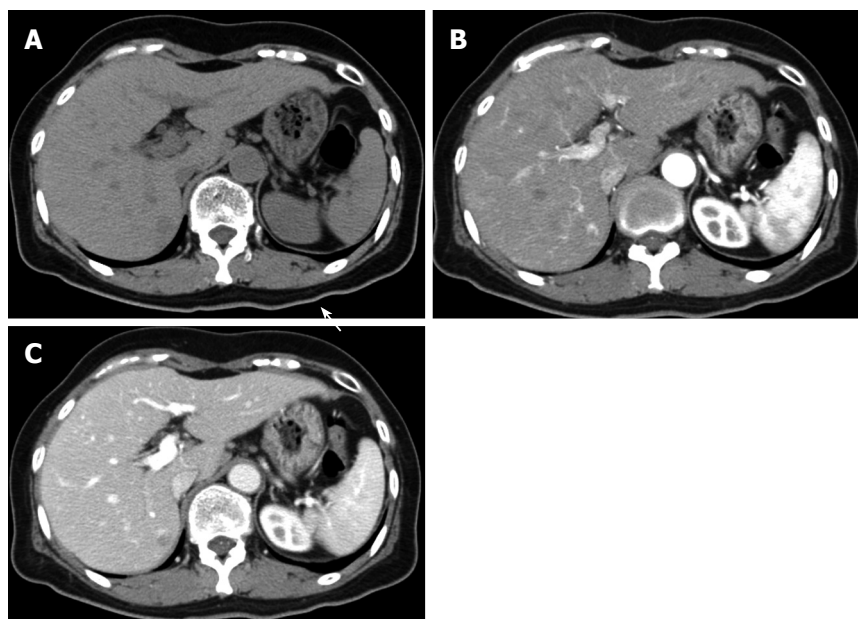


Figure 1 Computed tomography (CT) showing a 10 mm nodule in segment 7. A: A hypodense nodule in plane phase; B: A hyperdense nodule in the early phase after injection of contrast medium; C: A hypodense nodule in the late phase after injection of contrast medium.

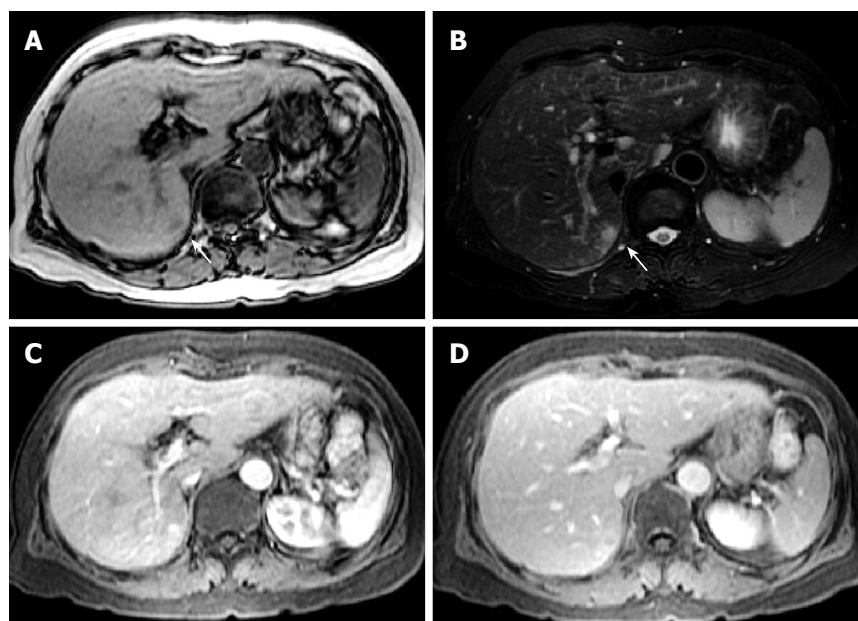


Figure 2 Magnetic resonance imaging (MRI) showing a 10 mm nodule in segment 7. A: A hypointense nodule on T1-weighted images; B: A hyperintense nodule on T2-weighted images; C: A hyperintense nodule in the early phase after injection of contrast medium; D: A hypointense nodule in the late phase after injection of contrast medium.

a hepatic lesion incidentally discovered in abdominal computed tomography (CT). The patient had taken only ursodeoxycholic acid 300 mg/d for PBC for 10 years and had not taken immunosuppressive agents. The patient was asymptomatic on admission and her condition was generally good. She had a surgical scar on her abdomen. We noted no hepatosplenomegaly, lymphadenopathy, or peripheral edema. Laboratory tests showed a prothrombin time of 11.8 s (normal, 10.8-13.3), a total bilirubin level of 0.6 mg/dL (normal, 0.3-1.2), and an albumin level of 4.0 g/dL (normal, 4.0-5.0). Her serum alkaline phosphatase level was 350 IU/L (normal, 115-359), γ -glutamyltransferase 48 IU/L (normal, 10-47), aspartate aminotransferase 24 IU/L (normal, 13-33), and alanine aminotransferase 16 IU/L (normal, 6-27). Serology examinations for hepatitis B and C viruses were negative. Rheumatoid factor antibodies, Sjögren syndrome-A antibodies, and Sjögren syndrome-B antibodies were negative. Antinuclear antibodies and antimitochondrial

antibodies were positive. Immunoglobulin G, immunoglobulin M, and immunoglobulin A were within normal ranges. Alpha-fetoprotein, protein induced by vitamin K absence, and carcinoembryonic antigen were within normal ranges. A urea breath test was negative. Abdominal ultrasonography showed a hypoechoic lesion, 10 mm in diameter in segment 7 (data not shown). CT demonstrated a 10 mm hypodense nodule that revealed hyperdensity in the early phase and hypodensity in the late phase after injection of contrast medium (Figure 1). Retrospectively, this nodule had previously shown up as a 4 mm nodule in a CT performed for examination of an adrenal tumor 4 years previously (data not shown). Magnetic resonance imaging (MRI) showed a hypointense nodule on T1-weighted images and a hyperintense nodule on T2-weighted images in segment 7. Following injection of contrast MRI, a hyperintense nodule in the arterial phase and a hypointense nodule in the portal phase were revealed (Figure 2). Superparamagnetic iron oxide-

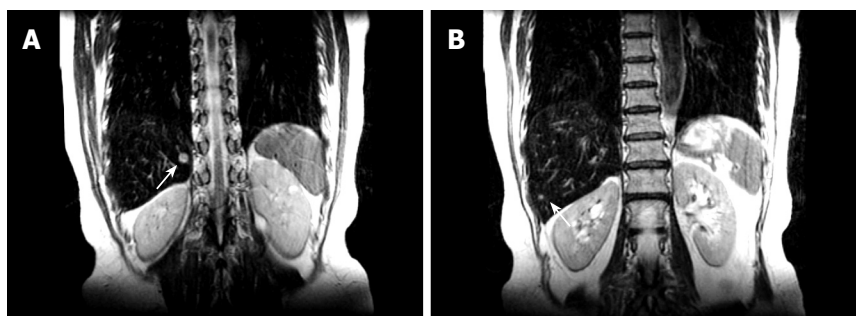


Figure 3 Superparamagnetic iron oxide-enhanced MRI showing hyperintense nodules. A: 10 mm nodule in segment 7; B: 4 mm nodule in segment 6.

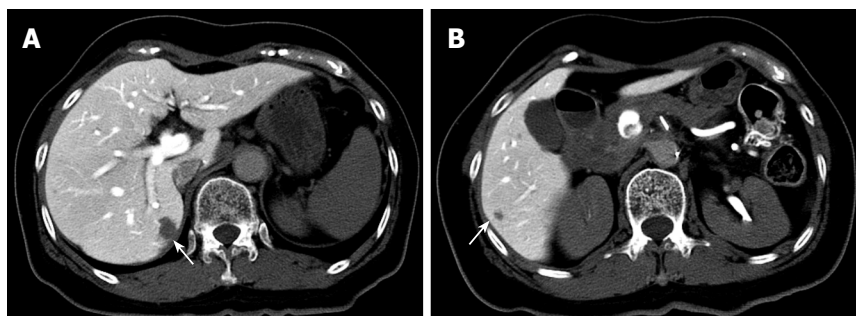


Figure 4 CT during arterial portography showing hypointense nodules. A: 10 mm nodule in segment 7; B: 4 mm nodule in segment 6.

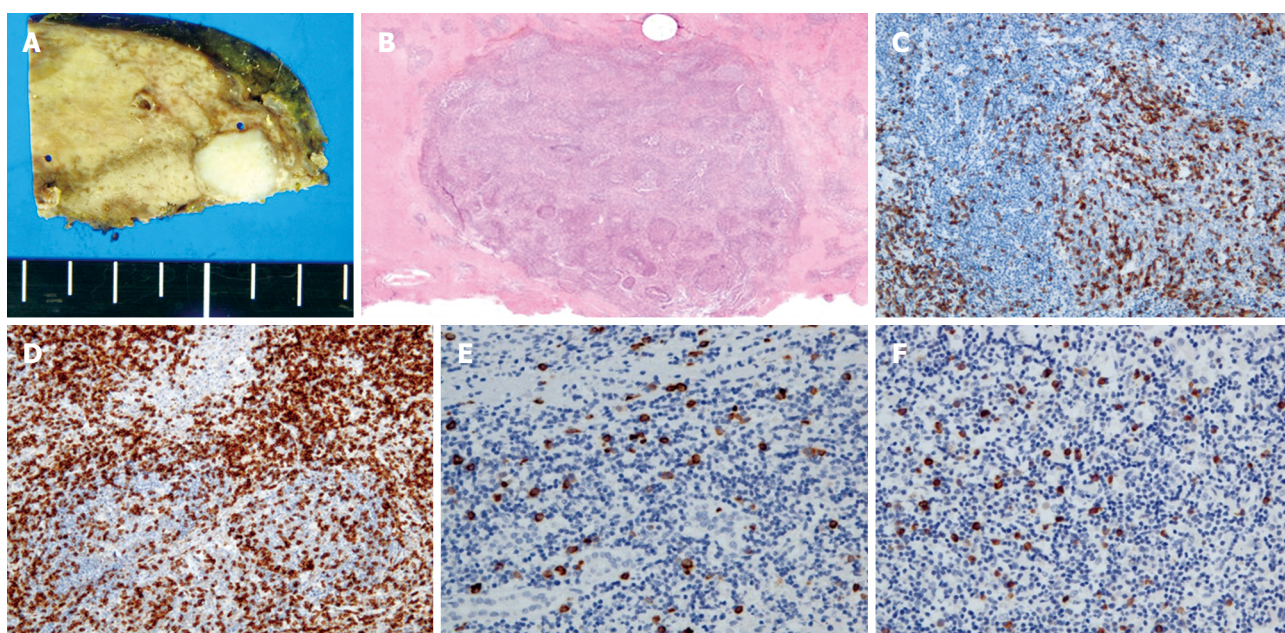


Figure 5 The pathological findings of the lesions. A: Macroscopically, the lesion was white and hard with a clear margin (a lesion in segment 7); B: Microscopically, the lesion consisted of a nodular lymphoid infiltrate with germinal centers (a lesion in segment 7), HE stain ($\times 40$); Lymphocytes in the lesions consisted of CD3-positive T-cells (C) and CD20-positive B-cells (D), Both $\times 100$; E: Stained for κ light chains with *in situ* hybridization ($\times 200$); F: Stained for λ light chains with *in situ* hybridization ($\times 200$).

enhanced MRI revealed another 4 mm hyperintense nodule in segment 6 in addition to the 10 mm hyperintense nodule in segment 7 (Figure 3). Although angiography *via* the common hepatic artery did not demonstrate tumor staining, CT during arterial portography revealed two hypointense nodules (Figure 4). Imaging findings suggested HCC, although no other hypervascular tumor could be excluded. The patient received an explanation of the results, including the possibility of a benign tumor, and expressed a desire for surgical extraction of the nodules. A right posterior segmentectomy was

performed. Macroscopically, the lesion in segment 7 was white and hard with clear margins (Figure 5A) and the lesion in segment 6 was not detected. Microscopically, two lesions showed similar histological features. The lesions exhibited a nodular infiltration of mature small lymphoid cells with many lymph follicles. No obvious atypical cells were identified in both lesions. Bile ducts were identified at the periphery of the lesions, although characteristic lymphoepithelial lesions could not be identified. No necrosis or granulomatous inflammation was identified (Figure 5B). Pseudolymphoma and extranodal marginal

Table 1 Reported cases of hepatic pseudolymphoma

No.	Author (reference)	Age	Sex	No.	Size (cm)	Pathological diagnosis	Association
1	Snover <i>et al</i> ^[32]	15	F	1		PL	Combined immunodeficiency, liver fibrosis
2	Grouls <i>et al</i> ^[1]	85	F	2	1.4, 0.8	PL	Gastric cancer
3	Tanabe <i>et al</i> ^[33]	30	F	1	1.5	PL	Acute enteritis
4	Isobe <i>et al</i> ^[34]	59	F	1	0.9	RLH	Diabetes mellitus
5	Ohtsu <i>et al</i> ^[6]	42	F	1	1.5	PL	Chronic hepatitis B, interferon- α therapy
6	Katayanagi <i>et al</i> ^[35]	66	F	2	1.5, 1.0	PL	Diabetes mellitus
7	Tanizawa <i>et al</i> ^[36]	67	F	1	2	RLH	Abnormal liver function
8	Endo <i>et al</i> ^[37]	38	F	1	1.8	PL	Pancytopenia
9	Kim <i>et al</i> ^[5]	72	M	1	1.7	PL	Chronic hepatitis C, gastric cancer
10	Fujinaga <i>et al</i> ^[38]	58	F	1	1.5	PL	Hypertension
11	Nishijima <i>et al</i> ^[39]	58	F	1	1.2	PL	Hypertension, diabetes mellitus
12	Sharifi <i>et al</i> ^[3]	52	F	1	0.4	NLL	Primary biliary cirrhosis
13		56	F	1	1.5	NLL	Primary biliary cirrhosis, CREST syndrome
14		56	M	1	0.7	NLL	Diverticulitis
15	Nagano <i>et al</i> ^[40]	47	F	1	1.7	RLH	Chronic thyroiditis, high titer of ANA
16	Pantanowitz <i>et al</i> ^[41]	69	F	2	1.7, 1.0	RLH	Renal cell carcinoma
17	Okubo <i>et al</i> ^[2]	49	F	1	2	PL	Sjögren's syndrome
18	Mori <i>et al</i> ^[42]	49	F	1	1.8	PL	Chronic hepatitis B
19	Okuhama <i>et al</i> ^[43]	70	M	1	4	PL	
20	Shiozawa <i>et al</i> ^[44]	51	F	1	2	PL	
21	Takahashi <i>et al</i> ^[45]	77	F	1	1.5	RLH	Colon cancer
22		64	F	2	0.9, 0.7	RLH	Colon cancer
23	Maehara <i>et al</i> ^[46]	72	F	2	1.3, 1.0	RLH	
24	Willenbrock <i>et al</i> ^[47]	36	F	1	1.8	NLL	Ovarian cyst, focal nodular hyperplasia of the liver
25	Sato <i>et al</i> ^[4]	75	F	1	1.4	RLH	Gastric cancer, colon cancers, metastatic liver tumor
27	Ota <i>et al</i> ^[20]	63	F	1	1.6	PL	Gastric ulcer, <i>Helicobacter pylori</i> infection
28	Machida <i>et al</i> ^[48]	53	F	3	1.5, 1.2, 1.0	RLH	Autoimmune thyroiditis
29	Matsumoto <i>et al</i> ^[49]	67	F	1	1.5	PL	Hypertension
30	Jiménez <i>et al</i> ^[50]	34	F	1	2.3	NLH	Hypothyroidism
31	Park <i>et al</i> ^[51]	46	F	2	1.0, 1.0	RLH	Renal cell carcinoma
32	Present case	63	F	2	1.3, 0.4	PL	Primary biliary cirrhosis, primary aldosteronism

RLH: Reactive lymphoid hyperplasia; PL: Pseudolymphoma; NLL: Nodular lymphoid lesion; NLH: Nodular lymphoid hyperplasia; CREST: Calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia; ANA: Antinuclear antibody.

zone B-cell lymphoma (MALToma) were differential diagnosis. Then, we performed immunohistochemical analysis and *in situ* hybridization. Immunostainings for CD3, CD20, and CD79 revealed regularly distributed T cells and B cells (Figure 5C and D). Plasma cells were not many in number in the lesions, but *in situ* hybridization for immunoglobulin light chains was performed because it is one of the useful tools to discriminate between reactive lymphoid lesions and MALToma. *In situ* hybridization revealed no significant difference between the numbers of cells positive for kappa-chain and lambda-chain (Figure 5E and F). Based on these histological features, the lesions were diagnosed as pseudolymphoma.

In the background liver, liver parenchyma showed bridging fibrosis with lymphoid infiltrate in portal tracts. Granulomas cholangitis and ductopenia were identified. Those histological features were consistent with Stage 2 PBC. The patient had an uneventful postoperative course and has shown no sign of recurrence for 11 mo.

DISCUSSION

In the liver, pseudolymphoma has also been variously termed as reactive lymphoid hyperplasia and nodular lymphoid lesion, and shows histological features of hyperplastic lymphoid follicles with polymorphic and polyclonal cell populations composed of small mature

lymphocytes, mature plasma cells, macrophages, stroma fibrosis and often numerous germinal centers. It is usually localized and well demarcated from surrounding tissue^[1]. Pseudolymphoma may occur at numerous sites including the stomach^[7], lung^[8], ocular adnexa^[9], hard palate and oral mucosa^[10], skin^[11], and breast^[12].

To the best of our knowledge, 32 cases (including ours) of hepatic pseudolymphoma have been reported in the English and Japanese literature (Table 1). There was a strongly asymmetric male-to-female ratio of 1:9.7 (3 M/29 F). While other sites of pseudolymphoma also tended to show asymmetry, only pseudolymphoma of the breast approached that of the liver in terms of asymmetry magnitude. All seven reported cases of breast pseudolymphoma were female^[12], but breast examination is limited almost exclusively to females, which could bias these findings. Reported male-female ratios of pseudolymphoma of the hard palate and oral mucosa^[10], lung^[8], and skin^[11] were 1:2.8, 1:1.2 and 1:1.8, respectively, while ocular adnexal lymphoid hyperplasia affects men and women about equally^[9]. Thus the extreme female asymmetry of hepatic pseudolymphoma is a unique characteristic.

Eight of 32 cases of hepatic lymphoma were associated with autoimmune disease and eight with malignant tumor (Table 1). In the lung, cases of pseudolymphoma associated with autoimmune disorders

such as Sjögren's disease were reported^[13], which is similar to the liver. In other organs, several factors are thought to be associated with pseudolymphoma, i.e. Epstein-Barr virus (hard palate and oral mucosa^[14]), mechanical stimulation (ear^[15]), anticonvulsant drugs (skin^[16]) and *Helicobacter pylori* (*H. pylori*) (stomach^[17,18]). Regarding the association of *H. pylori* with gastric pseudolymphoma, successful treatment of the pseudolymphoma by eradication of *H. pylori* has been reported^[19]. Ota *et al.*^[20] reported a case of hepatic pseudolymphoma in which the diameter decreased after *H. pylori* eradication. In the case reported here, the patient had no *H. pylori* infection.

Transformation of pseudolymphoma to lymphoma has been discussed. Malignant transformation of pseudolymphomas in the lung^[21] and stomach^[22] have been reported. These reports, however, either predated or did not include the use of immunofluorescent techniques. It is likely that these cases were in fact the early stage of primary lymphoma misinterpreted as benign^[8]. On the other hand, evidence of progression from histologically benign, immunohistochemically polyclonal lymphoid infiltrates to malignant lymphoma in cutaneous pseudolymphoma is well delineated in the literature^[23]. A potential association between lymphoma and PBC is suggested on the basis of individual reports in the literature^[24-29]. In a retrospective study by Panjala *et al.*^[30] based on an estimated 2,912 patients evaluated at their institution during a 22-year period, only 13 (an estimated 0.6%) patients were evaluated in referral visits for evidence of lymphoma. Although we could find one case of transformation of hepatic pseudolymphoma into lymphoma^[31], we were unable to deduce the long term natural course of hepatic pseudolymphoma since most reported cases of hepatic lymphoma underwent surgical resection.

In conclusion, we have reported a case of hepatic pseudolymphoma associated with PBC. Hepatic pseudolymphoma appears unique in its female preponderance and associated diseases. If hypervascular nodules in the liver of female patients with autoimmune disease are found, the possibility of pseudolymphoma should be considered. Cases of hepatic pseudolymphoma should be followed carefully as the exact nature of this disorder is still not fully understood.

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S- Editor Li LF L- Editor Cant MR E- Editor Ma WH

Repetitive response to gemcitabine that led to curative resection in cholangiocarcinoma

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Received: June 30, 2009 Revised: July 20, 2009

Accepted: July 27, 2009

Published online: September 28, 2009

Abstract

This study reports a case of unresectable intrahepatic mass-forming cholangiocarcinoma which showed a dramatic response to gemcitabine that led to curative resection and a long-term survival of more than five years. Six and five cycles of gemcitabine monotherapy were administered separately over a three-year period and a radical excision was performed at 4.5 years after diagnosis. This case indicates the role of gemcitabine as a neoadjuvant chemotherapeutic agent for cholangiocarcinoma and guarantees a randomized controlled prospective study.

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Key words: Cholangiocarcinoma; Gemcitabine; Neoadjuvant chemotherapy

Peer reviewer: Wei Tang, MD, EngD, Assistant Professor, H-B-P Surgery Division, Artificial Organ and Transplantation Division, Department of surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Kim SH, Kim IH, Kim SW, Lee SO. Repetitive response to gemcitabine that led to curative resection in cholangiocarcinoma. *World J Gastroenterol* 2009; 15(36): 4593-4595 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4593.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4593>

INTRODUCTION

Cholangiocarcinoma is a devastating malignancy with a

poor prognosis. Early diagnosis and extensive surgery offers the only chance for cure although other treatment modalities have been applied. Response rates to chemotherapy are relatively low although several kinds of chemotherapeutic agents such as gemcitabine are known to be effective. Furthermore, the role of neoadjuvant chemotherapy for unresectable cholangiocarcinoma has not been established either. Here we report the case of a patient with huge intrahepatic mass-forming cholangiocarcinoma who showed repetitive responses to a gemcitabine single regimen, and eventually underwent curative resection that led to long-term survival of more than five years.

CASE REPORT

A 54-year-old female patient was admitted with mild epigastric discomfort and a liver mass was detected by ultrasonography in January 2004. She had a history of cholecystectomy due to gallstone disease 20 years ago. She denied any recent history of medication or herbal treatments. She had never consumed raw fresh-water fish. An abdominal computed tomography (CT) scan showed a 10 cm sized irregular hypovascular mass occupying the left hepatic lobe and growing inferiorly to compress the gastric antrum and the pancreas (Figure 1A). Invasion of the middle hepatic vein and left portal vein were strongly suggested. Multiple lymph node enlargements were noted at the liver hilum and lesser omentum. Laboratory data showed a white blood cell count of $9560/\text{mm}^3$ (normal $4800-10800/\text{mm}^3$), hemoglobin 15.8 g/dL (normal 13-18 g/dL), platelet count of $203000/\text{mm}^3$, AST 33 IU/L (normal 12-33 IU/L), ALT 20 IU/L (normal 5-35 IU/L), total bilirubin 0.9 mg/dL (normal 0.2-1.2 mg/dL), and albumin 4.6 g/dL (normal 3.5-5.3 g/dL). A tumor marker study revealed AFP 2.3 ng/mL (normal 0-8 ng/mL), CEA 2.2 ng/mL (normal 0-5 ng/mL), and CA19-9 17.6 U/mL (normal 0-36 U/mL). A serologic study showed HBs Ag (-), ANA (-), HCV Ab (+) and HCV RNA PCR (-). Esophagogastroduodenoscopy and colonoscopy were nonspecific except for mild chronic gastritis and a small hyperplastic rectal polyp. An abdominal magnetic resonance imaging (MRI) reported T1 low and T2 slightly high lobulated contour solid mass showing a similar invasion pattern with the CT scan. Biliary dilatation at the upper portion of the mass was seen (image not shown). Percutaneous needle biopsy from the mass reported well differentiated cholangiocarcinoma (image not shown). Histo-

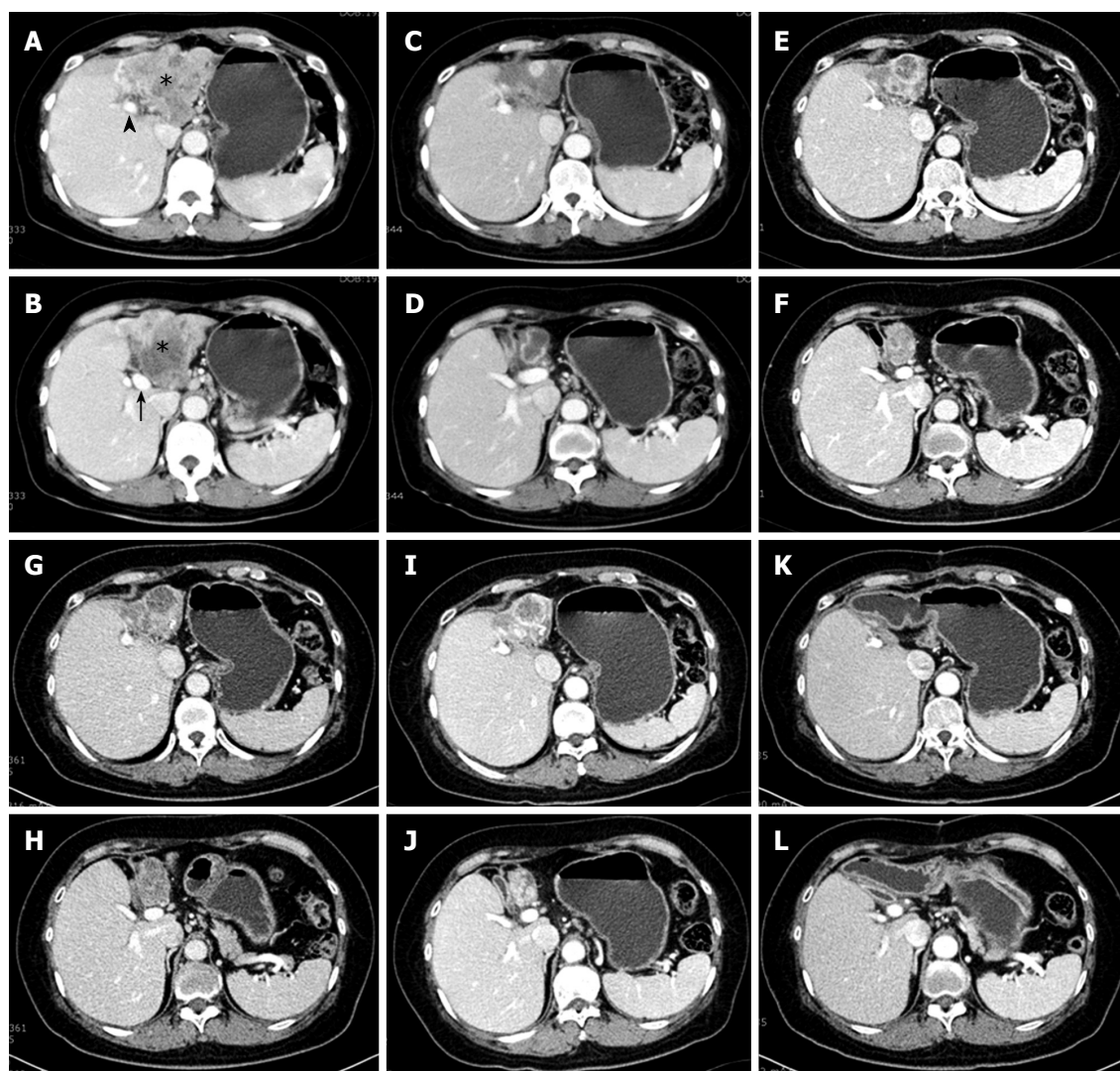


Figure 1 Abdominal CT scan images at two different levels are shown serially. A: At left portal vein level (black arrowhead), a 10 cm sized irregular hypovascular mass (asterisk) occupying left hepatic lobe is shown; B: At lower, main portal vein level (black arrow), the inferiorly grown mass is shown. Invasion of middle hepatic vein and left portal vein were strongly suggested with multiple lymph node enlargements at the liver hilum and lesser omentum; C and D: On CT scan taken after the end of the first 6 cycles (18 times) of gemcitabine chemotherapy, the tumor mass decreased in size dramatically, smaller than half of the previous diameter; E and F: On CT scan on 3 years and 3 mo from diagnosis, mass does not show any significant change except for a little sclerotic change; G and H: On CT scan on 4 years from diagnosis, tumor mass shows re-increment of the size than previous study; I and J: On follow up CT scan after second 5 cycles of gemcitabine chemotherapy, partial response is shown again with slight decrease of the mass size; K and L: On CT scan taken 11 months after R0 resection, there is no any recurrence or new lesion growing.

chemical and immunohistochemical study showed tumor cells were positive for CK-7, and negative for CK-19, and CK-20. An extended surgery was recommended to the patient after explaining the degree of tumor invasion and low possibility of curative resection. However, the patient declined any kind of treatment including surgery and chemoradiotherapy. After 3 mo from diagnosis, the patient was persuaded to undertake chemotherapy. Gemcitabine monotherapy was provided as intravenous 2 h infusions of 1000 mg/m² body surface area weekly every three out of 4 wk. A total of six cycles of chemotherapy were treated tolerably.

The CT scan taken on October 2004 (10 mo after diagnosis) showed that the tumor mass dramatically decreased in size and was smaller (half of the diameter) than that of the previous CT (Figure 1B). Surgery was again recommended to the patient. However, the patient

declined surgery and further chemotherapy. After ending chemotherapy treatment the tumor mass did not show any significant change on a regular CT scan over 3 years and 3 mo except for a little sclerotic change (Figure 1C).

Unfortunately, an abdominal CT scan in January 2008 showed a slight increase of the mass with a symptomatic complaint of fatigue (Figure 1D). Gemcitabine treatment was restarted under the same protocol as the previous one. The patient barely finished five cycles of treatment due to various symptoms such as weakness or edema. On follow up CT scan in May 2008, a partial response was repeated with a slight decrease in the mass size (Figure 1E). Radical surgery was recommended and the patient agreed to it. A curative resection of atrophied left liver including the mid-hepatic vein and extrahepatic bile duct was performed. A hepatico-enterostomy was followed after the resection. Histological findings

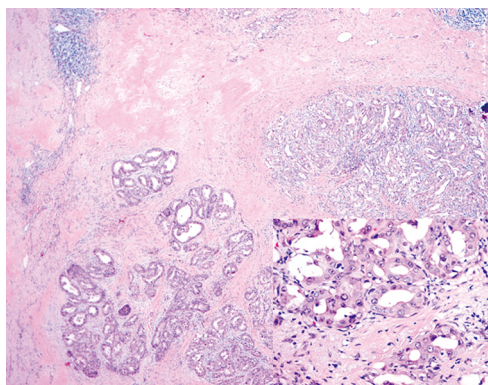


Figure 2 Histological finding of resected specimen is shown. Well differentiated adenocarcinoma is seen with glandular structure and a dense fibrous stroma. Scattered lymphocytes are also present (40 × magnification). In higher magnification, tumor is composed of glands lined by cuboidal mucin-producing epithelium resemble biliary epithelium (Inset, 400 × magnification).

of the resected specimen showed well differentiated cholangiocarcinoma composed of a glandular structure lined by cuboidal mucin-producing epithelium resembling cholangiocytes and a dense fibrous stroma with desmotic reaction (Figure 2). All resection margins were negative for malignancy microscopically. One cycle of adjuvant gemcitabine chemotherapy was administered due to concern about remnant tumor cells. On a CT scan taken 11 mo after R0 resection, there was no recurrence or new growing lesion (Figure 1F). In July 2009, 5 years and 6 mo from diagnosis (1 year from the surgical resection) the patient is living well without any symptoms.

DISCUSSION

The establishment of an optimal chemotherapeutic regimen for cholangiocarcinoma is necessary because most of the patients are diagnosed in an advanced stage and curative resection would not be viable. Gemcitabine is known as one of the most effective agents for cholangiocarcinoma and response rates were reported as 20%-35%^[1] for a single regimen^[2] or in combination

with other agents such as 5-FU (or capecitabine)^[3], mitomycin-C^[4], or a platinum analog^[5]. This case study showed repetitive responses to a gemcitabine single agent regimen given separately over a long-term interval. Tumors were observed to be strongly repressed for more than three years after the first cycle of treatment.

Studies in neoadjuvant settings for unresectable cholangiocarcinoma are very few although there was a remarkable success of a neoadjuvant protocol with the use of radiotherapy and capecitabine prior to liver transplantation^[6]. There was no report about gemcitabine based chemotherapy as a neoadjuvant approach combined with surgical tumor excision. In our case, a very large unresectable intrahepatic mass-forming cholangiocarcinoma showed a definite response to gemcitabine alone that eventually led the patient to a curative resection and long-term survival. This result suggests that gemcitabine based chemotherapy should be tried more actively for unresectable cholangiocarcinoma.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Ma WH



CASE REPORT

Post-gastrectomy acute pancreatitis in a patient with gastric carcinoma and pancreas divisum

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Received: July 11, 2009 Revised: August 6, 2009

Accepted: August 13, 2009

Published online: September 28, 2009

Abstract

Gastrectomy is commonly performed for both benign and malignant lesions. Although the incidence of post-gastrectomy acute pancreatitis (PGAP) is low compared to other well-recognized post-operative complications, it has been reported to be associated with a high mortality rate. In this article, we describe a 70-year-old man with asymptomatic pancreatic divisum who underwent palliative subtotal gastrectomy for an advanced gastric cancer with liver metastasis. His post-operative course was complicated by acute pancreatitis and intra-abdominal sepsis. The patient eventually succumbed to multiple organ failure despite surgical debridement and drainage, together with aggressive antibiotic therapy and nutritional support. For patients with pancreas divisum or dominant duct of Santorini who fail to follow the normal post-operative course after gastrectomy, clinicians should be alert to the possibility of PGAP as one of the potential diagnoses. Early detection and aggressive treatment of PGAP might improve the prognosis.

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Key words: Acute Pancreatitis; Gastrectomy; Pancreas divisum; Duct of Santorini; Laparoscopy

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Kuo IM, Wang F, Liu KH, Jan YY. Post-gastrectomy acute pancreatitis in a patient with gastric carcinoma and pancreas divisum. *World J Gastroenterol* 2009; 15(36): 4596-4600 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4596.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4596>

INTRODUCTION

Although gastrectomies for lesions of the stomach and duodenum have been performed safely for more than one hundred years, major complications such as anastomosis leakage, duodenal stump leakage and post-operative bleeding cannot be completely avoided even by experienced surgeons. In the past, most gastrectomies were performed for benign gastric or duodenal ulcer. One of the complications associated with these procedures was post-gastrectomy acute pancreatitis (PGAP). The pathogenesis of this condition is hypothesized to be related to pancreatic parenchymal injury secondary to severe adhesion of peripancreatic tissue, compromised pancreatic micro-circulation, hyperpressure of the duodenum, and edema or spasm of the major papilla^[1,2]. The incidence of PGAP has decreased in recent years, coinciding with the dramatic reduction in the need for surgical intervention of complicated peptic ulcer disease in the advent of improved medical and endoscopic management. Gastrectomies nowadays are performed mainly for patients with gastric malignancy. The "pancreatitis-like" presentation is mostly seen in patients with afferent loop obstruction after gastrectomies, mostly following Billroth II reconstruction. The true PGAP might be related to the extended lymph node dissection and resection of adjacent organs (such as splenectomy and distal pancreatectomy) in radical gastrectomies for gastric cancer^[3-5]. However, the correlation between pancreatic anomalies and PGAP has not been well documented in the literature. This article, therefore, reports a patient with concurrent advanced gastric cancer and pancreas divisum (PD) who had PGAP and died from multiple organ failure post-operatively.

CASE REPORT

A 70-year-old gentleman with a medical background of hypertension and asthma, presented with a 3 mo history

of poor appetite, 10 kg weight loss, postprandial fullness and nausea. There was, however, no history of tarry stool, hematemesis, fever or abdominal pain. The physical examination did not reveal any palpable abdominal mass, lymphadenopathy, anemia, or jaundice. All laboratory data, including biochemistry examination and hemogram, were within normal ranges except for a low hemoglobin level (10.3 g/dL). Esophagogastroduodenoscopy (EGD) was performed and one ulcerative mass over the posterior wall of antrum was identified (Figure 1). The histopathological examination of the biopsy of the gastric lesion confirmed that it was an adenocarcinoma. Dynamic CT was arranged for pre-operative staging. It revealed several cystic lesions of the liver and possible deformity of the duodenal bulb without definite evidence of metastatic disease (Figure 2). In addition, the gallbladder was contracted with suspicious wall thickening and heterogeneous content. At laparotomy, one unsuspected nodule was seen in segment 3 of the liver. The lesion was confirmed to be a metastatic liver nodule by frozen section. At the time of the operation, severe adhesion between the head of the pancreas and the peripyloric tissue was noted. As a result, a palliative subtotal gastrectomy with Billroth II gastrojejunostomy was performed due to partial outlet obstruction.

Post operatively, the patient had low-grade fever and persistent epigastric pain without jaundice, tarry stools, or active upper gastrointestinal hemorrhage. The laboratory tests on the 3rd postoperative day demonstrated a raised white cell count with left shift (11 500/ μ L), a platelet count of 176 000/ μ L and a hemoglobin of 9.5 g/dL. Serum biochemistry revealed a normal liver function and renal function but a raised C-reactive protein (CRP) of 193.27 mg/L. Slightly elevated amylase (185 U/L) and normal lipase (39 U/L) level were also noted. Chest X ray and urinary analysis were obtained and disclosed no pneumonia or urinary tract infection. Although the drainage fluid was clear, there was some yellowish and cloudy discharge from the abdominal wound. We collected the fluid for microbial analysis only and culture, which grew a yeast-like organism. Fever persisted despite the administration of intravenous antibiotics. Abdominal CT arranged on the 8th postoperative day demonstrated irregular pancreatic contours with diffuse enlargement and fluid accumulation in the lesser sac with retroperitoneal extension (Figure 3), suggestive of either an acute pancreatitis or duodenal stump leakage. The laboratory tests on the 10th postoperative day demonstrated a white cell count with marked left shift (8400/ μ L, 86% in segment form and 10% in band form), a decreased platelet count of 58 000/ μ L, a raised CRP of 247.95 mg/L, and normal serum level of pancreatic enzymes (amylase level was 79 U/L and lipase level was 26 U/L). In view of continuing clinical deterioration associated with peritonitis and intra-abdominal sepsis from a possible anastomosis leak rather than acute pancreatitis (according to the normal amylase and lipase levels), the patient underwent an exploratory laparotomy instead of percutaneous drainage of the fluid 10 d after the first operation. At laparotomy, colorless turbid fluid accumulation was found at the same regions as reported on the abdominal CT scan. There was no

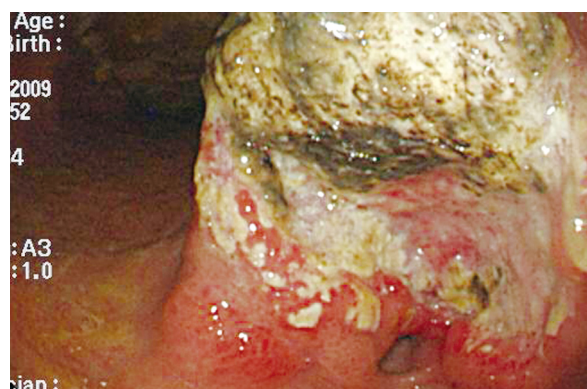


Figure 1 The esophagogastroduodenoscopy (EGD) showed an ulcerated, annular lesion over the gastric antrum.

evidence of bile leak, and the duodenal stump and anastomosis were intact. The necrotic tissues around the cavity of the loculated fluid collection had a similar appearance to fat necrosis in acute pancreatitis. Limited debridement was performed and a sump drain tube was placed for post-operative irrigation and drainage.

Bile-stained fluid was noted in the sump drain 2 d after the second operation, and the biochemistry study of the drainage fluid disclosed lower amylase and lipase levels (14 U/L and 9 U/L). Blood culture grew *Bacteroides fragilis* and hemogram showed progressive pancytopenia. A repeat CT scan suggested possible duodenal stump leakage with abscess formation (Figure 4). Despite total parenteral nutrition, hemodialysis for acute renal failure, and sump drain irrigation-drainage with intravenous antibiotics for septicemia and intra-abdominal infection, the patient eventually died of profound septic shock and multiple organ failure on day 30 after the first operation. A retrospective review of the patient's abdominal CT demonstrated a previously undiagnosed, asymptomatic PD (Figure 5).

DISCUSSION

Gastrectomy is a commonly practiced procedure for both benign and malignant lesions of the stomach. Major complications such as postoperative bleeding, anastomotic leak and delayed gastric emptying are well documented^[6]. In the past, most gastrectomies were performed for benign gastric or duodenal ulcer. The incidence of PGAP might be as high as 40.8% and the mortality rates in this group of patients ranged from 12.6% to 62.5%^[1,2]. Recently, gastrectomies have mostly been performed for gastric malignancies, and the complication and mortality rates are much less because of improved surgical technique and postoperative care. The incidence of PGAP nowadays, although difficult to accurately estimate, has been reported to be less than 5%^[7]. The mortality rate, however, can be up to 33.3%-50%^[8,9] and is higher than acute pancreatitis of other etiologies^[10]. Chen *et al.*^[3] reported a higher incidence of PGAP in patients having total gastrectomy compared with other types of gastrectomies (7.4% *vs* 0.8%), with a 33.3% mortality rate. Another study reported higher

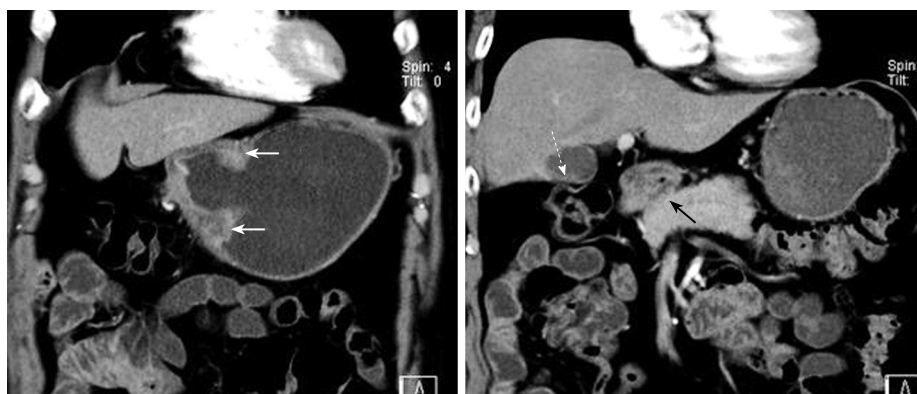


Figure 2 Preoperative abdominal CT images in coronary and transverse sections. The white solid arrows indicate diffuse wall thickening at the gastric antrum. The white dotted arrow indicates a contracted gallbladder with eccentric wall thickening. The black solid arrows point to the apparent deformity of the duodenal bulb with adhesion to the head of the pancreas. There is no evidence of intra-abdominal metastasis in this study.

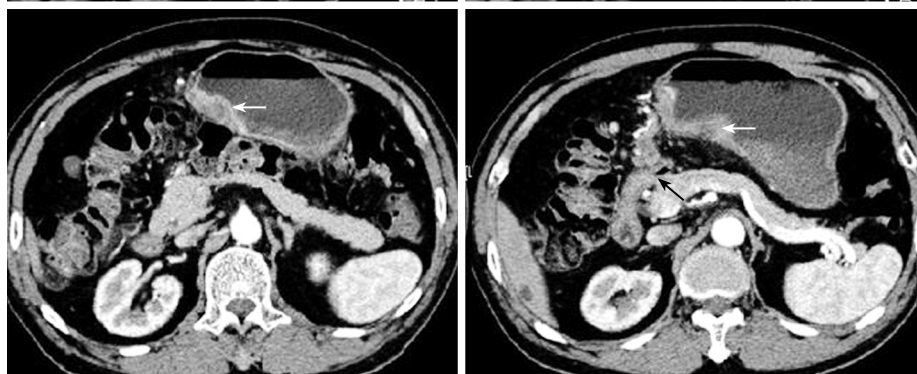


Figure 3 Abdominal CT 8 d after the first operation demonstrates massive fluid accumulation in the peripancreatic area and the lesser sac. The homogenous fluid extends to the retroperitoneal space. The status of the duodenal stump (black arrow) cannot be clearly assessed. The pancreas is well enhanced and enlarged, and the head shows an uneven and infiltrative margin (white arrow).

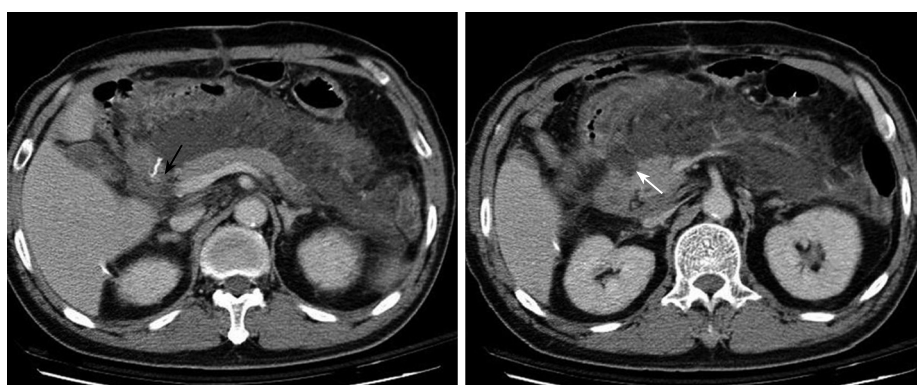


Figure 4 Follow-up abdominal CT after the second operation. Severe fat stranding is seen. An edematous duodenal stump is observed without a clear fat plane surrounding it. This is highly suspicious of a stump leak (black arrow). The loculated fluid with heterogeneous changes indicates the possibility of an abscess formation (white arrow).

PGAP rate in patients undergoing extended lymph node dissection^[4]. Despite the various advantages of laparoscopically assisted gastrectomy (LAG), the rates of acute pancreatitis after LAG have also been shown to range from 0.7% to 2.3%^[8,9,11]. The poorer prognosis of PGAP might be related to postoperative immunosuppression, increased pancreatitis-related hemorrhage because of extensive soft tissue/lymph node dissection, or anastomotic leak secondary to necrotizing pancreatitis

and intra-abdominal sepsis.

PGAP may be a result of several factors. The stomach/duodenum might adhere to the underlying pancreas because of severe peptic ulceration or desmoplastic change from malignancy. Sometimes, the pancreas might actually be the base of the ulcer. Injury to the pancreatic parenchyma by electrocauterization or traction, microcirculation compromise, and direct injury to the pancreatic duct might occur during gastrectomy^[7,11]. Other causes



Figure 5 The preoperative CT scan. The dominant dorsal duct (duct of Santorini) drains into the minor papilla (white arrow). The CBD is seen in the section (black arrow) but the ventral duct (duct of Wirsung) can not be traced. These features are consistent with pancreas divisum.

of PGAP such as duodenal hyperpressure^[1] and post-operative spasm of the major papilla^[2] have been hypothesized, but there is a general lack of evidence in the literature to substantiate these theories. Although some studies suggested insertion of a nasogastric tube or a duodenostomy tube after gastrectomy for decompression and releasing duodenal hyperpressure, Hsu *et al*^[12] reported no difference in PGAP in spite of NG tube insertion. On the contrary, the “pancreatitis-like” presentation (such as hyperamylasemia and epigastric pain) caused by afferent loop obstruction can be cured under careful management of operations or decompression procedures^[7,13]. The condition should be taken into consideration because the prognosis, timing of intervention and treatment options are far different from PGAP without A-loop obstruction.

The diagnosis of acute pancreatitis is mainly dependent on suggestive clinical features and laboratory studies. Physical examination may be variable and non-specific in postoperative patients because of pain and symptoms related to the operation. Elevated lipase levels 3-fold or more above the normal range appearing within 48 h is the most reliable test^[14]. However, normal serum pancreatic enzyme levels cannot exclude acute pancreatitis absolutely^[14]. Abdominal CT scan is particularly helpful in making a definitive diagnosis and excluding other differential diagnoses such as anastomotic leak, intra-abdominal abscess and hemorrhage^[15]. However, the anatomical distortion post-operatively often makes the interpretation of CT images difficult^[16]. This is exemplified by the present case report whereby a definitive diagnosis of PGAP was delayed because of equivocal pancreatic enzymes and non-specific CT findings of intra-abdominal fluid collection.

PD is a common congenital anomaly of the pancreas, with an incidence rate of 4% to 10%^[17-19]. Anatomically, the dorsal duct (duct of Santorini) becomes the dominant duct draining the majority of the pancreatic juice through the minor papilla. The ventral duct (duct of Wirsung) does not fuse with the dorsal duct and only drains a small portion of the pancreatic head through the major papilla. The diagnosis of PD is made with

endoscopic retrograde pancreatography (ERP) or magnetic resonance pancreatography (MRP). However, with the advent of multi-detector CT scans, high sensitivity and specificity for diagnosis of PD can be achieved by CT images in some particular conditions^[20-22]. The CT features consistent with PD include the presence of the dominant dorsal duct sign (the dorsal duct being larger than the ventral duct, or a missing ventral duct) and the miss-communication between the two pancreatic ducts^[20]. Both of these features were observed on the patient's CT images retrospectively (Figure 5). About 5% to 45.5% of patients with PD present with pancreatitis or chronic abdominal pain^[23,24]. The pathogenesis of this is attributable to the “relative stenosis” of the minor papilla as it drains the majority of the pancreatic juice through a small opening^[24], resulting in an increase in the intra-ductal pressure of the dorsal duct system (20-28 mmHg *vs* 8-14 mmHg in normal pressure of major papilla)^[25]. In addition, the unusual anatomical arrangement of the sphincter of the minor papilla could also contribute to the development of intra-ductal hypertension leading to pancreatitis^[26]. Kamisawa *et al*^[27] reported the high incidence of pancreatitis or pancreatic-type abdominal pain in patients with dominant duct of Santorini but without pancreas divisum.

According to these findings, it is reasonable to suppose that patients with PD might be more sensitive to minor injury of the pancreas and disturbance of its microcirculation. Once developed, it might progress precipitously to a more severe form and patients are slower to gain full recovery. Prophylactic administration of octreotide in the post-operative setting has been suggested in an attempt to prevent the development of PGAP^[7]. The roles of preoperatively prophylactic cannulation of minor papilla, stent placement, and sphincterotomy have not been reported yet. The potential benefits of these invasive procedures in patients with PD or dominant duct of Santorini undergoing gastrectomy warrant further investigation.

In conclusion, PGAP is a less frequent post-gastrectomy complication in the current era. However, diagnosis and intervention should be made as early as possible because of the relatively high mortality rate associated with PGAP. Theoretically, patients with pancreas divisum or dominant duct of Santorini might connect to PGAP but the absolute relationship should be explored. Although serum pancreatic enzymes play an important role in the diagnosis of acute pancreatitis, imaging study such as an abdominal CT scan is particularly helpful in patients at high risk for PGAP. The roles and benefits of prophylactic octreotide, preoperative cannulation/stenting of the minor papilla, and sphincterotomy of minor papilla have not been well documented and would therefore warrant further investigation.

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S- Editor Li LF L- Editor O'Neill M E- Editor Ma WH

Hemorrhagic hepatic cysts mimicking biliary cystadenoma

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Received: July 27, 2009 Revised: August 17, 2009

Accepted: August 24, 2009

Published online: September 28, 2009

Abstract

The hemorrhagic simple hepatic cyst is extremely rare and can sometimes be confused with biliary cystadenoma or cystadenocarcinoma. Here we present two cases of huge hemorrhagic simple hepatic cysts. Case 1 was a 43-year-old man with a cystic lesion measuring 13 cm × 12 cm in the right hepatic lobe. Ultrasound and computed tomography showed several mural nodules on the irregularly thickened wall and high-density straps inside the cyst. Case 2 was a 60-year-old woman with a huge cyst measuring 15 cm × 14 cm in the central liver. Ultrasound and magnetic resonance imaging showed the cystic wall was unevenly thickened and there were some flame-like prominences on the wall. The iconographic representations of the two cases mimicked biliary cystadenoma. Cystectomy and left hepatectomy were performed for the two patients, respectively. Both patients recovered quickly after their operations and showed no recurrence.

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Key words: Simple hepatic cyst; Intracystic hemorrhage; Biliary cystadenoma; Diagnosis; Treatment

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Zhang YL, Yuan L, Shen F, Wang Y. Hemorrhagic hepatic cysts mimicking biliary cystadenoma. *World J Gastroenterol* 2009; 15(36): 4601-4603 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4601.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4601>

INTRODUCTION

Intrahepatic cysts are generally classified as congenital cyst, traumatic cyst, infectious cyst, parasitic cyst, or neoplastic cyst. Congenital hepatic cysts include simple hepatic cysts and adult polycystic liver disease. A few simple hepatic cysts can reach large sizes and occasional complications, such as rupture, infection, hemorrhage, obstructive jaundice, or portal hypertension may occur.

Intracystic hemorrhage is an extremely rare complication of simple hepatic cysts and few cases have been reported worldwide. The hemorrhage usually occurs in solitary huge hepatic cysts in older patients. Massive bleeding into the cysts can significantly enlarge the cysts in a short time, which might partly contribute to the huge size of the cysts. The clinical manifestations of the hemorrhagic hepatic cysts are usually lack of specificity and the iconographic representations often mimic biliary cystadenoma or cystadenocarcinoma. Therefore, it is sometimes very difficult to make a precise diagnosis and select the appropriate treatment for this disease in clinical practice.

Here we present two cases of huge hemorrhagic simple hepatic cysts mimicking biliary cystadenoma, and discuss the diagnosis and the treatment of the disease.

CASE REPORT

Case 1 was a 43-year-old man admitted to hospital complaining of right upper quadrant abdominal pain for seven days. No positive clinical signs were found upon physical examination. The patient's serum carbohydrate antigen 19-9 (CA 19-9) concentration was four times higher than normal. Abdominal ultrasound examination showed a liquid anechoic area measuring 13 cm × 12 cm in right hepatic lobe. The cyst had an irregularly thickened wall with several mamelons on it. There were flocculation echoes inside the cyst. A computed tomography (CT) scan showed a low density cystic lesion with high density straps inside. The thickened cyst wall and mural nodules were not enhanced on contrast CT (Figure 1).

Case 2 was a 60-year-old woman admitted to hospital with a huge cystic liver mass found by regular ultrasound examination without any symptoms. The mass could be partly palpated below the right costal margin. The patient's serum concentration of CA 19-9 was normal. Abdominal ultrasound examination showed

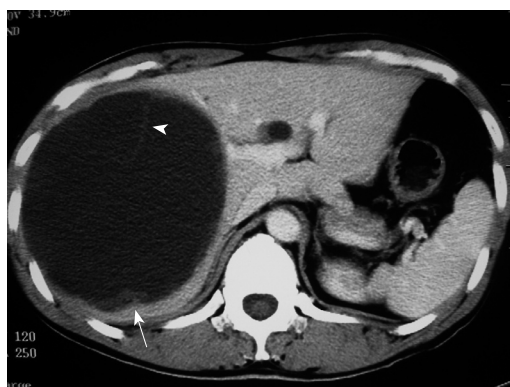


Figure 1 CT scan showing a huge low density cystic lesion in the right liver with high density straps (arrowhead) inside and mural nodules (arrow) on the cyst wall.



Figure 2 MRI (T2 weighted sequence) showing a huge cyst in the central liver with liquid content and flame-like prominences inside.

a huge echoless cyst measuring 15 cm × 14 cm in the central liver. The margin of the cyst was clear but its wall was unevenly thickened. There were some flame-like prominences on the wall. MRI showed the cyst content was liquid; the flame-like prominences were seen but not enhanced with contrast (Figure 2).

It was difficult to make a diagnosis in either case preoperatively. Biliary cystadenoma was highly suspected. Operations were performed for both cases. Case 1 received a cystectomy and case 2 received a left hepatectomy. The pathological diagnosis was hepatic simple cysts accompanied by intracystic hemorrhage. The mural nodules and prominences on the cyst wall were blood clots. Both patients recovered quickly after their operations and have been alive for six years and two years, respectively.

DISCUSSION

The clinical manifestation of this disease is lack of specificity. Generally the patients might have acute or chronic upper abdomen pain, but some patients have no symptoms^[1-4]. Extra attention should be paid to patients whose symptoms are relative to the severe complications caused by the intracystic hemorrhage. For example, Iguchi *et al*^[5] reported a 76-year-old man with severe edema of both lower extremities and a CT scan revealed an enlarged hemorrhagic hepatic cyst compressing inferior vena cava (IVC) in which massive thromboses formed. Buyse *et al*^[6] reported a patient with simple hepatic cyst developed acute dyspnoea. It was found that the intracystic bleeding led to the enlargement of the cyst and compression of the IVC, followed by pulmonary embolism and dyspnoea. The clinical signs of hemorrhagic simple hepatic cysts depend on the size, location, and complications of the cysts, in which abdominal mass is more common.

The iconographic representations of hemorrhagic hepatic cyst sometimes mimic biliary cystadenoma or cystadenocarcinoma. The common manifestation on ultrasound includes hepatic cystic mass, hyperechoic intracystic straps, and irregularly thickened cyst wall with flame-like prominences or convex palliates. The mural nodules can sometimes be seen to be mildly enhanced

on contrast enhanced CT and MRI^[2], which increases the difficulty in differential diagnosis with cystic neoplasms. In fact, as proved by surgery and histological examination, the mural nodules are usually blood clots or organized hematomas^[1,2]. In our cases, although the mural nodules and prominences on the cyst wall were not enhanced on contrast CT and MRI, the suspicion of biliary cystadenoma could not be precluded.

The distinction between hemorrhagic simple hepatic cysts and biliary cystic neoplasms can prove difficult to determine by clinical and iconographic features alone. For differential diagnosis, percutaneous transhepatic aspiration might be helpful if serosanguineous fluid is obtained^[4]. However, the puncture should be forbidden if malignancy is highly suspected. Naganuma *et al*^[7] reported that contrast-enhanced ultrasound clearly showed microbubbles oozing from the cyst wall into the cyst cavity in a case of hepatic cyst with intracystic bleeding, suggesting contrast-enhanced sonography might be a useful diagnostic tool for the disease. Akiyama *et al*^[8] also reported that Levovist ultrasonography imaging could play an important role in the correct diagnosis of a simple hemorrhagic cyst, by demonstrating the avascularity of the visualized intracystic structures. Horsmans *et al*^[9] found in a four-patient small group that both serum and cystic fluid CA 19-9 levels were elevated in the two patients with cystadenoma or cystadenocarcinoma, but remained normal in the other two patients with hemorrhagic simple cyst. This suggested that the determination of serum and cyst fluid CA 19-9 levels might be of help in distinguishing between hemorrhagic simple cyst and cystadenoma or cystadenocarcinoma. However, other studies revealed that the CA 19-9 levels in neither serum nor cyst fluid had a relationship to the benign or malignant property of hepatic cystic lesions^[10-12]. In the present two cases, the serum CA 19-9 level was high in one case but normal in the other case, which seems to agree with the latter standpoint. Except for biliary cystadenoma and cystadenocarcinoma, other hepatic lesions such as hemangioma^[13], abscess, and parasitic cyst should also be differentially diagnosed.

Treatment for the hemorrhagic simple hepatic cysts should be active because intracystic bleeding can enlarge

the cysts significantly and cause severe complications, such as rupture, infection, and compression of the IVC leading to venous return obstruction or thrombosis. For poorly conditioned patients, transcatheter arterial embolization, transhepatic cyst drainage, intracystic ethanol injection or noninvasive methods are indicated. These methods can obtain therapeutic effect in some patients, though the bleeding may recur afterwards^[3-5,14,15]. Surgery should be performed for most well conditioned patients, especially those whose diagnosis does not preclude malignancy. The operations can include partial hepatectomy, cystectomy, and fenestration. Partial hepatectomy and cystectomy can remove the whole cyst and reach curative effect^[1-4,8,9,11,13,16]. Occasionally, a fenestration operation is performed to simplify the procedure, which can also obtain a good therapeutic effect^[6].

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ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Meetings

Events Calendar 2009

January 12-15, 2009
 Hyatt Regency San Francisco, San Francisco, CA
 Mouse Models of Cancer

January 21-24, 2009
 Westin San Diego Hotel, San Diego, CA
 Advances in Prostate Cancer Research

February 3-6, 2009
 Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
 Second AACR Conference
 The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
 Hyatt Regency Boston, Boston, MA
 Translation of the Cancer Genome

February 8-11, 2009
 Westin New Orleans Canal Place, New Orleans, LA
 Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
 Hong Kong Convention and Exhibition Centre, Hong Kong, China
 19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
 Orlando, Florida
 AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
 Vienna, Austria
 EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
 Phoenix, Arizona
 AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
 Marriott Wardman Park Hotel
 Washington, DC
 13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
 Glasgow, Scotland
 British Society of Gastroenterology (BSG) Annual Meeting
 Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
 Silver Spring, Maryland
 2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
 Colorado Convention Center, Denver, CO
 AACR 100th Annual Meeting 2009

April 22-26, 2009
 Copenhagen, Denmark
 the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
<http://www.easl.ch/>

May 17-20, 2009
 Denver, Colorado, USA
 Digestive Disease Week 2009

May 29-June 2, 2009
 Orange County Convention Center
 Orlando, Florida
 45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
 Chicago, Illinois
 Endpoints Workshop: NASH

May 30-June 4, 2009
 McCormick Place, Chicago, IL
 DDW 2009
<http://www.ddw.org>

June 17-19, 2009
 North Bethesda, MD
 Accelerating Anticancer Agent Development

June 20-26, 2009
 Flims, Switzerland
 Methods in Clinical Cancer Research (Europe)

June 24-27, 2009
 Barcelona, Spain
 ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
 Beijing International Convention Center (BICC), Beijing, China
 World Conference on Interventional Oncology
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009
 Snowmass, CO, United States
 Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
 Aspen, CO, United States
 Molecular Biology in Clinical Oncology

August 1-7, 2009
 Vail Marriott Mountain Resort, Vail, CO, United States
 Methods in Clinical Cancer Research

August 14-16, 2009
 Bell Harbor Conference Center, Seattle, Washington, United States
 Practical Solutions for Successful Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
 Beijing International Convention Center (BICC), Beijing, China
 19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
 Taipei, China
 Asian Pacific Digestive Week
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009
 Boston Park Plaza Hotel and Towers, Boston, MA, United States
 Frontiers in Basic Cancer Research

October 13-16, 2009
 Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
 Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
 Versailles, France
 Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
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 The Liver Meeting

November 15-19, 2009
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 AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009
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 Gastro 2009 UEGW/World Congress of Gastroenterology
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Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua 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 PMID: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 1274 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 biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 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. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S,** Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious 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/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Current Contents®/Clinical Medicine, Science
Citation Index Expanded (also known as SciSearch®),
Journal Citation Reports/Science Edition, *Index
Medicus*, MEDLINE, PubMed, Chemical Abstracts,
EMBASE/Excerpta Medica, Abstracts Journals,
PubMed Central, Digital Object Identifier,
CAB Abstracts, and Global Health.
ISI, Thomson Reuters, 2008 Impact Factor: 2.081
(32/55 Gastroenterology and Hepatology).

Volume 15 Number 37
October 7, 2009

World J Gastroenterol
2009 October 7; 15(37): 4609-4736

Online Submissions

wjg.wjgnet.com
www.wjgnet.com

Printed on Acid-free Paper

世界胃肠病学杂志

World Journal of Gastroenterology®

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2007-2009



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^[1]Passed away on October 20, 2007

^[2]Passed away on June 14, 2008



World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 37
October 7, 2009



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NAME OF JOURNAL

World Journal of Gastroenterology

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Department of Science and Technology
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Taiyuan Research and Treatment Center
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PUBLISHING

The WJG Press and Beijing Baishideng
BioMed Scientific Co., Ltd., Room 903,
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District, Beijing 100025, China
Telephone: +86-10-59080039
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PRINTING

Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR

Beijing Bureau for Distribution of
Newspapers and Journals
(Code No. 82-261)
China International Book Trading
Corporation PO Box 399, Beijing,
China (Code No. M4481)

PUBLICATION DATE

October 7, 2009

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SUBSCRIPTION

RMB 50 Yuan for each issue, RMB
2400 Yuan for one year

CSSN

ISSN 1007-9327
CN 14-1219/R

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Involvement of interleukin-15 and interleukin-21, two γ -chain-related cytokines, in celiac disease

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Received: July 21, 2009 Revised: August 25, 2009

Accepted: September 1, 2009

Published online: October 7, 2009

15(37): 4609-4614 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4609.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4609>

Abstract

Celiac disease (CD), an enteropathy caused by dietary gluten in genetically susceptible individuals, is histologically characterized by villous atrophy, crypt cell hyperplasia, and increased number of intra-epithelial lymphocytes. The nature of CD pathogenesis remains unclear, but recent evidence indicates that both innate and adaptive immune responses are necessary for the phenotypic expression and pathologic changes characteristic of CD. Extensive studies of molecules produced by immune cells in the gut of CD patients have led to identification of two cytokines, namely interleukin (IL)-15 and IL-21, which are thought to play a major role in orchestrating the mucosal inflammatory response in CD. Here we review the current knowledge of the expression and function of IL-15 and IL-21 in CD.

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Key words: Interleukin-21; Interleukin-15; Celiac disease

Peer reviewers: Wolfgang R Stremmel, Professor, Department of Gastroenterology, University Hospital Heidelberg, Medizinische Universitätsklinik, Heidelberg 49120, Germany; Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia; Hitoshi Asakura, Director, Emeritus Professor, International Medical Information Center, Shinanomachi Renga Bldg.35, Shinanomachi, Shinjuku, Tokyo 160-0016, Japan

De Nitto D, Monteleone I, Franzè E, Pallone F, Monteleone G. Involvement of interleukin-15 and interleukin-21, two γ -chain-related cytokines, in celiac disease. *World J Gastroenterol* 2009;

INTRODUCTION

Celiac disease (CD) is a chronic gastrointestinal disorder caused, in genetically predisposed individuals, by ingestion of the gluten proteins of wheat, rye, and barley. The prevalence of CD is approximately 1% in the Western world^[1-3]. The disease is strongly associated with *HLA-DQ* genes. Most patients carry a variant of DQ2 (alleles DQA1*05/DQB1*02) and others carry a variant of DQ8 (alleles DQA1*03/DQB1*0302)^[4,5]. These haplotypes are relatively common in the healthy population, and only 1:20 individuals who express HLA-DQ2/DQ8 have CD^[4,5]. These observations and the fact that the concordance rate between HLA-identical siblings is much lower than between monozygotic twins suggest that most probably other non-HLA genes are involved in the pathogenesis of CD^[6-8].

In CD patients, ingestion of gluten triggers a mucosal inflammatory response, which leads to the tissue damage. The histological features of CD are villous atrophy, crypt cell hyperplasia, and increased number of intra-epithelial lymphocytes (IELs). The clinical manifestations can range from asymptomatic to severe malabsorption. Patients can also manifest symptoms of other immune-mediated diseases, which can associate with CD. The only treatment is a lifelong gluten-free diet, which results, in the vast majority of patients, in complete remission of symptoms and recovery of the normal mucosal histology^[9].

Although the pathogenesis of CD is not fully understood, it is known that gluten peptides are deamidated by tissue transglutaminase and presented by DQ2⁺ or DQ8⁺ antigen-presenting cells to lamina propria CD4⁺ T cells^[9,10]. Upon activation, CD4⁺ T cells polarize along the T helper (Th)1-type pathway, as substantiated by their ability to produce large amounts of interferon (IFN)- γ , the signature cytokine of Th1 responses^[11,12]. In CD patients on a gluten-free diet, IFN- γ production is as low as in healthy controls but it can be stimulated *in vitro* by gluten to reach levels of untreated CD patients. In these mucosal cultures, neutralization of IFN- γ prevents gliadin-mediated

morphological changes thus supporting the role of the adaptive immune response and IFN- γ in CD immunopathology^[11,12]. More recently it has become clear that some gluten peptides can induce mucosal damage by directly activating innate immune mechanisms^[13]. These observations collectively underline the complexity of the pathogenic mechanism in CD and suggest that the CD-associated mucosal damage relies on the activation of multiple rather than single cell pathways.

GLUTEN PEPTIDES STIMULATE INNATE IMMUNITY AND CAUSE EPITHELIAL DAMAGE VIA AN INTERLEUKIN (IL)-15-DEPENDENT MECHANISM

It has long been known that gluten peptides cause epithelial damage when added to *ex vivo* organ cultures of biopsies taken from CD patients on a gluten-free diet, but not from controls^[14]. As previously mentioned, this pathogenic response was initially thought to be secondary to the activation of lamina propria CD4⁺ T cells and production of inflammatory cytokines, such as IFN- γ ^[11]. However, more recently Maiuri *et al*^[13] showed that the gluten p31-43 peptide is able to induce mucosal damage by directly activating innate immune cells. In particular, it was shown that the p31-43 peptide elicits the production of IL-15 by lamina propria macrophages and dendritic cells in *ex vivo* organ cultures of CD biopsies, thus triggering a sequence of events that culminates in epithelial damage (Figure 1). These findings correlate well with the demonstration that IL-15 is over-expressed in both the lamina propria and intestinal epithelium of patients with active, untreated CD as compared with normal controls or inactive, gluten-free diet treated CD patients^[15,16].

The majority of intestinal IELs are T cell receptor (TcR) $\alpha\beta^+$ CD8⁺CD4⁻ and a significant proportion are TcR $\gamma\delta^+$ CD8⁺CD4⁻^[14]. IELs express a variety of natural killer (NK) lineage receptors, supporting their involvement in epithelial cell damage^[14]. Corroborating Maiuri's results, Hüc *et al*^[17] showed that treatment of biopsies taken from inactive CD patients with the gluten p31-49 peptide enhances the enterocyte expression of the non-conventional HLA molecules MICA/B. MIC molecules are known to be induced on enterocytes by stress and are up-regulated in active CD mucosa^[18]. MICA/B are ligands of the activating NKG2D receptor, which signals through the adaptor protein DAP10 and is expressed on most NK cells, CD8⁺ TcR $\alpha\beta^+$ and TcR $\gamma\delta^+$, but normally not on CD4⁺ T cells^[19,20]. Expression of NKG2D in IELs is increased in active CD mucosa, and IELs lyse epithelial cells *via* NKG2D^[17]. Up-regulation of MICA/B molecules also occurs in CD biopsies treated with exogenous IL-15, and a neutralizing IL-15 antibody blocks the gluten-mediated MIC-inducing effect in organ cultures of CD biopsies^[17,21]. Meresse *et al*^[22] showed that IL-15 also enhances the expression of both NKG2D and DAP10 in IELs, and that the cytolytic

attack of the epithelium by IELs may be perpetuated *via* NKG2D independently of TcR specificity (Figure 1). Taken together these findings emphasize the critical role of IL-15 in intestinal epithelial cell death induced by NKG2D-expressing IELs.

IL-15 AND COUNTER-REGULATORY MECHANISMS IN CD

In the gut, mucosal homeostasis arises from a highly dynamic balance between host protective immunity and regulatory mechanisms^[23]. One such counter-regulatory mechanism involves transforming growth factor- β 1 (TGF- β 1), a cytokine that is able to exert a number of negative effects on immune cells, including inhibition of T cell proliferation and differentiation, as well as down-regulation of macrophage activation and dendritic cell maturation^[24]. Consistently, mice with global TGF- β 1 defects, such as TGF- β 1-deficient mice or transgenic mice expressing a dominant-negative TGF- β RII chain that are unresponsive to TGF- β 1 signaling, develop intestinal mucosal inflammation^[25,26]. On the other hand, studies in mouse models of gut inflammation have shown that production of TGF- β 1 is consistently associated with greatly diminished severity of inflammation^[27]. Because TGF- β 1 regulates both lymphoid and myeloid cells^[24], several researchers have examined the expression and activity of TGF- β 1 in chronic gastrointestinal inflammatory diseases, including CD. Initial studies conducted by Lahat *et al*^[28] showed that in active CD mucosa there was enhanced expression of transcripts for TGF- β 1 as compared to controls. In contrast, Lionetti *et al*^[29] reported that the total RNA expression of TGF- β 1 did not differ between active CD and controls. However, in active CD, TGF- β 1 production was mostly confined to lamina propria T cells and macrophages, while in controls it was mostly produced by epithelial cells^[29].

TGF- β 1 initiates signaling through the ligand-dependent activation of a complex of heterodimeric transmembrane serine/threonine kinases, consisting of type I (TGF- β RI) and type II (TGF- β RII) receptors^[30]. Upon TGF- β 1 binding there is phosphorylation and activation of TGF- β RI by the constitutively active and auto-phosphorylating TGF- β RII. TGF- β RI in turn phosphorylates two proteins, termed Smad 2 and Smad3. Once phosphorylated, Smad2 and Smad3 associate with Smad4 and translocate to the nucleus where Smad protein complexes participate in transcriptional control of target genes^[30]. This pathway has been reported to be antagonized by inflammatory cytokines, which can impair the regulatory functions of TGF- β 1 and alter immune homeostasis^[31,32]. Therefore, it is conceivable that, during chronic inflammatory processes, there may be deficient TGF- β 1 activity despite this cytokine being highly produced. In line with this, Benahmed *et al*^[33] have recently shown that TGF- β 1 inhibited the proliferative response of normal intestinal IELs and lamina propria

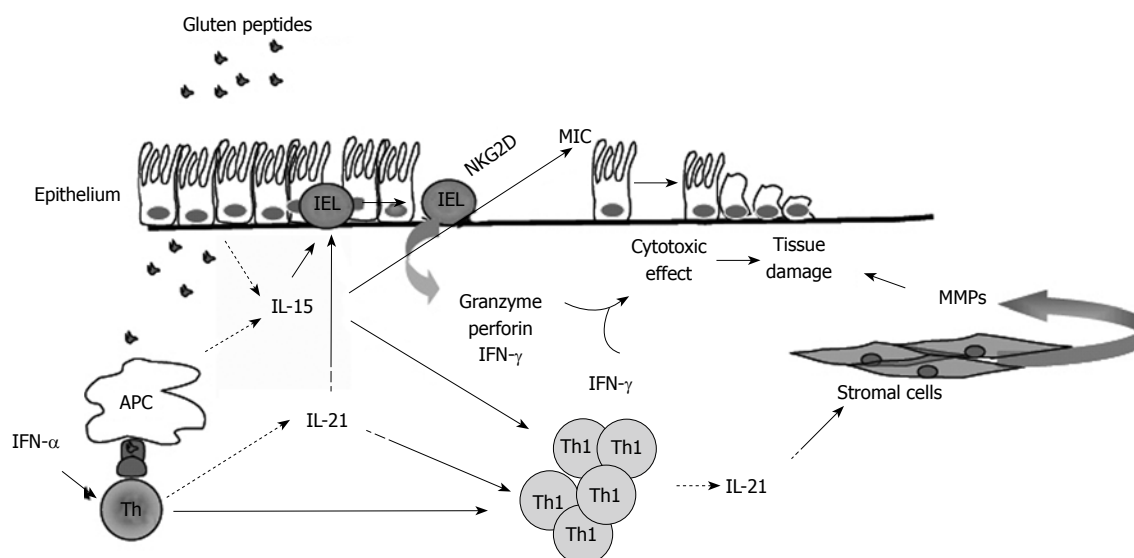


Figure 1 Hypothetical view of the pathogenic effects of IL-15 and IL-21 in celiac disease. In active celiac disease mucosa, IL-15 is produced by epithelial cells and antigen presenting cells (APC) in response to gluten stimulation. APC also produce interferon (IFN)- α , which stimulates CD4⁺ T cells to polarize along the Th1 pathway. IL-21 is produced by activated CD4⁺ T cells, including Th1 cells. IL-15 activates various pathways, which lead to the lysis and death of epithelial cells. IL-15 augments the production of granzyme B by IELs and IFN- γ by IELs and Th1 cells; enhances the expression of the activating NKG2D receptor on IELs; induces the NKG2D ligand, MICA, on epithelial cells. IL-21 seems to contribute to the tissue-damaging immune response in CD, given that this cytokine is able to enhance the production of perforin by IELs and IFN- γ by T cells, and to stimulate stromal cells to make extracellular matrix-degrading metalloproteinases.

lymphocytes induced by IL-2 but not that triggered by IL-15. The inhibitory effect of IL-15 on TGF- β 1 activity was substantiated further by the demonstration that IL-15 impaired the formation of Smad3-DNA complexes in response to TGF- β 1 stimulation^[33]. In contrast, IL-15 enhanced the activation of the Janus kinase (JNK) pathway thus promoting the inhibitory effect of phospho-c-jun on the formation of Smad-DNA complexes. Further analysis revealed that the transcription levels of tristetraprolin, a TGF- β target gene under the control of the Smad3 pathway, were decreased in duodenal biopsies from patients with active CD compared with controls. This defect was associated with no significant change in the intracellular levels of phosphorylated Smad3 and Smad7, clearly indicating that initial steps of TGF- β signaling are preserved in patients with active CD^[33]. This is in contrast to the Smad7-dependent inhibition of TGF- β 1/Smad3 signaling described in patients with inflammatory bowel diseases^[34,35].

IELs, lamina propria lymphocytes, and epithelial cells of active CD contain high levels of phospho-c-jun, and inhibition of this protein in organ cultures of CD biopsies enhances tristetraprolin^[33]. Moreover, treatment of *ex vivo* organ cultures of CD biopsies with anti-IL-15 reduces phospho-c-jun and increases tristetraprolin, thus confirming the prominent role of IL-15 in the phospho-c-jun-mediated inhibition of TGF- β 1 signaling^[33].

While NKG2C and NKG2D are activating NK receptors, NKG2A is considered an inhibiting NK receptor. NKG2A can associate with CD94 and bind the non-classical MHC Ib molecule, HLA-E, which is expressed on epithelial cells of CD patients but not on epithelial cells of healthy individuals^[36,37]. Although NKG2A competes with NKG2C for its interaction

with HLA-E, the former has a higher binding affinity for HLA-E compared with NKG2C. Upon binding to this ligand, CD94/NKG2A delivers negative signals to cytotoxic cells^[38]. A higher percentage of small intestinal TcR $\gamma\delta$ IELs express CD94/NKG2A compared with TcR $\alpha\beta$ IELs, and TcR $\gamma\delta$ IELs exert negative effects on the IL-15-mediated induction of IFN- γ , granzyme B, and NKG2D in CD8⁺ TcR $\alpha\beta$ IELs^[39]. These observations suggest that TcR $\gamma\delta$ IELs have the ability to suppress the cytotoxic programming of TcR $\alpha\beta$ IELs. This inhibitory effect requires interaction of NKG2A with its ligand HLA-E, a phenomenon which is followed by enhanced secretion of TGF- β 1. Notably, blockade of TGF- β 1 activity with a neutralizing human TGF- β 1 antibody partially abrogates the suppressive capability of TcR $\gamma\delta$ IELs, and exogenous TGF- β 1 dose-dependently inhibits the IL-15-mediated induction of IFN- γ , granzyme B, and NKG2D in CD8⁺ TcR $\alpha\beta$ IELs^[39]. Therefore, in this cell context, IL-15 is not sufficient to abrogate the immunosuppressive action of TGF- β 1. These findings conflict with the aforementioned data published by Benahmed *et al.*^[33]. It is likely that this discrepancy may simply be due to differences in cell culture conditions used in these two studies. Another possibility is that the IL-15-mediated negative regulation of TGF- β 1 signaling occurs only in specific cell types. Since TGF- β 1 can trigger both Smad-dependent and -independent intracellular pathways^[39,40], it is also plausible that IL-15 can interfere with some and not all TGF- β 1-activated signals.

Expression of NKG2A is decreased on TcR $\alpha\beta$ IELs and TcR $\gamma\delta$ IELs from CD patients as compared to normal subjects^[39-41]. The mechanism that removes this negative regulator from CD IELs remains unknown. NKG2A possesses in its promoter a binding site for

Smad3, and there is evidence that TGF- β 1 cooperates with the TcR in enhancing the expression of NKG2A on CD8⁺ T cells^[18,42]. Therefore, molecules that disrupt TGF- β 1 signaling could, at least in theory, contribute to the down-regulation of NKG2A in CD. IL-15 is not able to directly regulate NKG2A expression^[43], even though it remains possible that it could inhibit the TGF- β 1-induced NKG2A expression.

IL-21 as a positive regulator of Th1 cell response in CD

The demonstration that CD lesions are associated with a marked infiltration of IFN- γ -secreting cells has boosted intensive research aimed at identifying the factors that promote the ongoing mucosal Th1 cell response. Paradoxically, IL-12, the major Th1-inducing factor in man, is not over-produced in CD mucosa^[12]. However, analysis of transcription factors that drive Th cell differentiation revealed that in active CD mucosa there is enhanced expression of T-bet, a member of the T-box family of transcription factors that directs Th1 lineage commitment and is essential for IFN- γ production in CD4⁺ T cells^[43]. In contrast, no increase in GATA3 and active STAT6, two transcription factors that specifically regulate Th2 differentiation, is seen in active CD samples as compared to controls^[43]. Interestingly, active CD biopsies do not exhibit enhanced activation of STAT4^[43], an IL-12-dependent Th1-inducing transcription factor, thus confirming the lack of IL-12 activity. These data strongly support the existence of a regulatory pathway for Th1 differentiation that starts from activation of T-bet and is independent of IL-12-driven STAT-4 signaling. So the critical question is: what induces and sustains T-bet in CD? We have recently shown that in the duodenal mucosa of patients with active but not inactive CD there is enhanced production of IL-21^[44]. Interestingly, neutralization of IL-21 activity in *ex vivo* organ cultures of CD biopsies reduces both T-bet expression and IFN- γ production^[44]. Since IFN- γ enhances T-bet *via* a STAT1-dependent mechanism^[43], it is tempting to speculate that IL-21 is part of a positive feedback loop that helps amplify and stabilize the committed Th1 cell phenotype in CD (Figure 1).

Recent genome-wide association studies have provided convincing evidence that the chromosomal 4q27 region harboring the *IL-2* and *IL-21* genes is associated with CD^[7]. A similar genetic association has been described in other immune-mediated diseases, such as psoriasis, type 1 diabetes, and inflammatory bowel diseases^[45,46]. However, it is not yet known if such polymorphisms can influence the tissue levels of IL-21.

Biopsies taken from CD patients on a gluten-free diet over-express IL-21 when challenged *in vitro* with gluten peptides^[44]; however, the basic mechanisms that control the expression of IL-21 in CD mucosa remain to be elucidated. Previously, we showed that IFN- α is up-regulated in the mucosa of active CD patients, where it most probably contributes to intensifying IFN- γ production^[47]. Indeed, neutralization of IFN- α activity drastically reduces the gliadin peptide-driven

IFN- γ expression in biopsies of inactive CD patients^[48]. Interestingly, IFN- α enhances the mRNA expression of IL-21 in activated human T cells^[49], thus suggesting a role for this cytokine in the positive control of IL-21 in CD (Figure 1).

Potential involvement of IL-21 in the activity of other mucosal cells types

The biological functions of IL-21 are mediated by a cell-surface class I cytokine receptor, formed by the common γ -chain subunit (shared with IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 receptors) and its own unique receptor (designated IL-21 receptor)^[50]. Since this receptor is expressed by both immune and non-immune cells, it is plausible that IL-21 maintains chronic inflammation and/or favors tissue damage in CD by targeting additional cell types other than Th1 lymphocytes. In fact, IL-21 has been shown to stimulate epithelial cells to secrete chemokines and facilitate recruitment of immune cells within the inflamed tissue, induce fibroblasts to make tissue-damaging proteases, and make effector CD4⁺ T cells resistant to regulatory T cell-mediated immunosuppression^[51-53]. In light of the role of IL-21 in the control of B cell and plasma cell function^[54], IL-21 may also contribute to the production of CD-associated autoantibodies. Since its discovery, it has become clear that IL-21 is also able to modulate the proliferation and/or effector function of CD8⁺ T cells and NK cells^[55]. Using IELs isolated from human jejunal mucosa, Ebert has recently shown that IL-21 increases perforin-mediated cytotoxicity and serine esterase release without affecting the growth and survival of these cells^[56]. The relevance of this finding for CD pathogenesis remains to be ascertained, although IL-21-mediated cytotoxic T cell activation could contribute to the intestinal epithelial cell death and villous atrophy in CD.

CONCLUSION

A growing body of evidence suggests that IL-15 and IL-21 may play important roles in the immune response associated with inflammation and tissue damage in CD, even though some intriguing questions remain to be resolved. For instance, it remains to be determined whether these two cytokines contribute qualitatively and quantitatively differently to the initiation and progress of the inflammatory cascade in CD. The fact that IL-15 and IL-21 share a receptor subunit and target the same cell types raises the possibility that these cytokines may cooperate in regulating specific cell immune responses. Indeed, both IL-15 and IL-21 are known to stimulate the growth of T cells, enhance the production of IFN- γ by Th1 cells, and promote the activation of cytotoxic cells^[21,44,50,57] (Figure 1). As outlined in this article, these two cytokines might also act in concert to disrupt local mechanisms of immune tolerance. This hypothesis is supported by the demonstration that IL-15 inhibits TGF- β 1 activity, and IL-21 renders effector CD4⁺ T cells resistant to the suppressive effects of CD4⁺CD25⁺ regulatory T cells^[33,53]. Nonetheless it is noteworthy that

IL-15 and IL-21 seem to differentially modulate some aspects of both innate and adaptive immunity, such as dendritic cell maturation and T cell apoptosis^[50,57]. Therefore, we cannot exclude the possibility that, at least in some stages of the inflammatory process, IL-15 and IL-21 can exert opposing effects on the gluten-driven immune response.

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S- Editor Tian L L- Editor Logan S E- Editor Yin DH



Anemia and digestive diseases: An update for the clinician

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Received: July 23, 2009 **Revised:** September 7, 2009

Accepted: September 14, 2009

Published online: October 7, 2009

Abstract

Anemia and iron deficiency are so common in digestive diseases that often are underestimated and undertreated. Our goal is to review from classification to treatment of the diverse types of anemias in different digestive diseases to update our knowledge on diagnosis and treatment. With the goal of improving the prognosis and quality of life of digestive diseases patients, we will review current transfusion, intravenous iron, and erythropoietin roles in the treatment of anemia.

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Key words: Anemia; Iron deficiency; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

Gomollón F, Gisbert JP. Anemia and digestive diseases: An update for the clinician. *World J Gastroenterol* 2009; 15(37): 4615-4616 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4615.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4615>

The human mind is a marvellous albeit complex tool. It is not easy to understand how it works^[1] with no exception for the clinician^[2]. Inflammatory bowel diseases (IBD) are complex, often difficult to manage, and anemia is so common around the world^[3] that perhaps even the most astute clinician simply cannot always see that specific tree in the forest^[4]. However, anemia is really important for patients, and we should not forget this. Since publication of the landmark article by Gasche^[5], a study^[6]

has shown the importance of anemia that should be actively looked for and treated. Anemia in IBD is a good example^[6], and we will dedicate our specific attention to it, but anemia can also be a key point in many different clinical scenarios of gastroenterology and hepatology. Therefore, in this short article, we will attempt to review several aspects we consider interesting for the practicing clinician. There are many possible approaches and we will take a look at different perspectives. Hematologists, gastroenterologists, hepatologists and even nephrologists will contribute to our task.

Anemia is both a simple word and a complex one. There are many different diseases that could be included in the World Health Organization definition of anemia (a patient has anemia if his or her blood hemoglobin level is below standard for age and sex). However, the simple final result can be due to the consequence of many different causes and mechanisms that can even overlap in the same patient and at the same time. Our first goal is to furnish our readers with an up-to-date classification of anemia. To do this work, we have chosen the hematologists' point of view.

Iron is not only the most abundant chemical element on Earth, but also a key element for life from very ancient ancestors. Life beings often compete for iron, a key to the control of redox reactions in many living organisms^[7]. Perhaps, due to evolutionary reasons, iron regulation is important in inflammation. In many digestive diseases, iron is a principal player because absorption, loss and regulation of iron metabolism, can all be affected in different diseases. Most likely, the paradigm is Crohn's disease^[8], in which malabsorption, inflammation and blood loss, all contribute to iron deficiency and iron deficiency anemia in some cases. The regulation of iron metabolism has been a very interesting topic in the last few years, and some new molecules have been developed. Hepcidin is a rising star, but it is not alone^[9]. The clinician should know the basics of these mechanisms to understand the treatment modalities, and so we have asked the experts to summarize this topic.

It is a bit counterintuitive, but luminal contents are out of our body, in a bacterial world (in fact all the earth is a bacterial world). To get iron in, it has to be absorbed as many other elements or substances. The role of malabsorption in anemia is a very interesting topic for gastroenterologists.

Anemia is a rather common problem in liver units as well. Patients with severe liver disease often have anemia and need specific treatment. Blood transfusion is common

(perhaps too common) in these patients. Anemia in patients with liver disease has interesting particularities, such as the role of portal hypertension, renal failure or antiviral drugs. Thus, a specific article has been devoted to anemia in liver disease.

The story of intravenous iron is a complex one^[10]. Some old preparations are difficult to manage and cause significant risks in patients. So, when new safer preparations^[11] that are easier to use appear, clinicians are simply fearful of using intravenous iron. However, with increasing experience in nephrology, oncology, gastroenterology, and gynecology, IV iron is becoming a standard treatment for patients at all ages and conditions^[12]. If we want this to be a real life standard, clinicians need clear rules, namely “who”, “when”, and “how” are the classic questions that are addressed in a specific article on IV iron.

Intravenous iron is not always enough to treat anemia since erythropoietin can be very useful if used judiciously. For this topic, we prefer the nephrologists’ point of view because they have the most important experience with both sides: Dr. Jeckyll (the improvement in quality of life) and Mr. Hyde (mortality associated with a high hemoglobin level), who will tell us about their experience, which is very interesting with rather complex economic implications^[13].

Blood transfusion can be lifesaving in many clinical situations, and has been one of the greatest advances in medicine. However, blood transfusion is not a risk and/or cost-free, and may have been overused in many situations. Very important evidence from traumatology, surgical and intensive care units has been published in recent years^[14]. We should apply this update knowledge in our patients, following the most recent guidelines. The hematologists’ point of view can be important for the gastroenterologist and the hepatologist.

Anemia, intravenous iron, blood transfusion, iron metabolism, *etc*, have been a few of the changing topics over the last few years. To help our patients, we still need

to know many things about this very old tree with many roots and branches. We have selected to update our knowledge on some of them, with the aim of helping our patients. In Darwin’s year, it was always tempted to use the tree image, and so in a Freudian way to recognize him as the father of modern biology.

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S- Editor Tian L L- Editor Wang XL E- Editor Zheng XM



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An update on iron physiology

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Received: July 23, 2009 Revised: September 8, 2009

Accepted: September 15, 2009

Published online: October 7, 2009

Key words: Iron metabolism; Iron deficiency; Functional iron deficiency; Hepcidin; Anemia of chronic disease

Peer reviewer: Emiko Mizoguchi, MD, PhD, Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States

Muñoz M, Villar I, García-Erce JA. An update on iron physiology. *World J Gastroenterol* 2009; 15(37): 4617-4626 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4617.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4617>

Abstract

Iron is an essential micronutrient, as it is required for adequate erythropoietic function, oxidative metabolism and cellular immune responses. Although the absorption of dietary iron (1-2 mg/d) is regulated tightly, it is just balanced with losses. Therefore, internal turnover of iron is essential to meet the requirements for erythropoiesis (20-30 mg/d). Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency (ID) and iron-deficiency anemia. Hepcidin, which is made primarily in hepatocytes in response to liver iron levels, inflammation, hypoxia and anemia, is the main iron regulatory hormone. Once secreted into the circulation, hepcidin binds ferroportin on enterocytes and macrophages, which triggers its internalization and lysosomal degradation. Thus, in chronic inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling, which results in hypoferrremia and iron-restricted erythropoiesis, despite normal iron stores (functional ID), and anemia of chronic disease (ACD), which can evolve to ACD plus true ID (ACD + ID). In contrast, low hepcidin expression may lead to iron overload, and *vice versa*. Laboratory tests provide evidence of iron depletion in the body, or reflect iron-deficient red cell production. The appropriate combination of these laboratory tests help to establish a correct diagnosis of ID status and anemia.

INTRODUCTION

Erythropoiesis is a part of the larger process of hematopoiesis. In the normal adult human, the daily turnover of red blood cells (RBCs) exceeds 10^{11} cells. In periods of increased RBC loss caused by hemolysis or hemorrhage, the production of RBCs increases rapidly and markedly. However, an overproduction of RBCs (i.e. rebound polycythemia) does not occur even after the most severe loss of RBCs. Thus, erythropoiesis is a finely regulated yet rapidly responsive process that maintains the normal number of circulating RBCs within a narrow range^[1].

The major stages of differentiation in human erythropoiesis are depicted in Figure 1. The commitment of multipotent hemopoietic stem cells to erythroid progenitors is driven by several growth factors, such as stem cell factor, thrombopoietin, and interleukin (IL)-3^[2]. The most immature stage of committed erythroid progenitors is the burst-forming unit-erythroid, which differentiates into colony-forming unit-erythroid (CFU-E) in approximately 7 d, with declining proliferative potential as the progenitors approach CFU-Es. Each CFU-E develops a single cluster of 8-64 mature erythroblasts within 7 d, after several differentiation stages (proerythroblast, basophilic erythroblast, polychromatic erythroblast, and orthochromatic erythroblast). Orthochromatic erythroblasts do not divide but they enucleate, and form nascent RBCs, called reticulocytes, which are released into the bloodstream. After 1 d of circulation in the peripheral blood, reticulocytes mature into RBCs^[3]. The normal proliferation and differentiation of erythroid progenitor cells require several essential

nutrients, such as iron, folate, and vitamin B12, the interaction with the stromal cells in the bone marrow, and stimulation by erythropoietin (EPO)^[3].

IRON HOMEOSTASIS

For a 70-kg male individual, total body iron is about 3.5 g (50 mg/kg). Most of the iron in the body is distributed within RBC hemoglobin (65%; 2300). Approximately 10% is present in muscle fibers (in myoglobin) and other tissues (in enzymes and cytochromes) (350 mg). The remaining body iron is stored in the liver (200 mg), macrophages of the reticuloendothelial system (RES; 500 mg), and bone marrow (150 mg). In premenopausal women, total body iron (especially the stored fraction, 250-300 mg) is lower than in men. The normal diet contains 15-20 mg of iron, and the body absorbs 1-2 mg/d of dietary iron. This is balanced with losses *via* sloughed intestinal mucosal cells, menstruation and other blood losses. Therefore, internal turnover of iron is essential to meet the bone marrow requirements for erythropoiesis (20-30 mg/d)^[3-5].

On the other hand, the body has no effective means of excreting iron and thus the regulation of absorption of dietary iron from the duodenum plays a critical role in iron homeostasis in the body^[5]. This is extremely important as iron is essential for cellular metabolism and aerobic respiration, whilst cellular iron overload leads to toxicity and cell death *via* free radical formation and lipid peroxidation, thus, iron homeostasis requires tight regulation^[3,4,6]. A summary of proteins involved in iron homeostasis, as well as their most frequently used acronyms, is given in Table 1.

Iron absorption

Nearly all absorption of dietary iron occurs in the duodenum. Several steps are involved, including the reduction of iron to a ferrous state, apical uptake, intracellular storage or transcellular trafficking, and basolateral release (Figure 2). Dietary iron is found in heme (10%) and non-heme (ionic, 90%) forms and their absorption occurs at the apical surface of duodenal enterocytes *via* different mechanisms. Dietary non-heme iron primarily exists in an oxidized (Fe^{3+}) form that is not bioavailable, and must first be reduced to the Fe^{2+} form by a ferrereductase enzyme, before it is transported across the intestinal epithelium by a transporter called divalent metal transporter 1 (DMT-1), which also transports other metal ions such as zinc, copper and cobalt by a proton-coupled mechanism^[3,4]. There is also a siderophore-like iron uptake pathway mediated by lipocalin-2 (that seems to exert an innate immune response to bacterial infection by sequestering iron) but its physiological role is not fully worked out.

The absorption of non-heme can be diminished by co-administration of tetracyclines, proton pump inhibitors and antacid medication, phytates (high-fiber diets), calcium, and phenolic compounds (coffee and

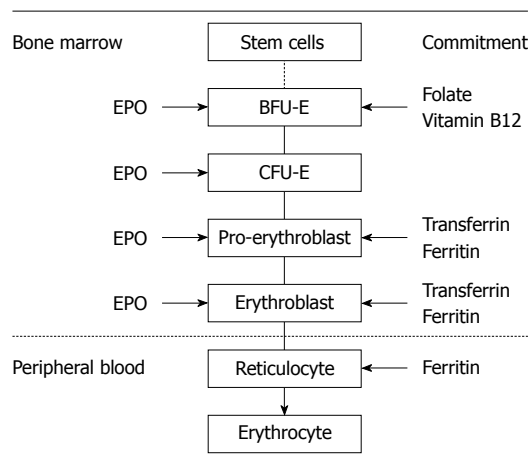


Figure 1 Major stages of human erythropoiesis showing the point of commitment, the period of EPO dependence and the requirements for essential nutrients. BFU-E: Burst-forming unit-erythroid; CFU-E: Colony-forming unit-erythroid; EPO: Erythropoietin.

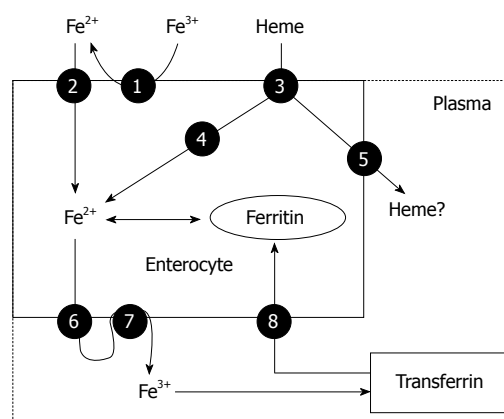


Figure 2 Main pathways of iron absorption by enterocytes in mammals. 1: Ferrereductase; 2: Divalent metal transporter 1 (DMT-1); 3: Heme protein carrier 1 (HPC1); 4: Heme oxygenase; 5: Heme exporter; 6: Ferroportin (Ireg-1); 7: Hephaestin; 8: Transferrin receptor-1 (TfR1) (for details see Table 1).

tea). In addition, infection with *Helicobacter pylori* (*H. pylori*) produces gastric atrophy that, even in the absence of significant bleeding, can lead to profound iron-deficiency anemia (IDA). As expected, this anemia is poorly responsive to oral iron therapy, but can be corrected by eradication of *H. pylori* infection^[7].

Heme iron is absorbed into enterocytes by a putative, not totally identified heme carrier protein 1, which is a membrane protein found in the proximal intestine, where heme absorption is greatest^[8]. Once internalized in the enterocytes, it is likely that most dietary heme iron is released as ferrous iron by heme oxygenase to enter a common pathway with dietary non-heme iron before it leaves the enterocytes^[3,4] (Figure 2). However, it remains uncertain whether some heme might traverse the cells intact, leaving the enterocytes through the action of the recently characterized heme exporters, Bcrp/Abcg2 and feline leukemia virus C receptor (FLVCR)^[8]. If this does occur, the subsequent disposition of plasma heme

Table 1 Main proteins involved in iron homeostasis in mammals

Protein name (alternative name)	Acronyms	Function	Localization
Divalent metal transporter 1 (divalent cation transporter 1, NRAMP 1 and 2)	DMT1 DCT1 NRAMP1* NRAMP2	Traffics divalent metal ions such as iron, zinc, copper and cobalt across the membrane by a proton coupled mechanism	Enterocyte (apical membrane) Erythroblast (siderosome) Macrophage (plasma membrane, phagocytic vesicles*) Hepatocyte Kidney cells
Ferriredutase	Dcytb STEAP3*	Reduction of Fe ³⁺ to Fe ²⁺	Enterocyte (apical membrane) Erythroblast (siderosome*)
Heme carrier protein 1	HCP1	Putative transporter that traffics heme across the membrane by an unknown mechanism	Enterocyte (apical membrane) Hepatocyte Kidney cells
Heme oxygenase		An enzyme that disassembles heme to liberate iron	Enterocyte (microsomal fraction) Macrophages
Ferroportin 1 (Iron regulatory protein 1)	FPN 1 Ireg1 MTP1	Transmembrane Fe ²⁺ transporter (exporter)	Enterocyte (basolateral membrane) Macrophages
Hephaestin	Hp	Membrane-bound multicopper ferroxidase, similar to plasma ceruloplasmin, which oxidizes Fe ²⁺ to Fe ³⁺ to load it onto transferrin Maintenance of cell-surface localization of ferroportin	Hepatocytes Enterocyte (basolateral) Macrophage Hepatocyte?
Transferrin	Tf	Plasma Fe ³⁺ binding protein Ligand for transferrin receptors 1 and 2	Plasma
Transferrin receptor 1	TfR1	Cellular uptake of transferrin bound iron	Ubiquitously expressed
Transferrin receptor 2	TfR2	Sensor for diferric transferrin; regulates hepcidin expression; may participate in a signaling complex with HFE	Enterocyte Hepatocyte Erythroblast
Mitoferrin	SLC25A37	Mitochondrial iron importer that plays a critical role in supplying iron to ferrochelatase for insertion in protoporphyrin IX to form heme	Erythroblast (mitochondria)
Ferritin	Ft	Iron storage protein (H and L chains) Ferroxidase activity (H chain)	Enterocyte Erythroblast Macrophage Hepatocyte Myocytes and cardiomyocytes
Hemosiderin		Iron storage protein; breakdown product of ferritin that occurs when iron levels are high	Macrophage (lysosomes) Hepatocytes (lysosomes)
Heme exporters	LFLVCR* Bcrp/Abcg2** Abcb6***	ATP-independent heme export at the cell membrane* ATP-dependent heme export at the cell membrane** and mitochondrial membrane***	Erythroblast* Ubiquitously expressed***
HFE	HFE	Regulates hepcidin expression, mechanism uncertain; may participate in a signaling complex with TfR2; interacts with TfR1 & β -2-microglobulin	Enterocyte Macrophage Hepatocyte
Hemojuvelin Lipocalin 2	HFE2	Acts as a BMP co-receptor to stimulate hepcidin transcription Mediates a siderophore-like iron uptake pathway. Its an innate immune response to bacterial infection by sequestering iron, but its physiologic role in iron absorption is not fully worked out	Hepatocytes Enterocytes (apical membrane) Macrophages Adipocyte
Hepcidin	HEP HAMP LEAP1	Iron regulatory hormones, binds ferroportin to cause its internalization and degradation	Hepatocytes Adipocytes (low secretion) Enterocytes?
Erythropoietin	EPO	Upregulates the expression ferroportin in macrophages, TfR1 in erythroblasts, and DMT1 and hephaestin in enterocytes Downregulates hepcidin expression in hepatocytes and DMT1 expression in macrophages	Kidney (interstitial peritubular cells) Hepatocytes (low secretion)

BMP: Bone morphogenetic protein.

is unknown. In addition, it is not yet known whether heme carrier protein 1 has physiological roles in tissues other than the intestine. The protein is also expressed in the kidneys and liver, which suggests that it may act at those sites. It might, for example, scavenge free heme or mediate cellular uptake of heme from its circulating carrier protein, hemopexin^[9].

Once inside the intestinal epithelial cell, iron may

either remains in the cell for use or storage (this iron is never absorbed into the body; rather, it is lost when enterocytes senesce and are sloughed into the gut lumen) or exported across the basolateral membrane of the enterocyte into the circulation (absorbed iron). Ferroportin 1 is the only putative iron exporter identified to date. Ferrous iron once exported across the basal membrane by ferroportin 1, is then oxidized

by a multi-copper oxidase protein called hephaestin (an enzymatic protein similar to plasma ceruloplasmin) before being bound by plasma transferrin. Ferroportin 1 is also the putative iron exporter in macrophages and hepatocytes (Figure 2)^[3,4].

The absorption of iron is dependent on the body's iron stores, hypoxia and rate of erythropoiesis. Two models have been proposed to explain how the absorption of iron is regulated: the crypt programming model and the hepcidin model.

The crypt programming model: This model proposes that enterocytes in the crypts of the duodenum take up iron from the plasma. The intracellular iron level of the crypt cells corresponds to the body's iron stores, which in turn determines the amount of iron absorbed from the gut lumen, as these crypt cells migrate upwards to become absorptive cells at the brush border. The crypt cells express both transferrin receptor 1 (TfR1) and TfR2, which mediate the cellular uptake of transferrin-bound iron from plasma^[3,5].

TfR1 is expressed ubiquitously and transferrin mediated iron uptake is thought to occur in most cell types. HFE, an MHC-class 1-like molecule that interacts with $\beta 2$ -microglobulin and forms a complex with TfR1, is highly expressed in crypt cells. Its role in the regulation of TfR1-mediated transferrin-bound iron uptake remains unclear, but it seems to enhance transferrin-bound iron uptake from the plasma into crypt cells *via* TfR1, and may also inhibit the release of iron from the cell *via* ferroportin 1. In contrast, TfR2 is restricted to hepatocytes, duodenal crypt cells and erythroid cells, which suggests a more specialized role in iron metabolism. The intracellular iron concentration controls the interaction of cytosolic iron regulatory proteins (IRPs) 1 and 2 with iron regulatory elements (IREs; which act as iron sensors in mammalian cells and regulate translation or stability of mRNA-encoding proteins) in the 3' and 5' regions of different mRNA molecules. In the absence of iron, IRP1 binds to IREs of TfR1, DMT-1, and ferroportin 1 mRNA, the transcript is stabilized, translation proceeds, and the proteins are synthesized. Thus, a high IRP binding activity reflects low body iron stores and results in upregulation of these proteins in the duodenum and increased dietary iron absorption. When IRPs bind to IRE of ferritin mRNA, translation of the transcript is blocked and synthesis is halted. Thus, ferritin levels are regulated reciprocally - being increased in iron-replete states and decreased in iron-deplete states^[3].

The hepcidin model: Liver hepcidin is a 25-amino-acid cysteine-rich peptide with antimicrobial properties, which is regulated by a number of factors such as liver iron levels, inflammation, hypoxia and anemia. The hepcidin model proposes that hepcidin is secreted into the blood and interacts with villous enterocytes to regulate the rate of iron absorption, by controlling

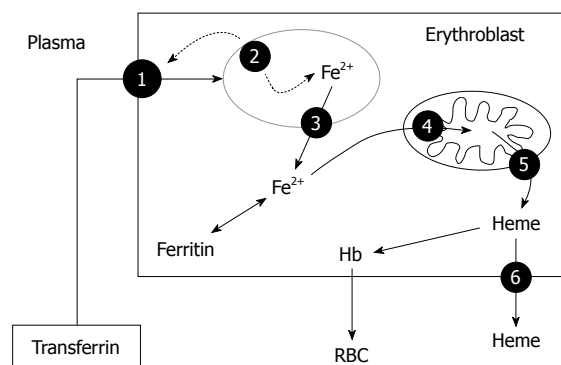


Figure 3 Main pathways of iron utilization by erythroblasts in mammals. 1: TfR1; 2: Diferric-transferrin-TfR1 complex; 3: Natural resistance macrophage protein (NRAMP-1); 4: Mitoferrin; 5: Mitochondrial heme exporter (Abcb6); 6: Heme exporter (FLVCR, Bcrp/Abcg2) (for details see Table 1).

the expression of ferroportin 1 at their basolateral membranes. The binding of hepcidin to ferroportin 1 results in internalization of ferroportin 1 and loss of its function. Ferroportin 1 molecules present in macrophages and liver are also targets for hepcidin. Thus, it is hypothesized that when hepcidin levels are increased in iron overload (by the uptake of transferrin bound iron *via* TfR1/HFE and TfR2) or inflammation (*via* IL-6), iron release from intestinal crypt cells, liver and macrophages is reduced. In contrast, when hepcidin levels are reduced, as in iron deficiency (ID), anemia or hypoxia, it is likely that ferroportin 1 expression and iron release from intestinal cells, liver and cells of reticuloendothelial system is increased^[10]. In contrast, a mutation in the *ferroportin 1* gene is responsible for type IV hemochromatosis.

There is evidence to support both models and it is possible that both control mechanisms may contribute to the regulation of iron absorption. In this regard, there is emerging evidence that hepcidin may act directly on mature villous enterocytes rather than crypt enterocytes. There are several situations (e.g. acute phase response) when iron absorption can be modulated more rapidly (within hours) than can be accounted for *via* the mechanism that involves the programming and maturation of crypt enterocytes (lag time of days)^[5].

Iron distribution

Iron released into the circulation binds to transferrin and is transported to sites of use and storage. Transferrin has two binding sites, binding one iron atom each (thus three forms can be found in plasma: apo-transferrin which contains no iron, monoferric-transferrin and diferric-transferrin). About 30%-40% of these sites are occupied under normal physiological conditions. Thus, transferrin-bound iron is about 4 mg, but this is the most important dynamic iron pool^[11]. Transferrin-bound iron enters target cells - mainly erythroid cells, but also immune and hepatic cells - through a process of receptor-mediated endocytosis (Figure 3). As diferric-

transferrin has a much higher affinity for TfR than does monoferric-transferrin, it binds to the TfR at the plasma membrane, and patches of cell-surface membrane that carry receptor-ligand complexes invaginate to form clathrin-coated endosomes (siderosomes)^[11]. After clathrin is removed, the siderosomes become acidified through an ATP-dependent proton influx, which leads to conformational changes in transferrin and TfR1, and promotes iron release of Fe^{3+} from transferrin. Fe^{3+} is then reduced to Fe^{2+} by a ferrireductase and transported to the cytoplasm through the DMT-1, whereas the TfR is recycled to the cell membrane and transferrin shed back to the circulation^[3,12] (Figure 3). Production of hemoglobin by the erythron accounts for most iron use. High-level expression of TfR1 in erythroid precursors ensures the uptake of iron into this compartment. To make heme, iron must again cross an ion-impermeable membrane to enter the mitochondria. The mitochondrial iron importer was recently identified as mitoferrin (also known as SLC25A37), a transmembrane protein that plays a crucial role in supplying iron to ferrochelatase for insertion into protoporphyrin IX to form heme^[12] (Figure 3). Recently, different human heme exporters have been identified in erythroblasts, and their activity seems to be essential for erythropoiesis, by transferring heme from the mitochondria to cytosol (Abcb6) and removing the excess of heme from the erythroid cells (FLVCR, Bcrp/Abcg2) (Figure 3).

In the erythroid precursors, the expression of TfR1, DMT-1 and ferritin are regulated reciprocally through IRP1 and IRP2, which act on the IRE present in their RNA. Thus, when increased iron uptake is needed, the expression of TfR1 and DMT-1 is increased, whereas the synthesis of ferritin is halted^[3]. In addition, there is evidence that EPO activates IRP-1, leading to upregulation of TfR1 expression in the erythroid precursors, which is maintained along with the differentiation process, and DMT-1 and hephaestin gene expression in the duodenum^[13]. To date, three patients have been reported with DMT-1 mutations that cause microcytic hypochromic anemia, as a result of decreased erythroid iron utilization, but lead to increased liver iron storage^[14].

A truncated form of the TfR can be detected in human serum. The serum concentration of this soluble form of TfR (sTfR; normal median concentration: 1.2-3.0 mg/L, depending on the assessment kit used) is proportional to the total amount of surface TfR. Increased sTfR concentrations indicate ID even during the anemia of chronic disease (ACD), as well as increased erythropoietic activity without ID, whereas lower sTfR concentrations may reflect decreased numbers of erythroid progenitors^[3,15].

Iron storage

Hemoglobin iron has substantial turnover, as senescent erythrocytes undergo phagocytosis by RES macrophages. Within the phagocytic vesicles, heme is metabolized by

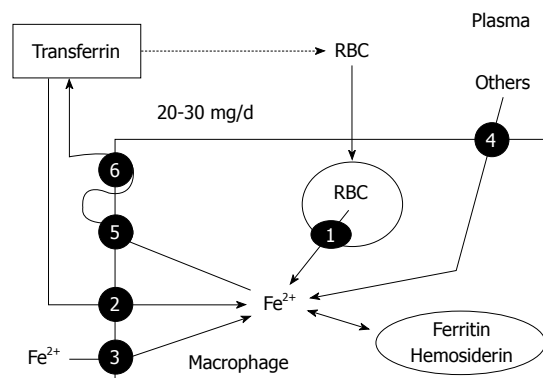


Figure 4 Main pathways of iron storage and exportation by macrophages in mammals. 1: NRAMP-1; 2: TfR1; 3: DMT-1; 4: Others: others: bacteria, lactoferrin, hemoglobin-haptoglobin, heme-hemopexin; 5: Ferroportin (Ireg-1); 6: Hephaestin (for details see Table 1).

heme oxygenase and the released iron is exported to the cytoplasm through the action of natural resistance-associated macrophage protein-1, a transport protein similar to DMT-1 (Figure 4). Macrophages can also obtain iron from bacteria and apoptotic cells, from plasma through the action of DMT-1 and TfR1, and from other sources (Figure 4). Within the cell, iron can be stored in two forms: in the cytosol as ferritin and, after breakdown of ferritin within the lysosomes, as hemosiderin. Hemosiderin represents a very small fraction of normal body iron stores, mostly in macrophages, but increases dramatically in iron overload^[11]. Iron export from macrophages to transferrin is accomplished primarily by ferroportin 1, the same iron-export protein expressed in duodenal enterocytes, and hephaestin^[3] (Figure 4). The amount of iron required for daily production of 300 billion RBCs (20-30 mg) is provided mostly by recycling iron by macrophages^[4]. Importantly, iron storage at the macrophages is safe, as it does not lead to oxidative damage. EPO reduces iron retention in macrophages by decreasing DMT-1 and increasing ferroportin 1 expression^[16].

The liver is the other main storage organ for iron. In iron overload, free radical formation and generation of lipid peroxidation products may result in progressive tissue injury and eventually cirrhosis or hepatocellular carcinoma^[17]. Iron is sequestered in hepatocytes predominantly in the form of ferritin or hemosiderin. The uptake of transferrin-bound iron by the liver from plasma is mediated by TfR1 and TfR2 (Figure 5). In iron overload, TfR1 is downregulated in hepatocytes^[5]. TfR2 is expressed highly in human liver and is likely to play an important role in liver iron loading in iron overload states. Unlike TfR1, TfR2 lacks an IRE and thus is not regulated reciprocally in response to the level of plasma iron. Instead, TfR2 protein expression is regulated by transferrin saturation (TSAT), and is upregulated in iron overload. In normal and iron-loaded conditions, expression of TfR2 exceeds that of TfR1, which suggests that TfR2 plays an important role in hepatic iron loading in hemochromatosis^[5].

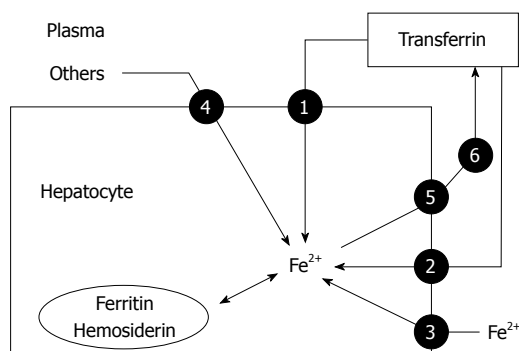


Figure 5 Main pathways of iron storage and exportation by hepatocytes in mammals. 1: TfR1; 2: TfR2; 3: DMT-1; 4: Others: hemoglobin, heme, ferritin; 5: Ferroportin (Ireg-1); 6: Ceruloplasmin (for details see Table 1).

In fact, a mutation in TfR2 is responsible for type 3 hemochromatosis^[14].

As transferrin becomes saturated in iron overload states, excess iron is also found as non-transferrin-bound iron is transported across the hepatocyte membrane *via* a carrier-mediated process consistent with DMT-1. The hepatocytes may also store iron from ferritin, hemoglobin-haptoglobin complexes, and heme-hemopexin complexes. In contrast, once again, ferroportin 1 is likely to be the only protein that mediates the transport of iron out of hepatocytes, which is then oxidized by ceruloplasmin and bound to transferrin^[3,4] (Figure 5).

Iron storage within cardiomyocytes is also of outstanding interest, as cardiac failure is the leading cause of death among patients with untreated hereditary hemochromatosis or transfusion-associated hemosiderosis^[3]. In cardiac cells, excess iron may result in oxidative stress and alteration of myocardial function because of DNA damage by hydrogen peroxide through the Fenton reaction^[3].

EFFECTS OF INFLAMMATION ON IRON HOMEOSTASIS AND ERYTHROPOIESIS

Anemia is a frequent complication of chronic inflammatory diseases (e.g. cancer, rheumatoid arthritis, inflammatory bowel diseases, and congestive heart failure), as well as sepsis and chronic renal failure. In addition to blood loss, hemolysis, hepatic or endocrine disorders, nutritional deficiencies, bone marrow infiltration (cancer cells), or vitamin consumption (bacteria), this anemia may be the result of activation of the immune system by the underlying process, and certain immune and inflammatory cytokines including tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and IL-1, 6, 8 and 10^[18,19].

As for chronic inflammatory diseases (and sepsis), these inflammatory mediators lead to anemia through several of the following pathophysiological mechanisms^[19] (Figure 6): (1) decreased RBC half-life because of dyserythropoiesis, RBC damage and increased erythrophagocytosis (TNF- α); (2) EPO responses are inadequate for the degree of anemia in most, but not

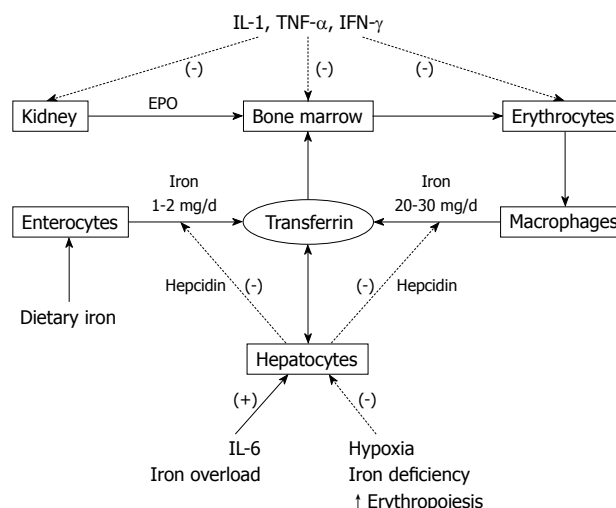


Figure 6 Effects of inflammation on erythropoiesis and iron homeostasis in mammals. (-): Negative effect; (+): Positive effect.

all (e.g. systemic-onset of juvenile chronic arthritis) (IL-1 and TNF- α)^[20]; (3) impaired responsiveness of erythroid cells to EPO (IFN- γ , IL-1, and TNF- α); (4) inhibited proliferation and differentiation of erythroid cells (IFN- γ , IL-1, TNF- α , and α -1-antitrypsin); and (5) pathological iron homeostasis caused by increased DMT-1 (IFN- γ) and TfR (IL-10) expression in macrophages, reduced ferroportin 1 expression (IFN- γ and IL-6-induced high hepcidin levels) in enterocytes (inhibition of iron absorption) and macrophages (inhibition of iron recirculation), and increased ferritin synthesis (TNF- α , IL-1, IL-6, IL-10) (increased iron storage). All these lead to hypoferremia through iron diversion to the RES [functional iron deficiency (FID) that is characterized by low serum iron and decreased TSAT], iron-restricted erythropoiesis, and mild-to-moderate anemia.

Thus, the immunological pathophysiology of ACD includes disturbances of iron homeostasis, impaired proliferation of erythroid progenitor cells, and a blunted EPO response to anemia^[19]. However, the pathophysiology of acute inflammation-related anemia (e.g. trauma or surgery) is somewhat different. In this setting, inflammatory responses are mediated mainly by IL-6 and IL-8 (with transient contribution of TNF- α and IL-1 in some visceral surgery, such as gastrointestinal or cardiac procedures), whereas IFN- γ plasma levels are undetectable or within the normal range^[21-23]. Therefore, in most of these conditions, the two major mechanisms that lead to anemia are perioperative or traumatic blood loss and blunted erythropoiesis caused by decreased iron availability (caused by IL-6-induced high hepcidin levels), whereas EPO levels are normal or near-to-normal^[24]. Finally, with persisting decreased iron absorption and/or chronic blood loss, ACD may evolve to ACD with true ID (ACD + ID).

On the other hand, it must be borne in mind that iron is not only required for erythropoiesis and oxidative metabolism. Cellular immune responses are also

dependent on the presence of iron, and specific defects in cell-mediated immunity have been described in detail, even in mild ID, including the impaired proliferation and function of lymphocytes and natural killer cells, and a depressed neutrophil respiratory burst^[25,26]. Thus, ID or FID may lead not only to a blunted erythropoiesis and chronic fatigue, but also to an inappropriate immune response. For this reason, systemic inflammatory response episodes last longer in critically ill patients with FID, and result in prolonged stay in the intensive care unit and increased morbidity^[27]. On the other hand, the effectiveness of the administration of iron sucrose, alone or in combination with EPO, has been assessed in a population of anemic, critically ill patients^[28]. Compared to those in the control group who only received folic acid, patients treated with iron sucrose experienced an amelioration of systemic inflammatory response [decreased C-reactive protein (CRP) levels]. These beneficial effects were not as evident in patients who received iron sucrose plus recombinant human EPO (rHuEPO), probably because of the persistence of FID caused by rHuEPO-enhanced erythropoietic activity.

LABORATORY ASSESMENT OF IRON STATUS

Under physiological conditions, there is a balance between iron absorption, iron transport and iron storage in the human body. However, ID and IDA are common conditions among medical, surgical and critically ill patients, and result from the interplay of three distinct risk factors: increased iron requirements [e.g. growth or use of erythropoiesis-stimulating agents (ESAs), pregnancy and post-bleeding recovery], limited external supply (e.g. malnutrition, malabsorption caused by inflammatory bowel disease, use of gastric antiacid agents, infection with *H. pylori*, even in the absence of significant bleeding), and increased blood loss (e.g. chronic gastrointestinal bleeding)^[28]. ID can be either absolute or functional. In absolute ID, iron stores are depleted; in FID, iron stores, although replete, cannot be mobilized as fast as necessary from the macrophages of the RES to the bone marrow. As stated above, FID occurs in anemia of inflammatory diseases because iron is trapped in the RES^[3,29].

Thus, laboratory tests for investigating ID fall into two categories: measurements providing evidence of iron depletion in the body, and measurements reflecting iron-deficient RBC production^[30] (Table 2). The appropriate combination of these laboratory tests will help to establish a correct diagnosis of anemia and ID status^[19].

ID without anemia

Normal hemoglobin level does not exclude ID, because individuals with normal body iron stores must lose a large amount of body iron before the hemoglobin falls

Table 2 Main laboratory tests for the diagnosis of anemia and iron deficiency

Laboratory test	Normal values, units	Conversion to SI units
Iron depletion in the body		
Serum iron	50-180 µg/dL	× 0.179 µmol/L
Transferrin	200-360 mg/dL	× 0.01 g/L
TSAT	20%-50%	
Ferritin	30-300 ng/mL	× 2.247 pmol/L
sTfR	0.76-1.76 mg/L	6.4-25.7 nmol/L
Ratio of sTfR to serum ferritin (sTfR/log ferritin)	< 1	
Iron deficient red cell production		
Hemoglobin	12-16 g/dL (female) 13-17 g/dL (male)	× 0.6206 mmol/L
MCV	80-100 fL	
RDW	11-15	
MCH	28-35 pg	
HYPO	< 5%	
CHr	28-35 pg	

TSAT: Transferrin saturation; sTfR: Soluble transferrin receptors; MCV: Mean corpuscular volume; RDW: Red cell distribution width; MCH: Mean corpuscular Hb; HYPO: Hypochromic red cells; CHr: Reticulocyte Hb content.

below the laboratory definition of anemia. According to WHO criteria, laboratory definition of anemia is Hb < 12 g/dL for female and < 13 g/dL for male. However, although we will refer to these values in the following paragraph, it is worth noting that WHO criteria have been challenged recently. Analysis of the large NHANES-III (the third US National Health and Nutrition Examination Survey) and Scripps-Kaiser databases has indicated that a hemoglobin concentration < 13.7 g/dL in a white man aged between 20 and 60 years would have only an approximately 5% chance of being a normal value. The corresponding value for female of all ages would be 12.2 g/dL^[31]. Should these new definitions of anemia be applied to our inflammatory bowel disease patient population, the prevalence of anemia would be higher than that reported in several previous studies.

In non-anemic patients, the most important clinical clue of ID is the symptom of chronic fatigue (iron is required for the enzymes involved in oxidative metabolism). However, it is of little screening value because clinicians rarely consider the presence of ID in patients who are not anemic, and therefore ID is invariably diagnosed in the laboratory^[30]. A normal hemoglobin level with a mean corpuscular hemoglobin (MCH) in the lower limit of normality (normal range: 28-35 pg), or an increased RBC distribution width (RDW, normal range: 11-15) point to mild ID without anemia, but the main laboratory finding is low ferritin level. Although ferritin is an intracellular iron storage protein, small amounts of ferritin are secreted into the circulation and can be measured in the laboratory, and 1 ng/mL serum ferritin corresponds to approximately 8 mg of stored iron. Thus, measurement of ferritin

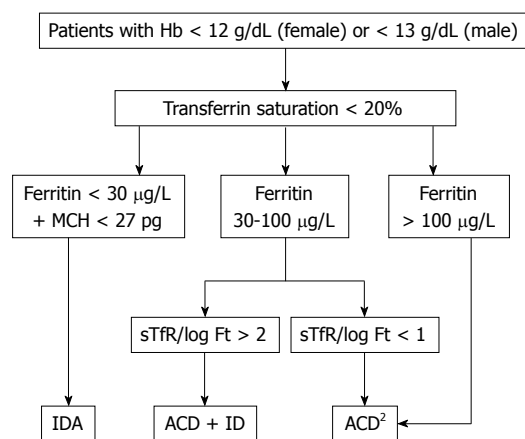


Figure 7 A simplified algorithm for the diagnosis of IDA (modified from Weiss *et al*^[19]).

provides the aID can be defined by a ferritin level < 30 ng/mL in the absence of inflammation (normal serum concentrations of CRP: < 0.5 mg/dL) (true ID). In the presence of inflammation, a normal ferritin level (acute phase reactant) does not exclude ID, and TSAT also should be measured. As transferrin is the only iron-binding protein involved in iron transport, TSAT reflects iron availability for the bone marrow. Thus, in the presence of inflammation, ID should be better defined by normal ferritin concentrations and low TSAT (FID). FID may also occur in response to the therapeutic use of ESAs, such as epoetin or darbopoetin, which place a significant demand on iron stores that may surpass the iron-release capacity of the RES^[32].

IDA

Patients should be considered to suffer from IDA when they presented with low Hb (male < 13 g/dL and female < 12 g/dL), TSAT (< 20%) and ferritin concentrations (< 30 ng/mL) but no signs of inflammation^[30]. The MCH rather than mean corpuscular volume (MCV) has become the most important RBC marker for detecting ID in circulating RBCs (Figure 7). MCV is a reliable and widely available measurement but is a relatively late indicator in patients who are not actively bleeding. In addition, patients may present with IDA and without microcytosis, with coexisting vitamin B12 or folate deficiency. sTfR levels are usually high or very high, but they are not usually required for the diagnosis of uncomplicated IDA.

ACD

Patients should be considered to suffer from ACD when they have: (1) evidence of chronic inflammation (high CRP level); (2) hemoglobin concentration < 13 g/dL for male and < 12 g/dL for female; and (3) low TSAT < 20%, but normal or increased serum ferritin concentration (> 100 ng/mL) or low serum ferritin concentration (30-100 ng/mL), and an sTfR/log ferritin ratio < 1^[19,33-35] (Figure 7). ACD, as well as FID, are frequent among

patients with inflammatory disease without apparent blood loss (e.g. rheumatoid arthritis, renal failure or chronic hepatitis).

On the other hand, although ACD is typically mild to moderate, and RBCs may not show any stigmata of ID (normochromic, normocytic anemia), the underlying iron etiology is evident: macrophages that normally recycle iron are found to sequester it, intestinal iron absorption is interrupted, and erythroid precursors respond very rapidly when iron-transferrin is made available, especially by the administration of iv iron preparations. Thus, it can be speculated that the normocytic RBCs result from the combination of iron insufficiency and an as-yet-unexplained tendency to macrocytosis (e.g. alterations in folate or B12 metabolism in response to inflammation)^[12].

ACD + ID

Patients should be considered to have ACD + ID when they have: (1) chronic inflammation (high CRP level); (2) hemoglobin concentration < 13 g/dL for male and < 12 g/dL for female; and (3) low TSAT < 20%, serum ferritin concentration > 30 and < 100 ng/mL, and an sTfR/log ferritin ratio > 2^[19,33-35] (Figure 7). This type of anemia is more frequent in patients with inflammatory diseases and chronic blood losses (e.g. inflammatory bowel disease). There are two important hematological indices that may also help in the diagnosis of ACD + ID: reticulocyte hemoglobin content (CHr) and hypochromic red blood cells (HYPO). In non-ferropenic patients, the 2.5 percentile values were 28 pg for CHr and 5% for HYPO^[15]. However, these hematological indices are only available in specific hematological analyzers. The Advia 120 hematology analyzer determines CHr and RBC hemoglobin content (≥ 27 pg), whereas the Sysmex XE-2100 hematology analyzer determines RET-Y, which can be considered as the reticulocyte hemoglobin equivalent, as well as RBC-Y, which can be considered as the RBC hemoglobin equivalent^[36,37]. These hematological indices (CHr and HYPO) are direct indicators of FID, in contrast to the majority of biochemical markers, which measure FID indirectly *via* iron-deficient erythropoiesis and demonstrate weaknesses in the diagnosis of FID as defined by hematological indices^[15]. New hematological indices are being developed for other hematological analyzers (e.g. Beckman-Coulter LH 750) and their clinical utility in the diagnosis of ID will be evaluated in the near future.

Although hepcidin affects iron traffic in ACD and ACD + ID, individuals suffering from ACD + ID have significantly lower hepcidin levels than ACD subjects, and ACD + ID individuals, in contrast to ACD subjects, are able to absorb some dietary iron from the gut and to mobilize some iron from macrophages. Thus, hepcidin determination may also aid differentiation between ACD and ACD + ID and in selecting appropriate therapy for these patients^[33].

CONCLUSION

Iron is an essential micronutrient, as it is required for an adequate erythropoietic function, oxidative metabolism and cellular immune response. Although the absorption of dietary iron (1-2 mg/d) is regulated tightly, it is just balanced with losses. Therefore, internal turnover of iron is essential to meet the requirements for erythropoiesis (20-30 mg/d). Hepcidin, which is primarily made in hepatocytes in response to liver iron levels, inflammation, hypoxia and anemia, is the main iron regulatory hormone for iron absorption and recirculation. Increased iron requirements, limited external supply, and increased blood loss may lead to ID and IDA. During inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling, which results in hypoferrremia and iron-restricted erythropoiesis, despite normal iron stores, and finally, in ACD, which can later evolve to ACD + ID. An appropriate combination of laboratory tests that provides evidence of iron depletion or reflects iron-deficient RBC production will help to establish a correct diagnosis of ID status and anemia^[38].

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Classification of anemia for gastroenterologists

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Received: July 23, 2009 Revised: September 11, 2009

Accepted: September 18, 2009

Published online: October 7, 2009

Peer reviewer: Giulio Marchesini, Professor, Department of Internal Medicine and Gastroenterology, "Alma Mater Studiorum" University of Bologna, Policlinico S. Orsola, Via Massarenti

Moreno Chulilla JA, Romero Colás MS, Gutiérrez Martín M. Classification of anemia for gastroenterologists. *World J Gastroenterol* 2009; 15(37): 4627-4637 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4627.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4627>

Abstract

Most anemia is related to the digestive system by dietary deficiency, malabsorption, or chronic bleeding. We review the World Health Organization definition of anemia, its morphological classification (microcytic, macrocytic and normocytic) and pathogenic classification (regenerative and hypo regenerative), and integration of these classifications. Interpretation of laboratory tests is included, from the simplest (blood count, routine biochemistry) to the more specific (iron metabolism, vitamin B12, folic acid, reticulocytes, erythropoietin, bone marrow examination and Schilling test). In the text and various algorithms, we propose a hierarchical and logical way to reach a diagnosis as quickly as possible, by properly managing the medical interview, physical examination, appropriate laboratory tests, bone marrow examination, and other complementary tests. The prevalence is emphasized in all sections so that the gastroenterologist can direct the diagnosis to the most common diseases, although the tables also include rare diseases. Digestive diseases potentially causing anemia have been studied in preference, but other causes of anemia have been included in the text and tables. Primitive hematological diseases that cause anemia are only listed, but are not discussed in depth. The last section is dedicated to simplifying all items discussed above, using practical rules to guide diagnosis and medical care with the greatest economy of resources and time.

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Key words: Anemia; Microcytic anemia; Iron; Deficiency diseases; Macrocytic anemia; Normocytic anemia

INTRODUCTION

Erythrocytes and the digestive system are linked closely from the very beginning of life. The yolk sack is the origin of the first generation of erythrocyte precursors. Yolk-sac-derived progenitor cells may seed the developing liver *via* the circulation and produce mature red blood cells that are required to meet the metabolic needs of the fetus. By week 8, liver-derived red cells are evident and the liver is the only source of erythrocytes until the 18th week of gestation. Afterwards, the spleen and bone marrow take over, but a small percentage of hepatic erythropoiesis remains until the postnatal week 6.

In adult life, the esophagus, stomach, bowel and liver are involved in the pathogenesis of different types of anemia caused by nutritional deficiency, bleeding or chronic inflammation.

Several diseases of the esophagus may produce chronic or acute bleeding: varicose veins, diverticula, diaphragmatic hernia, tumors, ulcers, esophagitis and Mallory-Weiss syndrome. The stomach plays a decisive role in the protection of vitamin B12 until it is absorbed in the final portion of the ileum, and contributes to an optimum absorption of iron reducing Fe^{3+} to Fe^{2+} . The stomach also may be a source of bleeding in peptic ulcer, gastritis and tumors, and treatment with acetylsalicylic acid and nonsteroidal anti-inflammatory drugs. The absorption of iron is a sophisticated and specialized process located mostly in the duodenum. The integrity of the small-intestinal mucosa is essential for folate absorption. Vitamin B12 absorption takes place chiefly in the ileum. In consequence, several gastrointestinal diseases, hereditary or acquired, including surgical resection, may produce nutritional deficiency anemia. Small-bowel inflammatory disease is a widespread cause of chronic disease anemia. Chronic bleeding is a crucial

sign in the diagnosis of malignant and benign lesions (such as diverticula) in the colon and rectum.

Splenomegaly is a relatively common cause of chronic hemolysis, and Zieve's syndrome is an infrequent cause of acute hemolytic crisis. Last, but not least, the liver plays an essential role in the control of iron metabolism through the hepcidin pathway, and accounts for most vitamin B12, folate and iron stores.

The links between anemia and digestive system are strong and multiple. Because of this, hematologist and the specialist in digestive system should work together to acquire a profound know of common pathogenic processes.

CONCEPT OF ANEMIA

Anemia is defined as a lower than normal hemoglobin concentration. Low hematocrit is a subrogate value for anemia, but it is not measured directly by the hematological analyzer. Instead, it is calculated from hemoglobin and other parameters. The erythrocyte count may be misleading in the evaluation of anemia. In fact, in some cases of microcytic anemia, such as thalassemia, there is usually an elevated erythrocyte count (spurious polycythemia).

In order to make a generalized approach to the diagnosis of anemia, the World Health Organization (WHO) has established a reference range for normal blood hemoglobin concentration, depending on age and sex^[1]. According to this criterion, anemia is present if the blood concentration of hemoglobin falls below 130 g/L in men or 120 g/L in women. This rule does not apply to infants, children and pregnant women, who have their own tables of lower limits of hemoglobin concentration. The WHO criterion has been accepted widely for diagnosis and publication, but its universal application has been questioned mainly because of racial differences. Beutler has proposed a lower limit of hemoglobin (1-2 g less) in African Americans than in Caucasians. The reference range of hemoglobin concentration in blood may vary depending on the population analyzed, age, sex, environmental conditions and food habits^[2,3].

Anemia causes tissue hypoxia and triggers compensating mechanisms. Both processes together produce the signs and symptoms characteristics of anemic syndrome. Patients with anemia may present with fatigue, dizziness and dyspnea; however, mild anemia shows few clinical signs or symptoms. The signs of anemia include pallor of the conjunctivae, face, nail beds and palmar creases, although the absence of pallor does not rule out anemia^[4].

Anemia is one of the most frequent causes of medical visits because of the high incidence in children, young women and elderly people, especially if malnutrition is present. Moreover, anemia is one of the leading signs in many diseases or is the first evidence of disease observed in routine blood cell enumeration. Anemia is unusually prevalent in developing countries because of malnutrition, and genetic, parasitic or infectious

diseases^[5,6]. The prevalence of anemia varies greatly, from 2.9% to 61%, depending on population, age, sex, and normal limits of hemoglobin used by the author^[7].

The most frequent cause of chronic anemia is gastrointestinal pathology, and this explains why patients with anemia are often sent for consultation to a gastroenterological specialist^[8].

Anemia is a syndrome, not a disease, and therefore, the etiology must always be investigated and therapy must be directed mainly to the causal disease, and not only to restoring a normal hemoglobin concentration. In a patient with anemia, whose clinical and laboratory data are insufficient to find the cause, examination of the digestive system is a priority for two reasons. The first one is the high frequency of anemia in bowel disease, and the second is the opportunity to diagnose a malignant disease before it is too advanced^[9-11].

CLASSIFICATION

Anemia can be classified from three points of view: pathogenesis, red cell morphology, and clinical presentation. All are important to guide the diagnosis. Pathogenic mechanisms involved in the production of anemia are very simple: inadequate production and loss of erythrocytes as a result of bleeding or hemolysis. Based on these pathogenic mechanisms, anemia can be divided into two types. (1) Hypo-regenerative: when bone marrow production is decreased as a result of impaired function, decreased number of precursor cells, reduced bone marrow infiltration, or lack of nutrients; (2) Regenerative: when bone marrow responds appropriately to a low erythrocyte mass by increasing production of erythrocytes.

In practice, classification based on basic parameters of red cell morphology such as mean corpuscular volume (MCV), allows for a quicker diagnostic approach.

Anemia also can be classified according to the form of clinical presentation as acute (usually bleeding or hemolysis) or chronic.

Anemia can be classified as microcytic, normocytic or macrocytic, depending on MCV. As stated above, it can be hypo-regenerative or regenerative, which depends on the number of reticulocytes. Using both, the list of possible diagnoses in the individual patient is reduced considerably. Both parameters can be supplied routinely by most of the automatic hematological cell counters.

PATHOGENIC CLASSIFICATION

The reticulocyte count is useful to distinguish anemia in which there is an appropriate bone marrow response from that in which there is a decrease in the production of erythrocytes. The concentration of reticulocytes reports on the bone marrow response to anemia. This approach is especially useful when MCV is normal.

A decrease in hemoglobin stimulates erythropoiesis through an increase in circulating erythropoietin. Therefore, when the bone marrow shows a normal

regenerative capacity, there should be an inverse relationship between the decrease in hemoglobin and the increased number of reticulocytes (regenerative anemia). The expected reticulocyte count is much higher than normal to compensate for the anemia. However, when the hemoglobin decreases and the bone marrow does not have regenerative capacity, the expected increase in reticulocytes fails, despite the increase in erythropoietin plasma level (hypo-regenerative anemia)^[12].

The reticulocyte count is expressed as a proportion of the number of erythrocytes, which must be corrected for anemia, and for increased lifespan of reticulocytes in peripheral blood. Most hematological analyzers provide directly the number of reticulocytes per mm³, which is a better estimation of erythropoietic activity than a percentage. However, the absolute reticulocyte enumeration per mm³ overestimates the actual activity of erythropoiesis, since reticulocytes are released earlier and remain longer in the circulating blood. To avoid these confounding events, it is recommended to calculate the reticulocyte index.

The next step is the calculation of reticulocyte production index (RPI):

$$\text{RPI} = \frac{\text{corrected reticulocyte count (\%)} \times (\text{haematocrit observed/normal haematocrit})}{\text{F (reticulocyte maturation times in vivo)}}$$

RPI in a healthy person is 1. An RPI > 3 in a patient with anemia suggests an appropriate bone marrow response, and therefore, regenerative anemia^[13,14]. Nevertheless, the best estimate of the actual erythropoietic activity^[15], and easiest to calculate, is to divide by two the number of reticulocytes per mm³.

Regenerative anemia

This is characterized by increased generation of erythropoietin in response to decreased hemoglobin concentration, and generally reflects a loss of erythrocytes, due to bleeding or hemolysis (Table 1). In both cases, there is a typical increase in reticulocytes. The bleeding can be intense, with a sharp drop of hematocrit and obvious clinical signs; or of small intensity and chronic, with progressive decrease in hematocrit and MCV, which may go unnoticed. Over time, chronic hemorrhagic anemia becomes hypo-regenerative and microcytic because of depletion of iron stores.

Hemolysis, can be acute (usually intravascular) or chronic (usually extravascular). Acute hemolysis is characterized by sudden episodes with very obvious clinical signs (fever, chills, back pain, dark urine) and typical laboratory data (hemoglobinuria and reduced plasma haptoglobin)^[16].

Hypo-regenerative anemia

This is caused by alteration of bone marrow progenitor cells, which can be located at different stages of differentiation and maturation. The impairment of pluripotent stem cells usually produces pancytopenia (anemia, leukopenia and thrombocytopenia). Pancytopenia may be caused by intrinsic [bone marrow aplasia, leukemia,

Table 1 Etiopathogenic classification of anemia

Regenerative anemia
Acute or chronic bleeding
Hemolytic anemia
Hereditary (hemoglobinopathy, enzymopathy, membrane-cytoskeletal defects)
Acquired (autoimmune, mechanical destruction, toxic-metabolic, drugs, infectious, PNH, hypersplenism)
Hypo-regenerative anemia
Bone marrow failure caused by stem cell pathology
Quantitative disorder
Selective: erythroblastopenia (pure red cell aplasia)
Global: aplastic anemia
Qualitative disorder (dysmyelopoiesis)
Inherited: hereditary dyserythropoiesis
Acquired: myelodysplasia
Bone marrow infiltration
Leukemia, lymphoma, multiple myeloma
Solid tumors
Myelofibrosis
Thesaurismosis (Gaucher disease)
Inflammatory chronic diseases, microorganisms (Histoplasma, HIV)
Drugs, hypothyroidism, uremia
Erythropoietic factors deficiency
Iron
IDA
ACD
Cobalamin and folate
Megaloblastic anemia
Hormones: erythropoietin, thyroid hormones, androgens, steroids

PNH: Paroxysmal nocturnal hemoglobinuria; IDA: Iron-deficiency anemia; ACD: Anemia chronic disease.

myelodysplastic syndrome (MDS) or myelofibrosis] or extrinsic (metastasis, Gaucher disease and other thesaurismosis, tuberculosis, histoplasmosis, viral and parasitic infections). All of them are capable of displacing normal hematopoiesis or changing the microenvironment necessary for regeneration, differentiation and proliferation of stem cells^[16,17].

Less frequently, progenitor cells committed to the erythroid line (burst-forming unit-erythroid and colony-forming unit-erythroid) are affected selectively, and the result is pure red cell aplasia. When progenitor red cells are impaired selectively, bone marrow erythroblasts are much reduced or absent, while other hematological cellular lines remain normal. Pure red cell aplasia, hereditary or acquired, is very rare. However, a qualitative alteration of the red cell line (dyserythropoiesis) is often seen in clinical practice. In contrast to pure red cell aplasia, the bone marrow is rich in erythroblasts in patients with dyserythropoiesis. In these cases, the erythropoiesis is abnormal morphologically and functionally. Erythroblastopenia as dyserythropoiesis may have a hereditary or acquired origin. The former is extremely rare. Thymoma is the most common cause of acquired pure red cell aplasia. MDS is the most common cause of acquired dyserythropoiesis. In MDS patients, the lesion is located at the level of very primitive multipotent progenitors, and thus, the morphological and functional alterations can affect all blood cell lines (erythrocyte, granulocyte-monocyte and megakaryocyte)^[18-20].

Table 2 Morphological classification

Microcytic anemia (MCV < 82 fL)
IDA
Thalassemia
Non thalassemic conditions associated with microcytosis
ACD (e.g. rheumatoid arthritis, Hodgkin's lymphoma, chronic infection, neoplasia)
Sideroblastic anemia (e.g. hereditary, lead poisoning)
Normocytic anemia (MCV = 82-98 fL)
Nutritional anemia (iron deficiency, cobalamin y/o folate)
Anemia of renal insufficiency
Hemolytic anemia
Red cell intrinsic causes: membranopathy, enzymopathy, hemoglobinopathy
Red cell extrinsic causes: immune-mediated, microangiopathic, associated with infection, chemical agent (spider venoms), metabolic
ACD
Primary bone marrow disorder
Causes that are intrinsic to hematopoietic stem cells: bone marrow aplasia (idiopathic, PNH, Fanconi syndrome), pure red cell aplasia (acquired, congenital, Diamond-Blackfan syndrome), myelodysplastic syndrome
Extrinsic causes: drugs, toxins, radiation, viruses, immune-mediated, bone marrow infiltration (metastatic and lymphoma)
Macrocytic anemia (MCV > 98 fL)
Drugs (hydroxyurea, zidovudine, methotrexate)
Nutritional (vitamin B12 and folate deficiency)
Drug-induced hemolytic anemia
Dyserythropoiesis, myelodysplastic syndrome, clonal hematologic disorder
Hereditary hematologic disorders
Mild macrocytosis (MCV = 100-110 fL)
Reticulocytes
Excess alcohol intake, liver disease, smoking
Hypothyroidism, Waldenström's macroglobulinemia
Copper deficiency, bone marrow aplasia, erythroidopenic anemia
Down syndrome
Chronic obstructive pulmonary disease

MCV: Mean corpuscular volume.

Anemia caused by nutritional deficiencies or decreased production of erythropoietin is much more frequent than that caused by a primitive defect in bone marrow.

MORPHOLOGICAL CLASSIFICATION

Pathogenic classification is very important to understand the mechanisms involved in the genesis of anemia (Table 2). However, in daily clinical practice, it is more useful to start with the analytical parameters of the hemogram. MCV allows us to classify anemia as microcytic (MCV < 82 fL), normocytic (MCV = 82-98 fL) and macrocytic (MCV > 98 fL)^[21,22].

MCV has a relationship with mean corpuscular hemoglobin (MCH), which reports on the mean hemoglobin per erythrocyte expressed in picograms (normal range: 27-32 pg). Therefore, MCV and MCH decrease (microcytic, hypochromic anemia) or increase (macrocytic, hyperchromic anemia) jointly. The MCH concentration (MCHC) reports on the average concentration of hemoglobin in each erythrocyte expressed as a percentage (normal range: 32%-36%), and its variations are very small, even in the presence of hypochromia. MCHC increases only in a few rare

Table 3 Classification of anemia as RDW and MCV

	↓ MCV	Normal MCV	↑ MCV
Normal RDW	β-thalassemia	Normocytic	Bone marrow aplasia
Increased RDW	α-thalassemia Iron deficiency	Inflammatory anemia Hypothyroidism	Megaloblastic anemia

RDW: Red blood cell distribution width.

diseases such as hereditary spherocytosis, and therefore, its practical utility is scarce.

It should always be borne in mind that MCV is an average value and therefore does not provide information about the homogeneity of the erythrocyte population. To resolve this problem, hematological analyzers provide the erythrocyte distribution curve, with an index of dispersion: red blood cell distribution width (RDW) (normal range: 10%-14%). RDW is a rough indicator of anisocytosis and is an essential complement to MCV (Table 3).

Microcytic anemia

Faced with microcytic anemia, the three main diagnostic possibilities include iron deficiency anemia (IDA), thalassemia, and anemia of chronic disorders (ACD). A fourth possibility, sideroblastic anemia, is so rare that is not considered in the initial diagnosis, unless there is a history of contact with lead (Figure 1).

Iron deficiency is the most common cause of anemia, so the first step in diagnosis should be directed toward confirmation or exclusion of IDA. Serum ferritin allows us to confirm the diagnosis. Despite ferritin being an acute phase reactant, the diagnosis of IDA is unlikely with normal or elevated ferritin levels. Other parameters of iron metabolism (serum iron, total transport capacity of iron, and transferrin) are unable to distinguish with certainty IDA from ACD^[23].

IDA occurs in 2%-5% of adult men and postmenopausal women in the developed world^[24,25], and is a common cause of referral to a gastroenterology clinic 4%-13%^[26].

It is important to note that microcytosis without anemia is characteristic of thalassemia trait, but also of polycythemia associated with iron deficiency^[27].

The simple analysis of different parameters provided by the hematological analyzer gives a diagnosis of microcytic anemia. RDW helps to distinguish thalassemia from IDA. RDW is normal in thalassemia; on the contrary, microcytic anemia with RDW > 15 is probably IDA.

In recent years, the importance of serum soluble transferrin receptor has been recognized in differential diagnosis of IDA and ACD. Serum soluble transferrin receptor is increased in IDA, without interference in case ACD is present^[28,29]. Intra-erythrocytic ferritin and erythrocyte zinc-protoporphyrin also help to differentiate IDA from ACD. These parameters allow the diagnosis

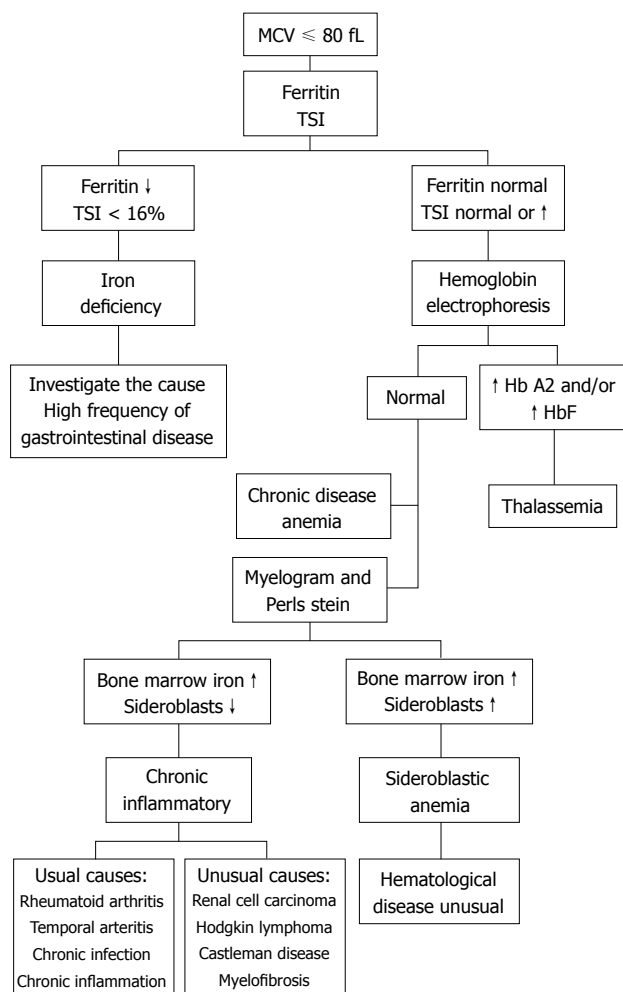


Figure 1 Evaluation of microcytic anemia. TSI: Transferrin saturation index; MCV: Mean corpuscular volume; Hb: Hemoglobin.

of borderline patients who, at another time, would have undergone unnecessary investigation of iron in the bone marrow^[30-32].

In men and postmenopausal women, the bigger concern should be to rule out the presence of occult bleeding. If positive, the first exploration should be a colonoscopy, mainly in men, because of the frequent association of occult blood with adenocarcinoma^[33,34]. If the colonoscopy does not reveal the cause of the anemia, gastroscopy should be performed. The study of the small bowel is more controversial, but it is convenient to keep in mind that celiac disease is a cause of IDA^[35].

In premenopausal women, genital bleeding is the most frequent cause of anemia. Therefore, gastrointestinal exploration is controversial. An anamnesis directed towards the characteristics of menstrual bleeding, although a subjective criterion, may be useful for distinguishing a subgroup of women without excessive genital bleeding, who require direct assessment of occult bleeding, followed by gastrointestinal exploration if positive. In cases in which occult bleeding is negative, the exploration should be directed to the genital system^[36-38].

Microcytosis associated with normal ferritin guides the

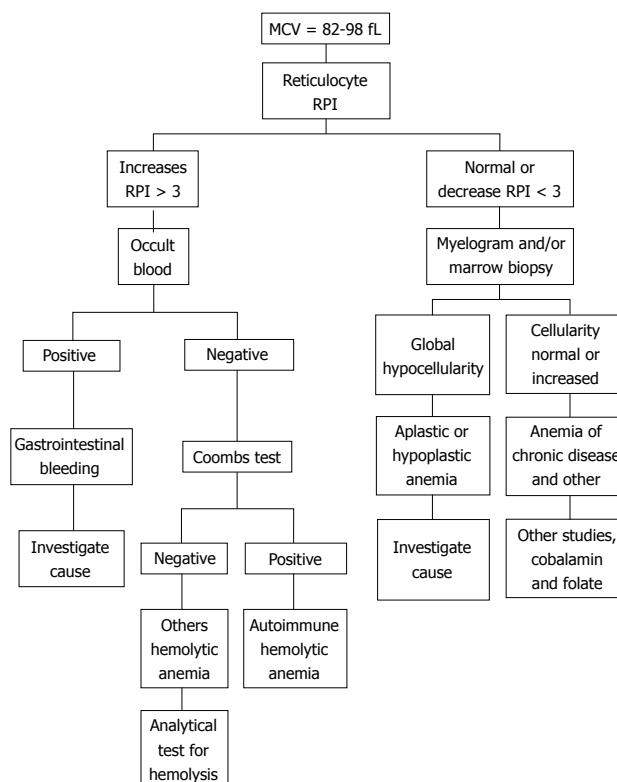


Figure 2 Evaluation of normocytic anemia. RPI: Reticulocytes production index.

diagnosis toward hereditary diseases, mainly thalassemia. If there are no family antecedents of microcytosis, it is necessary to investigate acquired causes of non-iron deficiency microcytosis, mainly ACD and sideroblastic anemia. Normal RDW guides the diagnosis toward the former and a high RDW toward the latter.

Normocytic anemia

The fundamental question in normocytic anemia is to recognize the causes and susceptibility to treatment as soon as possible. Among the causes are nutritional deficiency, renal failure and hemolytic anemia^[21]. Nutritional mixed anemia that combines deficiency of vitamin B12, folic acid and iron is frequent. In consequence, these three parameters should be requested in the initial phase of the diagnosis of normochromic anemia.

In order to differentiate among regenerative (hemolysis and bleeding) or hypo-regenerative anemia (bone marrow aplasia, chronic disease, nutrition deficiency and hemopathy), it is necessary to determine the corrected RPI (Figure 2). In anemia of renal failure, morphological alterations in the blood are scarce and serum erythropoietin may be normal, but inappropriately low for the degree of anemia. The severity of anemia is not evident until the disease is very advanced.

It will always be necessary to rule out hemolysis, by performing easily accessible laboratory tests [lactate dehydrogenase (LDH), indirect bilirubin, haptoglobin and reticulocytes]. These parameters do not inform us

Table 4 Serum levels that differentiate ACD, IDA and mixed anemia

	ACD	IDA	Mixed anemia
Iron	↓	↓	↓
Transferrin	↓ or N	↑	↓
Transferrin saturation	↓	↓	↓
Ferritin	N or ↑	↓	↓ or N
Serum transferrin receptor	N	↑	N or ↑
Ratio: soluble receptor of transferrin/log ferritin	< 1	> 2	> 2
Cytokine levels	↑	N	↑

N: Normal; ↑: Increase; ↓: Decrease.

about the origin of hemolysis, and it is necessary to evaluate others, such as schistocytes in peripheral blood (intravascular hemolysis), the Coombs test (autoimmune hemolysis), tests of osmotic fragility (extravascular hemolysis), and tests to rule out hemolysis induced by drugs^[39]. A detailed anamnesis and Coombs test, with and without the suspected drugs, are very useful in the investigation of drug-induced hemolytic anemia.

When the cause of normocytic anemia is not any of the previously mentioned causes, it is necessary to guide the diagnosis toward a chronic disease or to primitive hematological disease^[21,22]. It is difficult to arrive at the definitive diagnosis. In this situation, it is fundamental that a careful clinical evaluation is carried out to rule out other causes of normocytic anemia: alcoholism (more frequently macrocytic), effects of drugs (chemotherapy, immunosuppression), radiotherapy, neoplasia (bone marrow infiltration), infections (mainly in hospitalized patients), surgery, or recent trauma (first phase of bleeding). The association with pathological concomitant processes, elevation of erythrocyte sedimentation rate (ESR) and the absence of morphological alterations in peripheral blood smears supports the suspicion of ACD^[40]. ACD is the most frequent cause of anemia after ferropenia^[41,42]. It is observed in patients that have immune hyperactivity. Activation of cytokines and the reticuloendothelial system induces changes in iron homeostasis, erythroid precursor proliferation, erythropoietin secretion, and erythrocyte life span^[43]. All of these contribute to the pathogenesis of anemia.

ACD can be complicated with chronic bleeding, and in this case, the diagnosis is more difficult because of the presence of microcytosis and ferropenia. The biochemical parameter that better differentiates ACD from IDA is serum ferritin^[44] (Table 4).

The hematologist must revise the blood smear if primitive disease of bone marrow is suspected. Depending on the result, a bone marrow study may be necessary to detect hematological diseases or metastasis. The biopsy, bone marrow smear, or both inform us of: the morphology of the hematopoietic cells, on their quantitative distribution, especially the myelo-erythroid ratio (normal 3:1); fibrosis; the presence of non-hematopoietic cell; or possible bone marrow aplasia. Perls' specific iron stain informs us of the state of iron stores, and it allows quantification of siderocytes

and sideroblasts. In a patient with anemia and fever of unknown origin, we must carry out careful microscopic examination of bone marrow, some selective staining, and bone marrow cultures to diagnose any underlying infectious diseases. Some infectious or parasitic diseases that are diagnosed with relative frequency are: tuberculosis, histoplasmosis, kala-azar and malaria^[45-47].

Macrocytic anemia

Macrocytosis is observed frequently using blood cell analyzers (Figure 3). Its prevalence is 1.7%-3.9%, but 60% of the patients with macrocytosis do not have anemia^[48]. On the other hand, macrocytosis can be physiological in some circumstances (infants, pregnancy, some families). Even keeping in mind the precedent data is convenient to make a careful evaluation of macrocytosis in every patient, to rule out any underlying pathology. In vitamin B12 and folic acid deficiency, as well as in other diseases, macrocytosis (blood) is accompanied by megaloblastosis (bone marrow). In such cases, both terms can be used interchangeably^[49].

The starting point in the diagnostic process for macrocytic anemia will be to rule out therapy with drugs that interfere with nucleic acid metabolism, such as hydroxyurea, methotrexate, trimethoprim, zidovudine or 5-fluorouracil, as well as habitual intake of alcohol^[49,50]. Hydroxyurea is the drug that increases most the MCV (> 110 fL); the other drugs and alcohol induce a moderate macrocytosis (100-110 fL).

In the absence of intake of any of the drugs mentioned above or alcohol, the most frequent cause of microcytic anemia is nutritional deficiency^[50]. Therefore, serum vitamin B12 and folic acid levels should be evaluated. The last can be modified by the previous day's folic acid intake. A better alternative is intra-erythrocyte folate, which remains stable during the lifetime of red blood cells and gives a better indication of possible chronic folic acid deficiency. The measurement of erythrocyte folate is a difficult technique that is not available in every laboratory. The determination of homocysteine is an accessible alternative to intra-erythrocyte folate. In the absence of folic acid, homocysteine levels increase rapidly because it cannot be converted into methionine. Normal serum levels of homocysteine are highly unlikely in folate deficiency^[21].

In the same way, vitamin B12 deficiency is correlated with low serum levels, although there are clinical situations in which low serum levels are not correlated with vitamin B12 deficiency (pregnancy, elderly patients, and those with low white blood cell counts). In these circumstances, normal serum methyl-malonic acid levels, in the absence of congenital errors of metabolism or renal failure, preclude a deficiency of vitamin B12^[51].

To complete the study of vitamin B12 deficiency, it is necessary to look for antibodies to intrinsic factor, which if positive, confirms the diagnosis of pernicious anemia. If not present, it is necessary to investigate malabsorption by performing the Schilling test^[52] (Table 5).

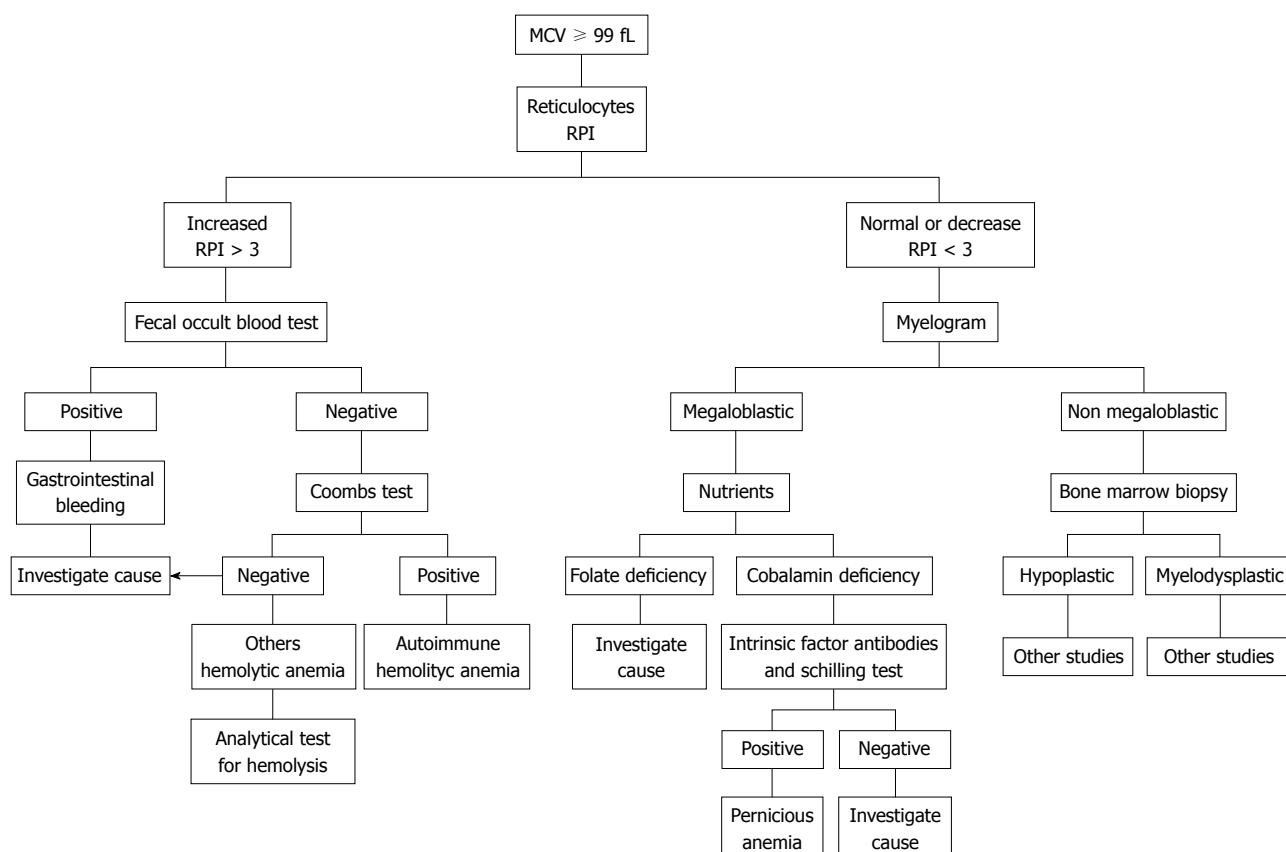


Figure 3 Evaluation of macrocytic anemia.

ANEMIA, A PRACTICAL APPROACH FOR THE GASTROENTEROLOGIST: STRATEGY TO DIAGNOSE PATIENTS WITH ANEMIA, AND PREVALENCE OF DIFFERENT TYPES OF ANEMIA

The strategy for establishing a diagnosis of anemia by gastroenterologists must follow a classic pattern that includes a clinical interview, a basic physical examination, and careful selection of complementary tests.

The clinical history should emphasize the causes of blood loss, including non-gastrointestinal diseases, recent history of gastrointestinal symptoms, gastrointestinal surgery, use of nonsteroidal anti-inflammatory drugs, acetylsalicylic acid and anticoagulant therapy, family history of hematologic diseases and colorectal neoplasia^[53-55]; all symptoms should be recorded, and not only the gastrointestinal ones.

A single blood sample can give information on the concentration of hemoglobin (essential for the diagnosis of anemia according to WHO criteria), the erythrocyte indices MCV and MCH (useful for the morphological classification of anemia), RDW (estimate of anisocytosis) and ESR, which reports on possible ACD.

The hematologist should examine a blood smear if any abnormal erythrocyte indices have been detected. A clotted blood sample (to obtain serum) allows the

determination of the rest of the analytical parameters that are useful in diagnosis, which are iron metabolism (mainly ferritin), urea, creatinine, bilirubin and total protein. In cases of suspected IDA, the concentration of soluble transferrin receptor should be included as a key parameter in differentiation between IDA and ACD^[56]. The most probable causes of microcytic anemia are IDA, thalassemia or thalassemia trait. RDW and iron metabolism are clues to differentiate between these processes in the initial step, as has been mentioned above. The most likely causes of normocytic anemia are ACD, renal failure and primitive hematological diseases (least frequently). Iron metabolism and routine biochemical tests may help to guide the diagnosis, but in some cases, erythropoietin serum levels and bone marrow examination are necessary.

In cases of macrocytic anemia, serum levels of vitamin B12 and serum and/or erythrocyte levels of folate will guide the diagnosis to deficiency of one of these. Most causes of folic acid deficiency are nutritional, although malabsorption should always be kept in mind. In contrast, vitamin B12 deficiency is almost always the result of malabsorption^[49,57]. The few exceptions are in strict vegetarians and hereditary transcobalamin II deficiency (a very infrequent disease). Among malabsorption causes of B12 vitamin deficiency, the most frequent is pernicious anemia. Therefore, the first step is to establish whether the serum levels of gastrin are highly elevated, because

Table 5 Causes of megaloblastic anemia

Cobalamin deficiency
Poor diet
Deficiency of intrinsic factor
Pernicious anemia
Total or partial Gastrectomy
Ingestion of caustic (lye)
Functional defect of intrinsic factor
Alteration of ileal (microenvironment)
Insufficient pancreatic protease activity
Inactivation enzyme (Sd. Zollinger-Ellison)
Competition for cobalamin
Alteration of ileal mucosa
Acquired
Surgical resection or by-pass
Regional enteritis (Crohn's disease)
Tropical sprue
Celiac disease, Tuberculosis
Lymphomatous infiltration
Induced by drugs
Congenital
Sd Immerslund-Gräsbeck
Congenital transcobalamin II deficiency or abnormality
Congenital methylmalonic acidemia and aciduria
Hemodialysis
Urinary losses (congestive heart failure)
Folate deficiency
Dietary
Old age, infancy, poverty, alcoholism, chronic invalids, psychiatrically disturbed, scurvy and kwashiorkor
Excess utilization or loss
Physiologic: pregnancy and lactation, prematurity
Pathologic: Hematologic diseases
Malignant diseases
Inflammatory disease
Metabolic disease
Excess urinary loss, congestive heart failure, active liver disease
Hemodialysis, peritoneal dialysis
Malabsorption
Congenital
Anti-folate drugs
Alcoholism
Tropical sprue, gluten-induced enteropathy
Extensive jejune resection, Crohn's disease, partial gastrectomy, systemic bacterial infection, Whipple's disease
Congenital abnormalities of folate metabolism
Cyclohydrolase, methionine synthetase
Combined deficit of folate and cobalamin
Celiac disease
Regional enteritis (Crohn's disease)
Congenital disorder of DNA synthesis
Disorders of DNA synthesis induced by drugs
Anti-folate
Purine antagonists
Pyrimidine antagonists
Alkylating
Eritroleucemia

Table 6 Differential diagnosis of anemia from a gastrointestinal point of view

Gastrointestinal causes of anemia
Microcytic anemia
Iron deficiency
Decreased iron absorption
Frequent: Celiac disease, gastrectomy, <i>H pylori</i> colonization
Infrequent: Bowel resection, bacterial overgrowth
Occult gastrointestinal blood loss
Frequent: aspirin and nonsteroidal anti-inflammatory drug use, colonic carcinoma, gastric ulceration, angiodysplasia, inflammatory bowel diseases
Infrequent: esophagitis, esophageal carcinoma, gastric antral vascular carcinoma, small bowel tumors, ampullary carcinoma, <i>Ancylomasta duodenale</i>
Non-gastrointestinal blood loss
Frequent: menstruation, blood donation, ACD
Infrequent: Hematuria, epistaxis
Sideroblastic anemia (alcohol, lead, drugs), vitamin B6
ACD
Normocytic anemia
Frequent: ACD (liver disease, renal insufficiency, malignancy, nutritional deficiency, drug effects, alcoholism, recent trauma or surgery, iron deficiency)
Infrequent: primary bone marrow disorder
Macrocytic anemia
Non-megaloblastic
Systemic disease:
Frequent: liver disease, alcoholism
Infrequent: primary bone marrow disease (myelodysplastic syndrome, aplastic anemia), metastatic infiltration, hemolytic anemia, hypothyroidism
Megaloblastic anemia
Vitamin B12 deficiency: pernicious anemia, gastrectomy, hereditary deficiency of intrinsic factor, inflammatory bowel disease, primary intestinal malabsorptive disorders, parasitic colonization, nutritional deficiencies
Folate deficiency: diet poor in folates, regional enteritis, Whipple's disease, scleroderma, amyloidosis, increase requirements (liver disease, hemolytic anemia)
Antifolate drugs: methotrexate

they are > 1000 pg/mL only in atrophic gastritis type A (pernicious anemia) and Zollinger-Ellison syndrome. In pernicious anemia, pepsinogen I levels are low, gastric pH is high and gastric acid secretion does not respond to stimulation with histamine or pentagastrin. Serum antibodies against intrinsic factor and parietal cells are not sensitive or specific enough to confirm the diagnosis. The exploration that leads directly to diagnosis is gastroscopy, with mucosal biopsy and study

of antiparietal cell antibodies. Gastric biopsy has the added advantage of giving information about possible metaplasia or precancerous lesions^[58]. In the absence of gastric disease, the most likely cause of malabsorption of vitamin B12 is Crohn's disease, with involvement of the terminal ileum^[59]. Hereditary causes of malabsorption (Immerslund syndrome) or transport (transcobalamin II deficiency) are extremely infrequent.

The list of causes of anemia is long and cumbersome, but most can fit into a very small number of diseases on which we must concentrate as a first diagnostic option (Table 6)^[60-66].

IDA is the most prevalent form of anemia worldwide, especially in women and children. Thirty-percent of the world's population, some 1300 million people, suffer from anemia. However, the prevalence of anemia worldwide is unequal (36% in underdeveloped and 8% in developed countries) About half of the patients with anemia have IDA, which is most prevalent in the general population and in outpatients. The most likely cause of IDA is malnutrition in children, bleeding in adult males (especially gastrointestinal), menstruation or lactation in fertile

women, and bleeding in the elderly. The distribution of nutrient-deficiency anemia in the elderly is: 48% iron alone, 19% folate alone, 17% vitamin B12 alone, and the rest have combined deficiencies. Therefore, in young male adults and in both sexes older than 65 years, the most likely cause of IDA is chronic bleeding, especially from gastrointestinal lesions^[67].

The hemogram, the concentration of iron, ferritin and transferrin in blood and investigation of fecal occult blood are sufficient to obtain a diagnosis in most cases.

ACD is the most prevalent form of anemia after IDA in the general population^[40,68], but is even more common in hospitalized patients, regardless of sex and age^[42,67,69,70].

The underlying causes of ACD vary greatly: acute and chronic infections, 18%-95%; cancer, 30%-77%; autoimmune diseases (including chronic inflammatory bowel disease), 8%-71%; rejection of solid organ transplantation, 8%-70%; and chronic renal disease, 23%-50%. ACD is found in 20% of elderly people with anemia (30% excluding nutritional deficiency), and a further 4.3% suffer from ACD and kidney failure. Therefore, we should keep in mind ACD as the most likely diagnosis in elderly and inpatients, and as the second most likely diagnosis in outpatients under 65 years of age^[68]. The laboratory tests needed to confirm the diagnosis include serum iron, transferrin, transferrin saturation, ferritin, urea and creatinine.

The gastroenterologist must rule out chronic inflammatory bowel disease, tumors of the digestive tract and liver disease. In some patients, the cause of anemia is complex (chronic disease and chronic hemorrhage). In these cases, it is difficult to confirm the diagnosis and additional testing is required: soluble transferrin receptor and microscopic examination of bone marrow with special stains for iron.

The most common type of anemia after IDA and ACD is megaloblastic anemia (vitamin B12 deficiency, folic acid or both). It is important in gastroenterology because intestinal malabsorption and pernicious anemia are common causes of this form of anemia. The diagnosis is suspected when MCV is elevated, and is confirmed by measuring serum vitamin B12 and serum and erythrocyte folate.

Hemolytic anemia follows next in order of frequency. Acute hemolysis presents a distinctive clinical picture, but chronic hemolysis may go unnoticed. If hemolytic anemia is suspected, the appropriate laboratory tests are antiglobulin test (direct and indirect), LDH, haptoglobin, total and conjugated bilirubin in serum, and salts and bile pigments in urine. These tests can give us the generic diagnosis of hemolytic anemia, immune or not immune, but the etiological diagnosis should be resolved in collaboration with the hematologist.

Anemia is common in hematological malignancies, but with limited gastrointestinal symptoms, except for gastric or intestinal bleeding caused by thrombocytopenia or lymphoma. It is rare, but it should be noted that gastric or intestinal lymphoma sometimes presents as

mild anemia with nonspecific gastrointestinal symptoms. Endoscopy usually provides the diagnosis.

Hereditary anemia is extremely rare, except for thalassemia and sickle cell disease in certain geographical areas. Sickle cell disease is restricted to blacks. When one suspects hereditary anemia, the cooperation of a hematologist should be sought, because the diagnosis is difficult and the laboratory tests should be carefully selected.

CONCLUSION

More than 100 diseases may cause anemia, but 90% belong to three groups: nutritional deficiencies (iron, vitamin B12 and folic acid), ACD (chronic inflammation, tumors), and bleeding (excluding chronic bleeding, which produces iron deficiency). Hemolytic anemia, although less frequent than the other, is the last option before considering the diagnosis of rare diseases.

A few laboratory tests, such as blood count, ESR, serum ferritin, and serum iron and transferrin, are sufficient to focus the diagnosis. According to initial results, additional laboratory tests should be ordered: serum vitamin B12 and serum and erythrocyte folate (in case of macrocytosis), tumor markers and acute phase reactants (if ACD is suspected), LDH, haptoglobin and antiglobulin test (if hemolytic anemia is suspected).

With these key data, the doctor can put in place more specific tests: imaging, endoscopy and additional laboratory tests. The next step should be to correct the cause of anemia when possible or correct anemia itself when the cause cannot be treated. The advantages are clear if the cause is a cancer that can be diagnosed at an early stage. On the other hand, we can improve the quality of life of patients by correcting the anemia with medical treatment or regular transfusions.

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S- Editor Tian L L- Editor Kerr C E- Editor Zheng XM



TOPIC HIGHLIGHT

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A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases

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Received: July 23, 2009 Revised: August 24, 2009

Accepted: August 31, 2009

Published online: October 7, 2009

Peer reviewer: Christa Buechler, PhD, Regensburg University Medical Center, Internal Medicine I, Franz Josef Strauss Allee 11, 93042 Regensburg, Germany

Bermejo F, García-López S. A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases. *World J Gastroenterol* 2009; 15(37): 4638-4643 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4638.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4638>

Abstract

Iron deficiency (ID), with or without anemia, is often caused by digestive diseases and should always be investigated, except in very specific situations, as its causes could be serious diseases, such as cancer. Diagnosis of ID is not always easy. Low serum levels of ferritin or transferrin saturation, imply a situation of absolute or functional ID. It is sometimes difficult to differentiate ID anemia from anemia of chronic diseases, which can coexist. In this case, other parameters, such as soluble transferrin receptor activity can be very useful. After an initial evaluation by clinical history, urine analysis, and serological tests for celiac disease, gastroscopy and colonoscopy are the key diagnostic tools for investigating the origin of ID, and will detect the most important and prevalent diseases. If both tests are normal and anemia is not severe, treatment with oral iron can be indicated, along with stopping any treatment with non-steroidal anti-inflammatory drugs. In the absence of response to oral iron, or if the anemia is severe or clinical suspicion of important disease persists, we must insist on diagnostic evaluation. Repeat endoscopic studies should be considered in many cases and if both still show normal results, investigating the small bowel must be considered. The main techniques in this case are capsule endoscopy, followed by enteroscopy.

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Key words: Anemia; Iron-deficiency; Iron deficiency anemia

INTRODUCTION

Iron deficiency (ID) is defined as the decrease of the total content of iron in the body. Iron deficiency anemia (IDA) occurs when ID is sufficiently severe to reduce erythropoiesis. This type of anemia is the most frequent chronic anemia. ID may be the result of either excessive loss or, less frequently, decreased absorption. In general, the iron absorbed daily equals the amount needed to compensate its loss, so that the overall iron pool remains stable. This fine balance is easily broken, because the capability to absorb iron orally is limited. When the inputs are less than necessary or, more frequently, when the outputs increase and cannot be compensated for, ID and finally IDA, develops. In many cases, these alterations will be secondary to gastrointestinal disease. IDA occurs in 2%-5% of adult males and postmenopausal women in the developed world^[1,2]. ID, with or without anemia, is even more frequent. It is a common cause of consulting a gastroenterologist (4%-13% of all referred patients)^[3]; however, there is only one clinical guide published that deals with this clinical entity and the evidence level for most conclusions is medium-low, due to the paucity of reliable clinical data^[1]. In addition, for similar reasons, many of the patients treated by gastroenterologists will develop ID and IDA. This data underscores the importance of anemia in our clinical practice. Using a therapeutic iron course has been suggested as diagnostic test of ID. This is a reasonable approach only for people at high risk of physiological ID, such as adolescents and pregnant women^[4]. However, in daily clinical practice it is not unusual to find patients who have received several cycles of iron treatment before assessing the potential cause of anemia. This strategy leads to belated evaluation, especially in young females and in patients with a

prior history of anemia, resulting in significant delay in etiological diagnosis of anemia^[5]. In this sense, except in very specific situations, ID with or without anemia should always be investigated because it can be caused by potentially serious diseases^[1]. We will review the etiology and laboratory diagnosis of chronic ID and IDA to establish recommendations on the practical management of this clinical entity.

ETIOLOGY OF ID OR IDA

There are many potential causes of ID and IDA and some of them are very relevant. These causes are clearly different in developing and in developed countries. Deficient intake is the most frequent etiology in the former, whereas other important diseases are potentially implicated in the case of the latter.

In developed countries, the likeliest cause of anemia in each patient depends on age and sex. In women of childbearing age, excessive menstrual loss is the most frequent etiology, while in postmenopausal women and in males, digestive diseases are the main causes^[3,6]. Taking both these data and a detailed clinical history into account, we will plan diagnostic strategy in each case. However, these assumptions should not lead to errors in dealing with IDA or ID, such as not to investigate anemia in women assuming non-diagnosed gynaecological problems, because that attitude leads to a significant delay in detecting important diseases^[5].

In addition to digestive disorders, gynaecological diseases, urological diseases and other specific situations, such as intravascular hemolysis, the etiology of IDA (Table 1) includes aspects that exceed the purposes of this article. Focusing on digestive diseases, the etiology of ID and IDA of gastrointestinal origin can be divided into two groups: situations with increased loss of iron (the most common in developed countries), and those with decreased iron absorption. In the former, the blood loss can occur in the form of visible bleeding (melena, hematemesis, rectal bleeding) or hidden bleeding, which might be more difficult to diagnose. Among the diseases causing the blood loss we should emphasize, by frequency and importance, benign or malignant gastrointestinal tumors of the colon, stomach, esophagus and small intestine, peptic ulcer and reflux disease, use of non-steroidal anti-inflammatory drugs (NSAIDs), and inflammatory bowel disease. The possible existence of a malignancy as the source of anemia, which leads to early completion of endoscopic examinations in this clinical scenario, is of great concern. In the National Health and Nutrition Examination Survey and Epidemiologic Follow-up Study carried out in the USA on a cohort of 9024 individuals (aged between 25 to 74 years old without prior diagnosis of gastrointestinal malignancy), hemoglobin levels and iron saturation were determined. No case of ID in premenopausal women was determined to be due to malignancies. Among men and postmenopausal women with IDA [Relative Risk (RR) = 31, 95% confidence interval (CI): 9-107] or ID without anemia (RR = 5,

Table 1 Causes of iron deficiency anemia

Digestive disorders
Increased losses of iron
Cancer/polyp: colon, stomach, esophagus, small bowel
Peptic ulcer, esophagitis
NSAID use
Inflammatory bowel disease: ulcerative colitis, Crohn's disease
Intestinal parasites
Vascular lesions: angiodysplasia, watermelon stomach
Meckel's diverticulum
Reduced iron absorption
Celiac disease
Bacterial overgrowth
Whipple's disease
Lymphangiectasia
Gastrectomy (partial and total) and gastric atrophy
Gut resection or bypass
Urological and gynecological disorders
Intravascular hemolysis
Prosthetic valves and cardiac myxomas, paroxysmal nocturnal hemoglobinuria, marathon runners, multiple blood donations
Deficient iron intake

95% CI: 1-21) an increased risk of being diagnosed with cancer within the subsequent two years was observed^[7]. Therefore, gastrointestinal malignancies are uncommon in premenopausal women with ID or IDA, but in men and postmenopausal women with ID or IDA gastrointestinal malignancies are more common than in individuals with normal hemoglobin and iron levels.

Reduced iron absorption is the second category of ID causes of digestive origin, and can be caused by celiac disease, atrophic gastritis, and postsurgical status (gastrectomy, intestinal resection) among others. Celiac disease is very relevant and specific evaluation to exclude it must be performed. In a study on patients referred to a specialized gastroenterological consultation because of ID or IDA, celiac disease was finally the diagnosis in 10% of cases^[8]; other authors described that at least 2%-3% of patients with IDA are finally diagnosed as celiac disease^[3,6]. The prevalence of this disease worldwide is approximately 1%, and it is probably under diagnosed^[9]. Microscopic alterations in the duodenal mucosa in non-treated celiac disease patients will lead to them becoming refractory to oral iron treatment. This has also been described in patients with autoimmune atrophic gastritis and gastritis due to *Helicobacter pylori* (*H. pylori*)^[10-12]. Gastroscopy with biopsies, allowing us to detect the presence of atrophy with or without *H. pylori*, is essential. The positivity of autoantibodies (anti-intrinsic factor or anti-parietal cell) supports the diagnosis of autoimmune atrophic gastritis^[13]. Regarding the possible role of *H. pylori* in IDA, a recent meta-analysis indicated that the infection is associated with depleted iron deposits. The mechanism by which *H. pylori* induces this alteration is not clear, but it appears to involve gastrointestinal blood loss, diminished iron absorption from the diet, and increased consumption of iron by the bacteria. The authors suggest that the impact of eradication of *H. pylori* in the improvement of the iron deposits must be evaluated in large controlled trials^[14]. Finally, it must be pointed out that in our environment,

a deficit of dietary iron not associated with any other pathology will rarely be the cause of ID or IDA.

CLINICAL MANIFESTATIONS

The clinical picture varies greatly from one case to another, and it is produced both by the anemia itself and by the lack of iron, which is essential for cellular energy metabolism. Symptoms depend greatly on the speed of onset of anemia, its severity, and the characteristics of the patient. Thus, IDA or ID can be detected in an asymptomatic individual on a screening-analysis, or in a person with symptoms that include general weakness, fatigue, irritability, poor concentration, headache, and intolerance to exercise. These symptoms appear even in the figures for ID with normal hemoglobin levels. Patients often show relatively few symptoms spontaneously. Although the impact of ID on the quality of life of the subject is high, they often get used to their symptoms and these are assumed as normal. The patient becomes aware of an improvement only when the symptoms disappear. Some iron-deficient patients, with or without anemia, might have alopecia, atrophy of lingual papillae, or dry mouth due to loss of salivation. Other symptoms, such as weakness or digging fingernails (koilonychia), chlorosis, or the syndromes of Plummer-Vinson or Paterson-Kelly (dysphagia with esophageal membrane and atrophic glossitis) have virtually disappeared. These changes were caused by reduction of iron-containing enzymes in the epithelia and the gastrointestinal tract. Pica, the eating disorder in which there is an irresistible desire to lick or eat non-nutritive and unusual substances, such as soil, chalk, gypsum, ice (pagophagia) or paper, might appear in some cases. Pagophagia is considered quite specific to ID and it responds quickly to treatment. In a study on a group of patients referred to a gastroenterology consultation, more than half had pagophagia. It was especially frequent in women, and was not related to the cause of bleeding^[15].

Physical examination might be normal or show pallor of varying intensity, there may be a systolic murmur in cardiac auscultation, and abdominal and rectal exploration will allow us to rule out the existence of masses at those locations.

LABORATORY DIAGNOSIS: ID WITH OR WITHOUT ANEMIA

The diagnosis of anemia is simple and objective: the World Health Organization defines it as the decline in blood hemoglobin to a concentration below 13 g/dL in men and 12 g/dL in women. However, to confirm that ID is the origin of the anemia is not always easy. Sometimes the simple blood cell count strongly suggests this origin, the typical pattern being microcytosis, hypochromia (perhaps the most important, even more than the microcytosis), and elevation of red cells distribution width (RDW). However, up to 40% of

“pure” IDA cases are normocytic. Therefore, a normal mean corpuscular volume (MCV) does not exclude ID from being the cause of the anemia. Moreover, the presence of microcytosis does not necessarily imply ID and can be produced by other anemias (chronic process, sideroblastic anemia) and diseases (e.g. thalassemia). RDW measures the degree of anisocytosis (size difference) of the population of red cells and its elevation is neither sensitive nor specific for ID. The next step is to determine the so-called iron metabolism (in addition to all other necessary determinations, including levels of vitamin B12 and folic acid) and in many cases the level of C-reactive protein. A typical pattern is a decrease in sideremia, plasma ferritin, and transferrin saturation. However, this is not the usual case. The least reliable parameter for diagnosis of ID is probably the determination of sideremia, because it could be detected as an artefact of contamination of laboratory equipment, it has a nocturnal rhythm and it can normalize hours after ingestion. Serum ferritin, in the absence of inflammation (usually defined as a normal C-reactive protein level), reflects total body iron deposits. Thus, a low serum ferritin (< 30 ng/L) unequivocally means ID, whether accompanied by anemia or not. However, as serum ferritin is an acute phase reactant, a normal or even elevated ferritinemia does not exclude the presence of ID. Thus, in the presence of an inflammatory process (usually defined by an elevated C-reactive protein level), ID could exist even with levels of ferritin up to 100 ng/mL. Another parameter of the normal “iron metabolism”, especially useful when the determination of ferritin is equivocal, is the transferrin saturation index. This shows the percentage of transferrin that transports iron and thus a decrease (< 20%) implies ID, either absolute or functional.

In some cases, even taking into account all these determinations, ID can be difficult to diagnose. It generally occurs in situations where the anemia has a multifactorial origin. This is typical in cases of anemia of mixed origin, a chronic process that coexists with ID, which is a frequent scenario in gastrointestinal inflammatory disease or cancer. Even with all the determinations previously described, it can be difficult to estimate the role of each factor in the genesis of anemia in this setting. In these cases, other values can help us to assess the pathogenesis of anemia. These other factors include the determination of soluble transferrin receptor, reticulocyte hemoglobin concentration, the percentage of hypochromic red cells, the concentration of erythropoietin (and its relation to the expected values), and even the determination of hepcidin. The soluble transferrin receptor is one of the most useful as it is the least influenced by the presence of inflammation and it correlates well with concentration of transferrin receptor in the cell plasma membrane^[16]. If the levels are high, ID is likely to be a major component of anemia, while in those cases with normal or low levels; anemia is probably not associated with ID. In Table 2, the values of different determinations in different clinical scenarios are shown.

Table 2 Differences between the serum values of iron-deficiency anemia, anemia of chronic diseases, and anemia of mixed origin

	Iron-deficiency anemia	Anemia of chronic diseases	Anemia of mixed origin
Serum iron	Decreased	Decreased	Decreased
Transferrin	Increased	Decreased or normal	Decreased
Transferrin saturation	Decreased	Decreased	Decreased
Ferritin ¹	Decreased (< 30 ng/mL)	Increased (> 100 ng/mL)	Normal
Soluble transferrin receptor	Increased	Normal	Increased or normal
C-reactive protein	Normal	Increased	Increased
Erythropoietin	Increased	Normal or slightly increased for the degree of anemia	Increased or normal

¹Ferritin values between 30 and 100 ng/mL: Make other determinations to differentiate the two entities.

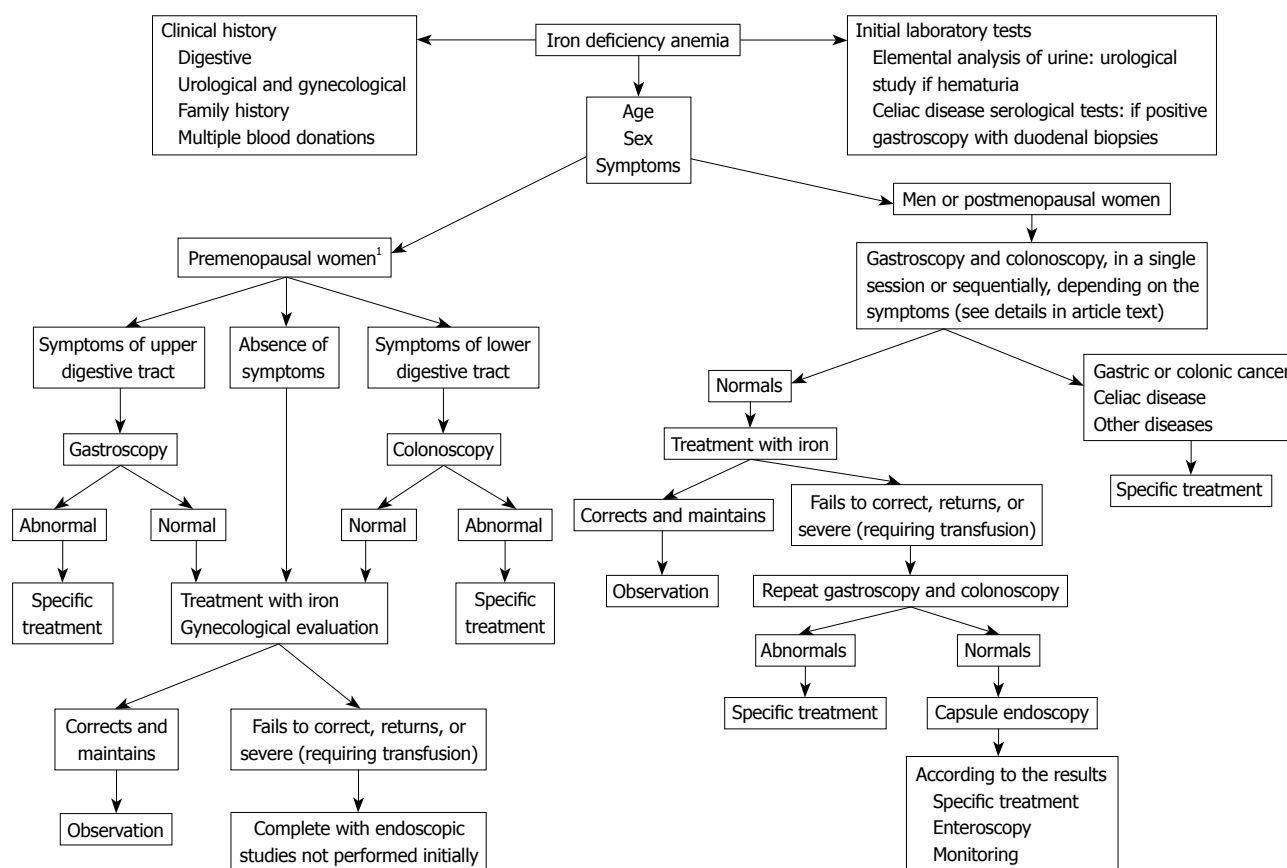


Figure 1 Etiologic diagnosis of iron deficiency anemia. ¹We must always consider gynecological cause.

ETIOLOGIC DIAGNOSIS OF ID ANEMIA

Once the diagnosis of IDA or ID without anemia has been established, it is necessary to investigate its origin (Figure 1), because it can be caused by very serious diseases. Taking into account the age and sex of the patient, and of course an adequate clinical history, we will plan a diagnostic strategy in every individual case.

In the clinical history, we should investigate any sign of digestive or urologic bleeding, and in the case of women, gynaecological history and symptoms should be included. Personal history of peptic ulcers and a family history of colon cancer or celiac disease should be investigated. The existence of suggestive symptoms in a location is a predictor of disease in that location. Therefore, the initial evaluation can be accompanied by the location of symptoms^[17].

The initial studies should include laboratory tests, with an elementary analysis of urine (up to one third of

the renal carcinomas have anemia). In the case of positive serological tests for celiac disease, it is also necessary to perform a gastroscopy with distal duodenum biopsies. In most patients, a dilemma will arise; is it necessary or mandatory to perform a gastroscopy, a colonoscopy or both? In patients over 50 years old, a colonoscopy is preferred to gastroscopy, and on the other hand, many of the patients under study for ID might have an indication of preventive colonoscopy screening, either because of family history or age (over 50 years)^[18]. It has been described that in the presence of a low ferritin value, the probability of finding any pathology at colonoscopy will be quadrupled^[19]. This supports the need for colonoscopy in the study of IDA and ID. It is important to underline that up to 15% of cases may have synchronous lesions in the upper and lower digestive tract, which means that it is necessary to perform both techniques, except in those cases in which celiac disease or a neoplasm is diagnosed

in the initial examination^[1]. Gastrointestinal endoscopies (gastroscopy and colonoscopy) might be performed in the same session or sequentially, according to clinical history (or serologic data). The combination of gastroscopy and colonoscopy is highly sensitive and specific for locating gastrointestinal lesions that produce anemia^[3,20,21]. However, the combination of both endoscopic techniques only determines the final cause of anemia in a little more than half of the patients. In a prospective study carried out on 100 consecutive patients with IDA, gastrointestinal endoscopies revealed at least one lesion potentially responsible for the loss of blood in 62 patients, 36% by gastroscopy, 25% by colonoscopy, and 1% by both^[17]. If examinations are normal, the anemia is not severe and symptoms do not suggest significant disease. The next step might be clinical follow-up, oral iron treatment (but iron supplementation may be harmful in some patients, e.g. in those with renal diseases) and cessation of any NSAIDs or aspirin consumption. Patients not responding to treatment with oral iron, those with severe anemia, or suspected serious illness, will require re-evaluation^[1,22]. Some authors describe that in the elderly, low MCV (≤ 60 fL) and a positive test for fecal occult blood are predictors of the presence of potentially bleeding lesions in endoscopy in patients with anemia without gastrointestinal symptoms^[23]. In premenopausal women, the most frequent endoscopic findings are gastritis by *H pylori* and celiac disease, and it has been suggested that in these patients initial diagnostic approach to IDA may include, in addition to the serologic celiac tests, a ¹³C-urea breath test, reserving endoscopic studies for cases where these tests are negative or anemia persists despite the eradication of *H pylori*^[24]. Finally, in the study of ID or IDA it must be pointed out that a barium enema is not a useful tool, being clearly inferior to colonoscopy^[25].

In those patients whose non-favourable clinical course after negative endoscopic studies advises further evaluation, repeating the endoscopic studies is justified because a proportion of lesions within the reach of conventional endoscopes might not be detected for several reasons. Repeating a gastroscopy might show erosions in a large hiatus hernia (Cameron lesions), peptic ulcer and vascular ectasy not detected in a previous exploration^[26]. Repeating a colonoscopy has a slightly lower yield, but it is a reasonable and necessary option if the previous colonoscopy was suboptimal because of incomplete or poor preparation. It has been shown that colonoscopy can fail in the diagnosis in 5% of colorectal tumors due to several reasons: an incomplete exploration, poor bowel preparation, misinterpretation of findings, inadequate biopsies of lesions^[27], or just not seeing the lesion.

In those cases in which repeated endoscopic examinations are all negative, we should investigate the small bowel as the source of anemia. In this scenario, the best initial approach is probably a capsule endoscopy^[28], reserving enteroscopy (single and double-balloon) for cases in which it is necessary to apply a treatment or to obtain biopsies of lesions localized by the capsule^[29,30]. This strategy significantly reduces the number of patients

requiring an alternative study after an initial investigation of the small intestine. Capsule endoscopy is a technique that explores the entire small intestine, something which is not always possible with enteroscopy. Capsule endoscopy has the disadvantage that it does not allow biopsies of detected lesions. The most common findings in patients with bleeding of obscure origin and/or IDA are angiodysplasia and Crohn's disease^[31]. According to the results of the meta-analysis of Triester *et al*^[32], diagnostic yield of capsule endoscopy (63%) in a study of patients with gastrointestinal bleeding of obscure origin was higher than that of push enteroscopy (26%), and of contrast studies with barium (8%). Enteroscopy should be considered as a second line technique in patients with a positive capsule endoscopy requiring sampling for histology or performing therapeutic endoscopy, and in patients in whom the suspicion of a small intestine lesion is high, despite the negativity of the capsule^[33].

Classical imaging studies of the small intestine (bowel through and enteroclysis) are much less sensitive for detecting lesions potentially causing anemia^[17] and have been reserved for those centres where the previous techniques are not available or are contraindicated. Radiological studies of the small bowel with computed tomography or magnetic resonance imaging techniques, which are very useful in the characterization of tumors from these locations and in the diagnosis of inflammatory bowel disease could, in experienced hands, increase the diagnostic yield^[34].

Finally, we should bear in mind that enteropathy by NSAIDs affects a significant number of people using these drugs, and that the amount of blood lost with the regular use of NSAIDs, such as ibuprofen, can be quite large^[35]. Injuries by the use of classic NSAIDs and selective inhibitors of COX-2 are very common (50%-70%) in the evaluation of the small intestine, i.e. by capsule endoscopy^[36,37]. We must remember that despite treatment with gastro-protective agents (proton pump inhibitors), aspirin and NSAIDs can cause gastrointestinal lesions of the lower bowel and thus cause bleeding and chronic ID.

CONCLUSION

IDA and ID are quite frequent in digestive pathology and they must always be taken into consideration for two reasons: (1) they have a clear impact on the patient's quality of life, and therefore they require adequate treatment and (2) they can be the consequence of significant or severe diseases, so it is essential to investigate their origin. Initial etiological evaluation must include celiac disease serological tests. In many patients, the usual endoscopic studies (gastroscopy and colonoscopy) will be prescribed, as they are necessary to rule out more severe diseases and they allow identification of the origin of anemia in more than half of the cases. In the rest of the patients, if anemia is severe or it does not respond to oral iron treatment, the first step will be to repeat those studies. If the results are still normal, it is necessary to investigate the existence of lesions in the small bowel by using capsule endoscopy. With all the diagnostic means

available nowadays, very few IDAs should be left without a diagnosis.

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TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, Series Editors

A short review of malabsorption and anemia

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Received: July 23, 2009 Revised: September 4, 2009

Accepted: September 11, 2009

Published online: October 7, 2009

Peer reviewer: Zvi Fireman, MD, Associate Professor of Medicine, Head, Gastroenterology Department, Hillel Yaffe Med Ctr, POB 169, 38100, Hadera, Israel

Fernández-Bañares F, Monzón H, Forné M. A short review of malabsorption and anemia. *World J Gastroenterol* 2009; 15(37): 4644-4652 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4644.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4644>

Abstract

Anemia is a frequent finding in most diseases which cause malabsorption. The most frequent etiology is the combination of iron and vitamin B12 deficiency. Celiac disease is frequently diagnosed in patients referred for evaluation of iron deficiency anemia (IDA), being reported in 1.8%-14.6% of patients. Therefore, duodenal biopsies should be taken during endoscopy if no obvious cause of iron deficiency (ID) can be found. Cobalamin deficiency occurs frequently among elderly patients, but it is often unrecognized because the clinical manifestations are subtle; it is caused primarily by food-cobalamin malabsorption and pernicious anemia. The classic treatment of cobalamin deficiency has been parenteral administration of the vitamin. Recent data suggest that alternative routes of cobalamin administration (oral and nasal) may be useful in some cases. Anemia is a frequent complication of gastrectomy, and has been often described after bariatric surgery. It has been shown that banding procedures which maintain digestive continuity with the antrum and duodenum are associated with low rates of ID. *Helicobacter pylori* (*H. pylori*) infection may be considered as a risk factor for IDA, mainly in groups with high demands for iron, such as some children and adolescents. Further controlled trials are needed before making solid recommendations about *H. pylori* eradication in these cases.

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Key words: Anemia; Celiac disease; *Helicobacter pylori*; Cobalamin deficiency; Gastrectomy

INTRODUCTION

Anemia is a frequent finding in most digestive diseases which cause malabsorption. In this review we will focus on some frequent entities producing anemia and malabsorption, which are of current interest because of recent advances regarding each of them. Celiac disease (CD) may be considered as an archetypal malabsorption syndrome, and it is a frequent cause of anemia without associated intestinal symptoms. Anemia due to isolated cobalamin deficiency is a frequent finding in the elderly, and its etiology goes beyond the classical pernicious anemia concept. On the other hand, physicians should be aware that anemia often follows gastric surgery procedures, mainly after bariatric surgery, in order to implement prevention strategies. *Helicobacter pylori* (*H. pylori*) infection is being recognized as a frequent cause of iron deficiency (ID), both in developed and developing countries. In these cases eradication of the infection could be a successful therapy of the anemia.

ANEMIA IN CD

CD is an immunologically-mediated enteropathy, triggered in genetically susceptible subjects, by the intake of certain proteins of wheat, barley and rye, and resulting in small-bowel mucosal villous atrophy with crypt hyperplasia^[1].

Anemia has frequently been reported as the only manifestation or the most frequent extra-intestinal symptom of CD^[2-4]. Although folate and cobalamin deficiency are known complications of CD, the most common nutritional anemia associated with CD is ID. ID anemia (IDA) was reported in up to 46% of patients with subclinical CD in one study, and its prevalence was higher in adults than in children^[4]. In a recent case-control study, only anemia (OR: 26.3; 95% CI: 6-120) and diarrhea

Table 1 Prevalence of CD in patients presenting with IDA

Study, yr	n	Positive serology (%)	Positive biopsy (%)	Comments
Corazza <i>et al</i> ^[16] , 1995	200	8	5	IDA. 8.5% if only obscure IDA considered. All patients with positive serology were biopsied
Carroccio <i>et al</i> ^[17] , 1998	85	5.8	5.8	IDA. 20% if only refractory obscure IDA considered. All patients with positive serology were biopsied. 80% of CD cases were women
Unsworth <i>et al</i> ^[18] , 2000	483	6.6	4.6	Blood donors with anemia, most with IDA. Not all seropositive patients were biopsied. 95% of CD cases were women
Haslam <i>et al</i> ^[19] , 2001	216	2.3	n/a	Pregnant women with anemia. Four of five anemic women with positive serology have IDA. Only serology was performed
Annibale <i>et al</i> ^[20] , 2001	190	n/a	13.7	Obscure IDA. All patients were biopsied. 85% of CD cases were women
Howard <i>et al</i> ^[21] , 2002	258	10.9	4.7	Majority of patients with previously not studied IDA (4% folate deficiency). Not all seropositive patients were biopsied. 83% of CD cases were women
Ransford <i>et al</i> ^[22] , 2002	484	3.5	2.3	Previously not studied IDA. Not all seropositive patients were biopsied. 73% of CD cases were women
Grisolano <i>et al</i> ^[23] , 2004	103	n/a	8.7	Previously not studied IDA. All patients were biopsied. 100% of CD cases were women
Mandal <i>et al</i> ^[24] , 2004	504	n/a	1.8	IDA with normal upper gastroscopy. Not all patients were biopsied
Hershko <i>et al</i> ^[25] , 2005	150	5.3	5.3	Previously not studied IDA. All seropositive patients were biopsied
Kalayci <i>et al</i> ^[26] , 2005	135	4.4	4.4	Previously not studied children with IDA. All seropositive patients were biopsied
Zamani <i>et al</i> ^[27] , 2008	206	15	14.6	Obscure IDA. All patients were biopsied
Carter <i>et al</i> ^[28] , 2008	116	n/a	6.5	Previously not studied premenopausal women with IDA. All patients were biopsied

n/a: Not available data; IDA: Iron deficiency anemia; CD: Celiac disease.

(OR: 4.5; 95% CI: 2-10) were identified as independent predictors of an eventual diagnosis with CD among the different clinical presentations in general practice during the 5 years prior to the diagnosis of CD^[5]. Similarly, among patients identified by population screening, 50% were anemic as the primary presentation^[6].

Iron is absorbed in the proximal small intestine and the absorption is dependent upon several factors, including an intact mucosal surface and intestinal acidity^[7,8]. ID in CD primarily results from its impaired absorption as a result of the villous atrophy of the intestinal mucosa. Consequently, IDA develops. The concept of abnormal iron absorption is supported by the failure to increase serum iron following oral iron supplementation.

Occult gastrointestinal bleeding has been described in CD in correlation with the severity of villous atrophy^[9]. More recent studies have found, however, that the rate of positive occult blood tests in CD is low and does not exceed that of the general population^[10,11].

Anemia of chronic disease has also been described in CD^[12,13]. It is well known that pro-inflammatory cytokines play an essential role in the pathogenesis of CD^[14]. In this regard, both interferon- γ and interleukin-6 are powerful mediators of hypoferrremia in inflammation, leading to the abnormalities in iron homeostasis associated with the anemia of chronic disease^[15]. Accordingly, Harper *et al*^[12] described that in the majority of CD patients with anemia, low serum ferritin levels were an indicator of ID. However, in 13% of patients with anemia, serum ferritin levels were increased, such abnormalities reverting to normal after a gluten-free diet. In a recent study, refined precision laboratory methods to identify anemia of inflammation were used^[13]. Among 65 anemic CD patients, 45 had IDA, two had cobalamin or folate deficiency, and 11 had anemia of chronic disease alone or in combination with ID, which implies

a prevalence of 17%. After 12-mo on a gluten-free diet, the response was equally favorable in the patients with either IDA or anemia of chronic disease, indicating that the suppression of inflammatory intestinal changes by the diet improves anemia both by correcting iron absorption and by blunting the inflammatory response.

CD is frequently diagnosed in patients referred for evaluation of anemia, and subclinical CD appears to be a relatively common cause of IDA. Studies using serologic tests and small-bowel biopsies in patients referred for evaluation of IDA have reported CD in 1.8%-14.6% of patients (Table 1)^[16-28]. These studies are heterogeneous in design, as some included only patients with IDA whereas others included both folate and iron deficient patients, and used different methods for diagnosis of CD, often in selected referral populations. Highest frequencies of CD were observed in patients with obscure IDA, and frequency may be as high as 20% in patients with refractory obscure IDA^[17]. The frequency of CD in women with IDA is higher than in other risk groups of CD, with a female:male ratio of 2:1^[4,29], and several studies have reported that 73%-100% of IDA patients diagnosed with CD were adult pre-menopausal women^[17,18,20-23]. The higher iron demand in adult pre-menopausal women as a result of menstrual loss in a condition of chronic iron malabsorption attributable to CD probably explains this higher prevalence of CD in this group of patients.

Clinicians should consider CD as a possible cause of anemia in all subjects with IDA of unknown origin, even in menstruating women. Recent guidelines from the British Society of Gastroenterology recommend that duodenal biopsies should be taken during endoscopy if no obvious case of ID can be found^[30]. The treatment of IDA associated with CD is primarily a gluten-free diet with iron supplementation until the iron stores have been restored.

Table 2 Stages of cobalamin metabolism and corresponding causes of cobalamin deficiency

Stage of cobalamin metabolism	Causes of cobalamin deficiency
Intake solely through food	Strict vegetarianism without vitamin supplementation
Digestion brings into play haptocorrin, gastric secretions (hydrochloric acid and pepsin), pancreatic and biliary secretions, enterohepatic cycle	Gastrectomy; pernicious anemia (Biermer's disease); food-cobalamin malabsorption
Absorption brings into play intrinsic factor and cubilin	Ileal resection; malabsorption; pernicious anemia; Imerslund syndrome
Transport by transcobalamins	Congenital deficiency of transcobalamin II
Intracellular metabolism based on various intracellular enzymes	Congenital deficiency of various intracellular enzymes

Several studies have shown that many untreated patients with CD are folate deficient^[7]. Deficiency of vitamin B12 is also common in CD and frequently results in anemia^[7]. The main site of vitamin B12 absorption is the distal ileum; a small proportion is also absorbed passively along the entire small bowel. The causes of vitamin B12 deficiency in CD may include associated autoimmune gastritis, bacterial overgrowth, decreased gastric acid or decreased efficiency of mixing with transfer factors in the intestine.

ANEMIA AND COBALAMIN DEFICIENCY

Literature of the last 10 years has provided several definitions of cobalamin (vitamin B12) deficiency, depending mainly on the population studied and on the particular assay kits used. Cobalamin deficiency is defined in terms of the serum values of cobalamin and of homocysteine and methylmalonic acid, two components of the cobalamin metabolic pathway. High homocysteine levels (hyperhomocystinemia) may also be caused by folate or vitamin B6 deficiencies, and these should be excluded before a diagnosis of cobalamin deficiency is made^[31]. Accordingly, definitions of cobalamin deficiency are^[32]: (1) Serum cobalamin levels < 150 pmol/L (< 200 pg/mL) with clinical features and/or hematological anomalies related to cobalamin deficiency; (2) Serum cobalamin levels < 150 pmol/L on two separate occasions; (3) Serum cobalamin levels < 150 pmol/L and total serum homocysteine levels > 13 µmol/L or methylmalonic acid levels > 0.4 µmol/L (in the absence of renal failure and folate and vitamin B6 deficiencies); (4) Low serum holotranscobalamin levels < 35 pmol/L.

Epidemiological studies show that in the general population of industrialized countries, cobalamin deficiency has a prevalence of around 20%, ranging from 5% to 60% depending on the definition of cobalamin deficiency used^[31,32].

Cobalamin metabolism and corresponding causes of deficiency

The metabolic pathway starts when dietary cobalamin, obtained through animal foods, enters the stomach bound to animal proteins. A typical Western diet contributes 3-30 µg of cobalamin per day towards the recommended dietary allowance of 2.4 µg/d for adults^[33]. Pepsin and hydrochloric acid in the stomach split the animal protein, releasing free cobalamin. Most

of the free cobalamin is then bound to R-protein which is released from the parietal and salivary cells. Intrinsic factor is also secreted in the stomach, but its binding to cobalamin is weak in the presence of gastric and salivary R-protein. In the duodenum, dietary cobalamin bound to R-protein is joined by cobalamin-R-protein complexes that have been secreted in the bile. Pancreatic enzymes degrade both biliary and dietary cobalamin-R-protein complexes, releasing free cobalamin. The cobalamin then binds with intrinsic factor. The cobalamin-intrinsic factor complex remains undisturbed until the distal 80 cm of the ileum, where it attaches to mucosal cell receptors (cubilin) and the cobalamin is bound to transport proteins designated transcobalamin I, II and III. Transcobalamin II, although it represents only a small fraction (about 10%) of the transcobalamins, is the most important because it is able to deliver cobalamin to all cells in the body. The cobalamin is subsequently transported systemically *via* the portal system. Within each cell, the transcobalamin II-cobalamin complex is taken up by means of endocytosis and the cobalamin is liberated and then converted enzymatically into its two coenzyme forms, methylcobalamin and adenosylcobalamin. The causes of cobalamin deficiency according to the stage of cobalamin metabolism are described in Table 2^[31,32].

Cobalamin deficiency in the elderly: clinical entities

Vitamin B12 or cobalamin deficiency occurs frequently among elderly patients, but it is often unrecognized or not investigated because the clinical manifestations are subtle^[33]. However, the potential seriousness of the complications (particularly neuropsychiatric and hematological) requires investigation of all patients who present with vitamin or nutritional deficiency. In elderly patients, cobalamin deficiency is caused primarily by food-cobalamin malabsorption and pernicious anemia^[31]. Food-cobalamin malabsorption accounts for about 60%-70% of the cases among elderly patients, and pernicious anemia accounts for 15%-20% of the cases. Other causes included dietary deficiencies (< 5%), malabsorption (< 5%) and hereditary cobalamin metabolism diseases (< 1%).

Intrinsic factor, which is released by parietal cells in the stomach, binds to vitamin B12 in the duodenum. This vitamin B12-intrinsic factor complex subsequently plays a role in the absorption of vitamin B12 in the terminal ileum. This mechanism is responsible for 60% of the absorption of cobalamin. In addition, an alternate system exists that is independent of intrinsic factor or

even an intact terminal ileum: cobalamin is absorbed by simple diffusion or mass action independent of intrinsic factor if 300-1000 µg/d cobalamin is administered orally or intramuscularly to patients with pernicious anemia. Approximately 1%-5% of free cobalamin (or crystalline cobalamin) is absorbed along the entire intestine by passive diffusion^[33,34].

Food-cobalamin malabsorption: Food-cobalamin malabsorption syndrome is characterized by the inability to release cobalamin from food or from intestinal transport proteins, particularly in the presence of hypochlorhydria, where the absorption of “unbound” cobalamin remains normal. This syndrome is defined by cobalamin deficiency in the presence of sufficient food-cobalamin intake and a negative Schilling test, where the latter rules out malabsorption or pernicious anemia. Thus in this syndrome, patients can absorb “unbound” cobalamin through intrinsic factor or passive diffusion mechanisms. The recognition of the syndrome permits new developments of oral cobalamin therapy^[31-33,35]. Researchers have supported the existence of this syndrome by using a modified Schilling test, which employs radioactive cobalamin bound to animal proteins and reveals malabsorption when the results of a standard Schilling test are normal.

Food-cobalamin malabsorption is caused primarily by gastric atrophy. Over 40% of patients older than 80 years have gastric atrophy that may or may not be related to *H pylori* infection^[31]. Other factors that contribute to food-cobalamin malabsorption in elderly people include: intestinal microbial proliferation; long term ingestion of biguanides (metformin) and antacids, including H₂-receptor antagonists and proton pump inhibitors; chronic alcoholism; surgery or gastric reconstruction; partial pancreatic exocrine failure; and Sjögren's syndrome^[31,32].

The partial nature of this form of malabsorption may produce a more slowly progressive depletion of cobalamin than does the more complete malabsorption engendered by disruption of the intrinsic factor-mediated absorption. The slower progression of cobalamin depletion probably explains why mild, preclinical deficiency is more frequently associated with food-cobalamin malabsorption than with pernicious anemia^[32,33,35]. When associated with *H pylori* infection, eradication of the infection alone may correct cobalamin levels^[36].

Pernicious anemia: This is the classic cause of cobalamin deficiency and one of the most frequent among elderly patients. Pernicious anemia is an autoimmune disease characterized by the destruction of the gastric mucosa, especially fundal mucosa, by a primarily cell-mediated process^[31,37]. Gastric secretions are neutral to slightly acidic even in the presence of gastrin and contain little or no intrinsic factor. The disease is also characterized by the presence of two antibodies, particularly in plasma and gastric secretions: few people who do not have the disease have antibodies

(specificity 98%), but only about 50% of patients will have anti-intrinsic factor antibodies (IFA) (sensitivity 50%). Anti-gastric parietal cell (GPC) antibodies can also be measured in the serum (sensitivity > 90% but specificity 50%). Moderate hypergastrinemia, and sometimes major hypergastrinemia, have also been associated with pernicious anemia, but this is not a pathognomonic finding. A positive Schilling test with the addition of a test for IFA virtually confirms the diagnosis (specificity > 99%)^[31].

The optimal testing strategy remains unclear and considerable controversy still exists as to whether both indirect immunofluorescence testing for GPC and enzyme linked immunosorbent assay for IFA need to be performed as screening tests for pernicious anemia. It has been suggested that both autoantibodies do not need to be tested simultaneously, as the finding of IFA alone is very rare. GPC testing is therefore the most appropriate means of screening for pernicious anemia, with IFA testing as a more specific, but less sensitive test, being reserved for confirmatory testing^[38].

Treatment

The classic treatment of cobalamin deficiency has been parenteral administration, usually by intramuscular injection, of the vitamin (in the form of cyanocobalamin and, more rarely, hydroxocobalamin or methylcobalamin). The recommended practice involves administration of 1000 µg of cyanocobalamin per day for 1 wk, followed by 1000 µg/wk for 1 mo, and then the dose is reduced to 1000 µg/mo, normally for the rest of the patient's life^[31,33].

Alternative routes of cobalamin administration have been used: oral^[32-35] and nasal^[32,33]. These other routes of administration have been proposed as a way of avoiding the discomfort, inconvenience and cost of monthly injections. An evidence-based analysis supports the efficacy of oral cobalamin therapy^[32,33,35,39]. Sublingual therapy (2000 µg/d for 7-12 d) is another treatment modality for cobalamin deficiency, applicable in patients who refuse parenteral treatment and present either diarrhea or vomiting, and/or are unable to take oral medication^[34].

The procedure for oral cobalamin therapy has, however, not been completely validated yet in clinical practice, most notably the long-term efficacy. The current literature does not suggest a strategy in terms of the optimal form (hydroxy- or cyanocobalamin), frequency or duration of treatment. The therapeutic schema for use of oral cyanocobalamin would be^[33]: (1) Intensification treatment: cyanocobalamin 1000 µg/d for 1 mo; (2) Maintenance treatment: cyanocobalamin 125-500 µg/d for intake deficiency and food-cobalamin malabsorption, and cyanocobalamin 1000 µg/d for pernicious anemia.

ANEMIA AND GASTRIC SURGERY

Gastrectomy, previously used for peptic ulcer and its complications, is the preferred operation for palliation of gastric cancer either as total or partial gastrectomy.

Anemia is a frequent complication of gastrectomy. There are many reports addressing iron, vitamin B12 or folate deficiencies either alone or in combination after gastric surgery. The most frequent is the combination of iron and vitamin B12 deficiency^[40]. Impaired absorption of iron following gastrectomy is probably due to operative bypass of the duodenum and to rapid intestinal transit. Reduction in gastric acid (necessary for the absorption of food iron), a common consequence of subtotal gastrectomy, has also been incriminated. Vitamin B12 deficiency develops as a consequence of the decreased production of intrinsic factor which is essential for vitamin B12 absorption in the lower small bowel, and also because of a defect in the separation of vitamin B12 from its transporter protein. It is a frequent deficiency which will appear 2-4 years or even longer after gastrectomy, when the vitamin stores are exhausted. Thus, gastrectomized patients should be followed carefully to avoid iron and vitamin B12 deficiencies and anemia.

Over the last few decades, bariatric surgery has been suggested as an effective treatment for obesity. There are several different procedures, including gastric bypass, laparoscopic adjustable gastric banding, vertical banded gastroplasty, biliopancreatic diversion, and biliopancreatic diversion with duodenal switch^[41]. All of these procedures may be associated with long-term sequelae including iron, vitamin B12 and folate deficiencies^[41,42]. ID and anemia can have a strong impact on quality of life, especially in menstruating women who make up the majority of bariatric surgery patients. Most studies report ID, ranging from 6% to 50% within months to years of follow-up^[43-45]. Vitamin B12 deficiency may appear 1-9 years after gastric bypass, and its prevalence has been estimated to be 12%-33%^[42].

The main causes of ID after bariatric surgery are similar to those described after gastrectomy; diminished gastric acid secretion and exclusion of the duodenum. In gastric bypass, patients experience decreased gastric acid production in their proximal pouch and, in addition, the duodenum is excluded from digestive continuity. Thus, banding procedures which maintain digestive continuity with the antrum and duodenum are associated with low rates of ID and other nutritional deficiencies^[41]. Conversely, the biliopancreatic diversion with duodenal switch, a gastric bypass procedure that may preserve some function of the proximal duodenum, may offer protection from ID, as compared with biliopancreatic diversion (which excludes the duodenum)^[46,47].

Physicians should be aware that folate, vitamin B12, and iron deficiencies occur after gastric bypass, though the time to development is variable. In an attempt to prevent nutritional deficiencies, multivitamin preparations are in general prescribed to all patients. Systematic prescription of such supplements may prevent most nutritional deficits. However, vitamin B12 and iron deficits require specific supplementation. In spite of a multivitamin, ID still develops postoperatively in some patients. Adherence to oral iron supplements is often low because of digestive intolerance, and unresponsive IDA

can be an important problem in these patients. Parenteral iron treatment is recommended in those patients refractory to oral iron supplementation. Intramuscular vitamin B12 supplementation is recommended only when a deficiency becomes clinically apparent.

ANEMIA AND *H PYLORI* INFECTION

The current evidence regarding the association between *H pylori* infection and either ID or IDA is mainly based on case reports^[48-53], observational epidemiologic studies^[54], and a very limited number of intervention trials^[55-64]. The mechanisms of *H pylori*-related anemia have not been well defined and it is not known why only a small population develops IDA despite a significant worldwide *H pylori* infection rate^[65]. Individuals with increased demands of iron needed for growth and tissue building such as children, pregnant, postpartum or premenopausal women, or those with chronic inflammatory disorders such as CD, are thought to be more likely to develop IDA associated with *H pylori* infection.

H pylori infection has been shown to be associated with ID in asymptomatic *H pylori*-infected subjects in several cross-sectional studies. However, a great variability was found across the studies and most of these have been conducted in countries with high prevalence of *H pylori* infection. Recently, Muhsen *et al*^[54] performed two different meta-analyses of observational epidemiologic studies aimed at examining the association between *H pylori* infection and either ID or IDA. These analyses yielded a 2.8-fold increased risk for IDA (95% CI: 1.9-4.2), and an 1.38-fold increased risk for ID (95% CI: 1.16-1.65) in *H pylori*-infected subjects as compared with non-infected subjects.

In clinical and interventional trials, the participants were mostly children and adolescents, and only three trials were conducted among ill people (those presenting with symptoms for investigation in clinical settings)^[58,59,62]. Small sample sizes^[56-59,62], lack of control groups, and other methodological issues, including the use of validated tests to confirm active *H pylori* infection, are among factors that limit the interpretation and ability to generalize the relevance of the results of these studies^[54]. Several studies reported resolution of IDA with eradication of *H pylori* infection, regardless of the absence of iron supplementation. The study of Kurekci *et al*^[59], in which all participants received *H pylori* eradication therapy without a control group, emphasized that resolution of both ID and IDA associated with *H pylori* infection may be achieved by *H pylori* eradication treatment alone. The antagonistic effect of asymptomatic *H pylori* infection on the response to iron supplementation was investigated in India among participants of a randomized, controlled trial of iron supplementation ($n = 169$, age 1-10 years). It was found that asymptomatic *H pylori* infection was not associated with higher rates of anemia or ID, but had a significant adverse effect on response to iron supplementation among children^[53].

Sarker *et al*^[61] completed a population-based,

randomized, double-blind, and placebo-controlled trial to evaluate the response of iron *plus* anti-*H pylori* therapy in children with IDA ($n = 200$). This trial was performed in Bangladesh, an area highly endemic for ID and *H pylori* infection. Results failed to observe any additional impact of combined anti-*H pylori plus* iron therapy over the iron therapy alone. These findings support those obtained by an Alaskan trial^[60], in another highly prevalent *H pylori* infection area, where a large therapeutic, randomized, controlled, unblinded trial in children ($n = 219$) was performed. There were no significant differences between the intervention and the control groups in the rates of ID and anemia, during 14 mo follow-up period. The authors hypothesized that 14 mo was too early to resolve *H pylori*-induced gastric damage. Consequently, in a follow-up study performed at 40 mo, 176 children were reevaluated. Re-infection occurred among 52% of children who had initially cleared their infection. However, *H pylori*-negative children had lower prevalence of ID (RR: 0.62; 95% CI: 0.38-1.01) and IDA (RR: 0.22; 95% CI: 0.03-1.50), compared with *H pylori*-positive children. It was concluded that the resolution of *H pylori* infection for > 14 mo modestly reduced the prevalence of ID and substantially reduced the prevalence of ID and IDA^[64].

In the light of the above mentioned studies, *H pylori* infection may be considered as a risk factor for IDA, mainly in groups with high demands for iron, such as some children and adolescents. However, the relationship between *H pylori* and ID may be stronger than that described, since most of the above mentioned studies have been performed in geographical areas where both ID and *H pylori* infection are highly prevalent, and where the etiology of ID is possibly multifactorial (malnutrition, vitamin deficits, chronic parasitic infections, malaria)^[66]. In this setting, poor response to *H pylori* eradication should be viewed with caution. Thus, further large and well-controlled trials will be of value. Both the impact of anti-*H pylori* therapy on the improvement of iron stores and the role the infection plays in interfering with iron supplementation in patients with IDA require further evaluation before making solid recommendations.

The biologic mechanism

The relationship between refractory IDA and *H pylori* infection may be explained by several hypotheses. One of the possible explanations is the gastrointestinal blood loss that may range from chronic occult bleeding from discrete gastric erosions^[67] to massive bleeding from peptic ulcers^[68] and gastric carcinomas. However, most patients with *H pylori*-associated IDA have no evidence of gastrointestinal bleeding. Data obtained through case series and case reports mostly described patients with IDA after a comprehensive investigation including laboratory tests, imaging, and endoscopic studies, and *H pylori* gastritis was the only pathologic finding^[55,61-63].

Intragastric acidic pH plays an important role in the reduction of the ferric to the ferrous form. This reaction is enhanced by ascorbic acid. Gastric juice and mucosal ascorbic acid concentrations were significantly lowered

in *H pylori*-infected subjects as compared to those non-infected; the lower concentrations were associated with more severe gastritis^[69]. This negative influence of *H pylori* on gastric ascorbic acid was reversed after eradication of the infection^[70].

The results of some studies support the hypothesis that the infection by *H pylori* influences iron absorption directly, and that iron absorption improves significantly after clearance of the infection^[71,72]. It has been suggested that there is a competition for iron between the bacteria and the host. Iron is an essential nutrient for bacterial growth. Therefore, an efficient iron uptake system is an important factor for the maintenance of virulence^[73]. Many pathogens use the siderophore-mediated iron acquisition system that removes iron from lactoferrin or transferrin. The regulation of iron uptake systems in *H pylori* are different from other bacteria since they are constitutively expressed, most probably as a part of the germ adaptation to the conditions in the human stomach, where iron starvation and iron overload can be encountered frequently^[74]. The siderophore of *H pylori* has not yet been identified. Several studies support this hypothesis^[75-78].

Bacterial genetic factors related to *H pylori*-associated IDA pathogenesis have been studied. Non-IDA and IDA strains can be distinguished by their protein expression profiles, suggesting that polymorphisms in *H pylori* strains may be one of the factors determining the occurrence of *H pylori*-associated IDA^[79]. A mutation in the *H pylori pfr* gene causing overproduction of *H pylori* ferritin protein (Pfr) has been proposed as playing a role in the imbalance of body iron^[80]. However, an analysis of the complete coding region of the *pfr* gene revealed three sites of mutation with no differences in the mutation among *H pylori*-positive patients with or without IDA^[80]. On the other hand, three heme-binding iron-repressible outer membrane proteins that may be involved in the uptake of heme from the host by *H pylori* were described in the presence of poor iron environment^[81]. Recently, *feoB* gene product, which was regarded as a high-affinity ferrous iron transporter, was proposed as a possible pathway related to the bacterium itself; however, its relation to IDA remains unclear^[82]. Although *H pylori* CagA strains were proposed to be involved in alteration of the host's iron stores in some studies, more recent work does not support this hypothesis^[72,83,84]. However, seropositivity to *H pylori* CagA antibodies was inversely associated with gastric ascorbic acid concentrations^[84].

CONCLUSION

CD should be considered as a possible cause of anemia in all subjects with IDA of unknown origin, even in menstruating women. Duodenal biopsies should be taken during endoscopy if no obvious case of IDA can be found. Vitamin B12 or cobalamin deficiency occurs frequently among elderly patients, but it is often unrecognized or not investigated because the clinical manifestations are subtle. In this age group, cobalamin

deficiency is caused primarily by food-cobalamin malabsorption (60%-70% of cases) and pernicious anemia (15%-20% of cases). The classic treatment of cobalamin deficiency has been parenteral administration of the vitamin. However, recent data support the efficacy of oral cobalamin therapy. Folate, vitamin B12, and iron deficiencies occur after gastric bypass, with an incidence ranging from 6% to 50% within months to years of follow-up. *H pylori* infection may be considered as a risk factor for IDA, mainly in groups with high demands for iron, such as some children and adolescents. However, further large and well-controlled trials will be required before making solid recommendations about *H pylori* eradication in these cases.

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S- Editor Tian L L- Editor Logan S E- Editor Zheng XM



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Spectrum of anemia associated with chronic liver disease

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Supported by CIBEREHD is funded by the Instituto de Salud Carlos III, Madrid, Spain

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Received: July 23, 2009 Revised: September 12, 2009

Accepted: September 19, 2009

Published online: October 7, 2009

Peer reviewer: Carla W Brady, MD, MHS, Duke University Medical Center, Division of Gastroenterology, DUMC Box 3913, Durham, NC 27705, United States

Gonzalez-Casas R, Jones EA, Moreno-Otero R. Spectrum of anemia associated with chronic liver disease. *World J Gastroenterol* 2009; 15(37): 4653-4658 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4653.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4653>

INTRODUCTION

Chronic liver diseases frequently are associated with hematological abnormalities. Anemia of diverse etiology occurs in about 75% of patients with chronic liver disease^[1].

A major cause of anemia associated with chronic liver disease is hemorrhage, especially into the gastrointestinal tract. Patients with severe hepatocellular disease develop defects of blood coagulation as a consequence of endothelial dysfunction, thrombocytopenia, deficiencies of coagulation factors and various associated disorders^[2].

In severe hepatocellular disease, decreased synthesis of liver-produced plasma proteins leads to reduced serum levels of several blood clotting factors. Hemorrhage may occur as a complication of chronic liver disease because of a lack of one or more liver-produced blood clotting factors, thrombocytopenia, and/or defective platelet function. Hemorrhage in such patients may also occur from esophageal or gastric varices secondary to portal hypertension. The biosynthetic pathways of blood coagulation factors II, VII, IX and X are within the hepatocyte and are dependent on vitamin K^[3]. Low serum levels of these factors are associated with prolongation of the prothrombin time (PT). When attributable to hepatocellular disease, they are not improved by administration of vitamin K; correction of the associated impaired blood coagulation necessitates infusion of preparations of the deficient factors.

Splenomegaly, which is usually caused by portal hypertension in patients with chronic liver disease, may lead to secondary hemolysis, an increase in plasma volume, macrocytosis and megaloblastic anemia. Alcohol, a common etiologic factor of chronic liver disease, is toxic to the bone marrow. Alcoholics often develop secondary malnutrition, a manifestation of

Abstract

Anemia of diverse etiology is a common complication of chronic liver diseases. The causes of anemia include acute or chronic gastrointestinal hemorrhage, and hypersplenism secondary to portal hypertension. Severe hepatocellular disease predisposes to hemorrhage because of impaired blood coagulation caused by deficiency of blood coagulation factors synthesized by hepatocytes, and/or thrombocytopenia. Aplastic anemia, which is characterized by pancytopenia and hypocellular bone marrow, may follow the development of hepatitis. Its presentation includes progressive anemia and hemorrhagic manifestations. Hematological complications of combination therapy for chronic viral hepatitis include clinically significant anemia, secondary to treatment with ribavirin and/or interferon. Ribavirin-induced hemolysis can be reversed by reducing the dose of the drug or discontinuing it altogether. Interferons may contribute to anemia by inducing bone marrow suppression. Alcohol ingestion is implicated in the pathogenesis of chronic liver disease and may contribute to associated anemia. In patients with chronic liver disease, anemia may be exacerbated by deficiency of folic acid and/or vitamin B12 that can occur secondary to inadequate dietary intake or malabsorption.

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Key words: Anemia; Liver disease; Liver failure; Aplastic anemia; Pegylated interferon; Ribavirin; Alcohol

which may be anemia caused by folic acid deficiency. In some patients, bone marrow failure and aplastic anemia develop after an episode of hepatitis. Finally, anemia is a recognized complication of treatment of chronic hepatitis C with a combination of interferon and ribavirin: anemia in this context is predominantly caused by ribavirin-induced hemolysis^[4].

The frequent association of anemia with chronic liver disease and/or hepatocellular failure provides a rationale for examining the role of the liver in the formation and destruction of erythrocytes. Indeed, the liver itself may be implicated in a variety of different mechanisms that contribute to the development of anemia in patients with chronic liver disease. This paper provides an overview of anemia that may complicate chronic liver diseases and the mechanisms responsible.

The frequent association of anemia with chronic liver disease and/or hepatocellular failure provides a rationale for examining the role of the liver in the formation and destruction of red blood cells. Indeed, a variety of different mechanisms may be implicated in the development of anemia in patients with liver disease.

PORTAL HYPERTENSION AND ANEMIA

Acute gastrointestinal hemorrhage is a common and potentially serious complication of portal hypertension^[5-8]. It is usually caused by rupture of an esophageal varix. Hemorrhage caused by this mechanism is the second most common cause of mortality in patients with cirrhosis. In such patients, a ruptured esophageal varix is the cause of approximately 70% of episodes of upper gastrointestinal hemorrhage^[6]. Acute hemorrhage may induce severe hypovolemia and subsequently secondary iron deficiency anemia. The initial aim of treatment is correction of hypovolemia and restoration of stable hemodynamic function; minimal values for mean arterial pressure and for hemoglobin of 80 mmHg and 8 g/100 mL, respectively, should be maintained. Initially, gelatin-based colloids or solutions of human albumin may be infused to correct hypovolemia. However, infusions of packed erythrocytes in plasma are ideal in this context since such infusions have the potential of correcting, not only hypovolemia, but also secondary anemia. First-line management involves institution of both medical and endoscopic treatments (Table 1)^[6]. Medical therapy includes administration of vasoactive drugs, such as somatostatin, octreotide or terlipressin. Optimal endoscopic treatment involves ligation of esophageal varices and obturation of gastric varices with tissue adhesives.

In some patients with cirrhosis, chronic hemorrhage into the gastrointestinal tract occurs. Esophageal and gastric varices and/or portal hypertensive gastropathy may be associated with slow chronic loss of blood into the gut and development of chronic iron deficiency anemia. The most important approach to management is prevention of variceal hemorrhage^[5,7,8]. The annual incidence of initial variceal hemorrhage in patients with cirrhosis is estimated to be about 4%, but for the group

Table 1 Management of acute gastrointestinal bleeding

Treatment of variceal bleeding

Pharmacological therapy
Somatostatin: initial bolus (24-48 h) and a perfusion (5 d)
Octreotide: initial bolus (24-48 h) and a perfusion (5 d)
Terlipressin: important side effects
Primary prophylaxis
β-blockers non-selective: propranolol, nadolol
Isosorbide 5-mononitrate
Secondary prophylaxis
β-blockers non-selective: propranolol, nadolol
Isosorbide 5-mononitrate
Endoscopy therapy: band ligation, sclerotherapy

with medium-sized or large varices, the incidence is about 15%^[6]. β-blockers or isosorbide 5-mononitrate may reduce the rate of transformation of small varices into large varices and decrease the incidence of variceal hemorrhage in patients with small varices^[5]. In patients who survive a first episode of variceal hemorrhage, the risk of recurrent hemorrhage is > 60%. Accordingly, all patients surviving variceal hemorrhage should receive active treatment aimed at preventing recurrence. Non-selective β-blockers or isosorbide 5-mononitrate and endoscopic therapy, including ligation of and/or sclerotherapy of varices, are the first-line treatments for preventing recurrence of variceal hemorrhage^[5,8]; a combination of both these approaches constitutes optimal management. Additional treatment with oral iron supplementation is indicated for iron deficiency anemia caused by chronic blood loss. In some cases of advanced chronic liver disease, intravenous iron formulations may be administered to increase plasma levels and tissue deposits of iron.

Hypersplenism secondary to portal hypertension is another mechanism of anemia in patients with chronic liver disease. Hypersplenism is associated with splenomegaly. In addition to chronic liver disease, thrombosis of the splenic vein may also be a cause of an increase in pressure within the portal venous system, which can lead to secondary hypersplenism. The main characteristics of hypersplenism are those attributable to pancytopenia. Hemolytic anemia occurs because of intrasplenic destruction of erythrocytes. Destruction of megakaryocytes and leukocyte precursors results in thrombocytopenia and leukopenia^[9]. Symptoms and signs of hypersplenism are influenced by the primary underlying disease; they include abdominal pain and/or discomfort, and, in advanced cases, gastrointestinal hemorrhage secondary to portal hypertension. There may be hyperplasia of the progenitor cells in the bone marrow. It is important to determine the cause of hypersplenism. The main therapeutic approach for this syndrome is management directed at the underlying primary disease, usually chronic liver disease. When chronic liver disease is advanced, additional therapeutic options may need to be adopted. After assessing the severity of impaired hepatocellular function in a patient with advanced chronic liver disease, splenectomy may

be considered if the splenic vein is thrombosed. An alternative approach is partial or total embolization of the splenic artery, which, in some recent studies, has been associated with good results, in particular, lower morbidity and mortality rates than those associated with surgery. Partial embolization preserves the immunological function of the spleen and is the preferred option for patients with cirrhosis^[10].

IMPAIRED BLOOD COAGULATION

The liver plays a central role in blood coagulation. Acute and chronic hepatocellular diseases are usually associated with defective blood coagulation due to a variety of different causes. These include: decreased hepatic synthesis of factors II, VII, IX and X; the presence of inhibitors of these factors; decreased clearance of activated coagulation factors; thrombocytopenia; impaired platelet function; hyperfibrinolysis; and disseminated intravascular coagulation^[11,12]. Coagulation defects complicating liver disease predispose to an increased bleeding tendency, which increases both morbidity and mortality^[11-13].

Defective blood coagulation associated with hepatocellular disease may be monitored using global screening tests, such as the PT and the activated partial thromboplastin time. In mild hepatocellular disease, PT usually is within the normal range or only modestly prolonged. In more advanced hepatocellular disease, prolongation of PT tends to reflect the severity of hepatocellular failure. Vitamin K routinely is administered parenterally (usually only once) to patients with liver disease and a prolonged PT, to exclude vitamin K deficiency as a cause of the prolonged PT^[11].

Thrombocytopenia (platelet count < 150 000/L) is common in patients with chronic liver disease; it has been reported in as many as 76% of patients with cirrhosis^[4,12]. The pathogenesis of the thrombocytopenia is complex; it includes splenic pooling, and increased destruction and impaired production of platelets (Figure 1). Impaired production of platelets is caused, at least in part, by low levels of thrombopoietin. Prolonged bleeding time, and impaired aggregation, reduced adhesiveness and abnormal ultrastructure of platelets reflect abnormal platelet function; these abnormalities have been attributed to an intrinsic platelet defect. Specific treatments to attempt to reverse the effects of this defect are not usually given, but platelet transfusions or platelet-stimulating agents have been administered in some cases.

An important coagulation defect associated with chronic liver disease is low levels of factor VIIa. In recent years, the hemostatic agent recombinant factor VIIa has become available as a potentially new therapeutic agent for use in the management of coagulopathy in patients with cirrhosis. This agent may enhance initial control of acute variceal bleeding^[14]. However, such therapy is associated with significant side effects, such as vascular injury and thrombosis.

Hyperfibrinolysis is another cause of impaired

hemostasis in patients with liver disease. In a non-randomized trial^[15], antifibrinolytic amino acids were administered to patients with acute or chronic liver disease, who had upper gastrointestinal bleeding and acquired defects of blood coagulation. However, administration of such amino acids does not have an established place in therapy.

Thrombotic events, although rare in patients with cirrhosis, may occur. They tend to involve particularly the portal and/or mesenteric veins.

A rational approach to managing disorders of blood coagulation in patients with liver disease is important because of the high risk of associated secondary hemorrhage.

APLASTIC ANEMIA

Aplastic anemia associated with liver disease is characterized by development of pancytopenia and hypocellular bone marrow in relation to the occurrence of hepatitis^[16]. The main feature of this syndrome is injury to or loss of pluripotent hematopoietic stem cells, in the absence of infiltrative disease of the bone marrow^[16-19].

Hepatitis-associated aplastic anemia (HAA) has been defined as a variant of aplastic anemia, which occurs concurrently with or within 6 mo of an increase in the serum level of alanine aminotransferase to at least five times the upper limit of the reference range. Severe marrow aplasia may be induced by hepatitis viruses, such as hepatitis B virus and hepatitis C virus (HCV), and also by other viruses, such as human immunodeficiency virus, Epstein-Barr virus, transfusion-transmitted virus and echovirus^[16,20]. Parvovirus B19 commonly infects pro-erythroblasts and may induce transient red-cell aplasia, particularly in patients with chronic hemolytic anemia. It has been postulated that viruses and/or antigens, through the mediation of γ interferon or the cytokine cascade, induce lymphocyte activation and ultimately apoptotic death of hematopoietic cells in the bone marrow^[17].

Clinical presentation includes symptoms and signs related to pancytopenia, such as pallor, fatigue, hemorrhagic manifestations, progressive anemia, and bacterial infections. The diagnosis of HAA is suggested by a complete blood count, which reveals pancytopenia (including anemia) together with absolute reticulocytopenia^[16]. A bone marrow biopsy typically reveals hypocellularity that affects red and white cell precursors and megakaryocytes; residual hematopoietic cells appear morphologically normal^[19].

The two major options for treating severe HAA are hematopoietic cell transplantation and immunosuppressive therapy. According to recent reviews, response rates to these approaches are 75%-88% and 75%-80%, respectively^[16,18]. Blood and platelet infusions are often necessary before instituting specific treatment; before administration blood products should be irradiated to avoid sensitization.

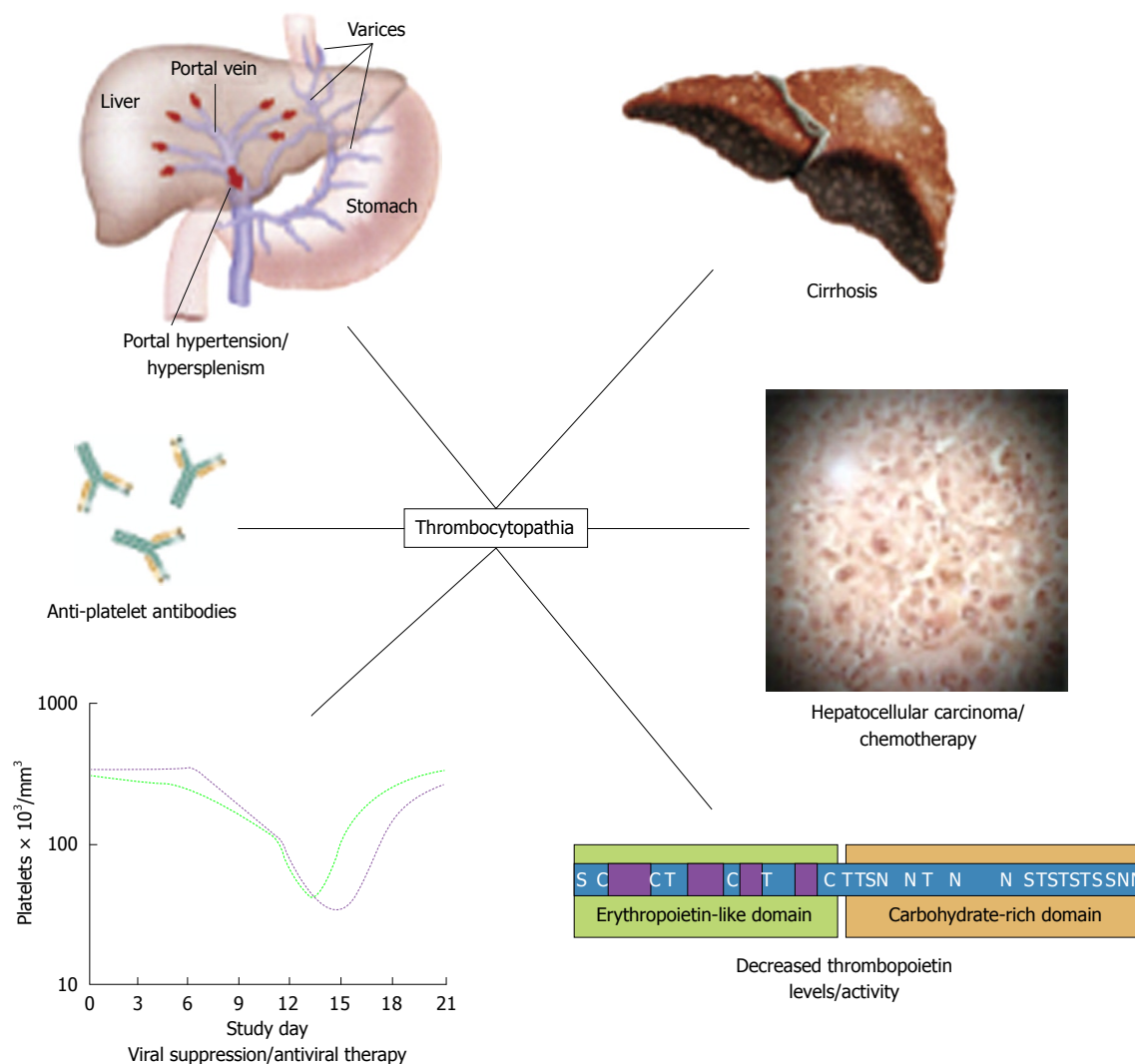


Figure 1 Development of thrombocytopenia in patients with chronic liver disease (adapted from Afdhal *et al*^[12]).

ANEMIA SECONDARY TO TREATMENT OF HEPATITIS

Currently, optimal treatment for chronic infection with HCV infection is a combination of therapy with pegylated interferon and ribavirin. Of hematological abnormalities that may be associated with such combination therapy, the most common is anemia^[21]. Significant anemia (hemoglobin < 10 g/dL) has been observed in 9%-13% of patients receiving interferon and ribavirin; moderate anemia (hemoglobin < 11 g/dL) occurs in about 30% of patients undergoing such treatment^[20-22]. There are several mechanisms by which anemia may occur during combination therapy for HCV infection, and ribavirin and/or interferons may contribute to anemia. In this context, hemoglobin concentrations decrease mainly as a result of ribavirin-induced hemolysis^[20].

Anemia caused to ribavirin leads to modifications of the dose in up to 25% of patients, and this type of anemia may be problematic in patients with HCV infection, especially those who also have renal or cardiovascular disorders. Adherence to ribavirin therapy is one factor that is critically important in the treatment

of HCV infection. Although ribavirin-associated anemia can be reversed by reducing the dose of ribavirin or by discontinuing the drug altogether, this approach compromises outcomes by significantly decreasing rates of sustained virological response. A recent study reviewed the predictors of anemia in patients undergoing treatment for HCV infection^[21]. Patients with impaired renal function may be at an increased risk of ribavirin-related anemia and, accordingly, should be monitored carefully. Furthermore, a decrease in hemoglobin concentration of ≥ 1.5 g/dL by week 2 of treatment has been found to be an excellent early predictor of subsequent substantial decreases in hemoglobin. This predictor might be applied to identify candidates for early intervention for management of anemia to facilitate maintenance of the dose of ribavirin. One of the specific approaches to manage ribavirin-associated anemia is administration of recombinant human erythropoietin^[21]. After 16 wk of ribavirin therapy, patients who had also been given erythropoietin alfa had significantly higher mean hemoglobin levels than patients in a control group. In patients with chronic hepatitis C, viremide, a prodrug of ribavirin that is

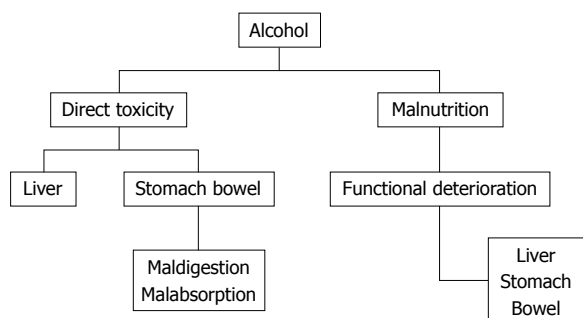


Figure 2 Scheme, in patients with alcoholic liver disease, depicting how different effects of alcohol may contribute to anemia (adapted from Moreno Otero *et al*^[23]).

selectively taken up by the liver, has the potential of maintaining the antiviral efficacy of ribavirin, while decreasing the risk of hemolytic anemia^[4].

Interferons may also contribute to anemia. Their main relevant action is induction of bone marrow suppression. This effect of interferon results in suppression of compensatory reticulocytosis associated with ribavirin-induced hemolytic anemia. Thus, the bone-marrow-suppressive effect of interferon may contribute to anemia, which complicates therapy with combination of interferon and ribavirin^[4].

ALCOHOL, LIVER DISEASE AND ANEMIA

Alcohol is implicated in the pathogenesis of chronic liver disease; it may contribute to anemia secondary to its direct effects on the liver and also to other diverse mechanisms (Figure 2)^[23].

Markers of iron overload tend to be higher among those who consume more than two alcoholic drinks per day than among non-drinkers, after adjusting for potential confounding factors^[24]. Consumption of alcohol appears to be associated with an approximately 40% reduction in the risk of development of iron deficiency anemia.

Folic acid and vitamin B12 deficiencies develop frequently in patients with cirrhosis. These deficiencies may be related to inadequate food intake or intestinal malabsorption. They are suspected when examination of a blood film reveals hypersegmented cells and oval macrocytes, in addition to round macrocytes characteristic of chronic liver disease. When anemia is caused by these deficiencies, the mean corpuscular volume is increased and bone marrow shows megaloblastic erythropoiesis.

Anemia due to folic acid deficiency may result, not only from a lack of folic acid in the diet, but also the weak antifolate action of ethanol. Folic acid deficiency is the most common cause of a low hematocrit in hospitalized patients who are alcoholics^[25,26]. Parenterally administered vitamin B12 not only corrects anemia caused by vitamin B12 deficiency, but may also induce improvement in the peripheral neuropathy that are associated with this deficiency^[23]. Supplements of vitamins A, B and C may be administered empirically to patients with advanced alcoholic disease.

Table 2 Etiologic factors that may contribute to anemia associated with alcoholism (adapted from Lewis *et al*^[26])

Cause of low hematocrit	Possible contributing factors
Hemorrhage and/or iron deficiency	Alcoholic gastritis Portal hypertension Peptic ulceration
Hemolysis	Chronic liver disease and/or cirrhosis Zieve syndrome Spur cell anemia of severe liver disease
Reduced erythropoiesis	Anemia of chronic disease Nutritional (e.g. folic acid deficiency) Sideroblastic anemia Alcohol toxicity
Hypersplenism	Portal hypertension
Hemodilution	Fluid retention of chronic liver disease Aggressive intravenous fluid therapy

Source: *Nat Clin Pract Gastroenterol Hepatol* © 2007 Nature Publishing Group.

Anemia in an alcoholic may also arise as a consequence of the direct toxic effects of alcohol on erythrocyte precursors in the bone marrow. Management of alcohol-induced suppression of erythropoiesis includes abstinence from alcohol and a nutritious diet with appropriate supplements.

Other factors that may contribute to anemia and a low hematocrit in alcoholic patients are given in Table 2.

CONCLUSION

Liver diseases are frequently associated with hematological abnormalities. Anemia of diverse etiology occurs in many of these patients. Bleeding is one of the most severe causes of anemia, with a high mortality, and defective blood coagulation contributes to the anemia. Other mechanisms of anemia include aplastic anemia secondary to previous hepatitis, or side effects of treatment of hepatitis with interferon and ribavirin. In patients with alcoholic liver disease, different effects of alcohol may contribute to anemia, such as malabsorption, malnutrition or direct toxic effect. The pathogenesis of the anemia in each case is different and it is important to begin the correct therapy.

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S- Editor Tian L L- Editor Kerr C E- Editor Zheng XM



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Anemia and inflammatory bowel diseases

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Author contributions: Gomollón F and Gisbert JP contributed equally to this work; Gisbert JP and Gomollón F performed the systematic review of the literature and wrote the paper.

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Received: July 23, 2009

Revised: August 12, 2009

Accepted: August 19, 2009

Published online: October 7, 2009

Abstract

Too often anemia is considered a rare or unimportant manifestation in inflammatory bowel disease (IBD). However, over the last 10 years a number of studies have been conducted and the most relevant conclusions obtained are: (1) anemia is quite common in IBD; (2) although in many cases anemia parallels the clinical activity of the disease, many patients in remission have anemia, and iron, vitamin B12 and/or folic acid deficiency; (3) anemia, and also iron deficiency without anemia, have important consequences in the clinical status and quality of life of the patient; (4) oral iron can lead to gastrointestinal intolerance and failure of treatment; (5) intravenous iron is an effective and safe way to treat iron deficiency; (6) erythropoietin is needed in a significant number of cases to achieve normal hemoglobin levels. Thus, the clinician caring for IBD patients should have a comprehensive knowledge of anemia, and apply recently published guidelines in clinical practice.

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Key words: Anemia; Inflammatory bowel diseases; Ferritin; Iron

Peer reviewer: Luis Rodrigo, Professor, Gastroenterology Service, Hospital Central de Asturias, c/Celestino Villamil, s.n., Oviedo 33.006, Spain

Gomollón F, Gisbert JP. Anemia and inflammatory bowel diseases. *World J Gastroenterol* 2009; 15(37): 4659-4665 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4659.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4659>

INTRODUCTION

In addition to the intestinal signs and symptoms (such as abdominal pain or diarrhea), inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are associated with a number of extraintestinal manifestations (at joints, skin, eyes, etc). Furthermore, systemic manifestations of IBD may also include malnutrition and anemia^[1,2].

Compared with the average awareness of other extraintestinal disease complications such as arthritis or osteopathy, the topic of anemia in IBD has received little attention: perhaps it is so common that sometimes it is considered an unavoidable manifestation of the disease^[2,3]. Although efficient therapeutic options have been developed for the treatment of IBD-associated anemia, treating anemia often has had a low priority for gastroenterologists^[4]. However, anemia is a clinically relevant condition which may affect quality of life or the ability to work^[5,6], and it is a comorbid condition which is associated with other diseases (such as transfusion-associated hepatitis C) or even death^[1,7].

Anemia is a disease and should be approached as such. Anemia in IBD is not just a laboratory marker, it is a condition which needs a specific diagnostic and therapeutic approach. Moreover, it is a complex condition, because there are many factors which can cause anemia in IBD patients.

The aim of the present manuscript is to review the main clinically relevant aspects of the diagnosis and management of anemia in the patient with IBD.

PREVALENCE OF ANEMIA IN IBD

Often anemia is not even mentioned, perhaps because some authors think it is a rather uncommon problem in IBD^[2]. On the contrary, anemia is very common in IBD patients, although the reported prevalence of this condition has been markedly variable, depending both on the definition and on the patient population considered (hospitalized patients vs outpatients)^[1,8]. In a systematic review published in 2004, the prevalence of anemia in

patients with IBD ranged from 9% to 74%^[8]. In a more recent systematic review^[1], which included 19 studies (mainly on Crohn's disease) the figures ranged from 6% to 74%. Even more recently, we reviewed published studies evaluating the prevalence of anemia in IBD^[9-30], and calculated a mean prevalence of 17%^[2]. However, as the prevalence was 16% in outpatients, this figure increased up to 68% when only hospitalized patients were included. Therefore, it may be concluded that anemia could be the most common systemic complication of acute IBD^[2].

PREVALENCE OF IRON DEFICIENCY IN IBD

It is also common to consider that iron deficiency is an exceptional finding in IBD^[2]. On the contrary, this condition is even more common than anemia, but to demonstrate it requires active investigation. In fact, iron deficiency is the main cause of anemia in IBD patients, as a consequence of dietary restrictions, malabsorption (in part as a result of inflammation), intestinal bleeding, and/or undertreatment of anemia (achieving normal hemoglobin values does not mean normal iron stores). In a recent systematic review^[1], the prevalence of iron deficiency ranged from 36% to 90% (depending on the definition of iron deficiency and on the type of cohort included)^[16,21,26,31-33]. In the most recent systematic review^[2], mean prevalence of iron deficiency in IBD was 45%, which underlines the fact that this condition may be considered the rule rather than the exception in these patients, especially in severe cases.

THE MULTIFACTORIAL ORIGIN OF ANEMIA IN IBD

Anemia in patients with IBD results primarily from iron deficiency because of chronic intestinal blood loss from inflamed mucosa, although in active disease more complex mechanisms involving absorption are also important^[34]. However, the anemia in IBD is likely to be multifactorial in origin, frequently being the result of a combination of iron deficiency (the first cause) and anemia of chronic disease (the second major cause)^[35]. In some cases, anemia may also be induced by drugs (sulfasalazine, thiopurines), hemolysis, and myelodysplastic syndrome. Finally, in some patients with Crohn's disease, impaired absorption of vitamin B12 and/or folate because of small intestinal inflammation and/or extensive bowel resection, may contribute to anemia^[35], and all these conditions frequently overlap. Therefore, anemia in IBD is often complex and commonly represents a particular example of the combination of, at least, iron deficiency anemia and anemia of chronic disease, and may be a challenge even to the most astute clinician^[4,24].

IRON METABOLISM PARAMETERS FOR THE DIAGNOSIS OF IRON DEFICIENCY IN IBD

The diagnosis of iron deficiency is traditionally based on

a combination of parameters, including hematological and iron metabolism indices^[36]. Pure iron deficiency is recognized by low iron, ferritin and transferrin saturation but increased transferrin concentrations. However, diagnosing iron deficiency in the setting of IBD may be difficult, particularly when both iron deficiency and the anemia of chronic disease are present (as previously mentioned, both conditions frequently coexist). In these circumstances, many of the laboratory measures of iron status may be unreliable, as inflammation influences parameters of iron metabolism^[24,37]. For example, in the presence of chronic inflammation, the elevation in transferrin levels typical of iron deficiency may not be found, as patients with low albumin tend also to have low transferrin concentrations^[38]. Similarly, iron and total iron binding capacity levels are often difficult to interpret in the presence of inflammation^[35]. Finally, serum ferritin, the most accessible and well known measure of stored iron and the most powerful tests for iron deficiency^[39], can be normal or even increased - in response to inflammation, as it is an acute phase reactant - even in the presence of severe iron deficiency^[24]. Therefore, although at present ferritin is generally considered as the most efficient indicator of iron deficiency, this parameter may not provide adequate information about the storage compartment in the setting of inflammatory conditions such as IBD^[24]. Testing for increased soluble transferrin receptor concentration distinguishes reliably between iron deficiency and anemia of chronic disease, but it is not yet widely available^[40,41].

Accordingly, it has been suggested that diagnostic criteria for iron deficiency need to be adapted to the level of inflammation. Thus, in patients without biochemical (C-reactive protein, *etc*) or clinical (diarrhea, endoscopic findings, *etc*) evidence of inflammation, the cut-off point for defining a low level of serum ferritin is < 30 µg/L; however, in the presence of inflammation, the lower limit of this parameter consistent with normal iron stores should be increased up to 100 µg/L^[37,42]. Some authors do suggest considering the presence of ferroopenia if there are low iron values and < 16% transferrin saturation^[43].

DRUG-RELATED ANEMIA IN IBD

Some drugs commonly used in the treatment of IBD can have myelosuppressive effects, both indirect (for instance the "antifolate" effect of salazopyrine) or even direct (such as azathioprine or mercaptopurine)^[44]. In particular, sulfasalazine affects erythropoiesis by several mechanisms including folate absorption, hemolysis and aplasia^[45]. Isolated anemia in patients on azathioprine or mercaptopurine is unlikely to be caused by these drugs; in some cases, however, a mild and asymptomatic reduction in hemoglobin may be detected in patients treated with thiopurine drugs.

THE IMPACT OF ANEMIA ON THE QUALITY OF LIFE OF IBD PATIENTS

The repercussion of anemia on quality of life in

both general patients^[46,47] and specifically in patients with IBD^[3,4,6,8] is substantial. Moreover, anemia may impair quality of life even in the absence of specific symptoms^[5,6]. As has accurately been noted by Gasche *et al.*^[3,4], for a long time it was thought that the clinical symptoms of anemia (such as fatigue, headache, dizziness, shortness of breath, or tachycardia) occurred only when the hemoglobin level dropped abruptly. It had been argued that patients would adapt to low hemoglobin levels if anemia developed slowly. This has led to the concept of “asymptomatic” anemia. In truth, the term “asymptomatic” seems to reflect the fact that impairments in physical condition, quality of life, and cognitive function may be unrecognized by both patients and their doctors. Therefore, the process of adaptation to chronic anemia would, in fact, be adaptation to a lower quality of life^[3,4]. These concepts have been thoroughly developed in other pathologies, especially in patients on dialysis: intravenous iron can be a key point in management of these patients^[48].

Remarkably, the quality of life in IBD patients may be as low as in anemic patients with advanced cancer^[49]. Moreover, chronic fatigue caused by anemia can debilitate, affect and worry these patients as much as abdominal pain or diarrhea^[4]. Therefore the beneficial impact on quality of life derived from anemia correction in IBD patients can be similar to the control of diarrhea^[4,6,50].

THE ROLE OF TREATMENT OF THE UNDERLYING DISEASE (IBD) ON THE CORRECTION OF ANEMIA

A general correlation exists between disease activity and the depth of the anemia^[38]. Active disease can cause anemia because of multiple factors, the most recently demonstrated being anemia of chronic disease and impairment of iron absorption in active Crohn's disease. Therefore, the most important measure for IBD anemia treatment is the treatment of the underlying disease^[1,24]. Although apparently obvious, sometimes this step is missed in actual clinical practice. Moreover, the long term effect to alleviate anemia depends on whether the bowel inflammation itself can be adequately treated. Every effort to accomplish this has to be undertaken in order to preclude recurrent anemia^[37,51].

WHEN TO START IRON SUPPLEMENTATION IN IBD ANEMIC PATIENTS?

There may be a tendency to look upon anemia as an unavoidable accompaniment to IBD^[38]. Only in recent years has correction of anemia been highlighted as a specific therapeutic aim in these patients^[38]. It should not be assumed that some level of anemia is a normal finding in IBD patients and consequently need not be treated^[3]. On the contrary, iron supplementation should be started as soon as anemia (hemoglobin < 13 g/dL in

males, and < 12 g/dL in females^[52]) is detected. Thus, the World Health Organization definitions of anemia apply to patients with IBD. In fact, it is possible that patients without anemia but with iron deficiency should be considered for treatment because even without anemia, iron deficiency can have clinical relevance. In summary, anemia in IBD patients should be aggressively diagnosed, investigated, and treated^[2].

WHEN TO STOP IRON SUPPLEMENTATION IN IBD ANEMIC PATIENTS?

Apart from the correction of hemoglobin levels, the primary therapeutic goal is to improve quality of life. Therefore, the therapeutic objective of the treatment with oral iron should be to completely correct both the anemia and iron deficiency, and not only to partially increase the hemoglobin levels. Thus, our final aim should be to achieve the previously mentioned normal values (hemoglobin > 13 g/dL in males, and > 12 g/dL in females), in accord with that recommended in patients without IBD^[3,4,53]. In fact, it is important to remember that the highest improvement in the quality of life is observed precisely when the hemoglobin levels increase from 11 to 13 g/dL^[4,54]. Moreover, all patients should receive enough iron supplementation to correct anemia and replenish body stores^[39,55]. In other words, the goals of anemia treatment, both in patients with and without IBD, are to normalize not only the hemoglobin value but also the iron stores, usually defined by the serum ferritin level^[2].

DOSE OF IRON SUPPLEMENTATION IN IBD ANEMIC PATIENTS

Although conventional wisdom “says” that up to 200 mg of elemental iron (and even 400 mg in some textbooks) per day is required to correct iron deficiency anemia, this is probably incorrect^[56]. Since a maximum of 10-20 mg of oral iron can be absorbed per day, very high doses and even high doses are questionable. In fact, there is no rationale to use “high” doses of iron to treat iron deficiency anemia (in IBD or in any other associated disease). There is no evidence to support high doses of iron in comparative trials^[57-59]. This makes sense from a physiologic standpoint since it is well known that the iron absorptive process is very efficient yet can be saturated^[56]. In this respect, a single tablet of most of the ferrous salt preparations (for example sulphate) provides more iron than the intestine is able to absorb in one day^[57,58]. On the other hand, non-absorbed iron salts can be toxic to the intestinal mucosa^[60-67], and perhaps could activate the disease^[1,24]. In any case, high doses of iron may cause diarrhea, which in turn not only impair quality of life but also may make it difficult to differentiate from an IBD relapse^[65,68]. Finally, non-absorbed iron salts may inhibit (i.e. by feedback) the intestinal iron absorption and decrease tolerance and compliance, which is difficult especially in young patients requiring several complex oral treatments.

Therefore, since absorption and efficacy of oral iron are no greater when high doses are used, and because adverse effects of this preparation are dose-related, oral iron, if used, should be recommended in low doses (e.g. 50-100 mg of elemental iron daily)^[2].

RESPONSE OF ANEMIA TO ORAL IRON SUPPLEMENTATION IN IBD PATIENTS

The main factor in favor of oral iron is convenience. However, even when iron treatment is correctly prescribed, the oral route has relevant limitations, such as^[2]: (1) only part of the iron is absorbed and, as previously mentioned, experimental and clinical evidence suggests that the non-absorbed iron salts can be toxic and proinflammatory, and perhaps could activate the disease^[1,24]; (2) absorption of oral iron can be severely compromised because of disease activity^[34], and in some Crohn's disease cases because of previous intestinal resections or involvement of the duodenum; (3) oral iron is often not well tolerated by patients. From a recent systematic review on the management of anemia in IBD which included several studies prescribing oral iron^[27,35,65-67,69,70], the intolerance rate (mainly because of nausea, abdominal pain, or diarrhea) was a common finding leading to discontinuation in up to 21% of the cases^[1]. Moreover, IBD patients often do need to take several oral drugs and overall compliance could be compromised by oral iron side effects^[71]; (4) oral supplementation results in a slow response, and in some patients persistent blood loss exceeds the capacity of intestinal absorption of iron^[72].

INTRAVENOUS IRON FOR THE TREATMENT OF IRON DEFICIENCY ANEMIA IN IBD

The efficacy of intravenous iron for the treatment of iron deficiency anemia in the general population (without IBD) has been demonstrated in numerous studies^[73]. Although the experience with intravenous iron in IBD is more limited, it is similarly encouraging^[6,16,50,51,66,70,74-78]. Iron sucrose was prescribed in most cases, which was effective in 50%-91% of the patients (depending on the criteria used for efficacy definition)^[1]. More recently, a mean response of iron deficiency anemia to the treatment with this intravenous iron formulation was calculated to be 73%, which is a considerably high figure^[2]. In summary, intravenous iron sucrose is more effective (in terms of faster and prolonged response) than oral iron supplements, and has a better safety profile which might positively influence the compliance of IBD patients. Accordingly, the inconvenience of intravenous iron is offset by the advantages in achieving better therapeutic results.

Following a widely recommended algorithm, the initial therapeutic strategy of iron deficiency anemia in IBD patients would be based on the level of hemoglobin. Patients with hemoglobin > 10/10.5 g/dL would initiate

treatment with oral iron, while in those with levels < 10/10.5 g/dL, which are generally considered to denote severe anemia, the intravenous route would be of choice^[1,3,8]. Intravenous iron should also be prescribed to patients with hemoglobin > 10/10.5 g/dL when intolerance to the oral formulation is present. In summary, the established indications for the use of intravenous iron are: severe anemia (generally defined as hemoglobin < 10 g/dL^[1,8,37], although some authors set the cut-off point at 10.5 g/dL), need for quick recovery in mild anemia, intolerance to oral iron, and failure of oral iron.

Although in IBD iron sucrose is the most used intravenous formulation; there are other new intravenous iron preparations which theoretically could be used, with an extremely low incidence of adverse effects, and in particular severe adverse effects^[1,73,79,80], but data in the specific IBD population is lacking, as reviewed by Auerbach^[79]. The experience with low-molecular-weight iron dextran is rather more extensive and encouraging^[79] and also a new molecule, iron carboxymaltose, merits mention because its pharmacokinetic characteristics and preliminary clinical experience seems very promising, and in this case was obtained directly in an IBD population^[81].

ROLE OF ERYTHROPOIETIN IN THE TREATMENT OF ANEMIA IN IBD

As previously mentioned, anemia in IBD patients results primarily from iron deficiency because of chronic intestinal blood loss. However, intestinal inflammation is mediated by overproduction of cytokines, which may contribute to the generation of anemia in chronic disease, accompanied by inadequate erythropoietin production^[16]. Thus, IBD-associated anemia is a unique example of a combination of chronic iron deficiency and anemia of chronic diseases. Since it was first used in chronic renal failure, recombinant human erythropoietin has been shown to be effective for treating the anemia that accompanies several chronic diseases^[41]. During the last few years, several studies have evaluated the efficacy of erythropoietin in IBD patients, reporting encouraging results^[16,20,27,50,75,82-84]. Nevertheless, as the cost of erythropoietin is much higher than the cost of intravenous iron, the latter formulation should be considered first-line therapy in patients with severe anemia, and erythropoietin therapy should be considered only for patients with low erythropoietin levels or who are unresponsive to intravenous iron^[37,85]. One must not forget to exclude or correct other causes of anemia in IBD patients before administering erythropoietin^[86]. Finally, erythropoietin should be reserved for patients in which aggressive management of IBD (including immunosuppressive therapy) has not suppressed inflammation, which underlines the idea that erythropoietin is an adjunct, - and not an alternative, -to appropriate treatment of IBD^[38].

Erythropoietic agents should always be combined with intravenous iron supplementation, because functional iron deficiency, -defined as an inappropriate availability of iron for erythropoiesis despite normal

body iron stores, is likely to develop^[76]. In the particular case of Crohn's disease, folic acid and vitamin B12 status should also be frequently checked^[43] and deficiencies adequately corrected. Accordingly, erythropoietin therapy has been accompanied by iron supplementation in all trials published so far^[1]. In summary, the enhancement of erythropoiesis by erythropoietin makes it mandatory to administer iron supplementation during therapy to meet the increased demand^[86].

CONCLUSION

Anemia is rather common in IBD. Particularly in Crohn's disease, it can be a very difficult clinical problem because iron deficiency, vitamin B12 and/or folic acid defects, malabsorption, malnutrition, inflammation, intestinal resection, and drug effects all can be the cause or contribute to a multifactorial and complex problem. The control of inflammation is a key point, but often is not enough to treat anemia. As anemia has a considerable impact on the quality of life of patients, a thorough and complete diagnostic and therapeutic strategy should be followed to help our patients have as normal a life as possible. Very recent evidence raises a very important problem for the clinician: anemia can be a chronic or at least a recurrent problem in IBD; patients should be followed up after completing treatment, and anemia and iron deficiency actively assessed in the standard investigations^[48].

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S- Editor Tian L L- Editor Cant MR E- Editor Lin YP



TOPIC HIGHLIGHT

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Intravenous iron in inflammatory bowel disease

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Received: July 23, 2009 Revised: September 17, 2009

Accepted: September 24, 2009

Published online: October 7, 2009

provide an excellent tool to prevent or treat anemia and ID in this patient population, which in turn avoids allogeneic blood transfusion and improves their quality of life.

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Key words: Inflammatory bowel disease; Anemia; Iron deficiency; Functional iron deficiency; Erythropoiesis stimulating agents; Oral iron; Intravenous iron

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Muñoz M, Gómez-Ramírez S, García-Erce JA. Intravenous iron in inflammatory bowel disease. *World J Gastroenterol* 2009; 15(37): 4666-4674 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4666.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4666>

Abstract

The prevalence of anemia across studies on patients with inflammatory bowel disease (IBD) is high (30%). Both iron deficiency (ID) and anemia of chronic disease contribute most to the development of anemia in IBD. The prevalence of ID is even higher (45%). Anemia and ID negatively impact the patient's quality of life. Therefore, together with an adequate control of disease activity, iron replacement therapy should start as soon as anemia or ID is detected to attain a normal hemoglobin (Hb) and iron status. Many patients will respond to oral iron, but compliance may be poor, whereas intravenous (IV) compounds are safe, provide a faster Hb increase and iron store repletion, and presents a lower rate of treatment discontinuation. Absolute indications for IV iron treatment should include severe anemia, intolerance or inappropriate response to oral iron, severe intestinal disease activity, or use of an erythropoietic stimulating agent. Four different products are principally used in clinical practice, which differ in their pharmacokinetic properties and safety profiles: iron gluconate and iron sucrose (lower single doses), and iron dextran and ferric carboxymaltose (higher single doses). After the initial resolution of anemia and the repletion of iron stores, the patient's hematological and iron parameters should be carefully and periodically monitored, and maintenance iron treatment should be provided as required. New IV preparations that allow for giving 1000-1500 mg in a single session, thus facilitating patient management,

INTRODUCTION

When body iron stores are depleted, iron supplementation seems beneficial, although the optimal route of administration remains controversial. Oral iron supplementation is adequate in some clinical conditions. Administration of oral iron, in the absence of inflammation or significant ongoing blood loss, can correct the anemia, provided significant doses can be tolerated. However, although conventional wisdom “says” that up to 200 mg of elemental iron per day is required to correct iron deficiency anemia (IDA), this is probably incorrect^[1]. Early studies indicated that the co-administration of iron with ascorbic acid (vitamin C) might be of benefit in enhancing iron absorption, since, in theory, more ferrous iron is maintained in solution. However reports indicated that such co-administration can induce severe toxicity in the gastrointestinal tract^[2]. Moreover, classically, oral iron intake separately from meals is recommended for increasing its absorption but this enhances digestive intolerance and, therefore, decreases compliance. In addition, the absorption of iron salts can be diminished by co-administration of some antibiotics (mainly quinolones, doxycycline, tetracyclines, chloramphenicol or penicillamine); proton pump inhibitors and anti-acid medication (aluminum,

bicarbonate, zinc or magnesium salts), levodopa, levothyroxine, cholestyramine, phytates (high fiber diets), soy products, ibandronate, etidronate, tannates, calcium, and phenolic compounds (coffee, tea), whereas amino acids seem to act as enhancers of iron absorption^[3].

On the other hand, non-absorbed iron salts may produce a variety of highly reactive oxygen species including hypochlorous acid, superoxides and peroxides that may lead to digestive intolerance, causing nausea, flatulence, abdominal pain, diarrhea or constipation, and black or tarry stools, and perhaps could activate relapsed inflammatory bowel disease (IBD)^[2].

Although preoperative oral iron has shown to be efficacious in uncomplicated IDA, in anemia of chronic disease (ACD) [e.g. Crohn's disease (CD)], as well as in that associated with acute inflammation (e.g. postoperative period after gastrointestinal surgery), the effectiveness of oral iron administration is rather limited since absorption is down-regulated, and the small amount of iron absorbed is directed to the reticulo-endothelial system (RES), where it is sequestered^[4].

In these situations, intravenous (IV) iron has emerged as a safe and effective alternative for IBD anemia management. This takes into consideration factors such as intolerance of or contraindications to oral iron, severe anemia (especially if accompanied by significant ongoing bleeding), short time to surgery, need of a fast recovery or the use of erythropoiesis-stimulating agents (ESAs)^[5]. Because daily iron absorption is only 1-2 mg (up to 10 mg in deep ferroplenia), for patients presenting with moderate to severe anemia at least three to 4 mo of oral iron administration are needed to correct hemoglobin (Hb) levels and replenish iron stores. As IV iron can allow up to a five-fold erythropoietic response to significant blood-loss anemia in normal individuals^[6], Hb starts rising in a few days, the percentage of responding patients is higher and iron stores are replenished. Boosting iron stores is an advantage, particularly for patients receiving ESAs^[7].

INTRAVENOUS IRON AGENTS

All IV iron agents are colloids with spheroidal iron-carbohydrate nanoparticles. Each particle consists of an iron-oxyhydroxide core (Fe^{3+}) and a carbohydrate shell that stabilizes the iron-oxyhydroxide core. Differences in core size and carbohydrate chemistry determine pharmacological and biologic differences between the different iron complexes, including clearance after injection, iron release *in vitro*, early evidence of iron bioactivity *in vivo*, and maximum tolerated dose and rate of infusion^[8,9]. Complexes can generally be classified as labile or robust (kinetic variability), and as weak or strong (thermodynamic variability), with all possible intermediates. Four different products are mostly used in clinical practice: iron gluconate, iron sucrose, iron dextran, and iron carboxymaltose^[2,10,11] (Table 1).

Iron gluconate

Iron gluconate has a core tightly bound to gluconate

and weakly associated with sucrose (molecular weight 38 kDa), and is a type III iron complex (labile and weak) with fast degradation kinetics and direct release to plasma proteins (apotransferrin, apoferritin, and others). The potential for acute adverse reactions related to labile iron release after IV injection, which is caused by oversaturation of the transferrin binding capacity, is higher with iron gluconate compared to the other available IV iron preparations. Non-transferrin-bound labile iron may induce acute endothelial cell injury and a transient capillary leak syndrome. Clinical symptoms of iron acute toxicity include nausea, hypotension, tachycardia, chest pain, dyspnea (lung edema), and bilateral edema of the hands and feet, and should not be misread as anaphylaxis^[9]. To avoid these side effects, the maximum recommended dose is 125 mg; whereas the administration of total dose is not recommended. The use of iron gluconate for iron deficiency (ID) in patients on dialysis has been found to be efficacious and safe^[8,9].

Iron sucrose

Iron sucrose has a core tightly bound to sucrose (molecular weight 43 kDa), and is a partially stable type with medium degradation kinetics and partial uptake of released iron by plasma proteins such as (apo)-transferrin but also by the RES (Type II: semi-robust and moderately strong). Its half life is relatively short (5-6 h) and the amount of iron transported by transferrin, calculated using the Michaelis-Menten model for a single dose containing 100 mg of iron, is around 30 mg $\text{Fe}^{3+}/24 \text{ h}$ ^[12]. Following a single IV injection of 100 mg iron sucrose to anemic patients, up to 95% of the injected iron was utilized within 2-4 wk. During the last few years, experience of using iron sucrose in various forms of ID has evolved. In spite of its safety profile, nowadays a test dose is still required at most European countries. Single doses of 100-200 mg as an IV injection^[13] or up to 500 mg over an infusion time of 3.5 h seem to be safe^[14]. The maximal recommended dosage is 600 mg/wk (200 mg iron as iron sucrose injected or infused intravenously no more than three times a week) but this amount exceeds the physiological needs of the proliferating erythroblast. If the infusion speed is too fast (above 4 mg $\text{Fe}^{3+}/\text{min}$) or the single total iron dose too high (above 7 mg Fe^{3+}/kg , with a maximum of 500 mg), non-transferrin bound labile iron may cause transient hypotension, tachycardia, and dyspnea, as described for iron gluconate. Paradoxically diarrhea, epigastric pain or dummy aches could appear within minutes to hours after infusion. Cases of phlebitis have been described, but they are probably secondary to longer lasting administration (rather than larger doses), the need to keep a venous access, or the use of solutions that are too dilute (iron concentration must be at least 1 mg/mL). Overall, iron sucrose is currently considered as the safest IV iron preparation^[15].

Iron dextran

Iron dextran is a stable parenteral iron product with

Table 1 Some characteristics of the different intravenous iron formulations

	Iron gluconate	Iron sucrose	Iron dextran (LMWID)	Ferric carboxymaltose
Complex type	Type III	Type II	Type I	Type I
	Labile and weak	Semi-robust and moderately strong	Robust and strong	Robust and strong
Molecular weight (kDa)	38	43	73	150
Initial distribution volume (L)	6	3.4	3.5	3.5
Plasma half-life (h)	1	6	30	16
Labile iron release	+++	± ¹	-	-
Direct iron donation to transferrin (% injected dose)	5-6	4-5	1-2	1-2
Test dose required	No	Yes/No ³	Yes	No
Maximal single dose (mg)	125	300	TDI	1000
Premedication	No	No	TDI only	No
Life-threatening ADE ² (× 10 ⁶ doses)	0.9	0.6	11.3	??
Death rate (× 10 ⁶ doses) ²	0.25	0.11	0.78	??

¹If the infusion speed > 4 mg Fe³⁺/min or dose > 7 mg Fe³⁺/kg; ²Data from patients with chronic kidney disease; ³Not required at USA and UK. ADE: Adverse drug events; LMWID: Low molecular weight iron dextran; TDI: Total dose infusion.

a molecular weight of 73 kDa (Low molecular weight iron dextran, LMWID) or 156 kDa (High molecular weight iron dextran, HMWID). This type I iron complex (robust and strong) shows high structural homogeneity and only slow and competitive delivery to endogenous iron binding proteins. Complexes are actively phagocytosed by macrophages of the RES before they are released and become available for Hb synthesis. Although the plasma half life of LMWID is 30 h (3 d for HMWID), the full process of iron release from the dextran complex in the RES, storage in ferritin and delivery as TBI to the bone marrow or other tissues may take several months^[16]. Iron dextran can be administered as intramuscular (i.m.) or IV injections and as IV infusion, but a test dose is always required before the first administration. The stability of the dextran complex allows administration of high single doses (so called “total dose infusion” which may be given over 4-6 h). In contrast, the bioavailability of iron following i.m. administration has not been studied extensively. There seems to be a risk of incomplete and variable absorption of the iron from the injection site, and a considerable amount (30%-50%) of iron can remain at the i.m. injection site for many months. Therefore i.m. injections are no longer recommended^[17]. However, these iron complexes may cause well known dextran-induced anaphylactic reactions, especially in patients receiving HMWID (not commercially available in Europe and considered as an obsolete and dangerous IV iron agent). The exact mechanism of the anaphylactic reaction to iron dextran has not been clarified yet, but it seems to be related to the antibody-mediated release of mediators by mast cells^[15].

Ferric carboxymaltose (FCM)

FCM is another stable parenteral iron product with a molecular weight of 150 kDa very similar to iron dextran in terms of stability and structure (Type I, robust and strong). The pharmacokinetic characteristics of FCM are similar but not identical to iron dextran. The distribution volume of both preparations corresponds

nearly to that of plasma, but half life is approximately 16 h for FCM as compared to 30 h for LMWID. It seems that FCM is broken down quicker than iron dextran because α -amylase does not affect dextran, or acts at a very slow rate^[2]. A study using positron emission tomography has shown that iron from FCM accumulates in the liver, spleen and bone marrow and substantial amounts were found in these organs within minutes. In addition, FCM is able to exchange iron rapidly with transferrin^[18]. As a result, the utilization of iron for RBC increased rapidly up to days 6 to 9, after which the utilization increased at a much lower rate. Patients with IDA showed iron utilization over 90% after 24 d compared to 60%-80% utilization for patients with renal anemia^[18]. FCM is designed to mimic physiologically-occurring ferritin, providing high iron utilization, without the disadvantageous characteristics associated with iron dextran (anaphylaxis) and iron sucrose (high pH, high osmolarity, dosage limitations, and the long duration of administration). Up to 100-200 mg FCM can be administered as IV injection and up to 1000 mg iron can be infused in at least 15 min and no test dose is required (Table 1). In comparison, the European Union (EU) prescribing information for other IV iron preparations indicates they can be administered only in low doses (e.g. usual recommended dose of LMW iron dextran is 100-200 mg of iron and the maximum EU dose of iron sucrose is 200 mg of iron) over a period of greater than 30 min, which results in the need for frequent infusions to administer the total calculated iron replacement dose^[19]. No serious adverse effects, including deaths, were considered related or likely related to FCM by trial investigators; however, the US Food and Drugs Administration has raised concerns about a potential mortality safety signal based on an increased of deaths in comparison to other arms across clinical trials^[20]. In contrast, since 2007 the use of FCM corresponds to over 17 000 patient-years (one patient corresponds to 2000 mg iron), and up to September 2008 no anaphylactoid reactions or death have been reported, suggesting a good safety profile for FCM^[2]. Therefore, information regarding

FCM safety in the clinical setting is somehow conflictive and further post authorization trials to confirm its benefit and safety are needed.

EFFICACY OF PARENTERAL IRON AGENTS IN IBD

Experience with the use of IV iron therapy is extensive in different clinical settings over the last 60 years. In the late 1980s, the introduction of recombinant human erythropoietin (rHuEPO) led to a revitalized interest in the use of iron therapy, either in combination with rHuEPO therapy, or alone. Intravenous iron therapy can be used in a variety of clinical settings, as long as iron parameters are carefully monitored. In a number of studies, IV iron was shown to be useful for the treatment of anemia associated with a variety of medical [IBD, chronic kidney disease (CKD), chronic inflammatory arthritis, congestive cardiac failure, pregnancy and postpartum, or cancer] and surgical conditions (orthopedic, cardiac, colorectal cancer, and gynecological surgical procedures)^[21,22]. Interestingly, in the settings of CKD or cancer related anemia, the use of IV iron resulted not only in a more rapid and complete response to rHuEPO, but also in a reduction of rHuEPO dose, and probably in a reduction of rHuEPO side effects, such as thrombosis^[23,24].

Approximately, one third of IBD patients suffer from recurrent anemia across different studies (ranging from 6% to 73%, depending on Hb cut-off for the definition of anemia; patient selection, IBD phenotype and year of publication), and the prevalence of ID is even higher [mean prevalence: 45%, 95% confidence interval (CI): 40%-50%]. A retrospective study found that the prevalence of mild to moderate anemia significantly decreased in the IBD population between 1993 and 2003 (33.8% *vs* 16.7%, $P = 0.013$), although the prevalence of severe anemia was similar (6.3% *vs* 5.6%, $P = \text{NS}$), and the only difference detected between the two cohorts was the increased use of immunosuppressive drugs (mainly azathioprine)^[25]. Both ID (due to intestinal blood loss that cannot be matched by duodenal iron absorption, creating a negative iron balance) and ACD (due to the inflammatory nature of the disease) contribute most to the development of anemia in IBD, whereas cobalamin or folate deficiency and various other causes of anemia such as hemolysis occur infrequently. Whatever the underlying mechanism, anemia is universally accepted as a condition having a significant impact on the affected patient's quality of life^[1,26].

Anemia control and recovery in patients with IBD has a beneficial impact on quality of life indices. Our goal is to attain Hb levels above 13 g/dL in males and 12 g/dL in females by the administration of iron supplements, with or without erythropoietin. However, it is worth noting that without an appropriate control of disease activity, the management of anemia associated to IBD is much more difficult. Thus, it is desirable to initiate the pharmacological treatment after adequate inflammation control^[27].

According to the recommendations of the Guidelines on the Diagnosis and Management of Iron Deficiency and Anemia in Inflammatory Bowel Diseases (Statement 2B), iron supplementation should be initiated when IDA is present (Grade A). For ID without anemia, different approaches to iron replacement should be considered and discussed with the patient. If patients are likely to develop IDA the monitoring frequency should be increased (Grade D)^[26], although it is unknown how often monitoring should be performed.

When body iron stores are depleted, iron supplementation seems beneficial, although the optimal route of administration remains controversial. Total iron deficit (TID) can be calculated using the Ganzoni's formula: $\text{TID (mg)} = \text{Weight (kg)} \times (\text{Ideal Hb} - \text{Actual Hb}) \text{ (g/dL)} \times 0.24 + \text{depot iron (500 mg)}$.

According to this formula, a person weighing 70 kg with an Hb level of 9 g/dL would have a body iron deficit of about 1400 mg. Nevertheless, Ganzoni's formula may underestimate iron depot in males, as in them it has been consistently reported to be 700-900 mg^[28]. Thus, a TID of 1600-1800 mg may be a more realistic estimation for this subject.

Following the administration of oral iron to a patient with uncomplicated IDA, it takes 2-2.5 wk for the Hb to start rising, 2 mo for it to reach normal levels and 6 mo for iron stores to be replete^[7]. However, the efficacy of oral iron therapy in IBD patients may be hindered by some IBD specific factors, such as reduced absorption of iron due to inflammation and gastrointestinal side effects of oral ferrous iron (due to the release of activated hydroxyl radicals that may lead to digestive intolerance, causing nausea, flatulence, abdominal pain, diarrhea or constipation, and black or tarry stools)^[2]. In addition, oral iron compounds are not all alike, as they may vary in composition, elemental iron concentration, absorption profile, efficiency or tolerance. As a non-written rule, the best tolerated oral agent is usually the one that contains or delivers less iron.

In a crossover study of 19 IBD patients randomly assigned to start treatment with ferrous fumarate 120 mg orally once daily or iron sucrose 200 mg IV three times during a period of 14 d, oral ferrous fumarate, but not IV iron sucrose, increased clinical disease activity in IBD patients^[29]. In contrast, iron sucrose, but not ferrous fumarate, increased intravascular oxidative stress^[29]. However, a prospective study comparing usage, tolerance, and efficacy of 4 wk therapy with oral iron therapy in patients with ID and IBD and patients with ID of non-inflammatory cause, intolerance to iron was reported in 24% of the patients who had IBD (non-active) and 29% of the patients who did not ($P = \text{NS}$), and only a tiny minority of IBD patients relapse in association with use of oral iron therapy^[30]. This data suggest that patients with IBD are no more intolerant to oral iron than other patients and have similar rates of repletion, but the low number of evaluable patients ($n = 47$) precluded the drawing of definite conclusions. Nevertheless, to avoid the risk of poisoning,

Table 2 Characteristics of the clinical trials involving IBD patients that compared IV iron administration with oral iron or no intervention included in this review

Study (yr)	n	Study design	Compound	Baseline Hb (g/dL)	Total dose, mg (schedule)	Duration (wk)	Response (%)	DCT (%)
Gasche <i>et al</i> ^[31] (2001)	103	Multicentre, open-label	Iron sucrose	≤ 10.5	1200 mg (6 × 200 mg)	4	65	0
Bodemar <i>et al</i> ^[32] (2004)	59	Retrospective	Iron sucrose	< 12	Mean 1400 mg (1-2 × 200 mg/wk)	8	60	0
Schröder <i>et al</i> ^[14] (2005)	46	Multicentre randomized open-label	Iron sucrose (22)	< 10.5 (F)	Mean 1418 mg (7 mg/kg + 5 × 200 mg)	6	55	4.5
			Ferrous sulfate (24)	< 11 (M)	Mean 5600 mg (100-200 mg/d)		53	20.8
García-López <i>et al</i> ^[33] (2006)	70	Single centre prospective observational	Iron sucrose	< 10.5 ¹	Mean 920 mg (200-1800 mg) (200 mg/1-3 times a week)	Mean 5 (1-9)	67	0
Kulnigg <i>et al</i> ^[37] (2008)	200	Multicentre randomized open-label	Ferric carboxymaltose (137)	≤ 10	1000-1500 mg (1-2 infusion of 500-1000 mg)	12	77	1.5
			Ferrous sulfate (63)		16800 mg (200 mg/d)		68	7.9
Lindgren <i>et al</i> ^[33] (2009)	91	Multicentre randomized investigator-blinded	Iron sucrose (45)	< 11.5	Mean 1700 mg (200 mg/1-2 wk)	20	66	7
			Ferrous sulfate (46)		Mean 38400 mg (200-400 mg/d)		47	22
Gisbert <i>et al</i> ^[34] (2009)	100	Multicentre, open-label	Iron sucrose (22)	< 10	Not reported (2 × 200 mg/wk if Hb < 10)	26	77	0
			Ferrous sulfate (78)	> 10	19000 mg (106 mg/d)		89	5.1

¹Also no response or intolerance to oral iron or clinical need of quick recovery of anemia. ΔHb ≥ 2 g/dL or normal Hb; DCT: Discontinuation due to serious adverse events; F: Female; M: Male.

other oral iron compounds (such as iron polymaltose which has very low toxicity and meets the requirements for a food supplement) might be used instead of ferrous salt preparations^[2] and lower doses (e.g. 50-100 mg of elemental iron) should be recommended^[1]. Additionally, the response and tolerance should be monitored and treatment changed to IV iron if necessary (Grade C)^[26].

Is this statement supported by the information reviewed above? Because of the limitations of oral iron therapy in IBD patients, parenteral routes of iron administration should be preferred, even though many patients will respond to oral iron. Intravenous iron is more effective, better tolerated, and improves quality of life to a greater extent than oral iron supplements (Grade A)^[26]. Absolute indications for IV iron include severe anemia (Hb < 10 g/dL), intolerance or inappropriate response to oral iron (once iron therapy has been initiated the response may be: “complete”, if Hb increases ≥ 2 g/dL; “partial”, if Hb increases 1-1.9 g/dL; or “no-response”, if Hb increases < 1 g/dL), severe intestinal disease activity, concomitant therapy with an erythropoiesis stimulating agent, or patient preference^[26]. In a prospective study of 103 patients with severe IBD-associated anemia who received IV iron sucrose for 4 wk (total dose 1200 mg), Gasche *et al*^[31] investigated the parameters that can predict effectiveness. Overall, a complete response at the end of the fourth week was observed in 67 (65%) patients, and the variables significantly associated with response were serum erythropoietin, soluble transferrin receptor, transferrin, and IL-6 levels. Once again, these data emphasized the need for an adequate inflammation control in IBD patients. We will review some studies assessing the efficacy of IV iron in anemic IBD patients (Table 2).

In this regard, a retrospective observational study in IBD patients with poor response or intolerance to oral iron, the administration of iron sucrose (200 mg once or twice per week to reach total ID) resulted in a “complete” response (Hb increment ≥ 2 g/dL or correction of anemia) in 60% of patients within 8 wk and in 90% of patients within 12 wk^[32]. However, a randomized, controlled, open-label, multicenter trial performed in 46 patients with anemia and transferrin saturation ≤ 20% and/or serum ferritin concentrations ≤ 20 µg/L found no differences in Hb increment within 6 wk between patients receiving IV iron sucrose and those receiving iron sulfate, but resulted in building up iron stores (about ferritin = 200 ng/mL, after 6 wk)^[14]. In addition, intractable gastrointestinal adverse events caused permanent study drug discontinuation in five patients (20.8%) receiving iron sulfate, whereas only one patient (4.5%) was withdrawn because of side effects due to IV iron sucrose^[14]. Thus, although being equal in short-term efficacy, these results suggest a better gastrointestinal tolerability for iron sucrose.

In a very recent study, 91 patients with IBD and anemia (Hb < 11.5 g/dL) were randomized to oral iron sulfate (*n* = 46) or IV iron sucrose (*n* = 45) treatment for 20 wk. More patients in the IV iron group completed the study (93% *vs* 78%, *P* = 0.001), increased their Hb ≥ 2 g/dL (66% *vs* 47%, *P* = 0.07), raised their ferritin levels to normal (74% *vs* 48%, *P* = 0.013), and recovered from anemia (84% *vs* 59%, *P* = 0.007) compared to patients in the oral iron group. In addition, treatment with IV iron sucrose improved iron stores faster and more effectively than oral iron (*P* = 0.002). Only 22 patients (48%) tolerated the prescribed oral dose, and 52% reduced the dose or withdrew from treatment because of poor tolerance^[33].

Finally, in a prospective multicenter study of 100 IBD patients with IDA [59 CD, 41 ulcerative colitis (UC)], those with Hb > 10 g/dL were prescribed oral ferrous sulphate ($n = 78$) and those with Hb < 10 g/dL received IV iron sucrose ($n = 22$). Hb normalization was achieved in 89% with oral and 77% with IV iron, and was associated with a relevant improvement in the patients' quality of life. IBD activity increase was not demonstrated in any patient. Four patients (5.1%) showed oral iron intolerance leading to discontinuation of treatment, whereas no adverse events were reported for IV iron^[34]. Thus, oral iron treatment was effective and well tolerated in most IBD patients, and did not exacerbate the symptoms of the underlying IBD, whereas IV iron was an effective and safe treatment in more severely anemic or intolerant patients.

At one author's centre, the safety and efficacy of IV iron sucrose therapy was evaluated in a preliminary study of 70 patients with digestive pathology (54 IBD: 27 CD, 18 UC, nine pouchitis)^[34]. IV iron sucrose in an "outpatient regimen" was used for patients with IDA due to digestive disorders with at least one of the following criteria: (1) no response or intolerance to oral iron; (2) IBD with severe anemia (Hb < 10.5 g/dL) and/or (3) clinical need of quick recovery of anemia. Average baseline Hb was 9.8 ± 1.7 g/dL, and 11.7 ± 1.5 g/dL at the end of treatment (mean increase 1.9 g/dL, range -2 to 5.5 g/dL), Hb increase exceeded 2 g/dL in 47% of treatments, and anemia was corrected in 67.8% of patients. No severe adverse events were witnessed. The authors concluded that IV iron sucrose should become the standard of care in IBD patients with ID^[35].

Therefore, treatment with IV iron sucrose is effective, safe, and well tolerated in correcting Hb and iron stores in patients with IBD, especially in those with severe anemia. The main disadvantage of IV iron sucrose is the need for multiple infusions as the maximum weekly dose should not exceed 600 mg. The availability of stable parenteral iron compounds allowing for TDI infusion may greatly facilitate iron replacement therapy in IBD patients.

As for children, the safety and efficacy of IV iron therapy was retrospectively evaluated in 70 pediatric patients with IBD (50 CD, 20 UC) who received a total of 119 TDI iron dextran infusions between February 1994 and February 2000. The average increase in Hb concentration was 2.9 g/dL. The authors concluded that TDI infusion of iron dextran, when appropriately used, is a safe and potentially efficacious treatment for children with IBD and IDA who are unresponsive to, or noncompliant with, oral iron therapy^[36]. However, as mentioned above, iron dextran, especially HMWID, has the disadvantage of potentially life-threatening dextran-associated anaphylactic reactions.

More recently, Kulnigg *et al.*^[37] randomized 200 anemic IBD patients (about Hb = 9 g/dL) to receive IV FCM (FCM, $n = 173$; maximum 1000 mg iron per infusion) at 1-wk intervals until the patients' calculated TID was reached or oral ferrous sulfate (100 mg *bid*)

for 12 wk. There were no differences between groups in Hb improvement at week 12 (3.8 ± 2.0 g/dL in both groups) or treatment-related adverse events, but response (defined as Hb increase of > 2.0 g/dL) was higher for FCM at week 2 ($P = 0.0051$) and week 4 ($P = 0.0346$), with a lower rate of discontinuation of study medication due to adverse events (1.5% and 7.9%, respectively), than for oral iron. Thus, FCM seems to be effective and safe in IBD-associated anemia.

Overall, from data depicted in Table 2, the mean response of IBD-associated anemia to treatment with IV iron (weighted mean) was (281/382) 73.6% and (140/215) 65.1% with oral iron, [odds ratio (OR): 1.49, 95% CI: 1.02-2.17, $P = 0.02$]. When the analysis was performed for data extracted from prospective randomized trials only, the response to IV iron was 72.5% (143/198) *vs* 58.2% (71/122) (OR: 1.87, 95% CI: 1.13-3.09, $P = 0.0097$). In addition, reviewed data strongly suggest that for patients with IBD, treatment with IV iron is effective, safe, well tolerated, provides a fast Hb increase and a sufficient refill of iron stores, and presents a lower rate of treatment discontinuation than oral iron. However, further research is needed to ascertain what is the appropriate timing to start treatment, which are the target Hb and ferritin levels to reach, and how IV iron may affect IBD clinical time course.

A TENTATIVE ALGORITHM FOR IRON REPLACEMENT IN IBD

Although further research is needed to ascertain what is the appropriate time to start treatment, which are the target Hb and ferritin levels to reach, or how IV iron may affect the IBD clinical time course, a tentative, easy to follow algorithm for iron replacement in IBD patients is depicted in Figure 1.

According to this algorithm, in which we assume that the severity of ID and anemia correlates with disease activity, the total iron dose and the route of administration rely upon baseline Hb, serum ferritin level and transferrin saturation. For male IBD patients (70-90 kg) with Hb > 13 g/dL and ferritin < 30 ng/mL, TID is estimated to be around 800-1000 mg. The corresponding value for TID in women (60-80 kg) with Hb > 12 g/dL would be 600-800 mg. If there are no contraindications, oral iron would be the simplest replacement therapy (50-100 mg/d, for 2-3 mo), and iron complexes are preferred to iron salts. If there is a contraindication for, or a non adequate response (ferritin < 100 ng/mL after 6-8 wk) to, oral iron, an IV iron preparation should be administered: iron sucrose (200 mg IV, 1-2 times/wk), LMWID (up to 1000 mg IV, single dose), or FCM (up to 1000 mg IV, single dose).

As for patients with Hb between 10 g/dL and 12/13 g/dL, ferritin < 100 ng/mL and transferrin saturation < 20%, TID is estimated to be 1300-1800 mg. Oral iron supplements could still be indicated (100 mg/d for 4-6 mo) but the IV route is preferred, as it will pro-

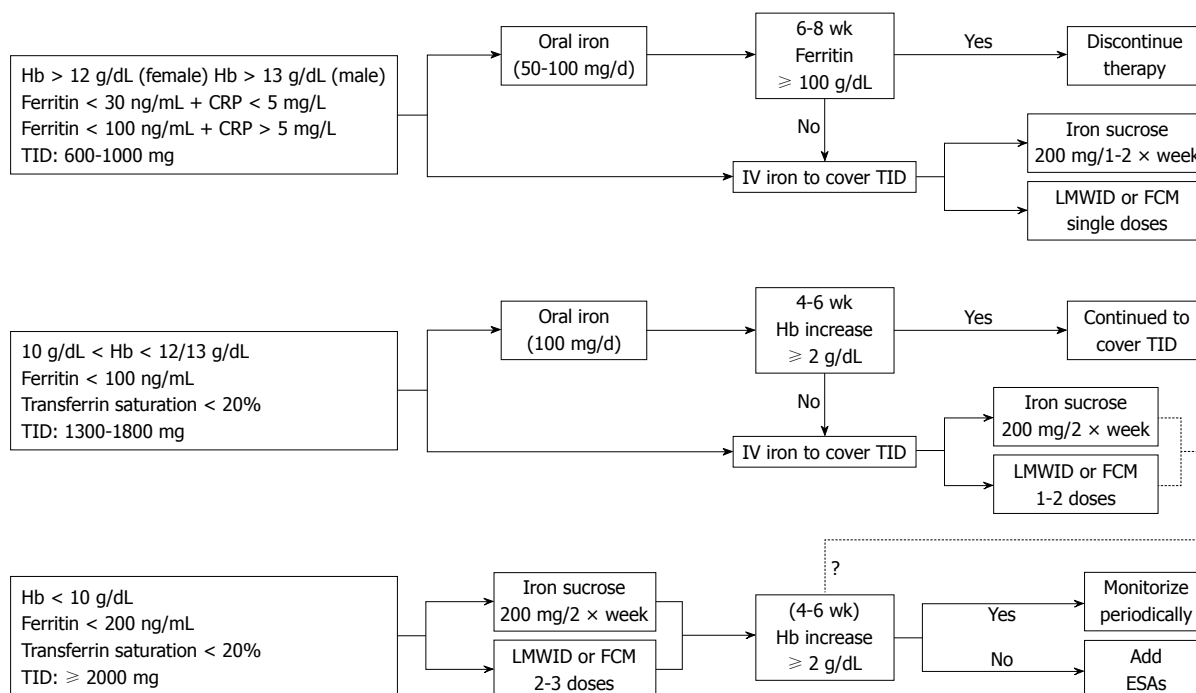


Figure 1 A tentative algorithm for iron replacement therapy in patients with inflammatory bowel disease. Hb: Hemoglobin; TID: Total iron deficit; CRP: C-reactive protein; LMWID: Low molecular weight iron dextran; FCM: Ferric carboxymaltose; ESA: Erythropoiesis stimulating agent.

vide a faster Hb recovery (iron sucrose 200 mg IV, twice a week; LMWID or FCM, two doses one week apart). Nevertheless, IV iron should be administered when there is intolerance to, or a non complete response to, oral iron, as defined by an Hb increase ≥ 2 g/dL or Hb normalization.

For patients with Hb < 10 g/dL and transferrin saturation < 20%, a higher ferritin trigger is selected (< 200 ng/mL), and a TID ≥ 2000 mg is estimated. These patients should receive IV iron (iron sucrose 200 mg IV, twice a week; LMWID or FCM, 2-3 doses). Patients should be re-evaluated after 4-6 wk; if there is not a complete Hb response, adjuvant treatment with erythropoiesis stimulating agents (e.g. epoetin, darbepoetin) should be considered.

SAFETY OF PARENTERAL IRON AGENTS

Nausea, abdominal pain, constipation, diarrhea, injection site reactions (pain, superficial phlebitis), metallic taste, headache, dizziness and rash may occur with all IV preparations, and were observed in clinical trials with an incidence of 1%-3%. However, the incidence of life-threatening adverse drug events (ADEs) associated with parenteral iron is much smaller.

Allergic and anaphylactic reactions

The numbers of non-CKD patients receiving IV iron are not large enough to draw definitive conclusions regarding the safety of IV iron agents in these clinical settings. Therefore, we will focus on ADEs associated with parenteral iron in CKD patients, as they are the largest collective receiving these drugs. According to data from the

United States Food and Drug Administration (FDA) on ADEs attributed to the provision of four formulations of IV iron (HMWID, LMWID, iron gluconate and iron sucrose) during 2001-2003, the total number of reported parenteral iron-related ADEs was 1141 amongst approximately 30 million doses administered (approx. 38 ADEs per million), with 11 deaths (seven iron dextran, three iron gluconate, one iron sucrose)^[15]. Relative to lower molecular weight iron dextran, total and life-threatening ADEs were significantly more frequent among recipients of higher molecular weight iron dextran and significantly less frequent among recipients of sodium ferric gluconate complex and iron sucrose. The absolute rates of life-threatening ADEs were 0.6, 0.9, 3.3 and 11.3 per million for iron sucrose, sodium ferric gluconate complex, lower molecular weight iron dextran and higher molecular weight iron dextran, respectively, whereas absolute rates of death were 0.11, 0.25, 0.75 and 0.78 per million, respectively (Table 1). However, there were no significant differences in mortality rates between LMWID and iron gluconate (OR: 0.3, 95% CI: 0.1-1.3) or iron sucrose (OR: 0.2, 95% CI: 0.1-1.0), and there are no conclusive data available regarding the safety of FCM. Therefore, the frequency of IV iron-related ADEs reported to the FDA has decreased, and overall, the rates are extremely low (Table 1). In addition, the rates of ADEs associated with IV iron, including iron-related deaths, are much lower than that of ABT-related severe side effects (10 per million) and ABT-related deaths (four per million)^[38].

IV iron and infection

Current information on the relationship between IV iron and infection, and between IV iron and oxidative

stress deserves special consideration. Elemental iron is an essential growth factor for bacteria with many species expressing iron transport proteins that compete with transferrin, and it has long been suggested that patients with iron overload are at increased risk of infection^[39]. In contrast, in the peritoneal dialysis population, no increased risk of peritonitis was found in patients receiving IV iron with respect to those not receiving IV iron^[40]. In addition, a meta-analysis of six observational studies (807 patients) revealed that the administration of IV iron to patients undergoing major orthopedic surgery led to a significant decrease in both transfusion rate [relative risk (RR): 0.60, 95% CI: 0.50-0.72, $P < 0.001$] and infection rate (RR: 0.45, 95% CI: 0.32-0.63, $P < 0.001$)^[41]. Nevertheless, despite this absence of definitive clinical data, it seems sensible to avoid IV iron administration in the setting of acute infection, and to withhold IV iron in patients with pre-treatment ferritin values > 500 ng/mL^[5].

IV iron and oxidant damage

Biologically active iron, which is released by all IV iron agents, also plays a role in inflammation, oxidative stress and the propensity for accelerated atherosclerosis. Persistent oxidative stress in CKD patients promotes inflammation and, in turn, atherogenesis, and increased cardiovascular morbidity and mortality. However, available evidence relating IV iron administration to atherogenesis is indirect, and there is little evidence that IV iron adversely affects survival in patients with dialysis-dependent CKD. Nevertheless, the evidence argues for caution, not complacency, in prescribing IV iron^[9].

IV iron and cancer development

The association between iron overload with cancer risk in humans has been under increased scrutiny in recent decades, although epidemiological studies on the association of iron with cancer remain inconclusive. The concerns are mostly focused on a possible risk associated with dietary iron in colorectal cancer, the increased risk of developing hepatocellular carcinoma in hereditary hemochromatosis and related hepatic iron overload and cirrhosis, and association between occupational exposure to iron and kidney, lung and stomach cancers. The risk of iron-induced sarcoma by repeated i.m. injections of iron dextran has also been raised. However, IV iron therapy has not been associated with an increase in tumor incidence^[42].

CONCLUSION

The prevalence of anemia across the studies on patients with IBD is high (30%) and that of ID is even higher (45%). However, the prevalence of anemia is decreasing, and this seems to be related with the availability and use of IV iron^[43].

Iron replacement therapy should start as soon as anemia or ID is detected (Grade D), and its goal is to attain a normal level of Hb, ferritin and transferrin saturation (Grade D)^[26]. Importantly, our efforts to correct anemia

should rely on adequate inflammation control, in the absence of which no proper approach to this condition is feasible^[27].

Although many IBD patients will respond to oral iron, IV iron is more effective, better tolerated, and improves the quality of life to a greater extent than oral iron supplements (Grade A)^[26]. Absolute indications for IV iron include severe anemia, intolerance or inappropriate response to oral iron, severe intestinal disease activity, use of ESAs, or patient preference^[26].

The use of ESAs should be restricted to those patients presenting with Hb < 10 g/dL and who do not appropriately respond to IV iron replacement for 4 wk (Grade B)^[26].

After the initial resolution of anemia and the repletion of iron stores, patients should be closely monitored, and maintenance iron treatment should be provided as required. New IV preparations that allows for giving up to 1000-1500 mg in a single session, provide an excellent tool to avoid or treat anemia and ID in the IBD patient population.

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S- Editor Tian L L- Editor O'Neill M E- Editor Zheng XM



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Use of agents stimulating erythropoiesis in digestive diseases

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Received: July 23, 2009 Revised: July 30, 2009

Accepted: August 6, 2009

Published online: October 7, 2009

and continuous erythropoietin receptor activator) will allow better dosing with the same efficacy and safety.

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Key words: Erythropoiesis-stimulating agents; Recombinant human erythropoietin; Darbepoetin; Continuous erythropoietin receptor activator; Inflammatory bowel disease; Anemia

Peer reviewer: Luis Bujanda, Professor, Donostia Hospital, Avda. Sancho El Sabio 21-3°C, San Sebastián 20010, Spain

Moreno López R, Sicilia Aladrén B, Gomollón García F. Use of agents stimulating erythropoiesis in digestive diseases. *World J Gastroenterol* 2009; 15(37): 4675-4685 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4675.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4675>

Abstract

Anemia is the most common complication of inflammatory bowel disease (IBD). Control and inadequate treatment leads to a worse quality of life and increased morbidity and hospitalization. Blood loss, and to a lesser extent, malabsorption of iron are the main causes of iron deficiency in IBD. There is also a variable component of anemia related to chronic inflammation. The anemia of chronic renal failure has been treated for many years with recombinant human erythropoietin (rHuEPO), which significantly improves quality of life and survival. Subsequently, rHuEPO has been used progressively in other conditions that occur with anemia of chronic processes such as cancer, rheumatoid arthritis or IBD, and anemia associated with the treatment of hepatitis C virus. Erythropoietic agents complete the range of available therapeutic options for treatment of anemia associated with IBD, which begins by treating the basis of the inflammatory disease, along with intravenous iron therapy as first choice. In cases of resistance to treatment with iron, combined therapy with erythropoietic agents aims to achieve near-normal levels of hemoglobin/hematocrit (11-12 g/dL). New formulations of intravenous iron (iron carboxymaltose) and the new generation of erythropoietic agents (darbepoetin

INTRODUCTION

Anemia is a frequent complication in patients with digestive diseases, especially chronic processes such as inflammatory bowel disease (IBD)^[1,2], and is associated with a higher rate of hospitalization and worse quality of life^[3,4]. The main cause of anemia associated with IBD is iron deficiency, but there is also a component of anemia that is associated with chronic diseases. Therefore, when there is no good response to intravenous iron therapy, combination with erythropoietic agents, erythropoietin (EPO) or darbepoetin, may assist in the management of anemia^[3,5-7]. The other subgroup of patients with digestive diseases in whom we can use erythropoiesis-stimulating agents (ESAs) are those being treated for chronic hepatitis C, whose anemia is caused by the use of rivabirin^[8,9].

Human EPO is a glycoprotein hormone of 30.4 kDa, which is composed of 165 amino acids, and is the main regulator of erythropoiesis. It inhibits the apoptosis of erythroid progenitors and induces the clonal proliferation of normoblasts^[10-12]. In adults, it is synthesized mainly in the kidney, which produces 90%-95% of the total circulating EPO; the remainder, about 10%, is synthesized by liver^[13,14]. In addition to kidney and liver, it has been shown that other organs can secrete EPO, including peripheral endothelial cells, vascular smooth

muscle cells, neurons, astrocytes, cardiomyocytes and microglia^[15].

The basal production maintains a relatively constant level of EPO in plasma, within a range between 5 and 29 U/L, which can increase up to 100 times in the presence of serious anemia^[16]. Tissue hypoxia is the most important trigger for increased synthesis of EPO^[10,17].

In 1977, Miyake *et al*^[18] purified EPO molecule, which enabled cloning of the gene in 1983, and the subsequent development of recombinant EPO in mammalian cell lines^[19]. The clinical use of EPO was started in 1986^[20] for the treatment of anemia of chronic kidney disease (CKD)^[21]. Treatment with EPO was able to eliminate almost completely transfusion dependence in hemodialysis patients, who in turn achieved a significant improvement in quality of life^[22-24]. Treatment of anemia with EPO has helped to reduce the need for transfusions and the associated risks: transmission of infection, allogeneic iron overload and immunological damage^[25].

There is a general consensus, as stated by the recommendations of panels of international experts (The National Kidney Foundation Dialysis Outcomes Quality Initiative^[26], the Canadian Society of Nephrology^[27], the European Renal Association/European Dialysis and Transplantation Association^[28,29], Kidney Disease: Improving Global Outcomes^[30] and the Japanese Society for Dialysis Therapy^[31,32]), that the partial correction of anemia in patients with CKD improves clinical parameters and quality of life compared with the lowest levels of hemoglobin^[26-32]. At present, for instance, in the United States, > 90% of patients on chronic dialysis and 30% on pre-dialysis are treated with EPO, with an average hemoglobin level of 12.0 g/dL^[33-35]. Subsequently, its use has been extended to the treatment of anemia associated with other diseases such as cancer^[36-38], IBD^[3,5-7], heart failure^[39,40] and the anemia associated with hepatitis C virus treatment^[8]. In addition to its role as an erythropoietic agent, EPO has other potential therapeutic uses currently under extensive investigation^[41,42], for use as a cytoprotective agent in the central nervous system^[43,44] and intestinal mucosa^[45-47]. Other possible applications include are cytoprotection in peripheral neuropathy, retinopathy and myocardial ischemia^[48,49].

TYPES OF EPO

In clinical practice there are several forms of EPO available for the treatment of anemia (Table 1). The first generation are two forms of recombinant human EPO (rHuEPO): epoetin α (Epogen, Amgen and Procrit/Eprex; Johnson & Johnson/Janssen-Cilag) and EPO β (NeoRecormon; Roche), which are administered three times a week^[50]. The development during the last decade of ESAs with a higher degree of glycosylation and prolonged half-life has allowed less frequent administration. A preparation called long-acting darbepoetin α (Aranesp; Amgen) produces a similar physiological response compared with rHuEPO^[51]. It has more chains of carbohydrates and sialic acid residues, which gives it a different

pharmacokinetic profile from that of rHuEPO, with a half-life approximately three times higher (25.3 h *vs* 8.5 h by iv administration), and a plasma clearance four times slower. This allows a frequency of administration of once weekly, or even every 2-4 wk^[52,53]. The equivalence relationship between rHuEPO and darbepoetin α from the molecular weight of both proteins is 1:200^[54]. In clinical practice, the multiplication factor is not so simple, as the required dose of rHuEPO can be higher, which probably is related to an increase in resistance to EPO^[55]. A pegylated derivative, the continuous EPO receptor activator (CERA) (epoetin β -methoxy polyethylene glycol, CERA; Roche) is another erythropoietic agent that activates repeatedly the EPO receptor. It has an elimination half-life in humans of about 130 h, and so can be administered every 3-4 wk^[56-59]. Several studies have shown an effect similar to EPO in maintaining hemoglobin levels^[60-65]. CERA is not available in United States, but is currently used in Europe. An additional advantage is that CERA can be kept out of the fridge and used for up to 1 mo (at < 25°C).

There is a new generation of erythropoietic analogues: synthetic erythropoiesis protein and peptide mimetics of EPO. These new agents stimulate erythropoiesis through activation of EPO receptors^[66-69]. Hematide (developed by Affymax) is a synthetic peptide agonist of the EPO receptor, and although it has no structural homology with EPO, it is able to activate the EPO receptor and stimulate erythropoiesis over a 1-mo period, with good tolerance and stability at room temperature^[66].

SIDE EFFECTS OF ESAs

The use of ESAs is related to several common side effects that should be well known by clinicians. Hypertension is the most common side effect, with an overall incidence of 5%-24%^[70]. Vascular access thrombosis occurs with higher incidence^[71,72], although it is not found in other small studies^[73,74]. The risk of serious cardiovascular events, such as thromboembolism and death, increases when hemoglobin levels increase rapidly (> 1 g/dL every 2 wk)^[75]. The development of anti-EPO antibodies is a very rare but serious complication of treatment with erythropoietic agents. From 1998 to 2004, it was apparent that there was a significant increase in the number of patients who developed pure red-cell aplasia (PRCA) secondary to the appearance of neutralizing anti-EPO antibodies^[76,77]. PRCA is characterized by severe anemia, high transfusion requirement and a total lack of response to increasing doses of rHuEPO. Most of the reported cases have been patients with chronic renal failure who received rHuEpo α (Eprex) subcutaneously. PRCA was related to a change of formulation of Eprex (replacement of human serum albumin by polysorbate 80, because of the risk of bovine spongiform encephalopathy) and formation of immunogenic micelles. Alternatively, it has been suggested that leachates released by the uncoated rubber stoppers of the pre-filled syringes may interact with polysorbate 80 and

Table 1 Erythropoietic agents available

	Half-life (h)		Periodicity	Initial dose	Target levels
	iv	sc			
Epoetin β	9	24	1-3 times per week	100-150 IU/kg per week, (max 300 IU /kg)	Hemoglobin: 11-12 g/dL, Hematocrit: 33%-36%, ↑ hemoglobin every 2 wk: 0.5-1 g/dL
Epoetin α	7	20	1-3 times per week	100-150 IU/kg per week, (max 300 IU /kg)	
Darbepoetin α	25	48	Every 1-2 wk	0.45 μ g/kg every 2 wk	
CERA	133	137	Every 2-4 wk	0.6 μ g/kg every 2 wk	

act as an adjuvant to the immune response. Failure in the cold chain is also a potential factor involved^[78-80]. The number of cases reported has dropped significantly from 2003, with none in 2007. This may have resulted from a change in the route of administration (at present the iv route is used in most patients on dialysis), maintenance of the cold chain, or elimination of uncoated rubber syringe stoppers^[75]. Should this complication occur, clinical guidelines for diagnosis and treatment are readily available^[81-84]. It has been suggested that the use of the new analogue hematide in these cases may be effective^[85].

Other possible side-effects are edema, fever, dizziness, insomnia, headache, pruritus, constipation, among other.

USEFULNESS OF ESAs IN DIGESTIVE DISEASES

IBD

Anemia is the most common complication of IBD^[86,87]. Inadequate monitoring and treatment leads to a worse quality of life^[88,89] and increased morbidity and hospitalization^[90-92]. Repeated loss of blood, and to a lesser extent malabsorption of iron are the main causes of iron deficiency in IBD^[86]. There is also a variable component of anemia that is related to chronic inflammation^[3,4,7,87,93]. This involves failure of iron transport that is mediated by inflammatory cytokines, such as hepcidin, which is the main negative regulator of iron absorption in the small intestine and of iron sequestration by macrophages^[94-101], and an inappropriately low production of EPO for the degree of anemia^[16,90,101-103]. The management of anemia in IBD should focus on proper control of the inflammatory process, as well as iron supplementation, and in cases of resistance, to assess iron therapy in combination with erythropoietic agents^[3,87,91,104]. Up to 25%-30% of patients with anemia associated with IBD combination therapy may require iron and erythropoietic agents to correct anemia^[3,105,106]. In other diseases (cancer, rheumatoid arthritis, AIDS), EPO levels < 500 mU/mL (some authors suggest < 100 mU/mL) may respond to administration of rHuEPO^[107-112].

In patients with anemia associated with IBD, high levels of transferrin (iron deficit indicators) as well as high levels of serum EPO (an indicator of a correct response to anemia) may predict a good response to treatment with iv iron. In contrast, low levels of serum EPO indicate the need to associate agents erythropoietic in addition to iron treatment^[3].

Since the increased production of hepcidin in anemia

of chronic diseases may limit the oral absorption of iron, it should be given by the iv route. The iv administration of iron has proven its efficacy, safety and tolerability, with iron sucrose^[7,89] as the new formulation iron carboxymaltose, that allows the administration of 1 g of iron in 15 min^[112,113]. The use of erythropoietic agents in the treatment of anemia associated with IBD is useful for patients who do not respond to treatment with iv iron, and in whom the aggressive treatment of IBD (including immunosuppressive therapy) has not abolished the mucosal inflammation, and who require additional blood transfusions^[5,7,114-116].

Hemoglobin target

Previous studies on the use of EPO in patients with CKD have found different results concerning the desirable target level of hemoglobin and its effect on cardiovascular prognosis. In 1997, the KDOQI guidelines^[117] recommended target levels for hemoglobin/hematocrit between 33% (11 g/dL) and 36% (12 g/dL). A similar recommendation was made in the 2006 update of the KDOQI guidelines^[26] although on that occasion, an upper limit for hemoglobin was set, because there was no evidence to maintain a target \geq 13 g/dL. The European Best Practice Guidelines Working Group did not recommend the complete correction of hemoglobin levels in patients with diabetes or cardiovascular disease^[29].

The clinical benefits and adverse effects associated with normal or near normal hemoglobin values were evaluated in multiple randomized studies that assessed mortality and morbidity from cardiovascular or cerebrovascular events, good control of blood pressure, quality of life, functional status and vascular access thrombosis^[71,118-126]. The results of these studies have not suggested any improvements after correction of anemia, except in quality of life. Despite differences in their populations, two large randomized studies published in November 2006, the CHOIR^[127] and CREATE^[128] studies have shown that attempts to correct anemia completely did not reduce mortality or cardiovascular disease in CKD patients, compared with partial correction. A meta-analysis that included these two studies concluded that patients with a higher target hemoglobin have a significantly higher risk of all-cause mortality and vascular access thrombosis^[129].

In light of these data, the KDOQI guidelines were reviewed and updated in 2007, with a recommended hemoglobin level of 11-12 g/dL, and not exceeding 13 g/dL^[130]. Also the European Best Practice Guidelines Working Group^[75] has concluded that hemoglobin

> 13 g/dL may be associated with cardiovascular events in these patients.

Factors that influence the increase in mortality with higher hemoglobin targets may include the impossibility of achieving the target hemoglobin, a too high hemoglobin target, the toxic effects of high doses of ESAs, the presence of comorbidity and other features^[131-135]. These factors were evaluated in a second analysis of the CHOIR study^[136] that found no clinical factor associated with risk, after adjustment and multivariate analysis, except for high dose of epoetin (> 20 000 U/wk), which was an independent risk factor for death, myocardial infarction, heart failure, or stroke. This increased risk was observed in the high and low hemoglobin groups, particularly among those who did not reach the target hemoglobin. These results suggest that increased mortality is due to high doses of ESAs rather than higher hemoglobin targets^[137]. Epoetin α dose should not exceed 20 000 U/wk in patients with CKD, and probably in other diseases. These patients should be assessed for other causes of poor response to treatment with EPO. Some studies have suggested the possible relationship between the variability of the hemoglobin level and the patient^[138,139]. Although there is variability in the results of different studies^[120,131,140-143], the most consistent observation is that there are better results in terms of quality of life, with no increase in adverse reactions of hemoglobin in the range of 11-12 g/dL (hematocrit 33%) compared with lower levels^[139-144]. There is a large study under way in relation to the normalization of hemoglobin. The Trial to Reduce Cardiovascular Events with Aranesp Therapy study is a randomized, placebo-controlled trial in pre-dialysis CKD patients with type 2 diabetes mellitus^[145,146], which is due to end in 2011.

In clinical practice, given the difficulty of maintaining standards within the narrow target range of 11-12 g/dL^[147-149], it's accepted the range 10-12 g/dL, particularly in patients with good tolerance.

The target haemoglobin level in patients with IBD is still to be determined. Data from recent studies in CKD patients indicate increased morbidity and mortality in relation to high levels of hemoglobin (normal)^[130-133], as in patients with cancer^[103,104]. It seems appropriate to establish a target hemoglobin level of 11-12 g/dL, which demonstrates greater benefit in quality of life and cost-effectiveness in renal patients^[120,133,139-145].

Dosage, monitoring and control

To maintain adequate levels of hemoglobin with erythropoietic agents iron stores must be normalized. Iron should be administered in sufficient quantity to achieve a transferrin saturation $\geq 20\%$ and a ferritin level ≥ 100 ng/mL^[7,87,150]. The response to EPO is dose dependent, but varies from patient to patient, depending on the frequency and route of administration (iv or sc), although to a lesser extent with darbepoetin^[151,152]. The hypertension may complicate treatment, particularly if hemoglobin level rises quickly (> 1 g/dL every 2 wk)^[153]. With the present preparations, sc and iv administration are indistinguishable; however, sc administration presents some advantages

over iv, such as a lower incidence of hypertension, and a 25%-50% reduction in dose compared with iv administration^[154-157]. Another important advantage of iv administration is a longer half-life (24 h vs 9 h)^[158,159]. Daily sc EPO is more effective than administration 2-3 times weekly^[160], although administration less frequently than every 2 wk is also effective^[161-163]. Discomfort at the injection site is minimal. It is important to remember to maintain the cold chain at 4°C to preserve a high effectiveness^[164].

EPO can be started at a dose of 100-150 U/kg per week sc with iron supplements. The dose can be increased by 25% every 2-4 wk to reach 300 U/kg per week. It is not worthwhile to continue increasing the dose in patients who do not respond after 12 wk, and in these patients, the dose should be kept to the minimum effective dose to avoid transfusion^[97,131]. The appropriate response should increase the hemoglobin level at least 0.5 g/dL at 2-4 wk. It is recommended analytical control in this period of time and if the hemoglobin increase is over target (> 10-12 g/dL) or > 1 g/dL in 2 wk, we must reduce dose by 25%. In contrast, if the hemoglobin level is < 10 g/dL (with adequate iron deposits) or hemoglobin increase < 1 g/dL in 4 wk, then we must increase dose by 25%^[101,104,165]. Children usually require higher doses than adults to achieve a similar response^[29,166]. In practice, most patients are dosed per unit dose (syringe) rather than kg. There are wide range of doses in pre-filled syringes.

Darbepoetin α can be administered iv and sc, with the main advantage of the half-life three times longer than epoetin. Effectiveness of Darbepoetin has been proved with administration weekly, every 2 wk^[167] and monthly^[117,168,169]. The initial dose of darbepoetin is 0.45 μ g/kg every 2 wk and that of CERA is 0.6 μ g/kg every 2 wk. Once the patient has been stabilized, the monthly dose may be doubled. Several well-designed prospective studies in renal patients have demonstrated the safety and effectiveness of weekly or even monthly treatment with epoetin α ^[170-174] in a similar way to new formulations of ESAs with long half-life^[175].

Resistance

Evidence of EPO resistance stems from studies with an inadequate response EPO in 5%-10% of patients^[176] or in patients who develop EPO resistance after a good initial response. There is a resistance to EPO when a sufficient dose of it, equal to or greater than 300 U/kg per week is not reached the desired concentration of hemoglobin^[28].

It has been shown that the most frequent and important cause of resistance to EPO is iron deficiency, but there are other less frequent factors^[28,177].

Blood loss is the most frequent cause of absolute iron deficiency, which is defined by ferritin levels < 100 ng/mL, transferrin saturation < 20%, and/or hypochromic red cells increased by 10%. Relative or functional iron deficiency results from difficulty in transferring stored iron to red blood cells and is defined by the existence of transferrin saturation < 20% while

maintaining high levels of ferritin > 100 ng/mL. The main causes of relative iron deficiency are acute and chronic inflammatory processes and chronic liver diseases^[178]. Elevated levels of parathyroid hormone in CKD patients are another cause of non-negligible resistance to EPO^[179]. The presence of an inflammatory disorder increases the resistance to treatment with erythropoietic agents, including the production of inflammatory cytokines that interfere with iron metabolism, reducing their availability in the bone marrow and causing functional iron deficiency. The process of dialysis may be associated with an increase in the induction of cytokines and the appearance of an inflammatory response syndrome^[180].

In patients undergoing hemodialysis, carnitine metabolism is altered and carnitine deficiency is more likely in patients with a protein-deficient diet and high dialysis dose^[181]. Carnitine may improve anemia in hemodialysis patients with EPO resistance^[182] by mechanisms not yet known^[183], or stabilizing the membrane as an antioxidant agent, or by stimulating erythropoiesis, by the increase in the number of reticulocytes demonstrated in some patients on hemodialysis treated with L-carnitine^[184]. The dose used in patients with EPO resistance is 1 g iv post-hemodialysis^[185].

Folic acid is involved in the process of regeneration and maturation of hematopoietic precursors. Folic acid deficiency is associated with ineffective erythropoiesis, and a megaloblastic anemia. Malnutrition, malabsorption, alcoholism, and various drugs that lower intestinal absorption, such as diphenylhydantoin, contraceptives and barbiturates, can cause folic acid deficiency. In dialysis patients with resistance to EPO, with normal ferritin levels, adjuvant treatment with folic acid may be attempted. It has been shown that the use of folic acid (10 mg/d) in hemodialysis patients improves the response to EPO, especially when presented with high mean corpuscular volume, even with normal levels of folic acid^[186].

The effectiveness of erythropoietic response to stimulation can be assessed by resistance index of ESAs. This expresses the relationship between the dose of erythropoietic agents and hemoglobin concentrations maintained (IU/kg per week divided by hemoglobin). Resistance index of ESAs varies from one patient to another and also in the same patient over time. These values range from 0 in patients able to maintain adequate hemoglobin level through the endogenous production of EPO, to > 50 IU/kg per week and per g/dL of hemoglobin in patients who cannot maintain adequate hemoglobin, even after high-dose EPO therapy (> 300 IU/kg per week)^[187-189].

Anemia following treatment with ribavirin in HCV patients

Hemolytic anemia is a frequent side effect of early use of ribavirin in the treatment of hepatitis C. It has a negative impact on quality of life, and it can, in extreme cases, cause deterioration in brain function and even death. Furthermore it is a common reason for reduction or discontinuation of antiviral therapy, which compromises the effectiveness and reduces the sustained viral response. The administration of EPO can

improve anemia, without the need to reduce the dose of ribavirin^[190-194].

FUTURE EXPECTATIONS WITH THE USE OF EPO

It has been discovered in recent years that the EPO is also synthesized locally by many tissues, especially in response to metabolic stress. EPO functions as a protective molecule that inhibits apoptosis in a wide variety of cell types, and reduces inflammation and local edema, as well as improving tissue regeneration^[195]. The mechanisms of tissue protection are mediated by a receptor. This receptor is different from that which mediates the effects of erythropoiesis of EPO^[196]. The future of EPO therapy in ischemic diseases, and as protective cytotoxic therapies appears promising. Of course clinical trials comparing its efficacy with other conventional therapies are needed^[197]. Also being evaluated is the effect of EPO on intestinal endothelial cells^[45], and in the maintenance and repair of the mucosa^[46].

SUMMARY AND PROSPECTS

For nearly 25 years, the anemia of chronic renal failure has been treated with rHuEPO, which has resulted in a significant improvement in quality of life and survival and avoidance of dependence on repeated transfusions^[22-24]. Subsequently, rHuEPO has been used in other conditions that occur with anemia of chronic diseases such as cancer, rheumatoid arthritis^[109] or IBD^[3,5,114-116], and anemia associated with the treatment of hepatitis C^[190-194]. It has also been investigated for its protective effects in other acute diseases such as myocardial ischemia and brain and kidney diseases^[198]. The classical dosing schedule of three times weekly has been simplified with the advent of new generations of erythropoietic agents such as darbepoetin α , which allows administration every 2 wk, and CERA, which has a longer half-life that allows prolonged administration monthly. Research continues to develop new biologically similar molecules such as agonists of the EPO receptor as hematein, which allows the treatment of patients with PRCA and monthly administration^[85]. As well as searching for orally active formulations that will simplify the management of anemia^[199-202]. There have also been negative aspects of treatment with EPO, as the cases of pure red cell aplasia^[64,80], or poor outcomes in relation to death and cardiovascular events in study of normalization of hemoglobin (in connection especially with high doses of EPO in patients with poor response)^[127-129,137] and worse survival in patients with cancer^[103,104]. However, EPO has been shown to be effective and safe in the treatment of anemia of chronic renal failure and has improved significantly quality of life of patients with chronic anemia^[22-25].

Similarly, the development of iv iron therapy was a major breakthrough in the management of chronic anemia. Iron sucrose has demonstrated its safety, efficacy and tolerance^[7,86,87]. Other new formulations of iron

that allow rapid iv administration of large doses with good efficacy and tolerance will make administration easier^[5,110,202].

CONCLUSION

To summarize we can say that erythropoietic agents come to complete the range available therapeutic for treatment of anemia associated with IBD that begins by treating the inflammatory disease basis, as well as intravenous ferroterapia first choice. In cases of resistance to treatment with iron may raise a combined therapy with erythropoietic agents to try to achieve near-normal levels of hemoglobin/hematocrit (11-12 g/dL). The new formulations of iv iron (iron carboxymaltose) and the new generation of erythropoietic agents (darbepoetin and CERA) will allow a more comfortable and spaced dosage schedule, with the same efficacy and safety.

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S- Editor Tian L L- Editor Kerr C E- Editor Ma WH



TOPIC HIGHLIGHT

Javier P Gisbert, Professor; Fernando Gomollón, MD, PhD, Series Editors

Blood transfusion for the treatment of acute anaemia in inflammatory bowel disease and other digestive diseases

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Supported by (In part) Grant ICS 08/0205 from Instituto Aragonés de Ciencias de la Salud (Zaragoza, Spain)

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Received: July 23, 2009 Revised: August 19, 2009

Accepted: August 26, 2009

Published online: October 7, 2009

Abstract

Allogeneic blood transfusion (ABT) is frequently used as the first therapeutic option for the treatment of acute anaemia in patients with inflammatory bowel disease (IBD), especially when it developed due to gastrointestinal or perioperative blood loss, but is not risk-free. Adverse effects of ABT include, but are not limited to, acute hemolytic reaction (wrong blood or wrong patient), febrile non-hemolytic transfusional reaction, bacterial contamination, transfusion-related acute lung injury, transfusion associated circulatory overload, transfusion-related immuno-modulation, and transmission of almost all infectious diseases (bacteria, virus, protozoa and prion), which might result in increased risk of morbidity and mortality. Unfortunately, the main physiological goal of ABT, i.e. to increase oxygen consumption by the hypoxic tissues, has not been well documented. In contrast, the ABT is usually misused only to increase the haemoglobin level within a fixed protocol [mostly two by two packed red blood cell (PRC) units] independently of the patient's tolerance to normovolemic anaemia or his clinical response to the transfusion of PRC units according to a "one-by-one" administration schedule. Evidence-based clinical guidelines may promote best transfusion practices by implementing restrictive transfusion

protocols, thus reducing variability and minimizing the avoidable risks of transfusion, and the use of autologous blood and pharmacologic alternatives. In this regard, preoperative autologous blood donation (PABD) consistently diminished the frequency of ABT, although its contribution to ABT avoidance is reduced when performed under a transfusion protocol. In addition, interpretation of utility of PABD in surgical IBD patients is hampered by scarcity of published data. However, the role of autologous red blood cells as drug carriers is promising. Finally, it must be stressed that a combination of methods used within well-constructed protocols will offer better prospects for blood conservation in selected IBD patients undergoing elective surgery.

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Key words: Anaemia; Blood transfusion; Autologous blood transfusion; Inflammatory bowel diseases; Risk assessment

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García-Erce JA, Gomollón F, Muñoz M. Blood transfusion for the treatment of acute anaemia in inflammatory bowel disease and other digestive diseases. *World J Gastroenterol* 2009; 15(37): 4686-4694 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4686.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4686>

INTRODUCTION

In surgical, medical and critically ill patients, allogeneic blood transfusion (ABT) is frequently used as the first therapeutic option for the treatment of acute anaemia, especially when it developed due to traumatic or acute blood loss or when the patient's compensatory mechanisms have a limited capacity of response^[1-4]. As a result, a high proportion of patients receive at least one ABT unit during their hospital stays.

However, many reports show variation in transfusion practice for comparable groups of patients between hospitals, and even between different departments

within a hospital. Variations in rates of transfusion may be due to many factors, but still differing opinions on the threshold level of haemoglobin (Hb) and the implementation of transfusion alternatives are the most important ones. The first may reflect uncertainty about the benefits and risks of ABT, and the second different perceptions of the value of stimulation of erythropoiesis and minimising blood loss and subsequent transfusion^[5].

Of note, the main physiological goal of ABT, i.e. to increase oxygen consumption by the hypoxic tissues, has not been well documented^[6-8]. In contrast, the ABT is usually misused only to increase the Hb level within a fixed protocol [mostly two by two packed red blood cell (PRC) units] independently of the patient's tolerance to normovolemic anaemia or his clinical response to the transfusion of each PRC unit according to a "one-by-one" administration schedule.

Unfortunately, good clinical studies and outcome data establishing the benefits and risks of ABT for a patient in different clinical settings are not available^[9]. To the best of our knowledge, there are very few situations or pathological entities where ABT and/or liberal transfusion criteria have shown to benefit our patients (drepanocytosis, thalassemic or low-weight premature infants)^[10,11]. Thus, to promote best transfusion practices and reduce variability, minimizing the avoidable risks of transfusion, and to help clinicians in choosing the appropriate treatment options, a number of clinical guidelines have been issued by different medical societies^[12-17]. In this article, we review the indications, benefits and risks of blood transfusion of the different blood components available, both allogeneic and autologous, with special emphasis on patients suffering from inflammatory bowel disease (IBD), either ulcerative colitis (UC) or Crohn's disease (CD).

INDICATIONS FOR ABT

Transfusion of PRC is indicated in order to achieve a fast increase in the supply of oxygen to the tissues, when the concentration of Hb is low and/or the oxygen carrying capacity is reduced, in the presence of inadequate physiological mechanisms of compensation. Tissue oxygenation depends on various factors: (1) the concentration of Hb; (2) the saturation of Hb, which, in turn, depends on the O₂ tension and the affinity of the Hb for O₂; (3) the O₂ requirements, that is, the volume of oxygen needed by the tissues to carry out their aerobic function. But, there are a number of clinical factors that affect the physiological mechanisms of adaptation to anaemia: (1) a reduced increase in cardiac output: hypovolaemia, coronary artery disease, disorders of heart valves, congestive heart disease, negative inotropic drugs; (2) decreased capacity to increase the extraction of O₂: acute respiratory distress syndrome (ARDS), sepsis, systemic inflammatory response syndrome (SIRS), traumatic ischemia-reperfusion syndrome; three, altered gas exchange: chronic obstructive pulmonary disease (COPD), ARDS; and

four, increased consumption of O₂: fever, pain, stress, sepsis, SIRS or hyperventilation syndromes^[17].

When there is an indication to correct anaemia, but the situation is not urgent, strategies other than transfusion are preferred, such as the use of haematopoietic drugs (iron, vitamin B12, folic acid, recombinant erythropoietin) (see another chapter).

Non-surgical IBD patients

Nowadays the administration of oral or IV iron supplements, with or without erythropoiesis-stimulating agents, but not ABT, is the standard therapy for the anaemia of the medical IBD patient (see corresponding article in this issue). According to the recommendations of the Guidelines on the Diagnosis and Management of Iron Deficiency and Anemia in Inflammatory Bowel Diseases (Statement 3A), the goals of anaemia treatment are to increase Hb, and iron studies above the lower threshold of normal, to prevent a further fall in Hb, to avoid the use of ABT, to relieve symptoms related to anaemia, and to improve the quality of life (Grade D)^[18]. Nevertheless, some IBD patients are still being transfused, mostly because of acute gastrointestinal haemorrhage, although the Guidelines do not provided any recommendations in this regard. In our modest opinion, these ABT are some times administered without strict criteria, just to raise a low Hb figure ("cosmetic" transfusion) or to treat a hypovolemic situation, but not to correct transitorily hypoxic symptoms or signs.

Fortunately, severe acute gastrointestinal haemorrhage is uncommon in IBD, although among children 0-5 years of age patients presented with more rectal bleeding than patients aged 6-17 years, despite the later having a more complicated disease course^[19].

Belaiche *et al*^[20] reviewed 34 cases of acute gastrointestinal haemorrhage in CD patients (defined as acute rectal bleeding originating in diseased bowel and requiring an ABT of at least 2 U of PCR within 24 h). When the bleeding source was identified, the bleeding lesion was an ulcer in 95% of cases, most often in the left colon. The treatment was surgical (20.6%), endoscopic (20.6%), or medical (58.8%), and there were no deaths. The authors concluded that given the potential efficacy of endoscopic or medical treatment, as well as the absence of mortality, a conservative approach may be suggested as first-line therapy in the majority of patients.

Similarly, Pardi *et al*^[21] characterized the clinical features and course of such haemorrhage in patients at their institution from 1989 to 1996. Thirty-one patients had acute lower gastrointestinal bleeding from IBD (three UC, 28 CD), representing approximately 1% of admissions for IBD. Again, the bleeding lesion presented with an ileocolonic or colonic localization in most cases (68%), and surgery was required in less than half of cases. However, in both patient series, recurrent haemorrhage was not rare, and for these cases surgery may be the most appropriate treatment.

More recently, Kostka *et al*^[22] characterized the clinical features and course of 11 separate episodes of severe haemorrhage in six of 156 patients with CD (3.8%),

treated between 1985 and 2003 at their institution. Emergency surgery was necessary to stop the primary or recurrent haemorrhage in four patients. The authors concluded that, although a conservative approach may be first-line therapy, surgery is inevitable in patients suffering from massive bleeding and in patients with recurrent bleeding. Similar conclusions were reached by Veroux *et al*^[23] in a previous series of five CD patients with severe gastrointestinal bleeding.

In a recent review of five years, Campos *et al*^[24] reported 301 episodes of massive transfusion occurred at a university hospital, and found that 51 out of these 301 episodes were due to upper gastrointestinal haemorrhage (17%), with a mortality rate of 52%. It is recommended that the Transfusion Services have a special protocol to manage severe bleeding emergency, however the blood component administration schedules are neither clear nor uniform. In the last few years, there has been a general review of the blood component schedule during massive transfusion^[25]. Nowadays, data from several large observational studies show that early beginning of plasma and platelet administration, including the use of the so-called “transfusion packs”, could improve mortality of patients with severe bleeding^[26].

Surgical IBD patients

As for acute gastrointestinal haemorrhage, other emergency complications of IBD are rare, but may be life-threatening, require surgery, and result in permanent end organ damage. The most common non-bleeding complications associated with UC are fulminant colitis and toxic megacolon, and often result in a total proctocolectomy. The most common non-bleeding complications associated with CD are abscesses and intestinal obstruction, and usually also require surgical intervention and intestinal resection^[27].

Despite pharmacological advancements, the management of IBD has become increasingly complicated. In a study of 2892 adults with CD and 5895 with UC who received care between 1998 and 2005, Herrinton *et al*^[28] found: a shift in gastroenterology-related visits from the gastroenterology division to primary care; an increased use of IBD-related drugs; a 36% decline in the prevalence of prolonged steroid exposure for CD patients, with a 27% increase for UC patients; a 30% decline in the hospitalization rates for CD and UC patients; and a 50% decline in the surgery rate for UC, but no significant change for CD. In this regard, it is worth noting that in UC patients, surgery remains an important part of the overall treatment plan, especially for prophylactic total colectomy in certain patients at high risk for colorectal cancer^[29].

As for other patients undergoing major surgery, in the surgical IBD patient, intraoperative and postoperative management of potential or actual blood loss should include (ASA)^[16].

Monitoring the amount of blood loss: A periodic visual assessment of the surgical field and communication with

the surgical team should be done to assess the presence of excessive microvascular bleeding (i.e. coagulopathy).

Monitoring for the presence of inadequate perfusion and oxygenation of vital organs: By using conventional (e.g. blood pressure, heart rate, oxygen saturation, urine output, electrocardiography) and/or special monitoring systems (e.g. echocardiography, mixed venous oxygen saturation, blood gasses, National Institute of Radiological Sciences).

Monitoring for transfusion indications: Measure Hb or haematocrit when there is substantial blood loss or there is any indication of organ ischemia. However, as the haematocrit value is subjected to a variety of bias, especially in hypovolemic anaemic patients, Hb concentration should be preferred^[30]. In this regard, it's worth noting that in most of the available hematological analyzers the “haematocrit” is an indirectly calculated parameter.

Transfusion of allogeneic red blood cells (RBCs): Maintain adequate intravascular volume and blood pressure with crystalloids or colloids until the criteria for ABT are met (see below). Adequate quantities of RBCs should be transfused to maintain organ perfusion (PCR units should be transfused one-by-one, and anaemia symptoms reassessed after each transfusion). When appropriate, pre-deposited autologous blood donation and other means to decrease blood loss (e.g. deliberate hypotension, antifibrinolytics) may be beneficial (see below).

Hb transfusion triggers

The only indication for the transfusion of PRC is to correct or prevent tissue hypoxia; thus, the parameter “of choice” for making decisions should be intracellular pO₂. However, this parameter is not useful for clinical purposes and it is, therefore, necessary to rely on “surrogate” parameters, such as Hb and the haematocrit. The indication for and the degree of urgency of PRC transfusions cannot, however, be defined only on the basis of the values of Hb or the Htc, but must be based on a complete evaluation of the patient's clinical condition and the possible presence of mechanisms compensating for the anaemia (see above)^[17].

According to ASA guideline on blood transfusion^[16], PRC should usually be administered when the Hb level is less than 6 g/dL, whereas they are usually unnecessary when the level is more than 10 g/dL. These conclusions may be altered in the presence of anticipated blood loss. The determination of whether intermediate Hb concentrations (i.e. 6-10 g/dL) justify or require PRC transfusion should be individualised. This indication should be based on any ongoing indication of organ ischaemia, potential or actual ongoing bleeding (rate and magnitude), the patient's intravascular volume status, and the patient's risk factors for complications of inadequate oxygenation. These risk factors include a low cardiopulmonary reserve and high oxygen consumption^[16,17].

Table 1 Transfusion haemoglobin threshold according to patient's age and characteristics and type of anaemia

Haemoglobin threshold (g/dL)	Patients characteristics and type of anaemia
< 5	Patients with chronic anaemia and without risk factors ¹
< 6	Patients with symptomatic ² chronic anaemia and without risk factors
< 7	Acute anaemia in younger patients Asymptomatic chronic anaemia in patients with risk factors
< 8	Acute anaemia in surgical and critically ill patients
< 9	Acute anaemia in surgical patients older than 65 yr
< 10	Acute anaemia in patients with organ dysfunction ³
< 10	Patients with massive transfusion
> 10	Do not transfuse

¹Risk factors; ²Symptomatic anaemia; ³Organ dysfunction.

These indications are in agreement with those proposed by most international clinical guidelines and documents issued by several medical societies, such as the American Consensus Conference on perioperative red blood cell transfusion (Hb < 8 g/dL)^[12], British Haematological Society (Hb < 8 g/dL)^[13], the Scottish Guideline on Perioperative Blood Transfusion (Hb < 7 g/dL)^[4], or the Spanish Society for Blood Transfusion (Hb < 7 g/dL and Hb < 5 g/dL in chronic anemia)^[15]. Recently, the Italian Transfusion Society^[17] proposed the same recommendations as ASA^[16], but giving a Grade of recommendation 1A. A tentative table of Hb transfusion thresholds as a function patient's age and characteristics and type of anaemia is given in Table 1.

Transfusion yield

As a rough guide, in adults one unit of PRC increases the Hb concentration by 1 g/dL and the haematocrit by about 3%. In children, the transfusion of 5 mL/kg increases the Hb concentration by about 1 g/dL. In the case of a lower than expected transfusion yield, conditions causing the loss, sequestration or destruction of RBCs should be looked for. Such conditions include: occult bleeding, repeated blood sampling (particularly in children), fever, hypersplenism, primary and secondary immunological causes, mechanical or other type of hemolysis^[17].

BENEFITS OF ABT

The experience with Jehovah's Witness patients suggests that, overall, postoperative anaemia is well tolerated if Hb > 7 g/dL, but it increases the risk of mortality when Hb falls below 5 g/dL, this effect being magnified by blood loss and cardiac disease (Hb < 10 g/dL), and indirectly suggests that ABT is life-saving in this context^[9]. Also, ABT may be life-saving in children with severe anaemia and signs of respiratory distress and, possibly, in very low birth weight infants^[9]. In contrast, for critically ill patients, severe anaemia (Hb < 8 g/dL) increases the risk of mortality by 1.54, whereas the transfusion of 1-2, 3-4, or more than 4 U increases this risk by 1.48, 2.68, and

Table 2 Theoretical reasons supporting the restrictive or the liberal use of allogeneic red cells in normovolemic patients

Rationale supporting the liberal use of red cells
Augmenting O ₂ delivery may improve patient survival and functional recovery
Increased risk of coronary ischaemia due to increased demand
Reduces respiratory work
Age, disease severity and drugs may interfere adaptation to anaemia
Improved safety margin if further blood loss
Increased safety of donor blood products
Rationale supporting the restrictive use of red cells
Moderate anaemia has not proved to increased mortality
Red cell transfusions impair microcirculatory flow
Progressive loss of red cell functionality during storage
Pathologic supply dependency is rare
Risk of pathogen transmission
Immunodepression causing increased infections and tumor relapse following transfusion
Risk of TRALI and TACO
Blood products are increasingly scarce and expensive

TRALI: Transfusion-related acute lung injury; TACO: Transfusion associated circulatory overload.

4.01, respectively^[31]. However, ABT may be life-saving in extremely ill patients with cardiovascular disease^[11]. Theoretical reasons for using a "liberal" or "restrictive" transfusion protocol are given in Table 2.

On reviewing the literature, it can not be concluded that ABT is beneficial for less sick patients and those without cardiovascular disease when Hb is above 7 g/dL^[11,32]. On the other hand, the effects of anaemia below 7 g/dL and the subsequent beneficial effects of ABT, if any, can also not be determined from published studies since patients either refuse to receive ABT for religious reasons or are transfused systematically, except in the case of chronically anaemic patients like those with thalassemia or drepanocytosis^[10].

As for patients with cardiovascular diseases, there appears to be some evidence that ABT, in small amounts, can reverse ischaemic changes and restore normal myocardial function. However, the randomized controlled trials conducted so far have offered contradictory results regarding the safety of restrictive transfusion triggers in older patients. Whilst four of them reported that a restrictive transfusion protocol resulted in the transfusion of appreciably fewer units of RBC, with no differences between groups regarding postoperative morbidity or mortality^[11,32-34], the last one which was designed to find differences in postoperative quality of life, found that in elderly patients undergoing surgery for hip fracture repair a restrictive transfusion threshold (Hb < 8 g/dL) may result in a higher incidence of postoperative cardiovascular complications (10% *vs* 2%, *P* = 0.05) and 30 d mortality (8% *vs* 0%, *P* = 0.02) when compared with a liberal transfusion threshold (Hb < 10 g/dL)^[35]. The Transfusion Trigger Trial for Functional Outcome in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair^[36], which has been planned to be a 2600 patient, multicentre clinical trial, will most probably address the question of whether patients with cardiovascular disease

or cardiovascular risk factors undergoing surgical repair of hip fracture benefit from a higher or lower transfusion trigger^[15]. While data from randomized trials are available, patients with suspected or proven myocardial ischaemia must be closely monitored and ABT should be given unit-by-unit until cardiac function is normalized, rather than to adopt a fixed transfusion trigger for all such patients^[15].

Packed red cell transfusion to a predetermined Hb in view of optimizing oxygen transport is definitely not supported by a large trial in critically ill patients [Transfusion Requirements In Critical Care (TRICC) trial]^[11]. In fact, in the study by Hébert *et al*^[11], patients submitted to a restrictive transfusion strategy seem to have better outcomes than those submitted to a liberal transfusion strategy, especially those who are younger (< 55 years old) or less ill (APACHE score < 20). However, the analysis by Deans *et al*^[37] suggests that the results of the TRICC trial were strongly influenced by non-comparable subgroups with different practice misalignments in each arm of the study. The excess risk incurred by each of these subgroups makes the comparison of mortality rates between the two treatment arms in the overall study difficult to interpret. Furthermore, as publications before this trial indicated that clinicians used higher transfusion thresholds in patients with ischaemic heart disease compared with younger, healthier patients, neither arm fully represented or was compared with current practices, and it remains unclear if the use of an absolute transfusion threshold is superior to adjusting therapy based on individual patient characteristics or implementing consensus guidelines for transfusion practices^[37].

The rationale behind ABT is to restore oxygen delivery and provide a reserve should further blood loss occurs. After PRC transfusion, an increase in Hb levels is readily observed, but controversial results were found when evaluating the influence of stored PRCs on tissue oxygenation, assessed by surrogate markers of oxygenation, such as gastric pH and CO₂^[7,8]. Very recently, Leal-Noval *et al*^[6] and Smith *et al*^[38] studied the effect of ABT on cerebral oxygenation in patients with severe traumatic brain injury and found erythrocyte transfusion to be associated with a variable and prolonged increment of cerebral tissue oxygenation. However, no relationship was observed between brain tissue partial pressure of oxygen (PtiO₂), cerebral perfusion pressure and Hb concentration. In addition, 3 h after transfusion, all patients with basal PtiO₂ < 15 mmHg showed an increment in PtiO₂, *vs* 74.5% of patients with PtiO₂ ≥ 15 mmHg, *P* < 0.01^[6]. Moreover, in a subsequent study, this group demonstrated that an increment in PtiO₂ was only observed in patients receiving blood stored for less than 19 d^[39]. Thus, low baseline PtiO₂ levels could define those patients who benefit the most from ABT.

However, ABT providing Hb of 10-11 g/dL might be required for patients with COPD, as it may reduce minute ventilation and the work of breathing, for patients bleeding to improve haemostasis in massive transfusion^[25,26], and for surgical patients with sickle cell disease, reducing HbS levels by direct transfusion

or by RBC exchange (partial or total) to avoid sickling crisis^[10,14].

In conclusion, while ABTs save lives, they save far fewer lives than we have been taught to believe. As ABT will never be a risk free therapy (see below), each and every unit transfused unnecessarily is, potentially, a noxious unit. In other words, each ABT which is not strictly indicated is severely contraindicated!

RISKS OF ABT

Nowadays, due to a careful donor selection and the introduction of highly sophisticated tests for pathogen detection, ABT is safer but more scarce and expensive than ever, and is still not risk-free. Transfusion therapy with PRCs can cause adverse reactions, which are classified on the basis of their etiopathogenesis and the time of occurrence with respect to the transfusion. Adverse effects of ABT (Table 3) include a broad panoply including, but not limited to, incorrect blood transfusion (“wrong blood”), acute or delayed haemolytic transfusion reaction, allergic reaction (from urticaria to anaphylactic reaction), bacterial contamination, transfusion-related acute lung injury (TRALI), transfusion associated circulatory overload (TACO), transfusion-related immuno-modulation (TRIM), and transmission of infectious diseases (viruses, protozoas and prions)^[40], which result in increased risk of morbidity and mortality. As the risks of ABT-transmitted viruses were reduced to exceedingly low levels in the US and Europe, TRALI, haemolytic transfusion reactions (HTRs), and transfusion-associated sepsis (bacterial contamination) emerged as the leading causes of ABT-related deaths^[41]. Since 2004, preventive measures for TRALI and bacterial contamination have been implemented, but their implementation remains incomplete^[41]. Infectious causes of ABT-related deaths currently account for less than 15% of all transfusion-related mortality, but the possibility remains that a new transfusion-transmitted agent causing a fatal infectious disease may emerge in the future^[40,41]. These, together with the possible advantages of implementing restrictive transfusion protocols, strongly indicate that transfusion practice in surgery and intensive care should be (and must be) modified, especially in terms of the level of pretransfusion Hb (“restrictive” *vs* “liberal” use of ABT; “acceptable Hb” *vs* “optimal Hb”) (Table 2). Briefly, we will describe several of the most important adverse reaction to transfusion.

Transfusion reaction: According to Serious Hazards of Transfusion report 1994-2004, 1832 out of 2628 reported incidents corresponded to episodes of incorrect blood component transfused (IBCT) (wrong blood product or wrong patient)^[42]. Thus, the incidence of reported IBCT is about 1:20 000-25 000 and the consequences can be disastrous. In Spain^[43], 246 “near-miss administration errors” and 134 of IBCT were reported during 2007. Of those, 49 led to HTRs (33 due to ABO incompatibility), with at least three deaths reported and confirmed^[4]. In a Spanish region (Catalunya) the incidences were estimated as 1:11 000 of IBCT, 1:77 000 of “ABO-mistakes” and 1:310 950 of death secondary to transfusion^[44]. As for

Table 3 Risks or hazards of allogeneic blood transfusion

Acute transfusion reactions
Immunologic reactions
Acute haemolytic reaction (or THRs)
Febrile non-haemolytic reaction
Allergic reactions: Urticaria and anaphylaxis
Acute non-cardiogenic pulmonary edema: TRALI
Alloimmunization with acute platelet destruction
Non-immunologic reactions
Bacterial contamination
TACO
Hypotensive reaction
Non-immunologic haemolysis
Others: Hypocalcemia, hyperkalemia (cardiac arrest), hypothermia, hyperglycemia, <i>etc</i>
Delayed transfusion reactions
Immunologic reactions
Delayed haemolytic reaction
Alloimmunization against blood cell antigens (also platelets and leukocytes)
Graft <i>vs</i> host disease
Transfusion-related immunomodulation
Post-transfusion purpura
Non-immunologic reaction
Transfusion-transmitted infection: viruses (Hepatitis A, B, C, E, VIH 1-2, West Nile virus, HTLV I - II, Citomegalovirus, Virus Herpes viridae, TTV, SEN-1, SARS, <i>etc</i>), protozoa (malaria, babe biosis, Chagas disease, <i>etc</i>), prion (new variant Creutzfeldt Jacob disease)
Post-transfusion hemosiderosis (iron overload)

TRALI: Transfusion-related acute lung injury; TACO: Transfusion associated circulatory overload.

the surgical patient, general anaesthesia may mask the symptoms of both HTRs and non-HTRs. Signs of HTRs include hypotension, tachycardia, haemoglobinuria and microvascular bleeding, but these may be erroneously attributed to other causes in the anaesthetized patient. The most common signs of a non-haemolytic transfusion reaction in awake patients include fever, chills, or urticaria. However, these signs may not be detectable during anaesthesia. Thus, checking for signs and symptoms of a THR should periodically be done in the anaesthetized patient, including urine output and colour and peak airway pressure^[16].

Bacterial contamination: Bacterial contamination of blood products, most frequently platelets, is one of the leading causes of death from ABT^[40,41] (seven deaths in UK from 1996 to 2004)^[42]. The increased risk of bacterial overgrowth is related to a storage temperature of > 20-24°C. Many blood banks are now culturing their platelet concentrates, or inactivating them, although this leads to a decreased functionality. If a patient develops a fever within 6 h of receiving platelets, sepsis from contaminated platelets may be a possibility. Between 2001 and 2003, an average of 11.7 years in the United States were reported to the Food and Drug Administration, whereas 7.5 per year were reported in 2004 and 2005 - a decrease attributable in part to the mandating of bacterial screening of platelets beginning in 2004^[40]. In Spain, during 2007 at least 17 cases, with one death, have been reported and confirmed^[43].

TRALI: TRALI is a well-characterized and serious adverse consequence of blood product transfusion; its overall occurrence is almost certainly more common than the quoted estimate of one case in 4000 U of blood transfused (as TRALI is generally unrecognized or misdiagnosed, its actual incidence is unknown)^[45]. TRALI is non-cardiogenic pulmonary edema resulting from immune reactivity of certain leukocyte antibodies a few hours after transfusion. Signs and symptoms will appear 1-2 h after transfusion and are in maximum force within 6 h. Hypoxia, fever, dyspnea, and even fluid in the endotracheal tube may occur. There is no specific therapy other than stopping transfusion and instituting critical care supportive measures. Most patients recover in 96 h, although TRALI is one of the top three most common causes of transfusion related deaths^[45]. In Spain, 32 cases have been reported and confirmed during 2007, with at least two deaths^[43]. The estimated rate was 1/32 000 (at Catalunya), but the authors suspected a great underreporting^[44]. For example, during preparation of this manuscript one of the authors diagnosed one highly probable case of TRALI during a plasma exchange, but no physician at the intensive care unit knew what TRALI meant.

TACO: TACO is a cause of hydrostatic pulmonary oedema with clinical and radiologic manifestations similar to those of TRALI. In fact, the distinction between TRALI and TACO after transfusion is difficult, in part because the two conditions may coexist^[44,46]. During 2007, in Spain at least 39 cases had been reported and confirmed, with at least one death confirmed^[43].

TRIM: TRIM is associated with increased risk of postoperative infection^[2,3,47-49]. The combined data from three studies including over 1700 patients undergoing elective cardiac surgery with cardiopulmonary bypass showed that (1) transfusion of RBC concentrates was independently associated with increased rates of postoperative pneumonia, mediastinitis and sepsis; (2) these effects were dose-dependent and storage-time-dependent; and (3) other blood components might be also involved^[50-52]. As for patients undergoing elective surgery for gastrointestinal cancer resection or urgent surgery for hip fracture repair, similar data have been reported^[4,49,53].

Infectious diseases: Another major adverse effect of transfusion therapy is the transmission of infectious agents^[40,41]. For the past 20 years, transfusion induced hepatitis and acquired immunodeficiency syndrome (AIDS) have been dominant concerns regarding ABT. These infectious risks are now very rare. One of the major reasons for the decrease in blood borne infections has been the use of nucleic acid technology (NAT). The human immunodeficiency virus (HIV), C hepatitis virus (CHV), and West Nile virus can now be detected by this technology. To date, malaria, Chagas disease, severe acute respiratory syndrome (SARS), and variant Creutzfeldt-Jakob disease (vCJD) cannot be detected^[16]. The possible safety intervention that might further

reduce the risk of transfusion-transmitted infection is not static, as new agents continue to emerge, old ones change their properties and epidemiologic patterns, and new information and technology become available to change our understanding of that risk^[40,41]. During 2007 in Spain, twenty five cases of possible hepatitis B or C were reported, but none of them were confirmed^[43]. Between 2005-2007 five cases of Chagas disease were diagnosed, although four of them retrospectively after a “look back”^[43].

PREOPERATIVE AUTOLOGOUS BLOOD DONATION (PABD)

PABD consists of obtaining the patient's own blood prior to surgery in order to administer it if necessary afterwards^[1,54]. Extraction frequency, type of blood component or bag for conservation, and volume of blood extracted are established by each Local Blood Bank on an individual basis^[1,54-56]. In the elective surgical setting, PABD is a convenient, predictable, safe and widely practised form of transfusion support^[4]. However, PABD cannot be made available to all patients (minimal Hb ≥ 11 g/dL), and it is contraindicated in the presence of several infectious, cardiac, oncologic or neurologic pathologies. Hospital admission and operative dates must be guaranteed, as donated blood has a limited storage life of 35 d (up to 42 d in additive SAG-mannitol solution). In addition, it used to carry some of the risks of ABT, especially IBCT, although in the European Union there are regulations aimed to reduce these risks (e.g. personalized unit identification, separate conservation and transport, de-referral for allogeneic use, *etc*), and these can often present logistical difficulties^[1,54,55]. Nonetheless, PABD avoids immunological and viral hazards of ABT. In spite these benefits, PABD use is decreasing worldwide^[56].

In accordance with the conclusion of the Spanish Consensus Statement on Alternatives to Allogeneic Blood Transfusion (Seville's Document)^[55]: PABD would be indicated in elective surgery if the risk of ABT > 20%-30%, and in patients with difficulties receiving ABT; PABD can be used safely in children and elderly populations^[54,57]; the administration of rHuEPO in patients with moderate anaemia, facilitates PABD^[57]; PABD contribution to ABT reduction is decreased when a transfusion protocol is adopted; PABD may have problems of over-collection and over-transfusion (in fact, PABD increases the total number of transfusion episodes). It is not without infectious risks; PABD erythrocytes undergo “storage lesion”. PABD has been classically associated with higher rates of “clerical errors”, and ABT may still be required (break-through transfusion).

In this regard, it is well known that surgery in IBD is frequently associated with a need for perioperative blood transfusions, but PABD is often limited by IBD-associated anaemia, although it is reversible by intravenous iron and rHuEPO. Consequently there is a paucity of studies on the use of PABD in surgical IBD patients

(and none in the use of perioperative cell salvage or acute normovolemic haemodilution). Mittermaier *et al*^[58] tested the feasibility of PABD (2-4 U; 350-450 mL blood per unit; 1 U/wk) in six patients (five CD, one UC) with indications for elective bowel resection IBD. Patients received 200 mg of iron sucrose IV after each donation, plus concomitant rHuEPO if there was preexisting anaemia or C-reactive protein > 2 mg/dL. Four patients received PABD transfusions intra- or postoperatively, and no patient needed ABT. No serious adverse events were observed during blood donations, perioperatively, and during the one year follow-up period. Thus, when appropriately indicated and supplemented with IV iron and rHuEPO, PABD seems to be safe and useful for surgical IBD patients. Since 2003, we have treated 2668 PABD patients in our hospital, only three of them with IBD (two CU and one EC) scheduled to prostatectomy, coxarthrosis and avascular femoral necrosis surgeries, donating two units each patient. The first patient required two doses of β -epoetin 30 000 IU plus iron sucrose iv 400 mg, and the second was transfused with two PABD units plus two ABT units.

Recently, PABD has been used, not as an alternative to ABT, but as a carrier of drugs, owing to the ability of the RBC membrane to be opened and resealed under appropriate conditions. In an uncontrolled pilot study, Annese *et al*^[59] investigated efficacy and safety of dexamethasone-encapsulated erythrocytes in 10 steroid-dependent adults IBD patients (five UC, five CD). Fifty milliliters of blood were drawn from each subject; dexamethasone 21-phosphate (Dex 21-P) was encapsulated into erythrocytes by means of specially designed equipment, and drug-loaded erythrocytes were infused into the original donors (5.5 ± 2.4 mg Dex 21-P). The procedure was repeated after 4 and 8 wk, and patients were instructed to withdraw corticosteroids. After the third infusion, all patients were in clinical remission. After a mean follow-up of 12 ± 3 mo, six patients relapsed, and the remaining four patients remained in remission. Pre-existing steroid-related adverse effects disappeared during the follow-up. The authors concluded that Dex 21-P loaded autologous RBC is a feasible technique, which is safe, maintains patients in clinical remission and allows steroid withdrawal.

More recently, infusions of autologous RBCs loaded with Dex 21-P, performed every 4 wk for 24 mo, to 18 consecutive paediatric patients with steroid-dependent CD resulted in a reduction of the CD activity index, 78% of patients discontinued steroids and endoscopic findings showed remission in 44% of patients, whereas none of the patients experienced serious side effects^[60]. Therefore, infusions of autologous RBCs loaded with Dex 21-P seem to be safe and useful for maintaining long-term remission in paediatric patients with moderately active CD. However, large randomized controlled trials are needed to confirm these promising results.

CONCLUSION

ABT used to be the first therapeutic option for the treatment of acute anaemia in IBD patients, especially

when it developed due to gastrointestinal or perioperative blood loss, but was not risk-free. Adverse effects of ABT include, but are not limited to, IBCT (wrong blood), THRs, bacterial contamination, TRALI, TACO, TRIM and transmission of infectious diseases, which might result in increased risk of morbidity and mortality. Evidence-based clinical guidelines may promote best transfusion practices and reduce variability, minimizing the avoidable risks of transfusion, and help clinicians in choosing the most appropriate treatment for the patient-ABT, PABD or pharmacologic alternatives. The acceptance of normovolemic anaemia is indeed one of the most effective measures to reduce ABT requirements. Thus, the adoption of restrictive transfusion criteria (lower threshold level of Hb) must be the first measure to implement in order to reduce both the number of transfused units and transfused patients. Obviously, the second measure must be the pharmacological treatment of anaemia. PABD consistently reduces the frequency of ABT, with a small incidence of adverse effects, although its contribution to ABT avoidance is reduced when performed under a transfusion protocol. In addition, interpretation of utility of PABD in surgical IBD patients is hampered by scarcity of published data. However, the role of autologous RBCs as drug carriers is promising. Finally, for selected patients, a combination of methods used within well-constructed protocols will offer better prospects for blood conservation in IBD patients undergoing elective surgery.

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Clonality and allelotype analyses of focal nodular hyperplasia compared with hepatocellular adenoma and carcinoma

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Supported by The National Natural Science Foundation of China (NSFC), Grants 30171052, 30572125 and 30772508 and the CAMS Cancer Hospital Clinical Research Project LC2007A21

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Received: July 7, 2009 Revised: August 17, 2009

Accepted: August 24, 2009

Published online: October 7, 2009

RESULTS: Nodules of altered hepatocytes (NAH), the putative precursors of HCA and HCC, were found in all the FNH lesions. Polyclonality was revealed in 10 FNH lesions from female patients, and LOH was not detected in any of the six FNH lesions examined, the results apparently showing their polyclonal nature. In contrast, monoclonality was demonstrated in all the eight HCAs and in four of the HCCs from females, and allelic imbalances were found in the HCAs (9/9) and HCCs (15/18), with chromosomal arms 11p, 13q and 17p affected in the former, and 6q, 8p, 11p, 16q and 17p affected in the latter lesions in high frequencies ($\geq 30\%$). Monoclonality was revealed in 21 (40%) of the 52 microdissected NAH, but was not found in any of the five ordinary nodules. LOH was found in all of the 13 NAH tested, being highly frequent at six loci on 8p, 11p, 13q and 17p.

CONCLUSION: FNH, as a whole, is polyclonal, but some of the NAH lesions derived from it are already neoplastic and harbor similar allelic imbalances as HCAs.

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Key words: Clonality analysis; Focal nodular hyperplasia; Hepatocellular adenoma; Liver tumorigenesis; Loss of heterozygosity; Nodules of altered hepatocytes

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Abstract

AIM: To identify clonality and genetic alterations in focal nodular hyperplasia (FNH) and the nodules derived from it.

METHODS: Twelve FNH lesions were examined. Twelve hepatocellular adenomas (HCAs) and 22 hepatocellular carcinomas (HCCs) were used as references. Nodules of different types were identified and isolated from FNH by microdissection. An X-chromosome inactivation assay was employed to describe their clonality status. Loss of heterozygosity (LOH) was detected, using 57 markers, for genetic alterations.

INTRODUCTION

Focal nodular hyperplasia (FNH) is defined as a lesion composed of hyperplastic hepatic parenchyma, subdivided into nodules by fibrous septa that may form stellate scars^[1,2]. As dystrophic vessels are observed in

the fibrous septa and an arteriole is present within most of the nodules, it is considered parenchyma overgrowth responsive to increased blood flow secondary to vascular malformations^[3]. Morphologically, FNH is classified into two categories, classical and nonclassical lesions^[4]. The latter includes telangiectatic, mixed hyperplastic/adenomatous types and lesions with cytologic atypia. The non-neoplastic nature appears to be supported by data from X-chromosome inactivation (XCI) analyses, which demonstrated polyclonality in all FNH lesions in most of the series^[5,6]. However, monoclonality was also observed in some of the lesions^[7], including telangiectatic FNH^[8,9]. FNH, as a descriptive term when proposed^[10], may represent a group of focal lesions with different morphologic phenotypes and genetic alterations, and may develop in different pathways. Chromosomal gains and losses have been observed in several lesions by comparative genomic hybridization, allelotyping or karyotyping^[9,11], but the molecular mechanism of FNH development remains unclear.

Hepatocellular adenoma (HCA) is a benign hepatic neoplasm, and its development is associated with the long-term use of steroids, mainly oral contraceptives^[2,12]. While HCA was proven to develop from foci of altered hepatocytes (FAH) in the liver of rodents^[13], the neoplastic features of human HCA have been confirmed by clonality analyses^[5,6,9]. Some HCA lesions tend to progress to hepatocellular carcinoma (HCC)^[2,13], and the malignant transformation has been linked to β -catenin activation^[14]. In contrast to HCA, FNH is a stable lesion in clinical phenotypes with a very low risk of malignant transformation, although FAH can be detected in FNH^[15] and HCC was identified in the liver harboring FNH as described in several case reports^[16-18].

The majority of FNH lesions are distinguishable from HCA by their morphologic features. However, the differential diagnosis is difficult for some lesions. A central scar is regarded as the most characteristic change, but it is detectable in less than half of FNH lesions. Even for classical FNH, its incidence was shown to be 62% (153/245)^[4,19]. More reliable procedures remain to be established for its distinction from HCA.

In the present study, we examined the clonality status of FNH, and compared its clonality with that of HCAs and HCCs, using assays based on XCI and polymorphism at the androgen receptor (AR) and phosphoglycerate kinase (PGK) loci. Secondly, these lesions were examined for loss of heterozygosity (LOH). In addition, 57 nodules were microdissected from FNH in female patients and used for the clonality analysis. Twenty-five nodules were also isolated from an FNH lesion and were used for LOH analysis.

MATERIALS AND METHODS

Tissue samples and histological examination

A total of 46 hepatic lesions were used, including 12 FNH lesions from 10 patients (Table 1), 12 HCAs from 11 patients and 22 well-differentiated HCCs (Table 2).

These patients were admitted to the Cancer Hospital, Chinese Academy of Medical Sciences in Beijing and Tangdu Hospital, the Fourth Military Medical University in Xi'an during the period from 1998 to 2007. All the lesions were resected by an operation. Representative formalin-fixed, paraffin-embedded tissue samples were retrieved from the archives for both tumors and the surrounding liver parenchyma. Sections of 4 μ m in thickness were prepared and stained by hematoxylin and eosin (HE).

All slides were reexamined independently by three pathologists (Su Q, Cai YR and Gong L), and their histologic features were reevaluated. FNH of the classical form was considered when the lesions showed characteristic features as proposed by other authors^[3,4,10]. All the HCA lesions were identified from livers without evidence of cirrhosis or diffuse fibrosis by their macroscopical and histological features using well-established criteria^[2]. FAH and nodules of altered hepatocytes (NAH) were identified on HE-stained sections^[20], and the lesions were further highlighted by a marked reduction or even absence of CK18-immunoreactivity in glycogenotic clear cells as described previously^[21]. Dysplasia, also designated as "small-cell change (SCC)"^[20,22] and "small-cell dysplasia"^[23], was recognized and graded into low-grade and high-grade lesions. The histological grades of HCCs were assessed as described by Hamilton and Aaltonen^[1], with the well-differentiated HCCs corresponding to all of the grade I and some of the grade II lesions by the criteria of Edmondson and Steiner^[24].

Immunohistochemical staining was performed using a streptavidin-labeled peroxidase (S-P) kit as in our previous study^[20]. The primary antibodies used in this study included those against cytokeratin (CK) 18, CK19, CD34, hepatitis B virus (HBV) surface antigen (HBsAg; Clone 3E7), p53 protein (DO-7) and Ki-67 antigen. All reagents were purchased from Dako (Glostrup, Denmark). Levels of Ki-67 antigen expression were expressed as Ki-67-labeling indices (Ki-67-LI). The nuclear accumulation of p53 protein was evaluated as many (3+, > 30%), moderate (2+, 5%-30%), few (+, < 5%) and absent (-, 0%), as described previously^[25].

Extraction of genomic DNA

Sections of 8 μ m in thickness were prepared. After staining with HE, the lesions of FNH, HCA and HCC were identified by microscopic examination. Then the lesional tissues, each covering an area of at least 1 cm \times 1 cm, were collected by a rubber policeman, and pooled into 1.5-mL tubes. The surrounding liver parenchyma and fibrous tissue were collected and used as controls. Previous XCI tests have demonstrated that monoclonality can be determined provided a given cell population contains at least 75% monoclonal cells^[26]. To guarantee reliability of the following assays, mesenchymal tissue areas containing inflammatory cell clusters were eliminated to enhance purity of target cells to at least 80%. Genomic DNA was then isolated with

Table 1 Clinicopathological features of 12 FNH lesions and data of immunohistochemistry, clonality and LOH assays

Case numbers	Age (yr)/gender	Lesion codes	Lesion sizes (cm)	Ki-67-LI (%)	p53-LI (%)	Clonality by XCIA	No. of LOH
01	40/F	01 ¹	5.0	1.6	0	PC ²	0
02	30/F	02	2.5	0.5	0	PC ²	0
		03	7.0	0.8	0	PC ²	0
03	22/M	04 ³	5.2	0.7	0	NT	0
04	44/M	05 ¹	1.2	0.5	0	NT	0
05	31/F	06 ¹	4.7	0.3	0	PC ²	0
06	43/F	07	3.2	1.0	0	PC ²	NT
07	44/F	08 ¹	2.0	0.8	0	PC ⁴	NT
08	53/F	09 ⁵	4.8	1.0	0	PC ²	NT
09	23/F	10 ^{1,5}	4.5	1.2	0	PC ⁴	NT
10	46/F	11 ^{1,5}	5.3	0.8	0	PC ³	NT
		12 ¹	1.5	0.9	0	PC ⁴	NT

¹Some small preneoplastic foci, composed of clear cells, identified in surrounding liver parenchyma; ²Tested on phosphoglycerate kinase (PGK) locus;

³Nodules of altered hepatocytes (NAH) isolated from FNH by microdissection for LOH detection; ⁴Noninformative at PGK locus and the data obtained by the assay on androgen receptor (AR) locus; ⁵NAH isolated from FNH by microdissection for clonality assessment by XCIA. FNH: Focal nodular hyperplasia; Ki-67-LI: Ki-67 antigen-labeling indices; p53-LI: p53 protein-labeling indices by DO-7; XCIA: X-chromosomal inactivation assay; LOH: Loss of heterozygosity; F: Female; M: male; PC: Polyclonality; NT: Not tested for its origin from a male patient.

Table 2 Clinicopathological features of 12 HCAs and 22 HCCs, with data of clonality and LOH assays

Case numbers	Age (yr)/gender	Lesion codes	Lesion sizes (cm)	Lesion types	Ki-67-LI (%)	p53-LI (%)	Clonality by XCIA	No. of LOH	Chromosomal arms affected by LOH
11	36/F	HCA01	3.5	HCA	1.3	0	MC ¹	3	8p, 11p, 16q
12	57/F	HCA02	3.0	HCA ²	1.3	0	MC ¹	1	17p
13	28/M	HCA03	7.3	HCA, SCC ²	2.5	0	NT	4	11p, 13q, 17p
14	52/M	HCA04	8.5	HCA	1.1	0	NT	3	11p, 17p
15	31/F	HCA05	7.0	HCA	3.1	0	MC ¹	4	11p, 13q
16	29/F	HCA06	1.0	HCA ²	2.0	0	MC ¹	NT	NT
		HCA07	12.0	HCA ²	1.8	0	MC ¹	5	1p, 13q, 17p
17	30/F	HCA08	1.0	HCA, SCC ²	1.5	0	MC ³	1	13q
18	29/F	HCA09	5.0	HCA	0.9	0	MC ¹	4	6q, 11p, 13q, 17p
19	37/F	HCA10	5.0	HCA	1.3	0	MC ³	2	13q, 17p
20	33/F	HCA11	2.0	HCA ²	2.3	0	MC ¹	NT	NT
21	40/F	HCA12	1.5	HCA ²	2.0	0	MC ³	NT	NT
22	31/F	HCC01	2.2	HCC, G1	2.0	10	NT	11	8p, 11p, 13q, 16q, 17p
23	46/M	HCC02	4.1	HCC, G1	65.0	0	NT	7	6q, 13q, 16q, 17p
24	42/M	HCC03	3.0	HCC, G1	2.5	0	NT	10	6q, 8p, 13q, 17p
25	51/M	HCC04	5.3	HCC, G1	8.2	80	NT	5	8p, 11p, 13q, 16q, 17p
26	49/M	HCC05	2.0	HCC, G1	2.0	0	NT	2	1p, 11p
27	27/M	HCC06	2.5	HCC, G1	1.2	0	NT	2	8p, 11p
28	56/M	HCC07	3.3	HCC, G1	1.5	0	NT	0	None
29	38/F	HCC08	8.3	HCC, G1	18.0	0	MC ¹	NT	NT
30	64/F	HCC09	3.6	HCC, G1	5.5	0	MC ³	NT	NT
31	53/F	HCC10	9.5	HCC, G2	8.5	0	NT	3	8p
32	33/M	HCC11	8.0	HCC, G2	5.2	0	NT	5	6q, 11p, 17p
33	48/F	HCC12	5.2	HCC, G2	35.0	0	NT	4	6q, 8p, 16q
34	44/F	HCC13	4.2	HCC, G2	65.0	80	NT	12	8p, 11p, 13q, 16q, 17p
35	29/F	HCC14	3.6	HCC, G2	0.9	0	NT	2	1p, 11p
36	61/F	HCC15	4.5	HCC, G2	3.7	0	NT	6	6q, 13q, 16q, 17p
37	48/M	HCC16	5.0	HCC, G2	1.4	0	NT	3	6q, 11p, 17p
38	39/M	HCC17	4.0	HCC, G2	1.5	0	NT	0	None
39	39/M	HCC18	5.0	HCC, G2	1.2	0	NT	0	None
40	50/M	HCC19	2.0	HCC, G2	2.0	0	NT	8	8p, 11p, 13q, 17p
41	51/M	HCC20	7.4	HCC, G2	12.2	0	NT	1	11p
42	55/F	HCC21	2.5	HCC, G2	28.5	0	MC ³	NT	NT
43	77/F	HCC22	5.6	HCC, G2	16.2	0	MC ³	NT	NT

¹Noninformative at PGK locus and the data obtained by the assay on AR locus; ²Some small preneoplastic foci, composed of clear cells, identified in surrounding liver parenchyma; ³Tested on PGK locus. HCA: Hepatocellular adenoma; HCC: Hepatocellular carcinoma; MC: Monoclonality; NT: Not tested for its origin from a male patient (for HCA) or its evidently malignant phenotypes; SCC: Small-cell change.

an extraction kit (QIAGEN, Chatsworth, CA, USA) following the manufacturer's instructions. The DNA samples of 50 µL each were stored at -20°C until use.

Microdissection of preneoplastic nodules from FNH

Selected FNH specimens with well demarcated NAH were subjected to microdissection as described previously^[27].

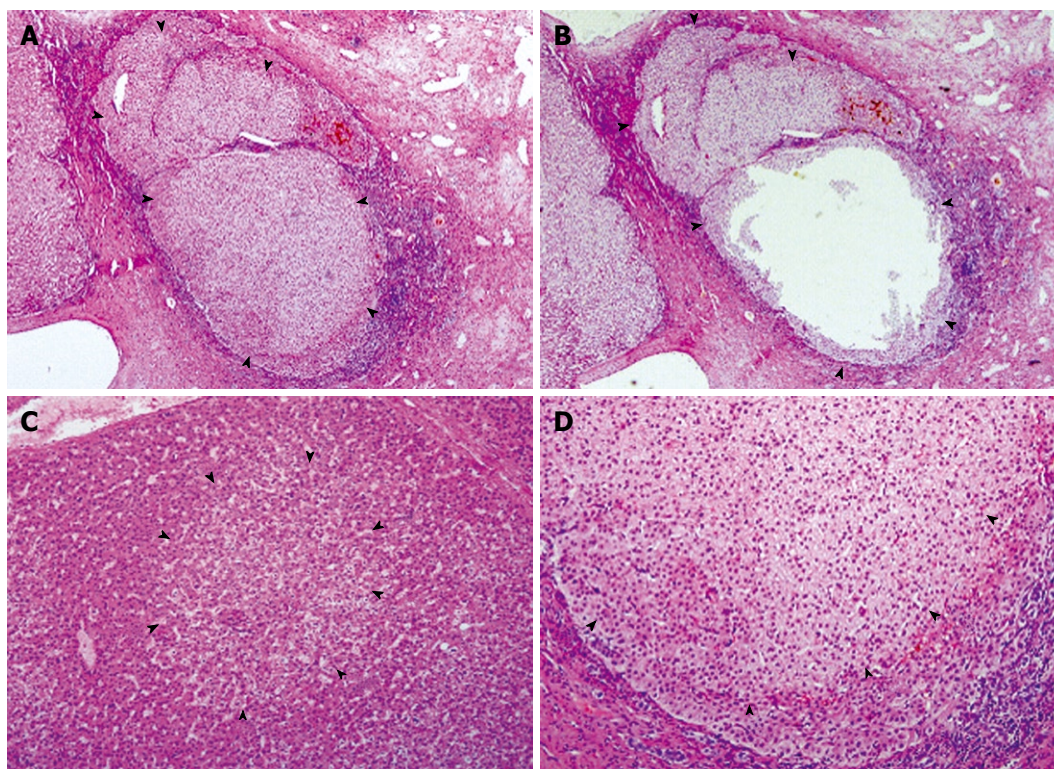


Figure 1 Premeoplastic lesions in classical FNH. A and B: FNH04 from Case 03, showing a nodule largely occupied by an expanding NAH (arrowheads), before (A) and after microdissection (B); C: Portion of FNH06, showing an FAH (arrowheads), in which the altered hepatocytes integrate well with the surrounding hepatic plate; D: Portion of an NAH composed mainly of clear hepatocytes (arrowheads), showing compression to surrounding liver parenchyma. HE, A and B, $\times 40$; C and D, $\times 100$.

Ten consecutive sections were deparaffinized with xylene, rehydrated by rinsing through graded alcohol, stained with HE and immersed in glycerol. Larger nodules, 2.5-5.0 mm in diameter, from these sections were identified and correlated. Target tissues were outlined and collected with a clean $4\frac{1}{2}$ needle under a microscope (Figure 1A and B). The tissues of the same nodules from the consecutive sections were pooled together and stored in 1.5-mL tubes. Using the same procedure, tissues of the same size were also collected from the surrounding liver parenchyma and were used as controls. Genomic DNA was isolated as described above.

Clonality assays based on XCI mosaicism in somatic tissues in female patients

The principles of XCI assays have been described previously^[28]. Briefly, there are two X chromosomes within each somatic cell in females, one from the father and the other from the mother. Both PGK and AR genes are located on X chromosomes, showing readily demonstrable polymorphism in different frequencies. The former carries a *Bst*XI restriction-polymorphic site, reflecting the G/A single-nucleotide polymorphism at exon 1, while the latter is polymorphic at the CAG short tandem repeat (STR). These allelic differences and the XCI mosaicism enable us to identify monoclonal, neoplastic lesions from normal or regenerating tissues composed of polyclonal cell populations^[28,29].

The DNA templates extracted from tumors and the non-neoplastic tissues were incubated with 5 U of *Hpa*II (Promega, Madison, WI, USA) at 37°C for 12 h in a volume

of 20 μ L containing 0.2 μ L of 10 mg/mL bovine serum albumin and 2 μ L of 10 \times reaction buffer, and heated at 95°C for 5 min to inactivate the enzyme. The digested DNA samples, 5 μ L each, were then subjected to nested PCR for amplification of exon 1 of PGK and AR genes, as described previously^[28]. The PGK products, 10 μ L each, were incubated with 5 U of *Bst*XI (Promega) at 47°C for 8-10 h. The digested products were then resolved in 2% (g/mL) agarose gel containing ethidium bromide (0.2 μ g/mL), and visualized under ultraviolet light. The fluorescence intensities of the products of these two alleles, at the positions of 530 and 433 bp separately, were assessed using LabWork 3.0 (UVP). Efficacy of the reaction was demonstrated by including a sample from a male patient with FNH (Case 03) who had been demonstrated to harbor a *Bst*XI restriction site.

The amplification products of AR exon 1, 3 μ L each, were mixed with the same volume of sample buffer (99% formamide, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol), resolved on 10% polyacrylamide gel containing urea (8.0 mol/L), and visualized by silver staining. Sizes of the products were determined using 100-bp and 10-bp DNA ladders (Gibco BRL).

LOH assays by conventional and multiplex PCR

A total of 57 loci, whose allelic loss has been frequently observed in HCC samples from various regions^[30-38], were chosen for the LOH assay. Amplification of the DNA samples from the whole FNH lesions, HCAs and HCCs was performed by conventional PCR. Sequences of the primer pairs (Table 3) were obtained from Genbank

Table 3 Sequences of primers for nested PCR-based LOH assays

Markers (locations)	External primer pairs ¹	Internal primer pairs ¹
D1S199 (1p36.13)	CCTGGGCAACATAGCAG TGACATCTTCTCCACCTC	GGTGACAGAGTGAGACCCTG CAAAGACCATGTGCTCCGTA
D1S2843 (1p36.12)	AGAAGGTGGGCACAAA TTCATCACCCACCAAAA	GGGCTGGGCATTACACAAC ATCAAATGGCTTCTCACC
D1S513 (1p35.2)	AGCTGAGACCCTGCCTTG AATGCCTGCTGTATGAAA	AGCTGAGACCCTGCCTTG AGCCTTCAGAGCCTGCC
D4S406 (4q31.21)	TCCCAGTTTACAACCACA GAAGGTGGCTAACTAAA	CTGGTTTAAAGGCATGTTTG TCCTCAGGGAGGTCTAAT
D4S426 (4q35.2)	CAAGTAATACAATATGGTAA ATACACTGCATCCATATATACAAGG	ATACACTGCATCCATATATACAAGG ACATTGTGAAATGACCACAG
D6S437 (6q25.3)	TTACTGACTTCTTGAAAGGGTG ACACGCCATCACAGGAAC	TGTCTGTTGGAGGCA GGTACAGTGTGTGACCCTAAGA
D6S305 (6q26)	AAAGCTGAGAAGCCATTCA GCAAATGGAGCATGTCACT	CACCAGCGTTAGAGACTGC GCAAATGGAGCATGTCACT
D6S1008 (6q27)	GCTACTTCAGCAGGGTT AATGACCACGAGTTCCTC	AAGAAAGACTAGAGAGACAGACAGC ATCATTGCCCATTACCAA
D6S297 (6q27)	CAAGAAGTGTCTCTAAAGATAAGG GCAAGATGTAGAAGGAGA	CAAGAAGTGTCTCTAAAGATAAGG CAACCAACCACGGTATATG
D8S277 (8p23.1)	GTTCCCTATGGCTAAAG AGTTTGCCTCCAGAAA	CCAGGTGAGTTTATCAATTCCTGAG TGAGAGGTCTGAGTGACATCCG
D8S1754 (8p22)	TTTGGGTAAGATACTTTAAGCG AAATAGTCGGTTCGGGAG	CAGGGAAGTCTCGGTTTG TCAGGGACACGATTACAGC
D8S1827 (8p22)	AGAAATAGCCAAAGAAGC CTGGGAAGGTCAGCAGATTG	GACAGAATCATGTGGCCTTT TTTTGTAAAATGTAAAATTGGCTTT
D8S261 (8p22)	GAGGCTACTAACCAACGTG TATTACAGTGGTATGGC	TGCCACTGCTTGAAAATCC TATGGCCAGCAATGTGTAT
D8S258 (8p21.3)	ACAATAATGATGAGGCCAGAA CAGCCAATACGAGGAGC	CTGCCAGGAATCAACTGAG TTGACAGGGACCCACG
D8S298 (8p21.3)	CTAGTTATTATGCAGAGGTGACATC GAGGCTGGTTAGCATCACG	AGGCTTACACCCATGGACC ACGCAGCACACAACATCAT
D8S1771 (8p21.2)	GGGGTGGGTGATGATATT TTGATTCTTTGCTGATGC	TTTACAAGAACCACCTGCC GATATAAAACATGACTTTGCTACCC
D8S1772 (8q22.1)	TTGATGCTCCTCTGTTCG AAGTCCTCCCTTTGCTGA	GATCTGAGCCTTCTACTGTC TGCCTTTTGGTGAATGG
D8S522 (8q24.12)	TATAGCAAATATGTTACAG CCATCTTGGCTCCTC	GAAAACAAAACAGGCTCC TGACCAAAACAACCTAGACAC
D11S1301 (11p13)	GCCACAGCACTCCAG GACAACCTCCCTCACC	GGCAACAGAGTGAGACTCA GTGTCTTTATGTGTAGTTC
D11S2008 (11p13)	ATTGAGGAGCACAGAACAA GCATCCATACGAAAAGTC	CATCCATCTCATCCCATCAT TTCACCCTACTGCCAACTTC
D11S907 (11p13)	GCTTATTGTCCATACCCAAA GAAAAGCAAAGGCATCGT	GCTTATTGTCCATACCCAAA AAAGNACCTTAATTTACGGC
D13S164 (13q12.3)	GGCGATCCACCCACCTT TGCCACGCACCTGTAGTCC	GCTGTGATTGCACCACC ATTACAGGCGTGACACACC
D13S289 (13q12.3)	AGGGCGTCACCGTGT GACTGAGGCAGGAGGATTG	CTGGTTGAGCGGCATT TGCAGCCTGGATGACA
D13S260 (13q12.3)	AATGGATCTGCTTC ATCACTCCAAATGTAAAT	AGATATTGCTCTCGTTCCATGA CCCAGATATAAGGACCTGGCTA
D13S171 (13q12.3)	CAGATACAGACATTTTGAA GCTCTACAGCATTGACCT	CCTACCATTGACACTCTCAG TAGGGCCATCCATTCT
D13S267 (13q13.1)	AGCTAATGGCCTGAAAGG AGAGGTCAAAGAGGAAGA	GGCCTGAAAGGTATCCTC TCCCACCATAAGCACAAG
D13S220 (13q13.2)	AATCACCTCCACCAG AGGGGTTCTTCATCC	CCAACATCGGGAACCTG TGCATTCTTAAAGTCCATGTC
D13S219 (13q13.3)	CTGGATGAAAAGGAAC TTATCTCATTCAGTTGCT	AAGCAAATATGAAAATTGTC TCTTCTGTTTCTTGACTTAACA
D13S218 (13q13.3)	TTCTCATAAGAAATCCCC TTTCATATCCCTGTTCAA	GATTTGAAAATGAGCAGTCC GTCCGGCACTACGTTTATCT
D13S325 (13q14.11)	ATGCAGCTTAAGTCCTTT CTGTGCTATCTCTCCAA	TCCTTTAAGTGCTAGAGAGGAGG TCTCTCTCAGAAGTTTGGAAGC
D13S291 (13q14.11)	GTCTGACGGGAAACAGC CACAAACAGAATCAACCTT	ATGGCCAGACTTCCCACT CCAGGCTCACATGCTAACA
D13S126 (13q14.2)	AGCTCCCAAAGTGCTA AGTCATCTGGTCCCTCAAT	TCACCAGTAAAATGCTATTGG GTGATTTTCAAATTGTCTCTG
D13S118 (13q14.2)	TGTAATAGCTTAGTTG CTACTGACATTGCTC	GAAATAGTATTTGGACCTGGG CCACAGACATCAGAGTCCTT
D13S153 (13q14.2)	AACCTGGCTGCGATGATAAGAA CCTGAGGTATTGACGAAGGGTC	AGCATTTGTTTCATGTTGGTG CAGCAGTGAAGGTCTAAGCC
D13S284 (13q14.3)	CCAGCTCGTGTTCATTT	AAAATCAGGTGGAACAGAAT

D13S137 (13q14.3)	GTACATTTTATAGATTTCATAGAGTTC GATGGTGGGTGGGACTT	AAAGGCTAACATCGAAGGGA CAGGAGGGATGGACTCACTTC
D13S321 (13q21.1)	GGAATATGTGGAGGATTTATCTCTG TAAATGCAATCTGAAT	TTTCCTCATCTTTCCCAATTG TACCAACATGTTCAATGTAGATAGA
D13S119 (13q21.1)	CATAGCAAGACTCTGTG TCTCATTTCCATAACAT	CATACACCTGTGGACCCATC TTATTGCCTTTGTAGATCATTG
D13S170 (13q31.1)	5' AGAGGAAAGATAGAACAA AGCTATTATGTAACCAAT	AAGACTTTGAATGAAATTCCT TTGCACTGTGGAGATAAACACATAG
D13S71 (13q31.3)	TGTTGTTCTAAGCCAC ACGCTCCTTCGTGGTG	TCACATGTCTTTTAAGGCAGGAG GTATTTTGGTATGCTTGTGC
D16S514 (16q21)	GTGCCTCTGTTTCTCCTATT TGACACGCAATTTACCCT	CTATTTTGGAAATATATGTGCCT CTATCCACTCACTTTCCAGG
D16S402 (16q22.1)	AATCACATTTTCCCCTG GAGGCAAAGAGGTATCCA	TCCCACGTACATCTTCTC TTTTGTAACCATGTACCCCC
D16S3029 (16q23.1)	CCCACTCATTCTGTCCC AGGGTGTGAGGTGTCTG	ATTTATAGGGCCATGACCAG ATAGAGTTGGGCTGCATAGA
D16S3040 (16q23.2)	ATAGGGTCTGCTGGGTT TATGTTGGTGGATGATT	CTTCTCTGAAATTTGGAAGTGA TACTCCGGCAAGGACG
D16S422 (16q24.3)	5' GTCAAGGAACACTGAAC ACAGCCACCTCTATTCA	GCTGCCTAGCACATGG CAGTGTAACTGGGGGC
D16S3121 (16q24.3)	AACATTTTACTATATCTTACTTTTC AAGTCACTGGGCTAACAAGG	CTTTGATTAGTTAGCAGAATGAG CATGTTGTACATCGTATGC
D16S303 (16q24.3)	TGGGTCTGAGGTACAAA GCAGGTTATGGATGTGTTGTT	AGCTTTTATTTCCAGGGGT GATCAGTGTCTGTTTGTGTTGG
D17S643 (17p13.3)	TTCGAAAGGCTGAGGCAGTA TGCCGTTCTCAGGTGGTT	CAACAAGAGCGAACTCGGTCTCAA CTTCCTGTCTCTAAACAGTCTTT
D17S849 (17p13.3)	TTCTCCCTGTCATGGATT TACAATACTGCTGCAATAAG	GTAGTCCCAGGAGCTGGAAGT CAATTCTGTTCTAAGATTATTTGG
D17S926 (17p13.3)	TACAATAACATCAGGAAACA GAAGTGGGAAGATTGCTT	CTCTGGCTGAGGAGGC GCAGTGGGCCATCATCA
D17S695 (17p13.3)	ATCCTCTGAACCGTATTT AGCCTGGGCAACAAGAGC	CCGCAGAAGGCTGTGT CTGGGCAACAAGAGCAAAATTC
D17S1840 (17p13.3)	CAGCCAACGGGCGTCATT GGCAACGTGGTGAAACCC	TTGTGTTGTGTCAITGACITCAGTCT GCCTGGGCGACAGATGA
D17S1529 (17p13.3)	ACCAGGACCGGTGCTCT GTTAGCCTCTTCTTGGACATTC	TGGGCGAGACTTGGCTCTT TTGTTTCTATCCACGCAGGC
D17S1574 (17p13.3)	ATCATTCCGTTTACCTTTGG CTTCTCTGCGTCAGGTATG	GATGGCTGTGCTTGTCTGGTA TCACTTATCGGCATCTGATCC
D17S831 (17p13.3)	CCAAAGTGCTGGGATTA GCCCAAATCAGAAGCAAG	CTGATGTGCCCTTTGTGTGTG CGCCTTCTCATACTCCAG
D17S654 (17p13.3)	GACATCCATTGGCACCAC GGGTACGCCTTTCCTCA	GCCAGACGGGACTTGAATTA GACCTAGGCCATGTTACAGCC
D17S796 (17p13.2)	GGTTGGCAAGACCACTGTTAGA GAGCAGTAGGATCAAGGG	GACATCCATTGGCACCACCCCAA CAATGGAACCAAAATGTGGTC
β-actin ²	AGAACGGCAACAACGAA CTGTGCCCATCTACGAGG	AGTCCGATAATGCCAGGATG AGAGATGGCCACGGCTGCIT
	AAAGGGTGTAACGCAACTAA	ATTGCGGTGGACGATGGAG

¹Nucleotide sequences were written in a 5' to 3' direction, with the forward and reverse primers for each reaction presented at the upper and lower lines, respectively; ²Amplified as an internal control with a product of 406 bp.

(<http://www.ncbi.nlm.nih.gov> and www.gdb.org). The reaction mixture was 50 μL in volume, containing 50 ng of DNA templates, 4 μL of dNTP (2.5 mmol/L each; Gibco BRL), 1 μL of 20 μmol/L primers each, 5 μL of 10 × buffer (100 mmol/L Tris-HCl, pH 8.3, containing 500 mmol/L KCl and 25 mmol/L MgCl₂) and 1.25 U of *Taq* DNA polymerase (Gibco BRL). Amplification was conducted for 35 cycles (94°C, 40 s; 46°C, 50 s; 72°C, 1 min) following the initial denaturation at 94°C for 5 min. The final elongation was at 72°C for 15 min. The efficacy and reliability of the reactions were ensured by amplification of the β-actin gene in a parallel reaction.

For microdissected samples, the amounts of DNA extracted from the minute tissues were too small to complete all the reactions, and a highly efficient amplification system was needed. Nested PCR was employed for this purpose. A multiplex PCR was performed, according to

the principle of Henegariu *et al*^[39], followed by a second-round reaction in separate tubes. For the first-round PCR, 57 external primer pairs, as listed in Table 3, were designed based on the genomic sequences (Genbank). They were classified into 11 groups according to calculated annealing temperatures and compatibility with the assistance of Beacon Designer (Version 5.0) software, allowing amplification of four to seven STR sequences in the same mixture. The reaction mixture was 50 μL in volume as described above, but it contained 20 ng of DNA templates. The amplification was performed for 35 cycles, and the annealing temperatures varied according to the primer groups. The second-round amplification was followed using the internal primer pairs (Table 3), with the 57 reactions in separate tubes. After initial denaturation at 94°C for 5 min, amplification was conducted for 15 cycles (94°C, 40 s; 50°C, 50 s; 72°C, 1 min) at first,

followed by 25 cycles with the annealing temperature adjusted to 46°C. The amplification products, 3 µL each, were mixed with the same volume of sample buffer, and then electrophoresed on a 15% polyacrylamide gel containing urea (8 mol/L). Gels were fixed in 10% acetic acid for 30 min, and the resolved products were visualized by silver staining.

Evaluation of data and statistical analyses

The images of the gels were subjected to analysis with a densitometer (Applied Syngene, Cambridge, UK). For clonality analysis based on polymorphism at the PGK and AR loci, a reduction of $\geq 50\%$ in their signal intensities for the products of either allele, as compared to the samples not treated with *Hpa*II or *Hba*I, is regarded as loss of XCI mosaicism^[28]. Each reaction was repeated at least once to guarantee reproducibility. Moreover, parallel assays were carried out using the surrounding liver parenchyma or fibrous tissue as controls. For the tests on microdissected lesions, special attention was paid to avoid interference from the XCI skewing patches^[28]. For each FNH lesion, four tissue samples of similar sizes as microdissected nodules were taken from the surrounding liver parenchyma and examined as controls.

For the heterozygosity test, normal tissue samples showing signals from two alleles migrating at expected locations and with similar intensities were considered informative, and those showing only one signal, were considered noninformative. Allelic imbalance was determined by comparing the intensity ratio between the signals for the two alleles. For a given informative marker, LOH was identified when one of two signals was absent or the allelic ratio was greater than 3 or less than 0.33. Reliability of the assays was guaranteed by including control tissue samples for all FNH lesions and microdissected nodules in a parallel way. Interpretation of LOH results of the lesions was considered reasonable when all of the control reactions from the same case gave rise to consistent allelic ratios.

Statistical computation was carried out using SPSS 13.0 software (SPSS, Inc, Chicago, IL, USA). Student and Kruskal-Wallis tests were employed to assess cell kinetic features of different lesion types reflected by Ki-67-LI, and the χ^2 test was used to compare LOH frequencies in HCA, FNH and HCC groups. $P < 0.05$ was regarded as statistically significant.

RESULTS

Clinical and pathological features

Twelve FNH lesions (FNH 01-12) were identified from 10 patients (Cases 01-10), eight patients had solitary lesions and separate lesions were identified in two liver specimens (Table 1). The patients included eight females and two males, aged 22-53 years (mean, 37.6 years). Grossly, the resected FNH lesions were 1.2-7.0 cm in diameter (mean, 3.8 cm), and were invariably described as circumscribed and solid lesions. On cut surfaces, a

multinodular appearance was observed, and a fibrous stellate scar was not recorded in any of the lesions. Histologically, all the lesions were classified as the classical form^[4], being well demarcated from the surrounding liver parenchyma but free of a fibrotic capsule. The lesions were subdivided incompletely into nodules with fibrous septa that contained malformed blood vessels, proliferating ductules, and scattered lymphocytes.

Most of the hepatocyte nodules appeared similar, about 1-5 mm in diameter, and histologically resembling regenerative nodules from cirrhotic livers. FAH, frequently of the clear cell type and clear/amphophilic mixed cell type, were identified in some of the nodules from all the lesions. The altered hepatocytes were arranged in plates frequently of two cells in thickness, with the sinusoids appearing crowded, but were integrated well with the surrounding hepatocytes in the majority of the lesions (Figure 1C). In some nodules, nearly all the parenchymal cells were replaced by the altered hepatocytes. These lesions became larger than other nodules, 2.5-5 mm in diameter, showing marked compression to the surrounding tissue (Figure 1D) and fulfilling the criteria for NAH^[20,22]. In all the FNH lesions, including the FAH and NAH areas, the thickness of hepatic plates did not exceed two cells and no SCC was detected. All the lesions were negative for p53 protein. Ki-67-LI ranged from 0.3% to 1.6%, with a mean of 0.8%. While this value was shown to be lower than those of HCAs ($P < 0.01$) and the well-differentiated HCCs ($P < 0.01$), these three lesion types showed overlap in their Ki-67-LI, with 6 (50%) of the 12 HCAs and 6 (27%) of the 22 HCCs within the range of FNH (Tables 1 and 2). FAH, mainly small-sized, and clear-cell lesions, were also identified in the surrounding liver parenchyma in six of the 10 livers (Table 1).

As listed in Table 2, 12 HCAs (HCAs 01-12) were resected from 11 patients (Cases 11-21), including nine females and two males. The ages of the patients ranged from 28 to 57 years (mean, 36.6 years), and none had a history of using oral contraceptives or other steroid hormones. Among the 12 lesions, 10 were solitary, and two were resected from the same liver (Case 16). The lesions ranged in size from 1 to 12 cm in diameter (mean, 4.8 cm). Histologically, the lesions were well demarcated from the surrounding liver parenchyma, but an intact fibrotic capsule was absent in all the lesions. The tumor was composed of hepatocytes arranged in plates usually of two cells in thickness. Low-grade SCC was identified in HCAs 03 and 08. The portal tract was not visible within the lesions. All these lesions were negative for p53 protein. Ki-67-LI ranged from 0.9% to 3.1%, with a mean of 1.8%. Phenotypic change of the sinusoid cells, as highlighted by emergence of CD34-immunoreactivity, was observed in all the HCA lesions, being diffuse in four and patchy in eight of the lesions. FAH, mainly of the clear cell type, were also identified in the surrounding liver parenchyma in six of the 11 livers.

A total of 22 well-differentiated HCCs were collected from 12 males and 10 females (Table 2). The age of the patients ranged from 27 to 77 years, with a mean of

Table 4 Nonrandom X-chromosomal inactivation revealed in NAH microdissected from 3 FNH and their inactivation patterns

Origin of lesions	No. of NAH tested	No. with signals	No. with MC (%)	No. of lesions with	
				Upper band	Lower band
FNH09	38	34	14 (41.2)	12	2
FNH10	6	6	2 (33.3)	0	2
FNH11	12	12	5 (41.7)	0	5
Total	56	52	21 (40.4)	12	9

46.6 years. All the patients were shown to be seropositive for HBsAg. Of the 22 HCC cases, 20 appeared solitary and well demarcated and two were multifocal. The lesions ranged in size from 2.0 to 9.5 cm, with a mean of 4.6 cm. Histologically, nine of them were classified into grade 1, and 13 into grade 2. Ki-67-LI ranged from 0.9% to 65%, with the mean and median being 13.1% and 5.4%, respectively. This value was shown to be higher than that of HCAs ($P < 0.05$), while the Ki-67-LI in 10 (45%) of the 22 HCCs were within the range of HCAs. Immunoreactivity for AFP was demonstrated in four of the HCC samples (HCC 01, 11, 21 and 22). Overexpression of p53 protein was observed only in three HCC lesions, with the percentages of positive tumor cells as high as 10% in Case 21 and 80% in Cases 25 and 34. HBV infection was confirmed by immunohistochemical demonstration of HBsAg in all of the cases. Cirrhotic changes were evident in surrounding liver tissues in all the cases, where numerous NAH were identified.

Nonrandom XCI revealed in HCCs and HCAs, but not in the FNH lesions

XCI analysis was performed using tissues from 4 of the female patients with HCC (Cases 29, 30, 42 and 43) and all the nine females with HCA (Table 2). The G/A polymorphism at the PGK locus was demonstrated in three of the HCC patients (Cases 30, 42 and 43) and in three of the nine HCA patients (Cases 17, 19 and 21), showing two bands at positions 530 and 433 bp. Pretreatment with *Hpa*II resulted in loss, or marked intensity reduction, of one band in all the tumor samples, while the allelic ratio was retained in the samples from surrounding liver parenchyma (Figure 2A). One HCC (Case 29) and the remaining HCA patients were not polymorphic at the PGK locus, but the AR locus showed CAG STR length-polymorphism. Pretreatment with *Hha*I resulted in loss, or marked intensity reduction, of one band in these two tumor samples, while the allelic ratios were not changed in the corresponding peritumorous liver parenchyma (Figure 2B and C). These results demonstrated monoclonality and confirmed neoplastic nature in all the HCC and HCA lesions.

Of the eight female patients with FNH, 5 (Cases 1, 2, 5, 6 and 8) showed G/A polymorphism at the PGK locus, and the remaining three (Cases 7, 9 and 10) were shown to be AR-polymorphic at the CAG STR. Pretreatment with *Hpa*II (for PGK locus) or *Hha*I (for AR locus) did not cause a remarkable allelic ratio change in any of the 10 FNH samples (Figure 3A). These results demonstrated polyclonality of classical FNH lesions.

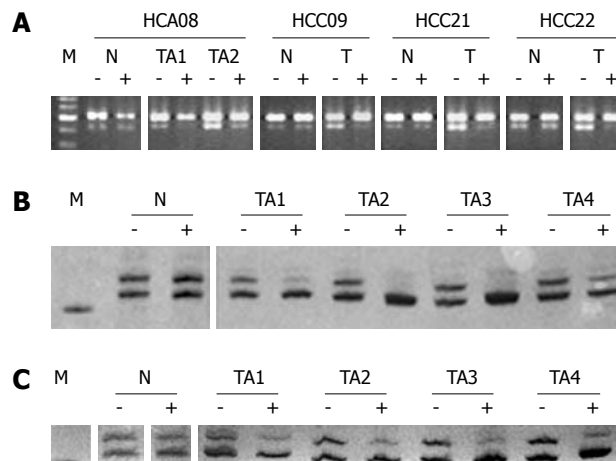


Figure 2 Representative data of X-chromosome inactivation (XCI) assays at PGK (A) and AR loci (B and C). Nonrandom XCI are present in HCAs (08 in A, 20 in B) and HCCs (09, 21 and 22 in A, 08 in C), revealing their monoclonality. A: The G/A single-nucleotide polymorphism at PGK exon 1 was demonstrated by *Bst*XI-digestion and electrophoresis on an agarose gel. The intact and cleaved amplification products migrate at positions of 530 (upper band) and 433 bp (lower band), respectively. Pretreatment with *Hpa*II (-: Before; +: After) resulted in marked reduction of the lower band in single tumor samples (T) and separate tumor areas (TA), but not in the surrounding liver parenchyma (N). M: DNA markers, with five bands at locations of 700, 600, 500, 400 and 300 bp; B and C: The length polymorphism of AR gene was resolved on a 10% polyacrylamide gel containing urea (8 mol/L) and visualized by silver staining, with one product migrating faster than the other. Pretreatment with *Hha*I (-: Before; +: After) resulted in loss or marked reduction of one band in tumor samples, but not in the non-neoplastic tissue. M: DNA marker at the location of 200 bp.

It appears that FNH is different in clonal composition from hepatocellular neoplasms including HCC and HCA.

Nonrandom XCI revealed in some NAH lesions microdissected from FNH

Three FNH lesions (FNH 09-11) with well-demarcated NAH from three women were chosen for microdissection. A total of 56 NAH lesions (NAH 01-56), ranging from 2.5 to 5 mm in diameter, and 5 ordinary regenerative nodules, of similar sizes and without involvement of FAH, were isolated from the FNH lesions for clonality analysis. Genomic DNA samples from 52 of the 56 dissected NAH were amplified successfully and subjected to AR (FNH 09 and 10) and PGK gene analysis (FNH11). Twenty-one (40%) of the 52 NAH showed monoclonality, including 14 (41%) of the 34 lesions in FNH09 (Figure 3B), 2 (33%) of the six in FNH10 and 5 (42%) of the 12 from FNH11 (Table 4). On average, the cross sectional areas of the 21 monoclonal

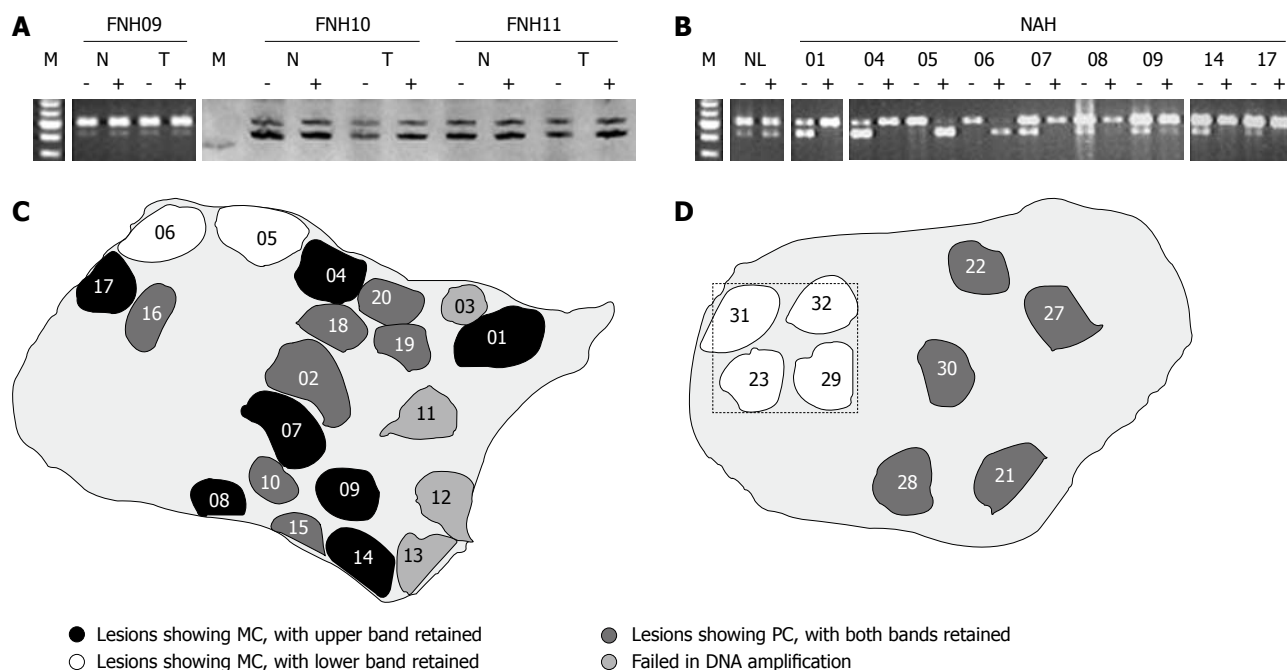


Figure 3 Clonality status of FNH lesions (A) and NAH (B), with XCI patterns revealed in different NAH (C and D). A: Polyclonality (PC) revealed in 3 FNH lesions, with FNH09 assayed at PGK locus and 10 and 11 at AR locus. *Hpa* II or *Hha* I pretreatment (-: Before; +: After) did not significantly change intensity ratios between the two bands of lesional tissues (T) compared to those of the surrounding liver parenchyma (N). M: DNA markers, with five bands at locations of 700, 600, 500, 400 and 300 bp for PGK assay, and a single band at the location of 200 bp for AR reaction; B: The PGK assay shows monoclonality (MC) in all of the nine NAH from FNH09, with the isolated reference tissue sample (NL), of a similar size to NAH, revealing polyclonality. XCI patterns of the NAH are different, with the upper band retained in NAH 01, 04, 07-09, 14 and 17, and the lower band retained in NAH 05 and 06; C: Sketch of a tissue section from FNH09, as illustrated in B, showing occurrence of monoclonal NAH with different XCI patterns and polyclonal lesions in the same section; D: Sketch of a tissue section from FNH11, showing four monoclonal and five polyclonal NAH. The clustered monoclonal lesions show the same XCI pattern, the XCI assays with small samples, as framed by hatch lines, may result in misinterpretation as monoclonality for the whole FNH lesion.

and 31 polyclonal lesions were 9.8 ± 7.0 and 9.5 ± 6.7 mm², respectively, the difference being insignificant ($P > 0.05$). Different alleles were retained among the 14 monoclonal NAH from FNH09 (Figure 3B and C), while identical inactivation patterns were observed in those NAH from FNH 10 and 11 (Figure 3D). All of the five ordinary nodules, four dissected from FNH09 and one from FNH11, were shown to be polyclonal in cell composition.

LOH detected in NAH, HCAs and HCCs, but not in whole FNH lesions

A total of 57 loci were examined in this study, located at 1p, 4q, 6q, 8p, 8q, 11p, 13q, 16q and 17p (Table 3). As shown in Table 2, the assay showed LOH, at least at one of the loci, in 15 (83%) of the 18 well-differentiated HCCs (01-07 and 10-20) and in all of the 9 HCAs examined (01-05 and 07-10, Figure 4A). Six of the FNH lesions (01-06), covering areas of at least 1.0 cm × 1.0 cm, were also examined, and no LOH was detected in any of them (Figure 4B).

Demonstration of clonal growth in some of the NAH from FNH, as described above, indicated that some genomic alterations may have occurred in these minute lesions. Twenty-five NAH were microdissected from an FNH lesion (FNH04) for LOH analysis. Genomic DNA samples from 13 of the microdissected lesions (NAH 57-69) were amplified successfully for the majority of the reactions, but for other samples

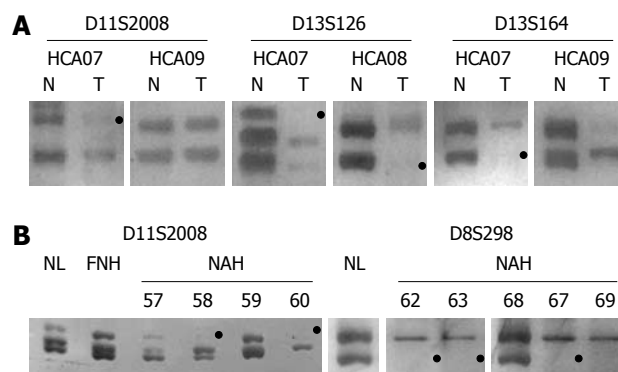


Figure 4 Representative data of amplification products at three length-polymorphic loci in HCAs 07-09 (A), FNH04 and NAH microdissected from the FNH lesion (B), with loss or marked reduction of the product from one allele (black dot) defined as LOH. A: Gels show the allelic imbalance at D11S2008, D13S164 and D13S164 in HCA07, at D13S126 in HCA08, at D13S164, but not in D11S2008, in HCA09 (T: Tumor; N: Peritumorous normal liver); B: The microdissected lesions NAH58 and NAH60, but not NAH57 and NAH59, show LOH at D11S2008. The lesions NAH62, NAH63, NAH67 and NAH69, but not NAH68, show LOH at D8S298. Both alleles are preserved in FNH04 as tested as a whole (FNH) and compared to those of the surrounding normal liver parenchyma (NL).

(NAH 70-81), the amounts of the extracted DNA were too small to complete all the reactions and the data obtained were not included. Allelic imbalance, at least at one of the loci, was demonstrated in all of the 13 NAH (Figure 4B, Table 5). The reliability of the assay was confirmed by full consistence between the results

Table 5 LOH profiles of 13 NAH microdissected from an FNH lesion (FNH04)

Lesion codes	Diameters (mm) ¹	Numbers of LOH	Loci affected ²
NAH57	3.8	6	D6S437, <u>D8S298</u> , <u>D13S118</u> , D13S153, D16S3029, D16S3040
NAH58	3.5	5	<u>D8S298</u> , <u>D11S1301</u> , D11S2008, <u>D13S321</u> , D16S3121
NAH59	1.8	2	<u>D13S164</u> , <u>D17S1840</u>
NAH60	2.7	2	<u>D11S1301</u> , <u>D13S321</u>
NAH61	3.8	6	D11S2008, <u>D13S118</u>
NAH62	3.2	4	<u>D8S298</u> , <u>D13S164</u> , <u>D13S321</u> , <u>D17S1840</u>
NAH63	2.8	5	D15S13, <u>D8S298</u> , <u>D13S118</u> , D13S153, D17S695
NAH64	3.8	5	D6S1008, D13S218, D16S514, D16S3040, D17S926
NAH65	3.2	5	D15S13, <u>D8S298</u> , D11S907, <u>D13S164</u> , D13S126
NAH66	2.0	1	D6S1008
NAH67	2.1	4	<u>D8S298</u> , <u>D11S1301</u> , D11S907, D16S422
NAH68	2.4	3	<u>D13S164</u> , D13S126, <u>D13S321</u>
NAH69	2.8	5	D6S437, <u>D8S298</u> , D13S118, <u>D13S321</u> , D17S926

¹The largest diameters as measured in cross sections of the lesions under a microscope; ²The markers written in underlined characters represent loci affected in high frequencies ($\geq 30\%$) in NAH.

obtained by the multiplex reactions and those obtained through conventional PCR.

Of the 57 loci examined, 39 (68.4%) showed LOH in at least one of the lesions tested. At the remaining loci, allelic imbalance was not detected in any of the samples examined. LOH was demonstrated in NAH, HCAs and HCCs at 21 (37%), 16 (28%) and 33 loci (58%), respectively. The allelic imbalance was more extensive among the locations tested in HCCs than in HCAs and NAH ($P < 0.01$), with their average numbers of LOH being 4.5 (0-12), 3.1 (1-5) and 4.1 (1-6), respectively. As shown in Figure 5, the most frequent LOH in NAH, HCA and HCC were at D8S298 (70%), D11S1301 (75%) and D6S1008 (50%), respectively. Several markers showed frequent LOH in the HCC samples but not in HCAs, including D6S1008, D8S1754, D8S261, D8S277, D16S3029, D16S303 and D17S796.

While LOH was demonstrated in NAH, HCAs and HCCs, its frequencies were found to vary with locations in the genome and lesion types (Figure 5), with those up to 20% and 30% considered frequent and highly frequent events, respectively. Of the 39 loci showing LOH, eight were found to be highly frequent in HCAs, clustering at 11p, 13q and 17p, six in the HCCs, located at 6q, 8p, 11p, 16q and 17p, and six in NAH, located at 8p, 11p, 13q and 17p.

Differences between these types of lesions were further demonstrated by their LOH frequencies at different locations at 8p, 11p and 17p (Figure 5). Among the four loci affected at 8p, D8S298 was the only one showing LOH in a high frequency (70%) in NAH. Its frequencies were lower in other clinically detectable hepatocellular neoplasms, being 20% in HCAs and 12% in the HCC samples. The difference between NAH and HCC was significant ($P < 0.01$), and that between NAH and HCAs did not reach statistical significance ($P > 0.05$). Among the three loci at 11p, D11S1301 and D11S2008 were affected more frequently in NAH, HCA and HCC.

As shown in Figure 5, allelic imbalances were found to affect more chromosomal locations in HCC specimens, with LOH demonstrated preferentially at D8S261 (3/8, 38%), D8S1754 (4/13, 31%), D8S277 (3/15, 20%) and

D17S796 (38%). Among the loci, D17S796 did not show allelic imbalance in any of the six NAH and 10 HCAs examined. The region 17p13.3, as shown using nine markers, was affected in 7 (39%) of the 18 HCCs, 6 (67%) of the nine HCAs and 6 (46%) of the 13 NAH examined, with the locus D17S926 involved frequently in all of the three lesions. The region 16q21-24, as tested using seven markers, was affected in 6 (33%) of the 18 HCCs, 1 (11%) of the nine HCAs and 5 (38%) of the 13 NAH examined, with D16S3029 and D16S3040 affected frequently in HCC and NAH, but not in HCA specimens.

DISCUSSION

FNH occurs within an otherwise normal liver, and is detected more frequently between the ages of 20 and 50 years. A careful survey of 168 patients in France revealed a marked female predominance, with the male to female ratio being 1/8 (18/150)^[4]. However, a male predilection was observed in a report from China involving 86 FNH cases, with the male to female ratio being 1.7:1 (54:32)^[19]. This may reflect the differences in lifestyle and eco-environmental factors between these two countries, as a similar tendency has also been noticed for HCA^[6]. The role of oral contraceptive use in its development remains a matter of debate^[40,41], while its progression was observed in women using oral contraceptives^[42-44]. The lesion is frequently solitary. A minority of cases ranging from 7% (6/86)^[19] to 24% (40/168)^[4] have been shown to have multiple (2 to 30) lesions.

The distinction between FNH and HCA may be difficult in some cases during pathological practice. A central stellate scar, when present, is helpful in establishing the diagnosis of FNH. However, this feature is undetectable in about half of FNH lesions^[4,19]. Moreover, a similar change was also observed in some well-differentiated HCCs^[45]. In our study, this hallmark feature was not recorded in any of the 12 lesions examined. Clearly, more reliable approaches are needed for the differential diagnosis.

The pathogenesis of FNH remains to be established. Wanless and collaborators proposed that FNH is a

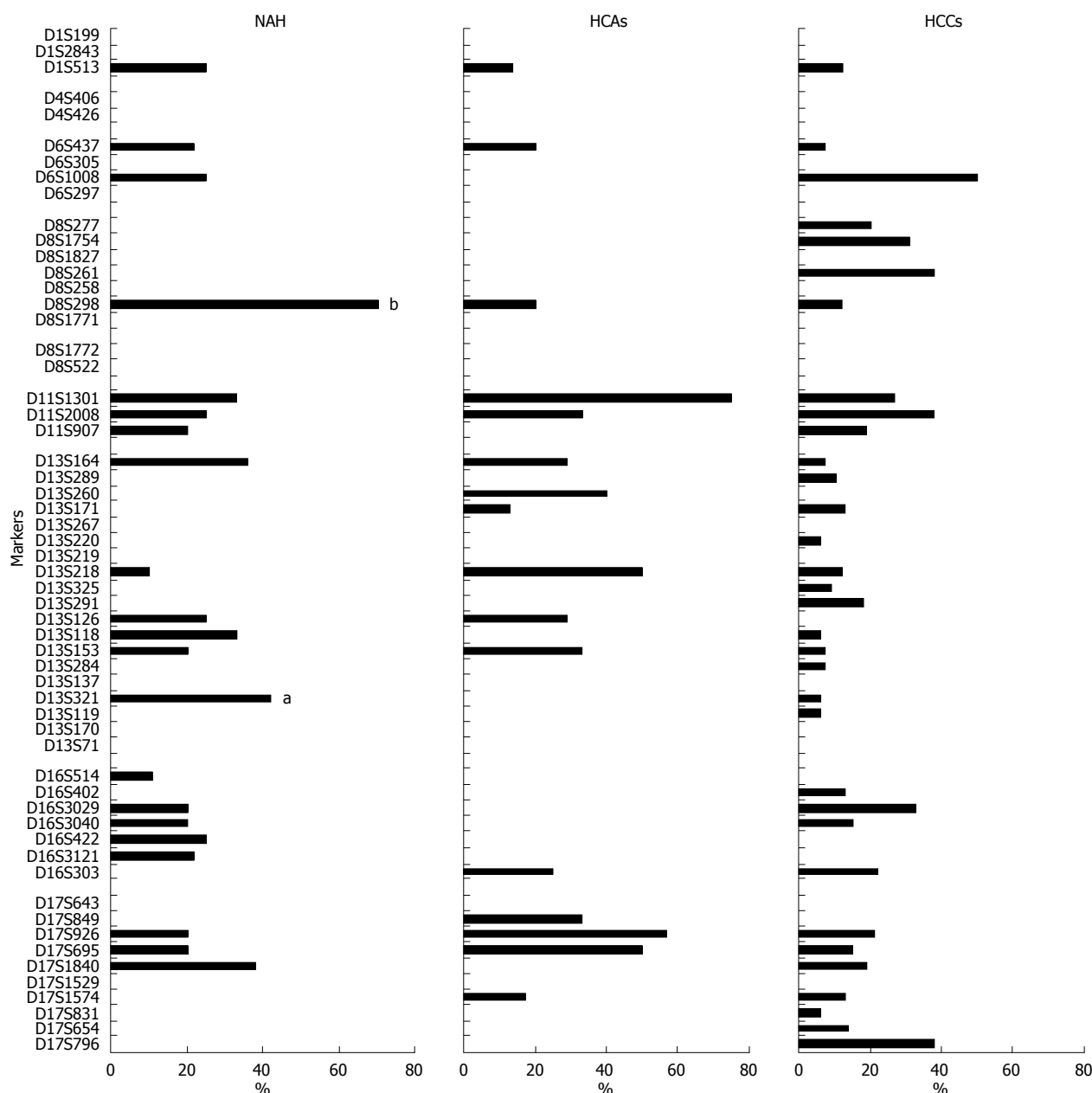


Figure 5 Frequencies of LOH as demonstrated using 57 microsatellite markers in NAH ($n = 13$), HCAs ($n = 9$) and well-differentiated HCCs ($n = 18$). ^a $P < 0.05$, ^b $P < 0.01$ vs HCCs.

proliferative response to an arterial malformation^[3]. Analysis of angiopoietin (ANGPT) 1 and ANGPT2 mRNA expression revealed an increase in the ANGPT1/ANGPT2 ratio in classical FNH, but not in HCA and telangiectatic FNH^[8,9,46]. This may provide a possible mechanism for alterations in proliferation and remodeling of vascular elements within these lesions. However, the nodular hyperplasia of the involved parenchyma needs to be explained. An observation by Paradis *et al*^[5], using the XCI analysis, revealed polyclonality in all of the 12 FNH lesions examined. However, data from other laboratories, obtained by similar assays, demonstrated monoclonality in FNH lesions at frequencies ranging from 39%^[9] to 75%^[7]. The reason for this discrepancy is currently unknown. Insufficient sampling may result in the occurrence of skewed XCI patterns during a test with

normal tissues^[28,47], which may lead to misinterpretation of the data. The phenomenon was associated with clonal cell clusters (patching) as described in other tissues^[28,47-49]. It may also be true that clonal proliferation occurs focally within some of the classical lesions.

In this study, the neoplastic nature of HCC and HCA was confirmed, however, a random XCI inactivation pattern was demonstrated in all of the eight FNH lesions examined. In order to avoid interference from clonal patches, large samples were analyzed for all the lesions. Our results demonstrate that classical FNH, when examined as a whole, is polyclonal in cell composition. The assay is useful for the differential diagnosis between FNH and neoplastic hepatocellular lesions including HCA and HCC.

Human hepatocarcinogenesis is a multi-step process,

including the occurrence of FAH and NAH, neoplastic formation and malignant transformation through high-grade SCC to a fully developed HCC^[20,22]. The sequential changes may involve inactivation of multiple tumor suppressors^[38]. LOH is one of the main mechanisms for inactivation of tumor suppressor genes. Its occurrence has been localized frequently at 1p, 6q, 8p, 11p and 16q in early-stage, small HCC, while the losses at 4q, 13q and 17p were linked to progression of the lesion^[30-38]. As some well-differentiated HCCs and HCAs with high-grade SCC share many morphologic features, it may be difficult to make the differential diagnosis between these disorders with full certainty. This is particularly true when the lesion occurs in a liver without chronic viral hepatitis and cirrhosis. Activation of β -catenin and Wnt-1 signaling pathway has been linked to HCA lesions showing various degrees of phenotypes indicative of malignant transformation^[11,14], however, these changes were also found in some HCCs^[50,51]. In this study, we examined the allelic integrity of these chromosomal arms using 57 microsatellite markers in nine HCAs and 18 well-differentiated HCCs. Frequent LOH was observed in these well-differentiated HCCs at loci on 6q, 8p, 11p, 16q and 17p. In HCAs, the alterations were observed on similar chromosomal arms, but at different loci (Figure 5). Among the eight loci selected for 8p, four showed LOH, located at D8S261, D8S1754, D8S277 and D8S298, in HCC, and only one in HCA, at D8S298. Among the seven loci for 16q, four showed LOH in HCC, located at D16S3029, D16S303, D16S3040 and D16S402, and only one, at D16S303, in HCA. It seems that the demonstration of 8p losses at D8S261, D8S1754 and D8S277, 16q losses at D16S3029 and D16S3040, and 17p loss at 17S796 is helpful for the identification of HCC from HCA.

In contrast to HCA, FNH did not show TCF1/HNF-1 α or CTNNB1 gene mutations and rarely showed DNA losses^[14,52,53]. Allelic imbalances are also present in the majority of HCAs, as shown in this study, but results of LOH detection in FNH are confusing. A genome-wide assay by Bioulac-Sage and colleagues demonstrated LOH in 5 (26%) of the 19 classical FNH samples^[9], but another group, using a similar approach, did not find any LOH among the 212 informative loci^[54]. In the present study, we did not find LOH at any of the 57 loci in the six FNH lesions, as tested using samples as large as 1 cm \times 1 cm. This is consistent to the polyclonal nature of FNH as revealed in most of the lesions.

Results from our previous studies on human liver has enabled us to identify FAH and NAH in both explanted and resected liver specimens, and these lesions were associated with SCC and a markedly increased risk of HCC development^[20,22]. In a recent study, various kinds of nodules from livers with HBV-associated cirrhosis were microdissected. XCI assay revealed monoclonality in all of the NAH with SCC, 35% of the NAH without SCC, but not in any of the ordinary regenerative nodules examined^[27]. These NAH with clonal expansion were considered neoplastic, designated microadenomas and

representing hepatic intraepithelial neoplasia (HIN). In this study, multiple FAH and NAH were also identified in FNH lesions, all of these lesions were composed mainly of clear hepatocytes with none showing SCC. A total of 56 NAH were isolated from four FNH lesions, and monoclonality was revealed in 21 (40%) of the 52 informative NAH by XCI analysis, demonstrating multifocal microadenoma formation within FNH. In contrast, all of the five ordinary nodules were proven to be polyclonal.

The occurrence of NAH with different inactivation patterns, as shown in FNH09 (Figure 3B and C), proves that microadenomas develop independently, as described for multiple leiomyomas of the uterus^[28]. It is conceivable that FNH is composed of numerous NAH and NAH-forming lesions. The synchronous relationship between different NAH may provide an explanation for the stable behavior of most FNH lesions, in contrast to HCA. In addition, the presence of multiple microadenomas (HIN) with similar XCI patterns in an FNH lesion, as shown in FNH11 (Figure 3D), may result in misinterpretation of the data if the sample examined is small. For this reason, we proposed that sample areas for clonality assessment should be as large as 1 cm \times 1 cm for larger FNH or cover the largest cross section for smaller lesions.

While some NAH were shown to be monoclonal, their pathogenesis is unknown. Our assay demonstrated allelic imbalance in all of the 13 lesions, providing additional evidence for their neoplastic nature. The alterations were highly frequent in six loci, involving similar chromosomal arms, as in HCA, including 8p, 11p, 13q and 17p. However, chromosomal arms 8p and 16q were affected in different frequencies in these two types of lesions. D8S298 was affected frequently in both NAH and HCAs, but LOH at this locus was revealed in only a minority of the HCC lesions. Allelic imbalance at D8S298 has been observed in oral and laryngeal squamous cell carcinoma and its precursor lesions. The change was proposed to be an early event in development of the cancer^[55]. This change was also observed in HCC specimens, with its frequencies ranging from 15%^[56] to 32%^[57]. It remains to be determined whether there is a tumor suppressor gene near D8S298 preferentially responsible for HCC development in HBsAg-negative patients.

In summary, classical FNH lesions were shown to be polyclonal by XCI and LOH assays and distinguishable from HCA and HCC. Secondly, multifocal HIN formation, in the form of NAH, was demonstrated within the FNH lesions. In addition, allelic imbalances were also identified in the microdissected NAH, with similar chromosomal arms affected as in HCAs. Therefore, classical FNH is considered a cluster of NAH. Elucidation of the process will be helpful for further understanding of early human hepatocarcinogenesis.

ACKNOWLEDGMENTS

The authors thank Dr. Lin Yang for her statistical assistance, Professor Peter Bannasch and Yan-Fang Liu for helpful

discussions, Dr. Su-Sheng Shi for providing valuable tissue samples, Xiu-Yun Liu, Xin-Hua Xue and Yong-Qiang Xie for their technical assistance.

COMMENTS

Background

Focal nodular hyperplasia (FNH) is a lesion found in an otherwise normal liver, and is considered parenchyma overgrowth responsive to increased blood flow secondary to vascular malformations. While its clinical outcomes are believed to be different from hepatocellular adenoma (HCA) and carcinoma, its pathogenesis is largely unclear and its distinction from HCA is sometimes difficult.

Research frontiers

While FNH was proposed to be a hyperplastic lesion, its clonality status has not been elucidated and the development of hepatocyte nodules within the involved parenchyma needs to be explained. In this study, the authors demonstrate that classical FNH, when examined as a whole, is polyclonal in cell composition. However, the formation of multiple nodules of altered hepatocytes (NAH), showing monoclonality and genetic alterations, was found within all the FNH lesions.

Innovations and breakthroughs

Clonality status of FNH has not been clarified and the mechanism for development of multiple hepatocyte nodules within FNH is unknown. In this study, classical FNH lesions were shown to be polyclonal and distinguishable from HCA and carcinoma. Secondly, the multifocal formation of NAH, representing early-stage hepatic intraepithelial neoplasia (HIN), was demonstrated within the FNH lesions. In addition, allelic imbalances were also identified in microdissected NAH. Classical FNH is considered a cluster of NAH.

Applications

The results of clonality analyses demonstrated polyclonality in all the classical FNH lesions, the approach being useful for the differential diagnosis of FNH from HCA and well-differentiated carcinoma. In addition, elucidation of the pathogenesis of the NAH lesions, representing hepatocytic microadenoma and early-stage HIN, may lead to further understanding of early human hepatocarcinogenesis.

Terminology

Most, if not all, of HCAs and carcinomas develop from a single focus of altered hepatocytes through nodular transformation (formation of NAH) and appearance of cellular and architectural atypia (small-cell change). Monoclonality and nonrandom genetic alterations similar to HCA, as demonstrated in NAH lesions in this study, enable us to consider these lesions hepatocytic microadenoma that represent early-stage HIN.

Peer review

This is an interesting report of clonality and genetic alternations in FNH. The article is well written and deserves publishing.

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Analysis of risk factors for central venous port failure in cancer patients

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Received: July 28, 2009 Revised: August 24, 2009

Accepted: August 31, 2009

Published online: October 7, 2009

Abstract

AIM: To analyze the risk factors for central port failure in cancer patients administered chemotherapy, using univariate and multivariate analyses.

METHODS: A total of 1348 totally implantable venous access devices (TIVADs) were implanted into 1280 cancer patients in this cohort study. A Cox proportional hazard model was applied to analyze risk factors for failure of TIVADs. Log-rank test was used to compare actuarial survival rates. Infection, thrombosis, and surgical complication rates (χ^2 test or Fisher's exact test) were compared in relation to the risk factors.

RESULTS: Increasing age, male gender and open-ended catheter use were significant risk factors reducing survival of TIVADs as determined by univariate and multivariate analyses. Hematogenous malignancy

decreased the survival time of TIVADs; this reduction was not statistically significant by univariate analysis [hazard ratio (HR) = 1.336, 95% CI: 0.966-1.849, P = 0.080]. However, it became a significant risk factor by multivariate analysis (HR = 1.499, 95% CI: 1.079-2.083, P = 0.016) when correlated with variables of age, sex and catheter type. Close-ended (Groshong) catheters had a lower thrombosis rate than open-ended catheters (2.5% vs 5%, P = 0.015). Hematogenous malignancy had higher infection rates than solid malignancy (10.5% vs 2.5%, P < 0.001).

CONCLUSION: Increasing age, male gender, open-ended catheters and hematogenous malignancy were risk factors for TIVAD failure. Close-ended catheters had lower thrombosis rates and hematogenous malignancy had higher infection rates.

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Key words: Central venous port; Chemotherapy; Risk factor; Cancer patient; Multivariate analysis

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Hsieh CC, Weng HH, Huang WS, Wang WK, Kao CL, Lu MS, Wang CS. Analysis of risk factors for central venous port failure in cancer patients. *World J Gastroenterol* 2009; 15(37): 4709-4714 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4709.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4709>

INTRODUCTION

Central venous access is necessary for patients who require long-term intravenous chemotherapy, parenteral nutrition, transfusion of blood components and repetitive blood sampling. Techniques for external cannulation of the subclavian and internal jugular veins were described by Broviac *et al*^[1] and Hickman *et al*^[2] in the 1970s. In 1982, Niederhuber *et al*^[3] introduced the

totally implantable access port. The principal advantages of these access ports are; no external dressing, lower infection rates than non-totally implantable devices and allowance of patients to perform normal physical activities. It is common practice to insert totally implantable venous access devices (TIVADs) in cancer patients beginning a course of chemotherapy to eliminate potential peripheral venous access problems^[4,5]; however, risk factors impacting the survival of TIVADs remain unclear. Accordingly, this retrospective cohort study analyzed the risk factors for failure of the TIVADs and compared adverse events among risk factors.

MATERIALS AND METHODS

Patient population

Between January 1, 2003 and December 31, 2006, this retrospective study enrolled 1280 cancer patients who underwent an operation for placement of TIVAD at Chang Gung Memorial Hospital, Chiayi, Taiwan. All devices were indicated for administering prolonged antineoplastic chemotherapy. Medical records for 1280 cancer patients provided data for age, sex, disease, catheter type, surgical procedure, origin of patients, reason of catheter failure, surgical complications and length of implantation. All patients were followed through December 31, 2006, until death or catheter removal due to complications.

Surgical procedure and device care

All procedures were conducted in an operating room by surgeons under fluoroscopic control for correct positioning of the catheter tip in the superior vena cava. The implantation of TIVAD required only local anesthesia (usually 10 mL of 2% mepivacaine hydrochloride) in almost all patients; general anesthesia was used in combination with major surgical procedures for 10 devices. Perioperative antibiotic prophylaxis consisted of a single dose of cephalosporin during the surgical procedure.

Surgical approaches were cephalic cut-down or subclavian vein puncture on either side depending on the surgeon's preference. The right side was usually selected for the TIVAD implantation due to shorter access route to superior vena cava than left side. Implantation from the jugular vein or femoral vein system was considered when there was failure of the cephalic vein and subclavian vein system. The TIVAD system was fixed to the underlying pectoral muscle fascia with one non-absorbable suture. Filling the port system with diluted heparin saline was performed routinely at the end of each procedure.

The TIVAD system was routinely flushed with diluted heparin saline by trained oncologic nurses, following each administration of chemotherapy agents. The device was maintained by flushing with a heparinized solution every 4 wk. Non-coring Huber needles were utilized for all injections into the TIVAD system.

Two catheter tips for port systems are used at our institution: the Groshong valved close-ended catheter

(Bard, Salk Lake City, UT, USA), and the open-ended catheter. Port systems were assigned to patients depending on surgeon's preference during the review period.

Study outcomes

The primary endpoint was failure of the TIVAD and the analysis of risk factors affecting device failure among the variables of age, gender, origin of patients, type of catheter, insertion site and type of malignancy using univariate and multivariate methods. The secondary endpoint was to compare the adverse events in relation to the risk factors.

The number of catheter-indwelling-days of TIVAD was calculated from day of insertion to one of the following observation end points: December 31, 2006; date of death; date of catheter removal due to adverse events.

Adverse events of the TIVAD system were divided into three categories: infection events (local erythematous skin change or catheter-related systemic febrile); thrombosis events (intraluminal thrombosis); and surgical complications (pneumothorax, hemothorax, distortion of port system, catheter fracture or malposition). The incidence rate of adverse event was defined as number of events per 1000 catheter-days.

Statistical analysis

The numeric variables were presented as mean \pm SD. The number of catheter-indwelling-days was presented as median with inner quartile range (IQR). Log-rank test was conducted for time of death, device removal due to adverse events. For other analyses, independent proportions were compared by using the chi-square test or Fisher's exact test. Using catheter-indwelling-days of TIVAD as a dependent variable and age, sex, catheter type, tumor type, origin of patients and insertion site as independent variables, Cox proportional hazards modeling with forward selection was performed using a two-step technique. First, univariate analysis was performed and included any potential prognostic factor. Thereafter, only variables with a value of $P < 0.10$ by univariate analysis were introduced in the Cox model. $P < 0.05$ indicated a significant statistical difference. All statistical analyses were performed using Stata Statistical Software version 9.2. (StataCorp. 2005. Stata Statistical Software: Release 9.2. College Station, TX, USA).

RESULTS

Distribution of device and device life

From January 1, 2003 and December 31, 2006, 1348 TIVADs were implanted into 1280 consecutive patients. Of the study population, 796 (62%) (842 TIVADs) were male and 484 (38%) (506 TIVADs) were female. The mean age of the subjects was 60.13 ± 13.06 years (range, 13-91 years). Patient origins for insertion of TIVADs were 967 (72%) inpatients and 381 (28%) outpatients. The devices were inserted into 1272 (94%) patients for treatment of solid tumors and 76 (6%) patients for

Table 1 Distribution of 1348 TIVADs and average catheter-indwelling-days

	<i>n</i> (%)	Median (range)
Sex		
Male	842 (62)	151 (60337)
Female	506 (38)	228 (88473)
Origin of patient		
Inpatients	967 (72)	157 (60358)
Outpatients	381 (28)	217 (93455)
Type of malignancy		
Hematogenous	76 (6)	148 (61303)
Solid	1272 (94)	180 (70403)
Type of catheter		
Groshong	830 (61)	218 (81478)
Open-ended	518 (39)	143 (55280)
Total	1348 (100)	178 (70399)

Table 2 Primary malignancy in 1280 patients with 1348 TIVADs for long-term intravenous chemotherapy *n* (%)

Malignancy	Patients	TIVADs
Colorectal	354 (27.7)	359 (26.6)
Lung	348 (27.2)	367 (27.2)
Head and Neck	139 (10.8)	150 (11.1)
Breast	103 (8.0)	109 (8.1)
Gastric	78 (6.1)	91 (6.8)
Hematogenous	70 (5.5)	76 (5.6)
H-B-P	69 (5.5)	72 (5.4)
Esophageal	41 (3.2)	43 (3.2)
Urologic	40 (3.1)	40 (3.0)
Gynecologic	12 (0.9)	14 (1.0)
Others ¹	26 (2.0)	27 (2.0)
Total	1280 (100)	1348 (100)

¹Others include skin, brain, bone, sarcoma and unknown primary origin. H-B-P: Hepato-biliary-pancreatic.

hematogenous tumors. The catheters used were 830 (61%) Groshong catheters and 518 (39%) open-ended catheters (Table 1). Table 2 lists the distribution of primary malignancies and TIVADs.

Table 3 lists the insertion sites, surgical procedures and catheter type used. Of the 1280 consecutive patients who required 1348 TIVADs, 1100 (81.6%) were suited to a cephalic vein cut-down approach and 196 (14.6%) to a subclavian vein puncture procedure. Of the remaining 52 devices, 23 (1.7%) were placed *via* the jugular vein system due to difficulty approaching the subclavian vein system. The final 29 (2.1%) devices utilized femoral vein placement with or without a saphenous vein approach due to previous neck/thorax radiotherapy or superior vena cava syndrome.

The median (IQR) number of catheter-indwelling-days was 178 (70399) d and total number of catheter-indwelling-days was 368373 d. There were 563 device expires in this study, including 461 deaths (331 males and 130 females) and catheters removed due to 102 adverse events.

Comparisons of risk factors and adverse events

Univariate analysis demonstrated that the following were

Table 3 Insertion site, surgical procedure and catheter type *n* (%)

Site	Surgical procedure	Open-ended catheter	Groshong catheter	Total
Cephalic vein	Cutdown			
Right		287	620	907 (67.3)
Left		111	82	193 (14.3)
Subclavian vein	Puncture			
Right		54	87	141 (10.5)
Left		38	17	55 (4.1)
Internal jugular vein				
Right		8	1	9 (0.7)
Left		0	1	1 (0.1)
External jugular vein				
Right		4	7	11 (0.8)
Left		0	2	2 (0.1)
Femoral vein				
Right		14	9	23 (1.7)
Left		2	4	6 (0.4)
Total		518	830	1348

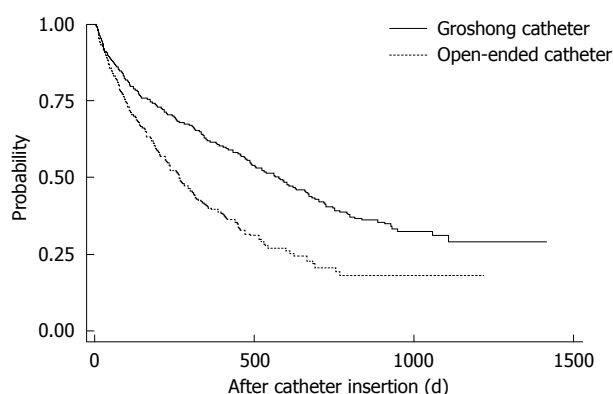


Figure 1 Kaplan-Meier survival curve showing that the Groshong catheter of the TIVAD had better survival time than open-ended catheters by log-rank test ($P < 0.001$).

significant risk factors for TIVAD failure: increasing age; male gender; and use of an open-ended catheter (Table 4). The remaining variables, such as patient origin, insertion site and malignancy type were not statistically significant. Increasing age, male gender, open-ended catheter and hematogenous malignancy [hazard ratio (HR) = 1.499, 95% CI: 1.079-2.083, $P = 0.016$] were significant risk factors by multivariate analysis for reduced TIVAD survival, although hematogenous malignancy (HR = 1.336, 95% CI: 0.966-1.849, $P = 0.080$) was not statistically significant by univariate analysis. The median numbers of catheter-indwelling-days for patients inserted with a Groshong or open-ended tube were 218 and 143 d, respectively. The log-rank test showed highly significant statistical differences between these survival curves ($P < 0.0001$) (Figure 1). Clearly, the patients inserted with open-ended catheter type had much lower survival rates than those with Groshong catheters.

The overall complications were 102 events (7.5%): 40 infection events; 47 thrombosis events; and 15 surgical complications. The overall infection rate was 0.108 events per 1000 catheter-days (40 cases, 2.96%), the thrombosis rate was 0.127 events per 1000 catheter-days

Table 4 Univariate and multivariate analyses of risk factors for TIVAD failure

	Univariate hazard ratio	95% CI	P value	Multivariate hazard ratio	95% CI	P value
Age (yr)	1.009	1.002-1.015	0.006	1.009	1.003-1.016	0.003
Sex						
Male	1.63	1.373-1.936	< 0.001	1.566	1.318-1.861	< 0.001
Female	1					
Origin of patients						
Inpatient	1					
Outpatient	0.926	0.779-1.100	0.385			
Type of catheter						
Groshong	1					
Open-ended	1.719	1.461-2.023	< 0.001	1.689	1.435-1.989	< 0.001
Insertion site						
RCV	0.891	0.754-1.053	0.179			
Others ¹	1					
Type of malignancy						
Solid	1					
Hematogenous	1.336	0.966-1.849	0.080	1.499	1.079-2.083	0.016

¹Others include left cephalic vein, right subclavian vein, left subclavian vein, femoral vein and jugular vein. RCV: Right cephalic vein.

Table 5 Comparisons of adverse events for open-ended *vs* Groshong catheter and solid *vs* hematogenous malignancy

	Open-ended catheter (n = 518)	Groshong catheter (n = 830)	P value ¹	Solid malignancy (n = 1272)	Hematogenous malignancy (n = 76)	P value ¹
Infection	14, 2.7% (0.130)	26, 3.1% (0.099)	0.651	32, 2.5% (0.091)	8, 10.5% (0.456)	< 0.001
Thrombosis	26, 5% (0.242)	21, 2.5% (0.080)	0.015	46, 3.6% (0.131)	1, 1.3% (0.057)	0.515
Surgical complication	9, 1.7% (0.083)	6, 0.7% (0.022)	0.084	12, 0.9% (0.034)	3, 3.9% (0.171)	0.048

¹ χ^2 test or Fisher's exact test. Parenthesis indicated events per 1000 catheter-days.

(47 cases, 3.48%), and the surgical complication rate was 0.04 events per 1000 catheter-days (15 cases, 1.1%).

Table 5 presents comparisons of adverse events for open-ended *vs* Groshong catheters and solid *vs* hematogenous malignancies. Open-ended catheter devices had a higher thrombosis rate than Groshong catheter devices (5% *vs* 2.5%, $P = 0.015$). Hematogenous malignancies had a higher infection rate (10.5% *vs* 2.5%, $P < 0.001$) and surgical complication rate (3.9% *vs* 0.9%, $P = 0.048$) than solid malignancies.

DISCUSSION

Notably, the TIVAD is designed to be a reliable, safe and dependable means of long-term venous access for administering chemotherapy or hyperalimentation nutrition. Several studies have documented the relative superiority of TIVADs over non-totally implanted devices^[3-6]. Nevertheless, factors affecting device survival remain a major concern. This study analyzed several risk factors including age, sex, patient origin, catheter type, insertion site and malignancy type to assess whether these factors influence the failure of TIVADs.

According to the literature, the average indwelling duration of the implanted devices varies from 61 to 512 d^[7-11]. In this series, the total number of catheter-indwelling-days was 368373 d and the median duration was 178 d.

The variable of age was a significant risk factor for TIVAD failure by univariate and multivariate analyses

(HR = 1.009, 95% CI: 1.003-1.016, $P = 0.003$), signifying that risk of TIVAD failure will increase 1.009 times for each additional year of age. Puig-la Calle *et al*^[11] reported an average of 512 catheter-indwelling-days for 123 patients with a mean age of 37 years. Hou *et al*^[10] identified an average of 395 catheter-indwelling-days for 298 patients with a mean age of 55 years. In this series, the median of catheter-indwelling-days was 178 d and mean age of 1280 patient was 60 years. Taken together, these three studies indicate that age affects TIVAD survival.

Male gender was another risk factor for TIVAD failure by univariate and multivariate analyses (HR = 1.566, 95% CI: 1.318-1.861, $P < 0.001$). Average number of catheter-indwelling-day for females (228 d) was longer than that for males (151 d). This finding may be due to a higher TIVAD failure rate for males (69%, 417/607 catheter) than females (31%, 190/607) in this series. The causes of TIVAD failure for males were 331 deaths and catheter removal due to 68 adverse events. Advanced stage of cancer with reduced life expectancy for male patients is the major reason for the decreased survival of the devices (data not shown).

The third significant risk factor was catheter type. Open-ended catheters (HR = 1.689, 95% CI: 1.435-1.989, $P < 0.001$) had shorter TIVAD survival time than closed-ended catheters (Groshong catheter). We utilized the log-rank test to compare the survival distributions of open-ended and closed-ended catheters. The results, depicted in Figure 1, show that the survival time for close-

ended catheters (Groshong catheter) is significantly better than open-ended catheters using log-rank test ($P < 0.001$). Hou *et al*^[10] reported these two catheter types had similar actuarial survival rates in their study. The Groshong catheter^[12] has a unique three-way, pressure-sensitive valve that allows infusion and blood aspiration while reducing risk of an air embolism, blood reflux, and clotting. This special design is supposed to increase TIVAD survival, which is confirmed in our study.

Hematogenous malignancy was the last risk factor analysed here. By univariate analysis, hematogenous malignancy decreased TIVAD survival, but was not statistically significant (HR = 1.336, 95% CI: 0.966-1.849, $P = 0.080$). Hematogenous malignancy became a significant risk factor by multivariate analysis (HR = 1.499, 95% CI: 1.079-2.083, $P = 0.016$) when correlated with age, sex and catheter type variables. Hematogenous malignancy has a hypercoagulable status when compared to solid tumors that is considered to be an etiology for shorter device life.

The reported total adverse event rate was 5.1% (13/296 catheters) in the study by Dillon *et al*^[13], 11% (33/298 catheters) in the study by Hou *et al*^[10], 12.8% (192/1500 catheters) in the study by Kock *et al*^[14] and 21% (14/66 catheters) in the study by Grannan *et al*^[15]. There was a 7.5% (102/1348 catheters) total adverse event rate in the present series. Groshong catheters (6.3%, 53/830 catheters) had a lower total adverse rate than the open-ended group (9.4%, 49/518 catheters), especially for thrombotic events (2.5% *vs* 5%, $P = 0.015$). This result supports the proposition that the valved tip of Groshong catheters prevents thrombotic events and prolongs catheter survival. Hematogenous malignancies had higher infection and surgical complication rates than solid malignancies ($P < 0.001$, $P = 0.048$, respectively). Vescia *et al*^[16] mentioned that sterile precautions are essential when implanting and accessing port systems; infections must be treated with adequate antimicrobial therapy and catheter-related thromboembolic complications constitute a major problem during the device life, but routine anticoagulation cannot be recommended based on the results of four clinical trials^[17-20].

Patients from an outpatient background for insertion of TIVAD had a lower risk for device failure than inpatients; however, the difference between the two groups was not statistically significant (HR = 0.926, 95% CI: 0.779-1.100, $P = 0.385$). The insertion site, i.e. right cephalic vein site or other sites, was not a significant risk factor.

In conclusion, increasing age, male gender, open-ended catheter use and hematogenous malignancy were all risk factors for reduced actuarial device survival time in this study by univariate and multivariate analyses. Close-ended (Groshong) catheters had a lower thrombotic rate than open-ended catheters. Hematogenous malignancies had higher infection and surgical complication rates than solid malignancies.

ACKNOWLEDGMENTS

The authors thank Mrs. Peggy Liu for data collection and management.

COMMENTS

Background

Central venous access is crucial for patients who have need of long-term intravenous chemotherapy, parenteral nutrition, transfusion of blood components and repetitive blood sampling. The main advantages of the totally implantable venous access device (TIVAD) are no external dressing, lower infection rates than non-totally implantable devices and allowance of patients to achieve normal physical activities.

Research frontiers

It is common practice to insert the TIVAD in cancer patients beginning a course of chemotherapy to eliminate potential peripheral venous access problems; however, risk factors impacting the survival of TIVADs remain unclear. Accordingly, the research hotspot is to investigate the risk factors for failure of the TIVAD and correlate adverse events with risk factors.

Innovations and breakthroughs

Recent reports have highlighted the relative superiority of TIVADs over non-totally implantable devices. Nevertheless, factors influencing device survival are a major concern. In this study, the authors found that increasing age, male gender, open-ended catheter use and hematogenous malignancy were significant risk factors reducing survival of TIVAD by multivariate analysis. Close-ended catheters (Groshong) had a lower thrombosis rate than open-ended catheters; hematogenous malignancies had higher infection rates than solid malignancies.

Applications

By understanding the risk factors that affect the survival of TIVADs and adverse events relating to the risk factors, this study may represent a future strategy for prolonging the survival time of TIVADs and help prevent the occurrence of adverse events when cancer patients need central venous ports for long-term chemotherapy.

Terminology

TIVAD is a port with a central venous line that does not have an external connector, instead, it has a small reservoir that is covered with silicone rubber and is implanted under the skin. Medication is administered intermittently by placing a small needle through the skin, piercing the silicone and into the reservoir.

Peer review

This is a large-scale analytic study in which authors investigated risk factors and their impact on failure of TIVADs in cancer patients receiving long-term chemotherapy. The findings suggest that increasing age, male gender, open-ended catheter and hematogenous malignancy are the factors that reduce the survival of devices. Additionally, the authors depict that close-ended catheters had lower thrombosis rates and hematogenous malignancies had higher infection rates. The results are interesting and informative, adding to existing literature.

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S- Editor Li LF L- Editor Logan S E- Editor Zheng XM



A systematic review of treating *Helicobacter pylori* infection with Traditional Chinese Medicine

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Received: June 15, 2009 Revised: August 18, 2009

Accepted: August 25, 2009

Published online: October 7, 2009

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Key words: Drug toxicity; *Helicobacter pylori*; Traditional Chinese Medicine; Treatment efficacy

Peer reviewer: Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Lin J, Huang WW. A systematic review of treating *Helicobacter pylori* infection with Traditional Chinese Medicine. *World J Gastroenterol* 2009; 15(37): 4715-4719 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4715.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4715>

Abstract

AIM: To evaluate the efficacy and safety of Traditional Chinese Medicine (TCM) in the treatment of *Helicobacter pylori* (*H. pylori*) infection.

METHODS: We electronically and manually searched electronic databases, references lists and conferences compilations, and included all randomized clinical trials comparing the treatment of *H. pylori* using TCM with proton pump inhibitor or colloidal bismuth subcitrate-based triple therapy as controls. The Jadad score was used to assess trial quality, *H. pylori* eradication rate and the incidence of side effects were taken as outcome measurements, and heterogeneity analysis, meta-analysis and funnel plot analysis were conducted.

RESULTS: Sixteen trials were included. The Jadad scores of all the trials were not more than 2. Clinical heterogeneity and substantial statistical heterogeneity existed among the trials ($P = 0.001$, $I^2 = 59\%$) and meta-analysis was not conducted. The average eradication rates following TCM and triple therapy were 72% and 78% and the incidence of side effects were 2% and 29%, respectively. The funnel plot was obviously asymmetric.

CONCLUSION: Available evidence is not convincing enough to show that TCM has the same efficacy as triple therapy in *H. pylori* treatment. TCM may be safer than triple therapy. TCM should not be recommended as monotherapy in *H. pylori* infection.

INTRODUCTION

Helicobacter pylori (*H. pylori*) plays a crucial role in the pathogenesis of chronic gastritis, peptic ulcer, gastric mucosa associated lymphoid tissue (MALT) lymphoma and gastric carcinoma. Clinical trials have shown that *H. pylori* eradication can relieve mucosa inflammation, accelerate ulcer healing, reduce complications and recurrence, alleviate MALT and decrease post-operative relapse of early stage gastric carcinoma. The eradication rates of *H. pylori* with proton pump inhibitor (PPI) or colloidal bismuth subcitrate (CBS)-based triple therapy, recommended in the Maastricht consensus report, are around 85%-90%. However, antibiotic resistance in *H. pylori* has increased over the past few years. The incidence of primary resistance to metronidazole is over 50%^[1] and to clarithromycin is about 18%^[2]. Antibiotic resistance decreases the eradication rates of triple therapies significantly. The eradication rate dropped by 38% for *H. pylori* resistance to metronidazole and by 55% for the strain resistant to clarithromycin^[3]. Additionally, the high dosages of antibiotics applied in triple therapies readily induce a high incidence of adverse effects in about 16%-29% of patients, and poor patient compliance decreases the efficacy indirectly^[4]. Researchers are exploring more effective and safer treatment regimens.

Since Warren and Marshall isolated *H. pylori* in 1982, many *in vitro* and *in vivo* studies on the treatment of *H. pylori* with Traditional Chinese Medicine (TCM) have been carried out. The *in vitro* studies showed that some

herbs such as *Radix scutellariae*, *Lonicera*, *Radix Isatidis Sen Baphicacanthi*, *Coptis chinensis Franch* and *Fructus Aurantii Immaturus* have obvious inhibitory effects on *H pylori*^[5,6]. The results of clinical studies are, however, variable. There have been no systematic reviews on the efficacy and safety of TCM in the treatment of *H pylori* infection. Therefore, we reviewed the clinical trials treating *H pylori* with TCM published between 1982 and 2008 which compared the efficacy and safety of TCM with those of triple therapies, in order to define the status of TCM in *H pylori* treatment.

MATERIALS AND METHODS

Inclusion criteria

Inclusion criteria were as follows: (1) Randomized, positive-controlled and parallel trials, irrespective of whether blinding was adopted; (2) Subjects with chronic gastritis, peptic ulcers, remnant gastritis or gastro-esophageal reflux disease who were *H pylori* positive. Subjects with bleeding ulcer or gastric cancer were excluded. (3) Determination of *H pylori* positive status was in accordance with the criteria of the 2007 3rd National Consensus Report on *H pylori* Infection (Consensus Report)^[7]; (4) The treatment groups received TCM including single herb, formulae or Chinese medicine products; (5) The control groups received the first line therapy regimens recommended in the Consensus Report; (6) Outcome measurements included *H pylori* eradication rate and the incidence of adverse effects. The definition of *H pylori* eradication must meet the requirements of the Consensus Report.

Literature searching strategy

The electronic databases of MEDLINE (1982-2008), Cochrane Controlled Trials Register (1982-2008), Wei-Pu database (1989-2008) and Wan-Fang database (1998-2008) were searched by using a combination of MESH subject headings of “*H pylori*”, “Medicine”, “Traditional Chinese” and “clinical trial” without language limitation. Reference lists from trials selected by electronic searching and conference compilations were hand searched.

Data collection and assessment

Two independent reviewers screened all retrieved trials according to the inclusion criteria. If the two reviewers disagreed, the difference was discussed. If a consensus could not be reached, a third reviewer was consulted. The quality of all the eligible trials was scored using the Jadad criteria^[8]. Allocation concealment was assessed. Central randomization, pharmacy controlled randomization or random numbers sealed in envelopes were regarded as appropriate concealment. If the method of allocation concealment was not mentioned or the subjects were allocated by the sequence produced by an open random number table, it was regarded as inappropriate concealment^[9].

Statistical analysis

H pylori eradication rate and the incidence of adverse

effects were used to evaluate the efficacy and safety of the regimens. The relative risk of *H pylori* eradication rate and the incidence of adverse effects were calculated using the original data and presented with 95% confidence interval. If there was no heterogeneity among the trials, a fixed effects model was applied in the meta-analysis. If there was heterogeneity among the trials and $I^2 < 50\%$, a random effects model was used instead in the meta-analysis. If $I^2 > 50\%$, the meta-analysis was aborted. Potential publication bias was analyzed by the funnel plot. All statistics were conducted using RevMan4.2 software.

RESULTS

Trials characteristics and quality

Three hundred and nine papers were obtained after initial searching. 293 papers were excluded after reading titles, abstracts or texts due to the non-clinical nature of the studies, repetitious publication or failure to meet the inclusion criteria. Sixteen papers^[10-25] were finally included with a total number of 1983 subjects (124/per trial). All the clinical trials were conducted in China and published in Chinese language. The characteristics of the included trials are presented in Table 1.

The Jadad scores of all the included trials were not more than two. One trial presented details of the randomization methods (random number table)^[22] while the rest did not present any such detail. All the trials were single-centered and non-blinded without reporting allocation concealment and sample size calculation. Three trials^[10,17,23] reported dropout cases and one of these trials^[23] conducted intention-to-treatment analysis.

Clinical efficacy assessment

Due to the wide variety of TCM treatment applied in each trial and the statistical heterogeneity of the trials ($\chi^2 = 36.97$, $P = 0.001$, $I^2 = 59.4\%$), the data could not be pooled to conduct a meta-analysis. The *H pylori* eradication rates of the TCM treatment in each trial are presented in Table 2. The average *H pylori* eradication rates following TCM treatment and triple therapy were 72% (36%-90%) and 78% (44%-93%), respectively. The eradication rates following *Chang Wei Qing* oral liquid and *Jian Pi Qing Hua* formula were lower than those of CBS-based triple therapies. The eradication rates following *Jian Wei Yu Yang* granule and *Wei Kang* formula were lower than those of PPI-based triple therapies. There were no differences in the eradication rates between the remaining TCM treatments and triple therapy. Worst-case scenario was performed in two trials^[10,17] which reported dropout cases and did not conduct intention-to-treat analysis. There was no significant difference between the new result and the original result.

Clinical safety assessment

Eleven trials^[10,11,14,16-20,23-25] reported adverse effects without presenting the methods of acquiring the information on adverse effects. Eight of 11 reported that TCM treatment had adverse effects with an average incidence of 2%

Table 1 Characteristics of randomized and controlled clinical trials treating *Helicobacter pylori* infection with TCM

Trials	No. of cases	Age (yr)	Gender (male) (%)	Jadad score	Regimen of TCM group	Regimen of triple therapies group
Chen <i>et al</i> ^[10] (2001)	419	23-68	62.1	2	Fixed formula × 7 d	(PPI + A + F) × 7 d
Hua <i>et al</i> ^[11] (2003)	155	19-65	54.2	2	Changweiqing oral liquid × 14 d	(CBS + A + F) × 14 d
Fan <i>et al</i> ^[12] (2006)	50	NA	NA	1	Anzhong Yin × 28 d	(CBS + A + F) × 14 d
Hua <i>et al</i> ^[13] (2006)	150	23-85	61.3	1	Jianwei Mieyou Inspissant × 60 d	(CBS + A + C) × 7 d + CBS × 49 d
Ma <i>et al</i> ^[14] (2006)	106	44.2/43.8	79.3	2	Weikang Capsule × 60 d	(RBC + A + M) × 14 d
Wang <i>et al</i> ^[15] (2006)	77	NA	47.7	1	Jianpi Qinghua formula × 30 d	(CBS + A + T) × 14 d
Wu ^[16] (2006)	71	19-65	67.6	2	Jiawei Liumo Decoction × 56 d	(PPI + A + M) × 10 d
Yang <i>et al</i> ^[17] (2006)	80	22-65	70	2	Weitongning Tab × 28 d	(CBS + A + M) × 14 d
Zhou <i>et al</i> ^[18] (2006)	56	23-70	47.2	2	Qingwei Decoction × 14 d	(PPI + A + M) × 14 d
Huang ^[19] (2007)	320	18-77	55.6	2	Maimendong Granule × 28 d	(PPI + A + M) × 14 d
Jin <i>et al</i> ^[20] (2007)	98	18-72	67.4	2	Maimendong Granule × 28 d	(PPI + A + M) × 7 d
Wang <i>et al</i> ^[21] (2008)	60	20-64	61.7	1	Mieyou Decoction × 14 d	(CBS + A + T) × 14 d
Ling <i>et al</i> ^[22] (2008)	46	33.2/35.1	71.7	2	Jianwei Yuyang Granule × 6 wk	(PPI + A + M) × 1 wk + PPI × 1 wk
Wang ^[23] (2008)	149	16-66	61.7	2	Formulae × 2 wk	(CBS + A + M) × 2 wk
Xiao <i>et al</i> ^[24] (2008)	80	19-77	62.5	2	Weiyan Decoction × 20 d	(PPI + A + C) × 7 d
Xin <i>et al</i> ^[25] (2008)	70	48.6/44.6	60	2	Weikang formula × 1 mo	(PPI + C + T) × 7 d

TCM: Traditional Chinese Medicine; PPI: Proton pump inhibitor; CBS: Colloidal bismuth subcitrate; RBC: Ranitidine bismuth citrate; A: Amoxicillin; M: Metronidazole; T: Tinidazole; C: Clarithromycin; F: Furazolidone. NA: No available.

Table 2 *H pylori* eradication rates comparison between TCM and triple therapies

Trials	TCM (n/N)	Triple therapies (n/N)	RR (95% CI)	P	Ref.
Fixed formula <i>vs</i> PPI + A + F	161/211	160/204	1.02 (0.91, 1.13)	0.60	[10]
Anzhong Yin <i>vs</i> CBS + A + F	19/30	14/20	0.90 (0.61, 1.34)	0.63	[12]
Jianwei Mieyou Inspissant <i>vs</i> CBS + A + C	60/100	22/50	1.43 (1.01, 2.03)	0.06	[13]
Weikang Capsule <i>vs</i> RBC + A + M	46/56	42/50	0.98 (0.82, 1.16)	0.80	[14]
Jiawei Liumo Decoction <i>vs</i> PPI + A + M	37/41	28/30	0.97 (0.84, 1.11)	0.64	[16]
Weitongning Tab <i>vs</i> CBS + A + M	32/40	31/40	1.03 (0.82, 1.30)	0.78	[17]
Qingwei Decoction <i>vs</i> PPI + A + M	13/29	19/27	0.64 (0.40, 1.02)	0.05	[18]
Maimendong Granule <i>vs</i> PPI + A + M	166/200	98/120	1.02 (0.91, 1.13)	0.76	[19]
Maimendong Granule <i>vs</i> PPI + A + M	43/50	40/48	1.03 (0.87, 1.22)	0.71	[20]
Mieyou Decoction <i>vs</i> CBS + A + T	22/32	19/28	1.01 (0.73, 1.43)	0.88	[21]
Formulae <i>vs</i> CBS + A + M	81/93	49/56	1.00 (0.88, 1.13)	0.94	[23]
Weiyan Decoction <i>vs</i> PPI + A + C	28/40	29/40	0.97 (0.73, 1.28)	0.80	[24]
Changweiqing oral lique <i>vs</i> CBS + A + F	53/103	36/52	0.74 (0.57, 0.96)	0.03	[11]
Jianpi Qinghua formula <i>vs</i> CBS + A + T	15/42	29/35	0.43 (0.28, 0.66)	< 0.01	[15]
Jianwei Yuyang Granule <i>vs</i> PPI + A + M	11/24	18/22	0.56 (0.35, 0.90)	0.01	[22]
Weikang formula <i>vs</i> PPI + C + T	26/40	26/30	0.75 (0.57, 0.98)	0.04	[25]

RR: Relative risk; CI: Confidence interval.

(21/903), while 10 of 11 reported that triple therapy resulted in adverse effects with an average incidence of 29% (204/697). The adverse effects associated with TCM included dizziness, loss of taste, nausea/vomiting, poor appetite, belching and loose stool/diarrhea. There were no abnormalities in routine blood tests, liver or kidney function tests in the TCM groups. Twenty subjects withdrew from the trials due to adverse effects and all were in the triple therapy groups^[10,11,17,23].

Funnel plot analysis

Funnel plot analysis was conducted using the eradication rate as the index. The figure was obviously asymmetrical (Figure 1).

DISCUSSION

Although the statistical data in this review showed that there were no differences in eradication rate between

TCM treatment and triple therapy in the majority of the trials (12/16), there is no powerful evidence to conclude that TCM treatment has the same efficacy as triple therapy in the treatment of *H pylori* infection due to the following reasons:

Poor quality of the clinical trials

All 16 trials were of poor quality and did not meet the requirements of the CONSORT statement^[26]. Although these trials claimed to adopt randomization, only one trial presented details of the randomization method and the remaining trials did not give any details of the randomization method. None of the trials described the methods of allocation concealment. Thus, whether the randomization was truly or effectively conducted in these trials is doubtful. Inappropriate randomization or allocation concealment can lead to selection bias. All trials were single-centered and non-blinded, and the treatment courses of the study groups were longer

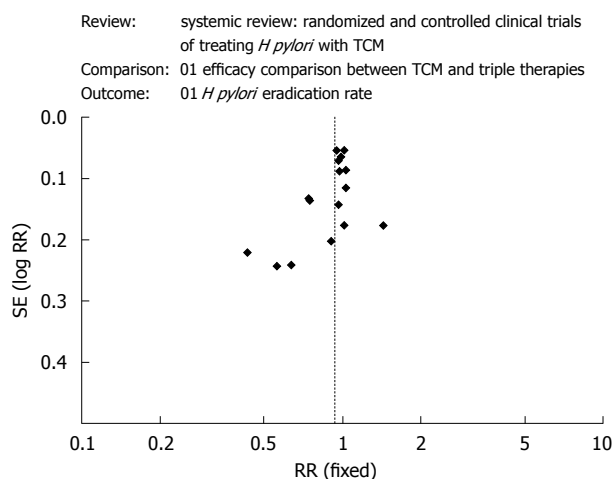


Figure 1 Funnel plot analysis of clinical trials treating *H pylori* with TCM. RR: Relative risk.

than those of the control groups in some trials, which may have resulted in performance bias. Dropout cases were reported in only three of 16 trials and were not mentioned in the other trials. It is not clear whether these trials had any dropout cases or whether they were simply not reported. Having dropout cases without reporting can result in attrition bias. Selection bias, performance bias and attrition bias may overestimate the efficacy of treatment groups. Additionally, none of the trials showed the calculation of sample size, thus, whether the sample size met the statistical requirement or the reliability of the statistical results cannot be judged. Therefore, confidence in the results obtained from these poor quality trials is questionable.

Asymmetrical funnel plot

The asymmetry of the funnel plot may have been caused by the poor quality of the trials and publication bias. All 16 trials were conducted in China and published in Chinese language. Fourteen trials were published in TCM journals, one in a journal of integrated Chinese and Western medicine and one in a college journal. TCM journals are inclined to publish only positive results showing efficacy of TCM treatment. Therefore, when we searched the literature, most of the papers we found reported positive results, with only a few presenting negative results.

Heterogeneity

The TCM formulae used in the 16 trials were composed of various herbs which can clear heat, strengthen the spleen, regulate *Qi* or promote blood circulation. The composition of the formula and the dosage of each herb were different in each trial. The treatment courses also varied in each trial, from 1 wk to 2 mo. This resulted in clinical heterogeneity. Heterogeneity analysis also showed that there was substantial statistical heterogeneity. Due to the limited number of trials and the variety of composition and treatment courses, these trials could not be classified into subgroups for meta-analysis. An overall comparison between TCM and triple therapy could not be conducted.

Eight of 11 trials showed that the incidence of adverse effects following TCM treatment was lower than those following triple therapy, while three trials showed that there was no difference between them. Although non-blinded and lacking in detail on how the information on adverse effects was obtained, due to the obvious difference in the incidence of adverse effects between the two groups (2% *vs* 29%), we speculate that TCM treatment might be safer than triple therapy.

On the basis of the above, we cannot conclude that TCM treatment has the same efficacy as triple therapy in the treatment of *H pylori* infection. TCM should not be recommended as monotherapy in the treatment of *H pylori* infection until stronger positive evidence is obtained. However, neither can we completely ignore the efficacy of TCM in the treatment of *H pylori* infection because of the poor quality of the trials. We suggest that a scientific proposal should be designed and clinical trials performed strictly in accordance with the requirements of evidence-based medicine, in order to truly show and objectively assess the efficacy of TCM in *H pylori* treatment. Additionally, based on the tenet that medical study originates from clinical practice and serves clinical practice, studies on TCM in the treatment of *H pylori* infection should focus on the problems faced in western medicine, such as antibiotic resistance, the high incidence of adverse effects and high cost of the treatment, in addition to comparing the efficacy between TCM and Western medical treatment.

COMMENTS

Background

As antibiotic resistance to *Helicobacter pylori* (*H pylori*) has increased over the past few years, the eradication rates following triple therapies have decreased. New approaches to eradicate *H pylori* are under development.

Research frontiers

Some herbs show obvious inhibitory effects on *H pylori* *in vitro*. Traditional Chinese Medicine (TCM) alone or integrated with western medicine have been used to treat *H pylori* infection in many clinical trials. However, the results of these trials are controversial and the efficacy of TCM in *H pylori* treatment is indefinite.

Innovations and breakthroughs

This is the first systematic review to assess the efficacy of TCM in the treatment of *H pylori* infection. It truly presents the quality of the clinical trials using TCM in *H pylori* treatment. The authors objectively concluded that the validity of TCM in the treatment of *H pylori* infection could not be determined due to the poor quality of the trials.

Applications

This study suggested that TCM should not be recommended as monotherapy to treat *H pylori* infection and the quality of TCM clinical trials need to be improved in the future.

Terminology

A systematic review is a summary of research that uses explicit methods to perform a thorough literature search and critical appraisal of individual studies to identify valid and applicable evidence.

Peer review

This is a good paper.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM



BRIEF ARTICLES

Correlation between anti-fibrotic effect of baicalin and serum cytokines in rat hepatic fibrosis

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Supported by Grants from National Key Technologies R&D Program of 11th five-year plan, 2009ZX09501-015

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Received: July 10, 2009 Revised: August 22, 2009

Accepted: August 29, 2009

Published online: October 7, 2009

Abstract

AIM: To investigate the correlation between the antifibrotic effect of baicalin and serum cytokine production in rat hepatic fibrosis.

METHODS: Forty male Sprague-Dawley rats were divided randomly into four groups: normal control group, model group, baicalin-treated group, and colchicine-treated group. Except for the normal control group, all rats in the other groups were administered with carbon tetrachloride to induce hepatic fibrosis. At the same time, the last two groups were also treated with baicalin or colchicine. At the end of the 8 wk, all animals were sacrificed. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), transforming growth factor (TGF)- β 1, tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-10 were measured. Liver index, hepatic hydroxyproline content and the degree of liver fibrosis were also evaluated.

RESULTS: The levels of ALT, AST and liver index in the baicalin-treated group were markedly lower than

those in the model group (ALT: 143.88 ± 14.55 U/L vs 193.58 ± 24.35 U/L; AST: 263.66 ± 44.23 U/L vs 404.37 ± 68.29 U/L; liver index: 0.033 ± 0.005 vs 0.049 ± 0.009 , $P < 0.01$). Baicalin therapy also significantly attenuated the degree of hepatic fibrosis, collagen area and collagen area percentage in liver tissue ($P < 0.01$). Furthermore, the levels of serum TGF β 1, TNF- α and IL-6 were strikingly reduced in the baicalin-treated group compared with the model group, while the production of IL-10 was up-regulated: (TGF- β 1: 260.21 ± 31.01 pg/mL vs 375.49 ± 57.47 pg/mL; TNF- α : 193.40 ± 15.18 pg/mL vs 260.04 ± 37.70 pg/mL; IL-6: 339.87 ± 72.95 pg/mL vs 606.47 ± 130.73 pg/mL; IL-10: 506.22 ± 112.07 pg/mL vs 316.95 ± 62.74 pg/mL, $P < 0.01$).

CONCLUSION: Baicalin shows certain therapeutic effects on hepatic fibrosis, probably by immunoregulating the imbalance between profibrotic and antifibrotic cytokines.

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Key words: Baicalin; Hepatic fibrosis; Hepatic stellate cell; Cytokines

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Peng XD, Dai LL, Huang CQ, He CM, Chen LJ. Correlation between anti-fibrotic effect of baicalin and serum cytokines in rat hepatic fibrosis. *World J Gastroenterol* 2009; 15(37): 4720-4725 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4720.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4720>

INTRODUCTION

Hepatic fibrosis is a common pathological process of chronic liver injury, regardless of etiology, and its progression leads to cirrhosis and liver cancer^[1]. Despite extensive efforts, its etiology and pathogenesis remain unclear, and effective therapy with limited side effects is still lacking^[2]. Baicalin is a major bioactive flavonoid contained in dried roots of *Scutellaria baicalensis*

Georgi (common name: *Huangqin* in China, a traditional Chinese herbal medicine) and it possesses a multitude of pharmacological activities. For instance, Baicalin exerts the inhibitory effects against several virus including influenza virus, human T cell leukemia virus and acquired human immunodeficiency virus type I [3-5]. It can act as potent anti-inflammatory, anti-allergic and anti-bacterial agent in a variety of inflammatory diseases [6-8]. It may also be potentially useful in the treatment of prostate and bladder cancers as well as hepatoma *via* multiple cellular mechanisms [9-11]. Most importantly, previous studies show that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injury induced by iron overload and CCl₄, suggesting that it is a potent free radical scavenger and hepatoprotective drug [12,13]. However, the exact mechanisms of anti-fibrotic effect of baicalin remain unclear. Therefore, it is necessary to be further elucidated.

CCl₄-induced hepatic fibrosis is a well-established animal model to study the pathogenesis and therapy of chronic liver injury. Zhang *et al.* [14] have reported that several profibrotic cytokines, including transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α and interleukin (IL)-6, play an important role in the initiation and perpetuation of CCl₄-induced liver fibrosis, whereas IL-10 plays an antifibrogenic role by counterbalancing the former effects. This study aimed to further investigate the effect of baicalin on hepatic fibrosis induced by CCl₄ and its relationship with the expression of TGF-β1, TNF-α, IL-6 and IL-10.

MATERIALS AND METHODS

Reagents and rats

Baicalin (7-glucuronic acid, 5,6-dihydroxyflavone, CAS No.: 21967-41-9) was provided by Sichuan Guanghan Bencao Plant Chemical Co., Ltd (Sichuan, China), and purity was assessed by HPLC (> 98%). Colchicine was purchased from Xiamen Sanland Chemical Co., Ltd (Fujian, China). CCl₄ was obtained from Chongqing Chemical Reagent Co., Ltd (Chongqing, China). Male Sprague-Dawley (SD) rats weighing 150-180 g were purchased from the Experimental Animal Center of Third Military Medical University. All studies involving rats were approved by the Institutional Animal Care and Use Committee.

Induction of liver fibrosis and baicalin treatment

Forty male SD rats were divided randomly into four groups: normal (*n* = 9); model (*n* = 11); baicalin-treated (*n* = 10); and colchicine-treated (*n* = 10). Except for the normal control group, all rats in the other groups were treated with subcutaneous injection of 40% CCl₄ (initial dose of 0.5 mL/kg, followed by 0.3 mL/kg), mixed with vegetable oil, twice weekly for 8 wk. The latter two groups were also treated with baicalin (70 mg/kg, dissolved in sterile saline water, intraperitoneal injection, once daily), or colchicine (50 μg/kg, dissolved in sterile saline water, intraperitoneal injection, once daily) on the same day as CCl₄ administration and continued for the

Table 1 Level of liver index and serum AST, ALT in different treatment groups (mean ± SD)

Group	<i>n</i>	Liver index	ALT (U/L)	AST (U/L)
Normal	9	0.026 ± 0.004	114.50 ± 8.16	183.09 ± 26.70
Model	7	0.049 ± 0.009 ^b	193.58 ± 24.35 ^b	404.37 ± 68.29 ^b
Baicalin	9	0.033 ± 0.005 ^{b,d}	143.88 ± 14.55 ^{b,d}	263.66 ± 44.23 ^{b,d}
Colchicine	9	0.031 ± 0.004 ^{a,d}	167.60 ± 21.66 ^{b,c}	325.61 ± 52.83 ^{b,c}

^a*P* < 0.05, ^b*P* < 0.01 *vs* normal control group; ^c*P* < 0.05, ^d*P* < 0.01 *vs* model group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

8-wk experimental period. The two drug doses were selected based on a previous study [13]. Simultaneously, normal control and model groups were intraperitoneally administered with the same volume of vehicle (sterile saline water) once daily. At the end of the 8-wk experimental period, all animals were anesthetized with 3% chloral hydrate and dissected. Blood and liver were obtained for further analysis.

Measurement of serum aspartate aminotransferase (AST)

Serum AST and alanine aminotransferase (ALT) levels were measured using an automated analyzer of biochemistry (Hitachi 7170, Tokyo, Japan) according to the manufacturer's instructions.

Liver index calculation

Liver index was measured according to the formula: (rat liver weight/rat weight) × 100% [15].

Histopathology

Samples were obtained from the same liver lobe in all animals and fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) or van gieson (VG) stain.

The degree of liver fibrosis was evaluated on HE-stained sections as described previously [16,17]. The collagen content of the sections was also determined on VG-stained sections by a computer image analysis system (CM2000B, Beijing University of Aeronautics & Astronautics, China). Five random fields were chosen in each section and the amount of total collagen was detected in the area stained by VG, and expressed as percentage relative to the total area [18].

ELISA for serum TGF-β1, TNF-α, IL-6 and IL-10

Cytokine levels in the serum samples were measured by a commercially available ELISA kit (Biosources, San Jose, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS for Windows, version 13.0 (Chicago, IL, USA). Parametric data were analyzed statistically by one-way ANOVA followed by post-hoc tests when appropriate. Degree of hepatic fibrosis was analyzed by Kruskal-Wallis nonparametric test. Data were expressed as the means ± SD. A significant difference was defined as *P* < 0.05.

Table 2 Degree of liver fibrosis in different treatment groups

Group	n	Degree of hepatic fibrosis					Average
		0	I	II	III	IV	
Normal	9	9	0	0	0	0	0
Model	7	0	0	0	4	3	3.43 ^b
Baicalin	9	0	3	4	2	0	1.89 ^{b,d}
Colchicine	9	0	3	5	1	0	1.78 ^{b,d}

^b*P* < 0.01 vs normal control group; ^d*P* < 0.01 vs model group.**Table 3** Comparison of collagen area and collagen area percentage in liver tissue from rats of different treatment groups (mean ± SD)

Group	n	Collagen area (μm ²)	Collagen area percentage
Normal	9	993.54 ± 145.31	1.97 ± 0.30
Model	7	2599.99 ± 488.32 ^b	10.15 ± 2.87 ^b
Baicalin	9	1407.74 ± 284.49 ^{b,d}	4.54 ± 2.08 ^{b,d}
Colchicine	9	1396.00 ± 276.07 ^{b,d}	4.65 ± 2.11 ^{b,d}

^b*P* < 0.01 vs normal control group; ^d*P* < 0.01 vs model group.**Table 4** Serum levels of TGF-β1, TNF-α, IL-6 and IL-10 in rats in the different treatment groups (mean ± SD) (pg/mL)

Group	n	TGF-β1	TNF-α	IL-6	IL-10
Normal	7	199.78 ± 18.92	157.62 ± 11.77	187.98 ± 51.97	700.52 ± 138.63
Model	7	375.49 ± 57.47 ^b	260.04 ± 37.70 ^b	606.47 ± 130.73 ^b	316.95 ± 62.74 ^b
Baicalin	7	260.21 ± 31.01 ^{b,d}	193.40 ± 15.18 ^{b,d}	339.87 ± 72.95 ^{b,d}	506.22 ± 112.07 ^{a,d}

^a*P* < 0.05, ^b*P* < 0.01 vs normal control group; ^d*P* < 0.01 vs model group.

RESULTS

Animals

Irritability, aggression and weight loss were present predominantly in the model group. At the end of the 8-wk experimental period, no death was found in the normal control group. There were four deaths in the model group, one in the colchicine-treated group, and one in the baicalin-treated group.

Liver index and serum aminotransferases

Liver index in the normal control group was 0.026 ± 0.004. However, 8 wk after CCl₄ injection, the liver index increased markedly. The increase was significantly attenuated by baicalin or colchicine treatment (*P* < 0.01, Table 1).

We then measured serum aminotransferase activity in different experimental groups. The levels of serum AST and ALT were significantly increased in the model group compared with those in the normal control group. In contrast, baicalin or colchicine treatment significantly suppressed upregulation of these parameters induced by CCl₄ (*P* < 0.05 or *P* < 0.01, Table 1).

Histopathology

Using HE staining, we observed that the liver tissue in normal control rats showed normal lobular architecture with central veins and radiating hepatic cords. However, liver sections taken from rats in the model group exhibited more inflammatory infiltration, steatosis, hepatocyte coagulative necrosis and fibrous septa compared with the normal control rats after 8 wk of CCl₄ treatment. In contrast, baicalin or colchicine treatment markedly ameliorated these histopathological changes (Figure 1A-D). The results were further supported by a significantly decreased staging score of hepatic fibrosis after baicalin or colchicine therapy (*P* < 0.01, Table 2).

We evaluated by VG staining the collagen level in the liver tissue from different treatment groups. Compared with the normal control group, both collagen area and collagen area percentage were significantly increased in the model group. The increases were reduced by baicalin or colchicine treatment, similar to the changes in hepatic histological examination (*P* < 0.01; Figure 1E-H, Table 3). Therefore, the above findings show that baicalin can prevent CCl₄-induced hepatic fibrosis in rats.

Effect of baicalin on serum TGF-β1, TNF-α, IL-6, IL-10 production

As shown in Table 4, the levels of serum TGF-β1, TNF-α and IL-6 in the model group were significantly higher than those in the normal control group (*P* < 0.01). Upregulation was markedly inhibited by treatment with 70 mg/kg baicalin (*P* < 0.01). On the other hand, IL-10 production in the model group was sharply decreased compared with that in the normal control group (55% reduction, *P* < 0.01). However, baicalin therapy significantly recovered the decrease induced by CCl₄ (*P* < 0.01).

DISCUSSION

In the present study, baicalin significantly lowered the levels of serum ALT, AST and liver index, reduced histological changes of liver fibrosis, suppressed the expression of cytokines, including TGF-β1, TNF-α and IL-6, and improved significantly the serum level of IL-10. Furthermore, our previous study showed that the increases of several fibrosis indices including serum hyaluronic acid, type IV collagen and hepatic hydroxyproline content after the CCl₄ injection can be notably inhibited by baicalin treatment^[13]. The above findings demonstrated that baicalin can effectively prevent CCl₄-induced hepatic fibrosis in rats and regulate the production of cytokines correlated with fibrosis.

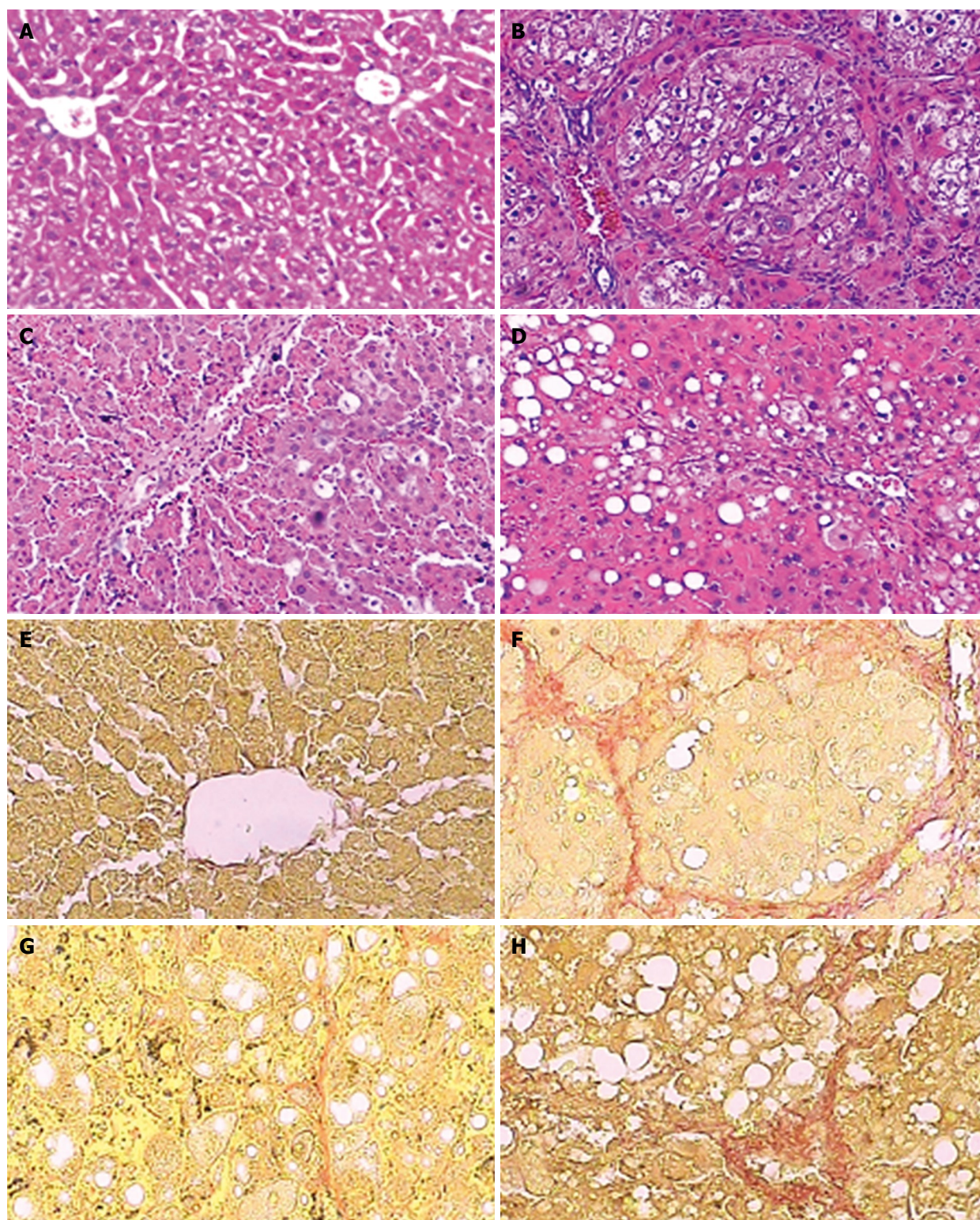


Figure 1 Representative pathological changes in liver sections taken from four experimental groups (A-D: HE, $\times 100$; E-H: VG, $\times 200$). A, E: Normal; B, F: Model; C, G: Baicalin; D, H: Colchicine.

Carbon tetrachloride (CCl_4), is a known hepatotoxin that can cause liver necrosis, fibrosis and cirrhosis when administered repeatedly. Hepatotoxicity is thought to involve two phases^[19,20]. The initial phase involves bioactivation by a microsomal cytochrome-P450-dependent monooxygenase system, which results in the formation of free radicals and oxidative

stress/lipid peroxidation which exhibits the increase of malondialdehyde (MDA) amounts and decrease of superoxide dismutase (SOD) levels^[19,21]. The second step involves the activation of Kupffer cells, which is accompanied by the production of profibrotic mediators such as TGF- β 1, TNF- α and IL-6^[22]. Hepatic stellate cells (HSC), activated by pro-fibrotic factors, lose

vitamin A and transform into myofibroblasts (MFB), expressing α -smooth muscle actin (α -SMA) and thus gaining the function of contractility, proliferation and fibrogenesis^[23,24]. In this study, we observed that baicalin significantly reduced the increase in profibrotic cytokines such as TGF- β 1, TNF- α and IL-6 induced by CCl₄. The reduction in profibrotic cytokines may be correlated closely with previous results that baicalin has good radical scavenging action (lessening the MDA level and activating the SOD activity) and can thus reduce the production of activated Kupffer cells^[12,13]. The down-regulation of pro-fibrotic cytokines induced by baicalin treatment then significantly inhibits the activation and proliferation of HSC and enhances HSC apoptosis *in vitro* or *vivo* studies^[13], which results in the extenuation of hepatic fibrosis. Thus, the reduction of pro-fibrotic cytokines such as TGF- β 1, TNF- α and IL-6 levels is one important mechanism associated with anti-fibrotic effect of baicalin.

IL-10 is a pluripotent cytokine produced by many activated immune cell types, including T-helper cells, B cells, macrophages, monocytes and keratinocytes^[25]. Recent studies have indicated that IL-10 might play an important role in antifibrogenesis during CCl₄-induced hepatic fibrogenesis^[26-28]. Our study showed that the level of circulating IL-10 in the model group was lower than that in the normal control group, which was consistent with a previous study^[14]. In contrast, baicalin significantly restored the decrease in IL-10 content induced by CCl₄, probably contributing to the antifibrotic effect of baicalin.

In conclusion, baicalin has significant antifibrogenic effects on CCl₄-induced liver fibrosis in rats. In addition to the inhibition of HSC activation and lipid peroxidation, as previously reported, immunoregulation of the imbalance between profibrotic and antifibrotic cytokines is one of the most important factors involved in the preventive effect of baicalin on CCl₄-induced liver fibrosis. The exact molecular mechanisms remain to be explored.

COMMENTS

Background

Hepatic fibrosis is a common pathological process of chronic liver injuries, regardless of etiology, and its progression leads to cirrhosis and liver cancer. Despite extensive efforts, its etiology and pathogenesis remain unclear and effective therapies with limited side effects are still deficient. Baicalin is a flavonoid purified from the medicinal plant *Scutellaria baicalensis* Georgi, a well known Traditional Chinese Medicine. The previous studies show that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injuries induced by iron overload and CCl₄, however, its exact mechanisms remain unclear.

Research frontiers

Baicalin is a flavonoid purified from the medicinal plant *Scutellaria baicalensis* Georgi, a well known Traditional Chinese Medicine. In the area of prevention of hepatic fibrosis with baicalin, one of research hotspots is that how is the actual mechanism of anti-fibrotic effect of baicalin.

Innovations and breakthroughs

The previous studies have shown that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injuries induced by CCl₄. In this study, the effect of baicalin on hepatic fibrosis induced by CCl₄

and its relationship with the expression of pro-fibrotic and anti-fibrotic cytokines were first investigated. The levels of liver index and serum aminotransferases in baicalin-treated group were markedly lower than those in model group. Baicalin therapy also significantly attenuated the degree of hepatic fibrosis, collagen area and collagen area percent in liver tissue. Furthermore, the levels of serum transforming growth factor- β 1, tumor necrosis factor- α and interleukin (IL)-6 were strikingly reduced in baicalin-treated group compared with model group while the production of IL-10 was up-regulated. The above results show that baicalin has certain therapeutic effects on hepatic fibrosis probably by immunoregulating the imbalance between pro-fibrotic and anti-fibrotic cytokines.

Applications

The study demonstrates that baicalin is a good hepatoprotective drug for preventing and treating human liver fibrosis probably by immunoregulating the imbalance between pro-fibrotic and anti-fibrotic cytokines.

Terminology

Hepatic fibrosis is characterized by elevated deposition and altered composition of extracellular matrix, which is a common stage in most chronic liver injuries. Baicalin is a bioactive anti-inflammatory flavone purified from the medicinal plant *Scutellaria baicalensis* Georgi, a well known Traditional Chinese Medicine.

Peer review

The authors analyzed the preventative effects and mechanism of baicalin in the treatment of liver fibrosis in rats. The results are interesting and suggest that baicalin may be a clinical useful agent for preventing and treating human liver fibrosis.

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S- Editor Li LF L- Editor Kerr C E- Editor Yin DH



CASE REPORT

Tumor lysis syndrome after transarterial chemoembolization of hepatocellular carcinoma: Case reports and literature review

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Received: June 28, 2009

Revised: August 17, 2009

Accepted: August 24, 2009

Published online: October 7, 2009

Abstract

Tumor lysis syndrome (TLS) is a potentially lethal complication in cancer therapy. It may occur in highly sensitive tumors, especially in childhood cancer and leukemia, whereas, it is rare in the treatment of solid tumors in adults. TLS results from a sudden and rapid release of nuclear and cytoplasmic degradation products of malignant cells. The release of these can lead to severe alterations in the metabolic profile. Here, we present two cases of large hepatocellular carcinoma (HCC) treated by transarterial chemoembolization (TACE) that resulted in TLS. Although TLS rarely happens in the treatment of adult hepatic tumor, only a few cases have been reported. We should keep in mind that all patients with HCC, particularly those with large and rapidly growing tumors, must be closely watched for evidence of TLS after TACE.

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Key words: Hepatocellular carcinoma; Therapeutic chemoembolization; Tumor lysis syndrome

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Hsieh PM, Hung KC, Chen YS. Tumor lysis syndrome after transarterial chemoembolization of hepatocellular carcinoma: Case reports and literature review. *World J Gastroenterol* 2009; 15(37): 4726-4728 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4726.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4726>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm worldwide, and the third most common cause of cancer-related death. Despite the development of several methods for unresectable HCC, transarterial embolization (TAE) is the most widely used^[1]. Although TAE is an effective modality, there are several potential side effects such as hepatic failure, internal bleeding, liver abscess, biliary tract injury, and renal failure^[2]. Among these varied complications, tumor lysis syndrome (TLS) is easily neglected and may be fatal.

TLS was first reported in 1929, and is defined by the release of intracellular components into the bloodstream as a result of massive lysis of malignant cells after effective therapy. The release of these components can cause severe metabolic alterations, characterized by hyperuricemia, hyperkalemia, hyperphosphatemia, and hypocalcemia^[3-5]. Hyperuricemia is caused by purine degradation and can lead to precipitation of uric acid crystals in the kidney collecting tubules, which results in obstructive nephropathy^[6]. Hyperkalemia, following potassium effusion from the cytoplasm, may lead to cardiac arrhythmia and arrest. Therefore, TLS can lead to acute renal failure and can be life-threatening, if it is not recognized early and treated. It is most commonly observed following chemotherapy for high-grade lymphoproliferative malignancy, such as acute lymphocytic leukemia and Burkitt lymphoma^[7,8]. Although TLS also has been reported in many different types of solid tumors, including lung and breast carcinoma^[9-11], it is still very rare in the treatment of HCC with transarterial chemoembolization (TACE). As far as we know, only a few cases have been reported in the English-language literature^[12,13].

Here, we report two cases of acute TLS in patients with HCC following TACE, and review the literature.

CASE REPORT

Case 1

A previously well 76-year-old woman suffered from epigastric fullness and dull pain for about 1 mo. Abdominal sonography revealed a large liver tumor that occupied the majority of the right lobe (Figure 1A), and biopsy of the tumor showed HCC. TACE with 20 mg adriamycin (Figure 1B) was performed during admission.

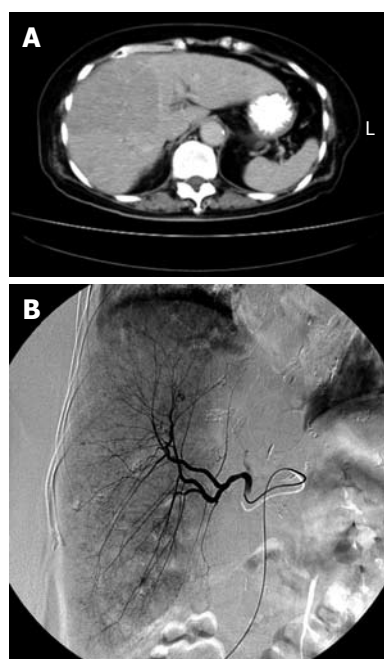


Figure 1 Computed tomography scan obtained before TACE showed a large HCC over the right lobe (A), and TACE revealed hypervascularization of the tumor (B).

The patient developed acute renal insufficiency on the next day. Contrast-induced acute renal failure was suspected in the beginning, but the symptom did not improved after adequate resuscitation. On the third day post-TACE, the acute renal failure worsened. In addition, hyperuricemia (16.6 mg/dL), hyperkalemia (5.7 mEq/L) and hypocalcemia (6.8 mg/dL) were noted later. Under suspicion of TLS, oral allopurinol and urine alkalization, combined with hemodialysis, were given immediately. The patient's renal function stabilized and the urine output increased progressively after treatment. Unfortunately, severe aspiration pneumonia developed during this period, and the patient expired because of sepsis on day 17 after TACE.

Case 2

A 56-year-old male patient was a hepatitis B virus carrier. Regular surveillance revealed a large hepatic tumor over segment 6 and a high α -fetoprotein level (247 ng/dL). Under suspicion of HCC, he was admitted for further treatment. After discussion with the hepatobiliary team, TACE (10 mg lipiodol + 20 mg adriamycin) was suggested as the treatment of choice. Oliguria occurred on the same night. The previous experience reminded us about the possibility of TLS in such a situation. We checked the laboratory profile for TLS immediately. Hyperuricemia (15.1 mg/dL), hyperphosphatemia (4.7 mg/dL) and hypocalcemia (6.6 mg/dL) were noted, and TLS was diagnosed. After standard treatment including oral allopurinol and urine alkalization, this patient's symptoms improved dramatically. Finally, he was discharged on day 6 post-TACE without any other complication.

DISCUSSION

HCC is the fifth most common neoplasm in worldwide, and the third most common cause of cancer-related

death. Only a minority of HCCs are diagnosed at an early stage for potentially curative therapy such as surgical resection or local ablation^[1]. Several methods have been developed for unresectable HCC, including radiotherapy, chemotherapy and TACE. Among the various treatments, TACE has shown evidence of a beneficial survival effect compared with other methods^[1,2]. As the most widely used treatment for advanced HCC, TACE still has some complications. Common adverse effects of TACE include liver dysfunction, post-embolization syndrome, hepatic infarction, and liver abscess. Besides these, TLS is a very rare and easily neglected complication.

The first two cases of TACE-induced TLS were reported by Burney in 1998, and both had large HCCs (> 5 cm in size)^[12]. As in our situation, the first case was diagnosed too late and the patient died. The second patient's syndrome was detected early and treated appropriately. Another case report was published by Sakamoto *et al*^[13]. This patient had a large HCC > 20 cm in size. After treatment with TACE, the patient also died of acute TLS. Both authors concluded that TLS is a possible and lethal complication of TACE for large HCC.

TLS is a known consequence of treatment of hematological malignancies such as leukemia and lymphoma^[7,8]. In general, TLS is less likely to occur in adult patients with solid tumors. However, some solid malignancies in adults, such as breast cancer, small cell lung cancer, seminoma, metastatic Meckel's cell tumor, medulloblastoma, and even hepatoma have been reported to be the cause of TLS^[9-11]. TLS may occur after various treatments of HCC, including radiofrequency ablation^[14], oral thalidomide^[15] and TACE^[12,13].

TLS arises from spontaneous cell death or within the first few days after cytotoxic therapy, and results from rapid tumor destruction. It leads to hyperuricemia, hyperkalemia, hyperphosphatemia, hypocalcemia, or often, acute renal failure^[3-6]. Early identification of patients at risk and prevention of TLS development is critical. Acute renal failure may occur from intravascular volume depletion or from deposition of uric acid and calcium phosphate crystals in the renal tubules. Without prompt and proper management, derangements of TLS can result in death, as in case 1. Early recognition and treatment will reduce the risk of this fatal complication. For early recognition, it is essential to monitor patients at high risk, especially during the early course of treatment. Traditionally, patients with large tumor burdens and/or rapidly dividing tumors are at greatest risk for TLS. Other risk factors include chemosensitive tumors, large area of tumor necrosis, and pretreatment renal dysfunction. Serum potassium, uric acid, calcium, phosphate and lactate dehydrogenase level, and renal function should be monitored intensively in these high-risk patients after cancer therapy^[3-5].

According to the Cairo-Bishop classification, TLS is defined as a 25% change or a level above or below normal for any two or more serum values of uric acid, potassium, phosphate, and calcium within 7 d after cancer therapy^[5] (Table 1). The principles of treatment of TLS

Table 1 Cairo-Bishop definition of TLS

Uric acid	≥ 8.0 mg/dL or 25% increase from baseline
Potassium	≥ 6.0 mEq/L or 25% increase from baseline
Phosphorus	≥ 4.5 mg/dL or 25% increase from baseline
Calcium	≥ 7.0 mg/dL or 25% decrease from baseline

should address three critical areas: hydration, correction of metabolic abnormalities, and aggressive treatment of renal failure. Aggressive fluid administration has been recommended in all high-risk patients. Volume expansion with isotonic saline can reduce serum concentrations of uric acid, phosphate and potassium. As renal blood flow, glomerular filtration rate, and urine output are all increased, the concentration of solutes in the renal tubules is decreased, which makes precipitation less likely^[16]. Prevention and treatment of abnormal metabolites are the next step, especially hyperuricemia. Uric acid crystals can precipitate in renal tubules and cause obstructive nephropathy and renal failure. Oral or intravenous allopurinol (100 mg/m² every 8 h) is recommended for the treatment of hyperuricemia. Recently, another urate oxidase (rasburicase; 0.05-0.2 mg/kg) was demonstrated to be more effective than allopurinol for the treatment of hyperuricemia^[3-6]. If obstructive nephropathy persists and progresses to acute renal failure, aggressive hemodialysis should not be delayed^[16].

Oliguria is not a rare occurrence post-TACE. The most common cause is radiocontrast-induced renal insufficiency. However, oliguria also can be one of the early clinical signs of TLS^[5]. The incidence of acute renal insufficiency caused by TACE *per se* has been shown to be 3%-8% for each treatment session^[17]. It is much more common than that induced by TLS. Adequate hydration is sufficient to overcome this complication. Therefore, many physicians may misdiagnose oliguria as a sign of contrast-induced renal insufficiency and delay proper treatment. Hydration alone is not sufficient for the treatment of TLS. Correction of abnormal metabolites, such as reduction of uric acid level, is another essential feature. If we overlook the potential risk of TLS, the results can be tragic.

In conclusion, although rare, TLS can occur after TACE of HCC in adults. Pretreatment evaluation of risk factors, including large tumor size, rapid tumor growth, and renal insufficiency should be completed. Close monitoring after TACE in these high-risk patients is warranted. When TLS develops, effective treatment, including adequate hydration, oral medication (allopurinol

and/or urate oxidase) or hemodialysis, should be undertaken immediately.

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S- Editor Tian L L- Editor Kerr C E- Editor Zheng XM



A rare case of periampullary carcinoma with ectopic ending of Vater's ampulla

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Received: July 21, 2009 Revised: August 10, 2009

Accepted: August 17, 2009

Published online: October 7, 2009

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Jin SG, Chen ZY, Yan LN, Zeng Y, Huang W, Xu N. A rare case of periampullary carcinoma with ectopic ending of Vater's ampulla. *World J Gastroenterol* 2009; 15(37): 4729-4731 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4729.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4729>

Abstract

A 71-year-old woman was referred to our department complaining of painless progressive jaundice for the last 3 mo. Magnetic resonance imaging and magnetic resonance cholangiopancreatography (MRCP) showed the ectopic hepatopancreatic ampulla draining into the fourth part of the duodenum adjacent to the duodenojejunal flexure; the irregular morphology of the duodenojejunal flexure likely due to a soft tissue mass. Laparotomy confirmed the presence of the abnormal ampulla of Vater located at the fourth part of the duodenum and a soft tissue tumor about 6 cm × 5 cm × 5 cm with a peduncle adjoining the ampulla. Resection of the tumor, including some peripheral tissue, and a Roux-Y loop anastomosis choledochojejunostomy were performed. Pathological examination indicated an intestinal villous adenoma accompanied by severe dysplasia and focal canceration. Periampullary carcinoma with ectopic ending of the Vater's ampulla into the fourth part of the duodenum is rather rare. The embryonic genetic background of this anomaly has not yet been fully explained. It is worth mentioning that MRCP is useful for demonstrating anomalies and anatomic variants of the biliary tract system and pancreatic duct.

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Key words: Ectopia; Ampulla of Vater; Periampullary carcinoma; Magnetic resonance cholangiopancreatography; Treatment

Peer reviewer: Kazuhiro Hanazaki, MD, Professor and

INTRODUCTION

The usual location of the major papilla of Vater is in the mid-portion of the descending duodenum, closer to the posterior wall than the anterior wall. On rare occasions it may be found in other sites, along a line extending from the stomach to the fourth part of the duodenum^[1-5]. Here we present a case of periampullary carcinoma combined with ectopic opening of the Vater's ampulla into the fourth part of the duodenum and a low confluence of the cystic duct into the common bile duct. To our knowledge, such a case has never been described previously, so the pathogenesis, diagnosis and treatment will be discussed in this report.

CASE REPORT

A 71-year-old woman was referred to our department complaining of painless progressive jaundice for the last 3 mo. Hypertension and diabetes mellitus were diagnosed 10 years previously, and a cholecystectomy was performed 18 years before admission. Physical examination did not show any abnormalities except jaundice. Laboratory data (Table 1) showed the levels of the serum total bilirubin, direct bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and glutamyl transpeptidase were 138.5 μmol/L, 120.0 μmol/L, 78 IU/L, 184 IU/L, 1031 IU/L, and 944 IU/L, respectively (significantly higher than normal); tumor markers including serum α-fetoprotein, carcinoembryonic antigen, carbohydrate antigen 199 (CA199), and carbohydrate antigen 125 (CA125) were 1.44 ng/mL, 3.27 ng/mL, 34.54 U/mL, and 89.87 U/mL, respectively (the latter two were a little higher than normal); the hepatitis B & C markers were negative; blood routine test (BRT) was normal. Sonography of the upper abdomen revealed only hepatic biliary and main pancreatic duct dilation. Subsequently magnetic resonance imaging and magnetic resonance cholangiopancreatography

Table 1 Some lab tests of patient

Lab items	Values		Normal values
TBIL ($\mu\text{mol/L}$)	138.5	↑	1.7-17
DBIL ($\mu\text{mol/L}$)	120.0	↑	0-6.8
ALT (IU/L)	78	↑	10-40
AST (IU/L)	184	↑	10-40
ALP (IU/L)	1031	↑	40-110
GT (IU/L)	944	↑	< 50
AFP (ng/mL)	1.44		< 20
CEA (ng/mL)	3.27		< 15
CA199 (U/mL)	34.54	↑	< 33
CA125 (U/mL)	89.87	↑	0-35

TBIL: Total bilirubin; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GT: Glutamyl transpeptidase; AFP: α -fetoprotein; CEA: Carcinoembryonic antigen.



Figure 1 Magnetic resonance imaging showed the expansion of hepatic biliary duct and the ectopic hepatopancreatic ampulla (arrow) draining into the fourth part of the duodenum.

(MRCP) were performed (Figure 1) which showed the expansion of the extrahepatic and intrahepatic biliary ducts and the main pancreatic duct, the ectopic hepatopancreatic ampulla draining into the fourth part of the duodenum adjacent to the duodenojejunal flexure, a low confluence of the cystic duct into the common bile duct with a distance of approx 4 cm to the Vater's ampulla, and the irregular morphology of the duodenojejunal flexure; likely due to a soft tissue mass, which did not rule out the possibility of periampullary tumor resulting in the common bile duct stricture. Gastroscopy revealed chronic superficial antral inflammation without orifice or mucosal ulceration in the stomach and without any duodenal papilla in the descending duodenum.

Laparotomy confirmed the presence of an abnormal ampulla of Vater located at the fourth part of the duodenum and a soft tissue tumor about 6 cm \times 5 cm \times 5 cm with a peduncle joined to the ampulla (Figure 2). Intraoperative choledochoscopic examination displayed no mass in the common bile duct. Consequently, complete resection of the tumor including some peripheral tissue was performed from an incision of the jejunum, close to the duodenojejunal flexure. Frozen pathological examination indicated an intestinal villous adenoma accompanied by severe dysplasia and highly suspect focal canceration, however, no incisional tumor cells were seen. A Roux-Y loop anastomosis

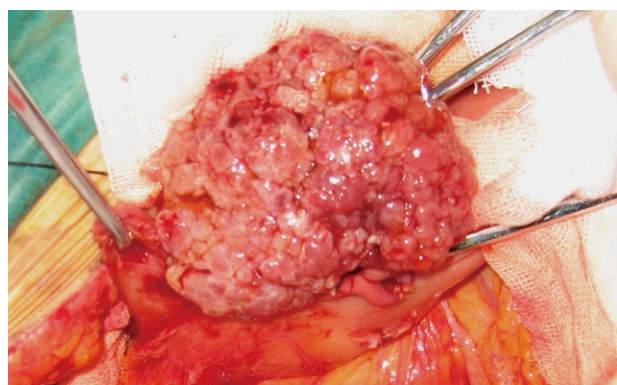


Figure 2 A soft texture tumor about 6 cm \times 5 cm \times 5 cm with a peduncle conjunct to the ampulla.

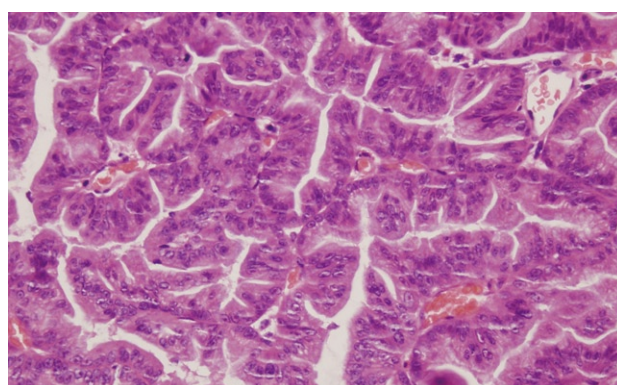


Figure 3 Microscopic examination (HE, \times 400) showed severe dysplasia and focal carcinomatous change.

at side-to-side choledochojejunostomy and end-to-side jejunojunctionostomy was carried out in order to prevent stricture at the end of the common bile duct. Pancreatoduodenectomy was not done. Paraffin block histopathologic examination confirmed a diagnosis of intestinal villous adenoma accompanied by severe dysplasia and focal carcinomatous change without incisional margin infiltration (Figure 3). After operation, the patient recovered without incident and liver function tests gradually reverted to normal levels.

DISCUSSION

The ectopic drainage of the biliary tract is a rare congenital anomaly, which consists of abnormal communication of the common bile duct, the cystic duct, or an intrahepatic duct with the gastrointestinal tract. As noted by previous authors, ectopic drainage of the common bile duct may be located along a line extending from the stomach to the fourth part of the duodenum^[1-5]. The frequency of this anomaly fluctuates between 0% and 13%^[1,6]. However, there only four cases with ectopic ampulla draining into the fourth part of duodenum were reported^[5,7]. Periampullary carcinoma combined with this anomaly has never described previously.

The embryogenetic background of this anomaly has not yet been fully explained. As is known, the extrahepatic biliary duct and the ventral pancreas arise

from the hepatic diverticulum of the end of foregut during the 4th to 6th week of embryo life^[8,9]. The dorsal pancreatic bud appears opposite the hepatic diverticulum. During development, as the duodenum rotates to the right and becomes C-shaped, the ventral pancreatic bud is carried dorsally with the bile duct. The ventral pancreatic duct joins the distal portion of the dorsal pancreatic duct to form the main pancreatic duct, which merges with the common bile duct, inserting into the duodenum *via* the papilla of Vater^[10]. Then, the proximal portion of the dorsal pancreatic duct gradually regresses and becomes obliterated at the junction with the ventral pancreatic duct. By the end of the 7th week, the proximal portion of the dorsal pancreatic duct may disappear or persist as the accessory pancreatic duct^[11,12]. Thus, we can make the hypothesis that the ampulla of Vater may be found in the sites along a line extending from the stomach to the fourth part of the duodenum, or in another words, along the end of the foregut. However, other factors may play a role in this process.

It is worth mentioning that MRCP is useful for demonstrating anomalies and anatomic variants of the biliary tract system and pancreatic duct^[13,14]. In our case, MRCP was of great value not only for depicting the ectopic common bile duct and pancreatic duct draining into the fourth part of duodenum but also for depicting the abnormal junction of the cystic duct and intraluminal small bowel lesion. According to Ruge's^[15] categorization, this variation of lower confluence of the cystic duct belongs to type II - the double-barreled type in which the cystic duct follows the hepatic duct for some distance before entering it. In addition to MRCP, gastroscopy should be used to assess the gastric mucosa and other malformations which could possibly exist.

Periampullary carcinoma includes cancer of the terminal end of the common bile duct, the ampulla of Vater and its adjacent duodenum. These lesions are similar to each other in many aspects, such as their clinical manifestations, changes noted by laboratory determinations and approach and technique of surgical excision. The prognosis is much better than for carcinoma of the head of pancreas. Endoscopic ampullectomy may be considered as a viable procedure in patients with small ampullary tumors who are unfit for surgery or who prefer a nonsurgical approach^[16-18]. But in our case, on the account of the ectopic hepatopancreatic ampulla and a likely major periampullary tumor located in the duodenojejunal flexure, endoscopic ampullectomy was unfit. So local excision, combined with frozen section, is a low morbidity and a valid alternative to pancreaticoduodenectomy^[19]. However, a choledochojejunostomy was carried out in order to prevent stricture of the lower end of the common bile duct after operation.

In conclusion, periampullary carcinoma with ectopic ending of the Vater's ampulla in the fourth part of the duodenum is rather rare. Although pathogenesis is not quite clear, accurate diagnosis and appropriate investigations are necessary for surgeons before operation. Use of MRCP is recommended for the diagnosis and

exclusion of associated anomalies of the biliary and pancreatic ducts.

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ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

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Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
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EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

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Phoenix, Arizona
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Marriott Wardman Park Hotel
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Email: bsg@mailbox.ulcc.ac.uk

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2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
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<http://www.easl.ch/>

May 17-20, 2009
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Endpoints Workshop: NASH

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June 17-19, 2009
North Bethesda, MD
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June 20-26, 2009
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August 1-7, 2009
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Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
Taipei, China
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October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
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October 13-16, 2009
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Issue with no volume

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Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (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 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; 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 quantum numbers can be found at: <http://www.wjgnet.com/wjg/help/14.doc>.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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ISI, Thomson Reuters, 2008 Impact Factor: 2.081
(32/55 Gastroenterology and Hepatology).

Volume 15 Number 38
October 14, 2009

World J Gastroenterol
2009 October 14; 15(38): 4737-4864

Online Submissions

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 14, 2008



World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 38
October 14, 2009



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World Journal of Gastroenterology

RESPONSIBLE INSTITUTION

Department of Science and Technology
of Shanxi Province

SPONSOR

Taiyuan Research and Treatment Center
for Digestive Diseases, 77 Shuangta
Xijie, Taiyuan 030001, Shanxi Province,
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PUBLISHING

The WJG Press and Beijing Baishideng
BioMed Scientific Co., Ltd., Room 903,
Building D, Ocean International Center,
No.62 Dongsihuan Zhonglu, Chaoyang
District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
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PRINTING

Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR

Beijing Bureau for Distribution of
Newspapers and Journals
(Code No. 82-261)
China International Book Trading
Corporation PO Box 399, Beijing,
China (Code No. M4481)

PUBLICATION DATE

October 14, 2009

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SUBSCRIPTION

RMB 50 Yuan for each issue, RMB
2400 Yuan for one year

CSSN

ISSN 1007-9327
CN 14-1219/R

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Etiology of chronic pancreatitis: Has it changed in the last decade?

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Received: August 3, 2009 Revised: September 7, 2009

Accepted: September 14, 2009

Published online: October 14, 2009

Abstract

The evidence from recent surveys on chronic pancreatitis carried out around the world shows that alcohol remains the main factor associated with chronic pancreatitis, even if at a frequency lower than that reported previously. It has further confirmed that heavy alcohol consumption and smoking are independent risk factors for chronic pancreatitis. Autoimmune pancreatitis accounts for 2%-4% of all forms of chronic pancreatitis, but this frequency will probably increase over the next few years. The rise in idiopathic chronic pancreatitis, especially in India, represents a black hole in recently published surveys. Despite the progress made so far regarding the possibility of establishing the hereditary forms of chronic pancreatitis and the recognition of autoimmune pancreatitis, it is possible that we are more inaccurate today than in the past in identifying the factors associated with chronic pancreatitis in our patients.

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Key words: Cohort studies; Combined modality therapy; Data collection; Genetics; Pancreatitis; Alcoholic; Population

Peer reviewer: Ross C Smith, Professor, Department of Surgery, University of Sydney, Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia

Pezzilli R. Etiology of chronic pancreatitis: Has it changed in the last decade? *World J Gastroenterol* 2009; 15(38): 4737-4740 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4737.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4737>

INTRODUCTION

Augusto Murri, Chair of Clinical Medicine at Bologna in 1875 was regarded as one of the most famous Italian physicians and clinical researchers of his time, wrote that "...to know a disease is different from recognizing it; we can recognize a disease only when we know its natural history"^[1]. This concept implies that it is important to know also the etiology of the disease and this is particularly true for chronic pancreatitis.

More than ten years ago, in 1998, Lankisch and Banks^[2] reported that the prevalence of chronic pancreatitis appeared to be in the range of 3-10 per 100000 people in many parts of the world, and underlined that the most important medical problems associated with the disease included abdominal pain, steatorrhea, diabetes mellitus and the possibility that chronic pancreatitis may be considered a premalignant condition^[3,4]. In 2002, Banks^[5] further pointed out that the two most important etiological forms of chronic pancreatitis were alcoholic and tropical.

There is no doubt that, in Western countries, alcohol is the most frequent factor associated with chronic pancreatitis, that alcoholic chronic pancreatitis presents clinically in young adults of 30-40 years of age, with a higher prevalence in males, that the histological lesions are chronic "*ab initio*" and that, from a clinical point of view, the disease is characterized by recurrent attacks of abdominal pain. In Western countries, in the period from 1940 to 2003, the frequency of alcohol as an etiological factor of chronic pancreatitis increased from 19%^[6] to 50%^[7] and even up to 80%^[8,9]. The results of the latter study regarding the etiology of chronic pancreatitis were subsequently confirmed by others in Europe^[10-17] as well as in Brazil^[18], Australia^[19] and South Africa^[20]. On the other hand, four consecutive surveys carried out in Japan (from 1970 to 1977, from 1978 to 1984, in 1994, and in 1999, respectively)^[21] showed that alcohol as an etiological factor accounted for fewer than 60% of chronic pancreatitis cases in this country. The study by Sarles *et al*^[9] reported that India is the most characteristic country in which patients with chronic pancreatitis were mainly malnourished in childhood, had a low fat and low protein diet and were not alcoholics. Thus, this particular form of the disease was named "tropical pancreatitis". Subsequent studies from India and Africa confirmed this finding as was reported in the review article published by Mohan *et al*^[22] in 2003.

THE IMPORTANCE OF THE ETIOLOGY

From a practical point of view, understanding the pathogenesis of chronic pancreatitis may lead to the identification of novel molecular targets and the development of new potential therapeutic agents. Thus, the role of alcohol is the cornerstone of the pathogenesis of chronic pancreatitis, at least in Western countries. Durbec *et al*^[8] clearly demonstrated that alcohol is a risk factor for chronic pancreatitis; in fact, they showed that the relative risk would be multiplied by approximately a factor of 1.4 when passing from one 20-gram intake to the next. Furthermore, the increase appears to be more rapid when passing from the class of non-drinkers to that of 20-g of alcohol intake per day. The mechanism which determines the main manifestation of chronic pancreatitis, i.e. fibrosis of the pancreatic gland, has been well-summarized by Talukdar *et al*^[23]: the oxidation of ethanol to acetaldehyde determines the activation of the pancreatic stellate cells in the quiescent state without any pre-activation; this process generates a state of oxidant stress within the pancreatic stellate cells which subsequently activates the downstream pathways of fibrogenesis. This finding implies that, in the human pancreas, pancreatic stellate cells may be stimulated early during chronic alcohol intake even in the absence of necroinflammation. The importance of oxidative stress in chronic pancreatitis patients has also been reported using breath analysis^[24]. Using a mass spectrometer on breath samples from 31 patients with chronic pancreatitis (mainly alcoholics) and without pancreatic pain as compared to 11 healthy subjects, we found that the volatile compounds H₂S, NO and malononitrile were significantly higher in chronic pancreatitis patients than in healthy subjects^[25]. These substances are the final products of ethanol and oxidative stress and they are able to initiate fibrogenesis of the pancreas. Regarding tropical pancreatitis, several hypotheses have been proposed, in particular, the malnutrition theory, the cassava hypothesis and the oxidant stress hypothesis^[26]. Thus, in this particular form of the disease, it is also possible that there is activation by certain substances in the pancreatic stellate cells.

However, according to this postulated pathogenesis, alcohol seems to induce pancreatic fibrosis as has frequently been found in autopsic series of alcoholics without clinical history of chronic pancreatitis^[25-27].

Furthermore, animal models of alcoholic chronic pancreatitis have not been able to induce pancreatic damage similar to that observed in human chronic pancreatitis; alcohol requires prior sensitization with other agents (viruses, obstruction) in order to produce damage similar to that found in humans.

In summary, alcohol represents a defined risk factor for chronic pancreatitis; it is capable of inducing pancreatic fibrosis by its action on pancreatic stellate cells, but its role in the etiopathogenesis of the disease is still being debated.

NEW ETIOLOGICAL FORMS OF PANCREATITIS

Genetic factors

The possibility of evaluating mutations of the cystic fibrosis transmembrane conductance regulator-gene (*CFTR*-gene)^[28], as well as the identification of mutations of the cationic trypsinogen gene (*protease-serine-1* gene, *PRSS-1*)^[29], the serine protease inhibitor and *Kazal type 1* gene (*SPINK-1*)^[30], has led to a better evaluation of the familial/hereditary forms as well as idiopathic forms of chronic pancreatitis in Western countries. In tropical pancreatitis it has also been noted that this disease has been highly associated with the SPINK-1 N34S mutation^[31,32], whereas the frequency of CFTR mutations was lower than in white subjects^[33]. The PRSS1 mutations appear capable of inducing chronic pancreatitis whereas CFTR and SPINK-1 seem to be “gene modifiers” capable of inducing the disease in the presence of a risk factor such as alcohol^[32,34].

Autoimmune diseases

In 1961, Sarles *et al*^[35] reported the case of a non-drinker suffering from pancreatitis associated with hypergammaglobulinemia. The authors hypothesized that the disease in this patient was an autonomous pancreatic disease of autoimmune origin. After this report, other authors around the world described similar cases. In 1995, Yoshida *et al*^[36] suggested the term “autoimmune pancreatitis” for this disease and, therefore, this term has become largely accepted for pancreatic disease of an autoimmune origin. In the past 10 years, an increasing number of cases have been reported in all countries^[37], and the frequency of autoimmune pancreatitis will probably increase in the next few years.

Changing lifestyle

The impact of changing lifestyle, especially in developing countries, may contribute to modifying the etiology of chronic pancreatitis. For example, alcohol consumption in developing countries may increase^[38] and this could change the etiology of chronic pancreatitis in these countries. On the contrary, in Europe, there was a progressive reduction in alcohol consumption from 1961 to 1991^[39]. Furthermore, taking into account the lifestyle of chronic pancreatitis patients, it has been reported that the pancreatic functional changes caused by alcoholic pancreatitis progress even after cessation of alcohol use, but the progression is slower and less severe when alcohol intake is stopped^[40].

THE FREQUENCY OF CHANGE IN ETIOLOGY

All these new factors and changing lifestyle may contribute to the changing frequencies of the various etiologies of chronic pancreatitis. This is the reason why,

from 2004 to the present, the etiological features of chronic pancreatitis have been reported to be different compared to those in the past. Four studies show examples of this. In Korea^[41], the main etiological factor remains alcohol (64.3%) followed by an unknown etiology (20.8%), obstruction (8.6%) and autoimmune pancreatitis (2.0%). In a recent survey on chronic pancreatitis in the Asian-Pacific region^[42], there was a great variability in the frequency of alcoholic pancreatitis, accounting for about 19% of chronic pancreatitis cases in China to 95% in Australia, whereas tropical pancreatitis was 46.4% in China and, obviously, was not present in Australia. In a recent survey of chronic pancreatitis in Italy^[43], chronic pancreatitis associated with alcohol abuse accounted for less than 50% of cases and this figure is lower than that reported by Gullo *et al*^[10] in 1977. However, some regional differences regarding the frequency of alcoholic chronic pancreatitis exist in Italy. In fact, in Bologna (located in Northern Italy), alcohol as an etiological factor remains high (80.4%)^[44], whereas in Sicily (located in Southern Italy), the percentage of alcoholic chronic pancreatitis is about 60%^[45]. In a survey of chronic pancreatitis in Italy^[43], alcohol as an etiological factor of chronic pancreatitis is followed by obstruction (27%), pancreatitis of unknown origin (17%), autoimmunity (4%) and hereditary/genetic factors (4%). The most surprising results come from India. In a prospective nationwide study in India^[46], the authors found that the majority of patients had pancreatitis of unknown origin (60% of the cases); alcoholic chronic pancreatitis accounted for a third of the cases, whereas tropical pancreatitis was present in only 3.8% of the cases. It seems that alcohol tends to be increasing in frequency in India, as is chronic pancreatitis of unknown etiology. However, the data reported by the Indian researchers (60% were idiopathic forms of chronic pancreatitis) need to be re-evaluated. In this regard, it is worth noting that the frequency of chronic pancreatitis of unknown origin was 17% in the Italian survey^[43] ranging from about 12% in Bologna to 38% in Sicily^[44,45].

CONCLUSION

The evidence from recent surveys on chronic pancreatitis carried out around the world shows that alcohol remains the main factor associated with chronic pancreatitis, even if at a frequency lower than that reported previously. However, it has further confirmed that heavy alcohol consumption and smoking are independent risk factors for chronic pancreatitis^[47].

Autoimmune pancreatitis accounts for 2%-4% of all forms of chronic pancreatitis, but this frequency will probably increase over the next few years. The rise in idiopathic chronic pancreatitis, especially in India, represents a black hole in recently published surveys. Despite the progress made so far regarding the possibility of establishing the hereditary forms of chronic pancreatitis and the recognition of autoimmune pancreatitis, it is possible that we are more inaccurate today than in the past in identifying the factors associated with chronic pancreatitis in our patients.

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S- Editor Tian L L- Editor Webster JR E- Editor Lin YP

Adult celiac disease with acetylcholine receptor antibody positive myasthenia gravis

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Received: June 1, 2009 Revised: September 8, 2009

Accepted: September 15, 2009

Published online: October 14, 2009

Key words: Acetylcholine receptor antibodies; Celiac disease; Myasthenia gravis; Transglutaminase antibodies

Peer reviewers: Epameinondas Tsianos, MD, PhD, Professor, 1st Division Of Internal Medicine & Hepato-Gastroenterology Unit, Medical school University of Ioannina, PO Box 1186 Ioannina 45110, Greece; Kazuichi Okazaki, Professor, Third Department of Internal Medicine, Kansai Medical University, 10-16 Fumizono-cho, Moriguchi City, Osaka, 570-8507, Japan

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Abstract

Celiac disease has been associated with some autoimmune disorders. A 40-year-old competitive strongman with celiac disease responded to a gluten-free diet, but developed profound and generalized motor weakness with acetylcholine receptor antibody positive myasthenia gravis, a disorder reported to occur in about 1 in 5000. This possible relationship between myasthenia gravis and celiac disease was further explored in serological studies. Frozen stored serum samples from 23 acetylcholine receptor antibody positive myasthenia gravis patients with no intestinal symptoms were used to screen for celiac disease. Both endomysial and tissue transglutaminase antibodies were examined. One of 23 (or, about 4.3%) was positive for both IgA-endomysial and IgA tissue transglutaminase antibodies. Endoscopic studies subsequently showed duodenal mucosal scalloping and biopsies confirmed the histopathological changes of celiac disease. Celiac disease and myasthenia gravis may occur together more often than is currently appreciated. The presence of motor weakness in celiac disease may be a clue to occult myasthenia gravis, even in the absence of intestinal symptoms.

INTRODUCTION

A range of neurological disorders have been identified in patients suffering from celiac disease^[1]. Occasionally, neurological changes first occur and celiac disease is only recognized later^[2]. Celiac disease is considered an immune-mediated disorder that affects the proximal small intestine and leads to reduced nutrient absorption, diarrhea and weight loss. Treatment with a gluten-free diet is usually sufficient. Marked fatigue and weakness may also occur in many chronic disorders, including celiac disease. However, here, concomitant myasthenia gravis was also discovered^[3].

Serological screening for celiac disease antibodies using stored frozen samples from a serum bank of 23 additional patients with acetylcholine receptor positive myasthenia gravis was also completed. From these banked serum samples, one was discovered with both positive IgA endomysial (EMA) and IgA tissue transglutaminase (tTG) antibodies. Subsequent clinical evaluation, including endoscopic biopsy studies, confirmed the findings of celiac disease.

Although rare and estimated to occur in only about 1 in 5000, myasthenia gravis may occur more frequently than is currently appreciated if celiac disease is also present. Ongoing fatigue and profound muscle weakness in celiac disease may be a clinical clue that this unusual immune-mediated neurological disorder, myasthenia gravis, is present.

CLINICAL CASE STUDY

A 40-year-old male orchidist was initially investigated in 2001 for diarrhea and weight loss of 10 kg with intermittent generalized fatigue. His IgA tTG antibody assay was increased to 89 units (normal, < 20 units). Gastroscopy and colonoscopy were visually normal, but small bowel biopsies showed changes of celiac disease with crypt hyperplastic villus atrophy (i.e. severe "flat" lesion, Marsh 3 lesion). Treatment with a gluten-free diet led to rapid resolution of diarrhea and weight loss. His IgA tTG antibody assay also subsequently normalized completely to 10.2 units.

By July 2003, however, his fatigue was persistent and his weakness became progressive and generalized. Although his physical attributes were well known locally, having previously been placed 6th in an international strongman competition, he stumbled and fell easily with weakness notably exacerbated by exertion. Marked leg fatigue developed, especially while standing on a ladder picking peaches. Once fatigue occurred, he was unable to step up to the next rung on the ladder, holding on with both arms.

While picking peaches, he also noted that he could only lift his arms above shoulder level for 15 min before he could no longer lift his arms. During the previous year, fatigue with chewing also developed along with right eyelid ptosis and diplopia.

Detailed neurological examination showed rapid muscle fatigue on repetitive exercise. Bilateral ptosis with a flattened facial expression, but normal speech function, was noted. Extra-ocular movements were abnormal with progressive eye elevation weakness after 20 to 30 s of sustained upward gaze along with worsening ptosis. Diplopia was also evident. After a minute of voluntary upward gaze, he was unable to elevate his eyes beyond the primary position. Facial muscles were strong. Examination of his upper extremities revealed deltoid fatigue after 10 to 15 repetitive movements, and examination of his lower extremities revealed that 9 stand-ups from a sitting position produced complete fatigue and an inability to stand upright. Reflexes and sensory studies were normal. His Quantitative Myasthenia Gravis (QMG) examination was at 18 (normal, 0; maximum deficit, 39)^[4]. A Mestinon test produced an evident response with return to normal strength. Repeated stimulation confirmed the existence of decrement. Computerized tomography of his chest revealed no evidence of a thymoma. Acetylcholine receptor antibodies were 22.0 nm/L (normal, < 0.1 nm/L). The final diagnosis was acetylcholine-receptor-antibody-positive, generalized myasthenia gravis class IIIb (Osserman classification)^[5].

Subsequent prednisone and Mestinon treatment provided partial improvement in motor weakness. Thymectomy was performed and the resected thymus was noted to be large (10 cm × 9 cm × 2 cm). Microscopic evaluation noted normal thymic tissue with no hyperplasia or evidence of thymoma. Post-operatively, his weakness on exertion persisted and his acetylcholine receptor antibodies increased by November

2004 to 38.4 nmol/L. Over the next 3 years, his symptoms improved with infusions of immunoglobulin along with the addition of mycophenolate mofetil and cyclosporin. His QMG score normalized to 3 with some persistent diplopia and minor facial weakness. This clinical improvement was accompanied by serological improvement with reduced levels of acetylcholine-receptor antibodies to 23 nmol/L.

SPECIAL SEROLOGICAL STUDIES

A total of 23 patients with acetylcholine receptor antibody positive myasthenia gravis were identified through the Neuroimmunology Laboratories in the UBC Brain Research Center (Dr. Joel Oger). Acetylcholine receptor antibodies were measured using a modified radioimmunoprecipitation assay^[6]. Approval to test samples for endomysial antibodies and IgA tissue transglutaminase antibody concentrations was obtained through the Clinical Research Ethics Board at UBC as previously noted^[7,8].

Residual frozen and stored serum samples from this myasthenia serum bank were quantitatively evaluated for IgA EMA and IgA tTG antibodies. IgA EMA was detected using indirect immunofluorescence against human umbilical cord using the method described by Ladinser *et al*^[9], measuring the serum at an initial dilution of 1:5. With this method, positive sera were repeated at increasing dilutions until they became negative. Titers of IgA tTG antibody were measured using an ELISA method based on that reported by Dieterich *et al*^[10] but modified to account for local differences in scientific supplies. During the initial evaluation of this assay, a reference range of up to 140 U/mL (3 SDs above the mean, 99% confidence limits) was calculated from titers on adult intestinal disease controls with normal small intestinal biopsies, and on sera from adults with biopsy-defined celiac disease as previously noted by Gillett and Freeman^[11]. During validation, all IgA-deficient patients were found to have tTG titers of 5 U/mL or lower. For this study, therefore, samples with titers of 5 U/mL or lower were tested for IgA deficiency using NOR-Partigen Total IgA kit (Behring Diagnostics Inc, USA).

RESULTS OF SCREENING

All 23 samples were examined for both IgA EMA and IgA tTG. One sample was EmA positive and tTG quantitation measured 1160 U/mL. All other serum samples were negative for EmA. For tTG quantitation using this assay method, all other serum samples were less than 95 U/mL. The single positive patient, a 16-year-old female, had endoscopic evaluation that revealed mucosal scalloping in the duodenum, consistent with celiac disease. Findings were confirmed on histologic evaluation of small intestinal biopsies and a gluten-free diet was initiated.

DISCUSSION

These studies suggest that myasthenia gravis, an

autoimmune neuromuscular disorder, may occur more often in adult celiac disease than is currently appreciated. In most patients with myasthenia gravis, circulating antibodies are thought to block acetylcholine receptors at the post-synaptic neuromuscular junction, inhibiting stimulatory effects of the neurotransmitter, acetylcholine, causing muscle weakness. Both generalized or localized muscle weakness with fatigue occur, particularly with exertion, but these might conceivably be ascribed to various chronic medical disorders, including celiac disease. In adults with celiac disease, weakness and fatigue could also result from changes in nerves (e.g. peripheral nerve lesion)^[12], or alternatively, skeletal muscle (e.g. rhabdomyolysis, myositis)^[13,14]. Alternatively, nutritional depletion associated with electrolyte alterations such as hypokalemia^[15] or a protein deficiency state such as hypoalbuminemia might be responsible. The present report further emphasizes that myasthenia gravis should also be considered as a specific association with both symptomatic or classical celiac disease as well as subclinical disease initially defined with screening measures.

Myasthenia gravis is uncommon, with estimates of about 1 in 5000 in the United States, while celiac disease has been estimated to occur in about 1 in 200. Although the appearance of these two disorders together could be coincidental, there is now other evidence available to suggest that these two distinct immune-mediated disorders occur together more frequently than is currently appreciated. First, early case reports from Europe recorded myasthenia gravis in celiac disease^[16-18]. Second, similar human leukocyte antigen (HLA) types (B8 and DR3) appear to independently predispose to both myasthenia gravis and celiac disease. Third, other historical serological survey studies with different, but less specific antibodies have been positive. For example, anti-reticulín antibodies (present in celiac disease) were previously recorded in myasthenia gravis^[19]. Finally, acetylcholine receptor antibodies have been recently detected by others in celiac disease^[20]. In the present study, both IgA EMA and IgA tTG antibodies in celiac disease were found in myasthenia gravis patients, while antibodies to acetylcholine receptors were defined in celiac disease before and after gluten-free diet treatment. In this case, seropositive patients were confirmed to have positive intestinal biopsies.

Celiac disease will have to be added to the increasing list of other autoimmune disorders that have been associated with myasthenia gravis, including diabetes mellitus, rheumatoid arthritis, thyroid disease, neuromyelitis optica (post-thymectomy myasthenia gravis)^[21] and multiple sclerosis^[22]. The suspicion of a common mechanism between these diseases is well supported by common HLA haplotypes, co-occurrence in the same families and confirmed by their occurrence in the same patients.

CONCLUSION

In conclusion, these studies suggest that celiac disease and myasthenia gravis may occur together

more frequently than is currently appreciated, in part because clinical changes may be subtle and difficult to recognize. The precise frequency of celiac disease in this local myasthenia population is unknown as serum was collected during the previous decade and additional celiac disease cases may have been subsequently detected. Future antibody screening studies exploring the frequency of celiac disease in myasthenia gravis, and *vice versa*, as well as their relatives remains to be elucidated.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Terry Maguire, Dr. David Israel, Dr. Kevin Farrell and Dr. DA Adams for assistance in clinical care.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Lin YP

Role of LPS/CD14/TLR4-mediated inflammation in necrotizing enterocolitis: Pathogenesis and therapeutic implications

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Received: July 23, 2009 Revised: August 24, 2009

Accepted: August 31, 2009

Published online: October 14, 2009

Abstract

AIM: To establish the roles of lipopolysaccharide (LPS)/CD14/toll-like receptor 4 (TLR4)-mediated inflammation in a rat model of human necrotizing enterocolitis (NEC).

METHODS: Six pairs of intestinal samples from human NEC were collected before and after recovery for histological and molecular analysis of inflammatory cytokines and signaling components. In the rat NEC model, we isolated 10-cm jejunum segments and divided them into six groups ($n = 6$) for sham operation, treatment with LPS, bowel distension, combined bowel distension and LPS stimulation, and two therapeutic groups. The potential efficacy of a recombinant CD18 peptide and a monoclonal CD14 antibody was evaluated in the latter two groups. The serum and tissue levels of several inflammatory mediators were quantified by real-time polymerase chain reaction, ELISA and immunoblotting.

RESULTS: Human acute phase NEC tissues displayed significant increases ($P < 0.05$) in levels of TLR4, CD14, myeloid differentiation protein (MD)-2, tumor necrosis factor (TNF)- α and nuclear factor- κ B when

compared to those after recovery. The histological and inflammatory picture of human NEC was reproduced in rats that were treated with combined bowel distension and LPS, but not in the sham-operated and other control rats. Serum levels of interleukin-6 and TNF- α were also elevated. The NEC pathology was attenuated by treating the NEC rats with a monoclonal CD14 antibody or an LPS-neutralizing peptide.

CONCLUSION: LPS and distension are required to produce the histological and inflammatory features of NEC. A potential treatment option is blocking LPS activation and leukocyte infiltration.

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Key words: CD14 antigen; Lipopolysaccharide; Necrotizing enterocolitis; Pathogenesis; Therapy; Toll-like receptor 4

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Chan KL, Wong KF, Luk JM. Role of LPS/CD14/TLR4-mediated inflammation in necrotizing enterocolitis: Pathogenesis and therapeutic implications. *World J Gastroenterol* 2009; 15(38): 4745-4752 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4745.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4745>

INTRODUCTION

Necrotizing enterocolitis (NEC) is a common gastrointestinal disorder in newborn and premature infants with a relatively high mortality rate (approximately 30%), and requires emergency medical treatment^[1]. While the molecular pathogenesis of neonatal NEC is multifactorial and remains to be elucidated fully, its known risk factors include prematurity and low birth weight^[1,2]. Ballance *et al*^[3] have reviewed pathology specimens from a cohort of 84 neonatal NEC patients, and have reported that the most consistent features of

the disease are ischemic necrosis, inflammation, and bacterial overgrowth. Since neonatal NEC does not develop *in utero* in the sterile gut, bacterial contamination and/or microbial involvement have been implicated in its pathogenesis^[4]. Furthermore, > 90% of preterm infants who develop NEC have received enteral feeds^[5], and there is an increased risk of NEC when these infants are overfed frequently^[6-9]. This could result in intestinal injury because of impaired digestion of nutrients, delayed transit time and bacterial overgrowth^[10].

Based on these recent findings, we believe that there is a connection between host immunity and pathogenesis of NEC, and the toll-like receptor (TLR)-mediated immunity apparently plays an important role. TLRs are evolutionarily conserved transmembrane molecules that help the immune system to recognize pathogen-associated molecular patterns, and TLR4 sensitizes immune cells to bacterial lipopolysaccharide (LPS). When stimulated by bacterial LPS, many intracellular signaling pathways are activated, and lead to the generation of nuclear factor (NF)- κ B, which in turn promotes pro-inflammatory cytokine production and release^[11]. Caplan *et al*^[12] have reported upregulation of MD-2, one of the bacterial LPS co-receptors, in patients with NEC.

In overfed or food-intolerant neonates, bowel loops can become distended and filled with milk, and this creates a good nutritive medium for bacterial growth and colonization. We hypothesized that bacterial LPS or endotoxin from cultured bacteria can trigger inflammation in NEC. In order to validate this hypothesis, we studied the TLR4-mediated signaling in the jejunal tissue from a rat model of human NEC, and then used the findings to design novel therapeutic agents for use in neonatal patients with NEC. In this model, we ligated the jejunum, and distended the ligated jejunum with bacterial LPS in order to induce the inflammatory reaction of NEC.

MATERIALS AND METHODS

Human tissues

Necrotic NEC intestinal tissues were collected from six human infants (two girls and four boys) with stage 3 NEC, who underwent curative surgery at Queen Mary Hospital, Hong Kong. The mean gestational age of these NEC patients was 30 wk (range: 25-32 wk), and their mean birth weight was 1.51 kg (range: 0.57-2.51 kg). NEC was diagnosed between 2 and 32 d after birth (mean: 17 d). For comparison, intestinal tissues were collected during the recovery phase at the time of ileostomy closure. After surgical resection, each intestinal tissue sample was divided into two: one was snap-frozen in liquid nitrogen for determination of mRNA/protein levels, and the other was fixed in 10% buffered formalin for histological examination.

Reagents

Bacterial LPS from *Salmonella typhimurium* was purchased from Sigma (St. Louis, MO, USA), and was used to prepare a 5 μ g/mL LPS solution with endotoxin-free

saline^[13]. A polyclonal antibody for use as an antagonist against the CD14 antigen (M-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The LPS-neutralizing peptide was prepared using our established procedures^[14,15]. The protein content in each solution was determined using Lowry's method^[16].

Animals

One-month old Sprague-Dawley rats were obtained from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited Laboratory Animal Unit of the University of Hong Kong (Pokfulam, Hong Kong). The rats were maintained in a pathogen-free facility with a 12-h light/dark cycle at ambient temperature and in a controlled humidity environment. The rats had *ad libitum* access to sterilized animal chow and water. All experimental procedures were reviewed, approved, and conducted in accordance with the ethical guidelines set forth by the University's Committee on Using Live Animals for Teaching and Research.

Experimental NEC model

The rat model of human NEC was modified from our published model^[17]. Before surgery, rats were fasted overnight, and anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). Once anesthetized, the abdomen was opened by a midline incision, and a 10-cm segment of the most proximal jejunum was isolated with its vascular pedicle intact. One end of the segment was closed by ligation. An 18-G angiocatheter was inserted and fixed to the other end. The angiocatheter was connected to an infusion set whose height was 100 cm above the operating table.

Each control and experimental group comprised six animals. For the sham-operated control rats, the angiocatheters were closed for 3 h using a plug. For the rats treated with LPS alone, 2 mL of 5 μ g/mL LPS solution was injected directly into the jejunal segment. To increase intraluminal bowel pressure, the jejunal segment was distended with sterile saline at a pressure of 100 cm H₂O for 3 h in four groups of rats. In two groups, the bowel was distended with sterile saline alone or combined with 5 μ g/mL LPS. In the remaining two groups, the bowel was distended in an identical manner, and contained 5 μ g/mL bacterial LPS and 0.8 μ g/mL CD14 antibody or 0.8 μ g/mL CD18 peptide, which has LPS-neutralizing capacity. After 3 h bowel distension, the animals were killed humanely by an overdose of sodium pentobarbital (100 mg/kg). Formalin-fixed and snap-frozen jejunal tissues were prepared for histological examination and determination of mRNA/protein levels, respectively. Blood was also collected from the inferior vena cava of all rats for serum/plasma preparation, and stored at -70°C until use.

Assessment of NEC severity

Sections (4 μ m thick) of the harvested jejunal tissues were prepared for hematoxylin and eosin (HE) staining and immunohistochemistry^[18,19]. The severity of NEC

was scored histologically on the appearance of the intestine, using a modification of the method that was described by Caplan *et al.*^[20]: 0, normal intestine; 1, mild hemorrhage; 2, moderate hemorrhage and necrosis; 3, severe hemorrhagic necrotic lesions with or without pneumatosis. The degree of macrophage infiltration was determined following overnight incubation at 4°C with a macrophage staining reagent (Dako, Glostrup, Denmark). The resulting images were reviewed with a Nikon epifluorescent upright microscope E600 (Nikon, Tokyo, Japan), and captured with 3-CCD color camera DC-330 (DAGE-MTI, Michigan City, IN, USA).

Real-time polymerase chain reaction (PCR)

Real-time PCR was employed to determine the relative expression levels of CD14, TLR4 and MD-2 mRNA in human and rat jejunal tissues. First-strand cDNA was synthesized from extracted RNA from jejunal tissue^[20], and amplifications were done using the Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) in an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA)^[21]. Normalization and comparison of mRNA transcript levels between the groups were then performed, as described previously^[22].

Immunoblotting of NF- κ B p65 and tumor necrosis factor (TNF)- α in the intestine of NEC patients

The nuclear levels of NF- κ B p65 and TNF- α in the jejunal tissue of NEC patients were detected by antibody against NF- κ B p65 (Invitrogen) and TNF- α (Santa Cruz Biotechnology), as described previously^[15].

Determination of serum pro-inflammatory cytokine levels

Serum TNF- α and interleukin (IL)-6 levels in the control, sham-operated, and treated rats were determined using commercially available ELISA kits that were purchased from Dakewe (Shenzhen, China) and eBioscience (San Diego, CA, USA), respectively, in accordance with the manufacturer's instructions.

Statistical analysis

Statistical analyses of the experimental data were performed using GraphPad Prism (version 4.0, GraphPad Software Inc, San Diego, CA, USA). Statistical significance was set at 5%, and data are presented as mean \pm SD.

RESULTS

Human NEC intestinal histology and inflammatory pathology

Serial samples of intestinal tissue from the acute and recovery phases of NEC were collected, prepared, and scored. Intestinal tissue from the acute-phase in which severe coagulative necrosis was observed histologically was given a score of 3 (Figure 1A). The appearance of the intestinal tissues from the recovery phase was normal, and given a score of 0. The extent of

macrophage infiltration in acute-phase intestinal tissues was greater than that in the recovery-phase tissues. To determine the potential causal immunological factors, we studied the expression of key signaling components that are mediated by LPS activation. The protein levels of NF- κ B p65 and TNF- α (Figure 1B) in acute-phase intestinal tissues were significantly higher than those in recovery-phase tissues. Furthermore, the gene transcript levels of CD14 and MD-2 (Figure 1C) were significantly increased in the acute-phase tissues, when compared to those in recovery-phase tissues. The TLR4 mRNA level was also higher in the acute-phase tissues than in the recovery-phase tissues, but this increase was not significant statistically.

Molecular pathogenesis of experimental NEC

Rat jejunal histology: Compared to the sham-operated control specimens (Figure 2A), there was no apparent histological damage (score 0) in jejunal loops that were treated with LPS alone (Figure 2B) or distended with normal saline (Figure 2C). We observed histological changes (Figure 2D) (score 2), such as sloughing of the villi and vascular congestion in jejunal specimens from rats that were treated with a combination of saline distension and bacterial LPS. Although the changes were less severe, they did resemble those that were observed in acute-phase intestinal specimens from NEC patients, and were associated with substantial macrophage infiltration (Figure 3A-D).

Jejunal distension requires bacterial LPS to trigger inflammatory reactions: To determine whether the inflammatory responses could be induced in the experimental NEC model, we assessed the key LPS-mediated signaling components or reporters (CD14, TLR4, MD-2, TNF- α and IL-6) at the transcript or protein level in the jejunal segments and plasma from each experimental group. The results were consistent with the histological findings. Combination treatment of intestinal distension and bacterial LPS jejunal injection significantly raised the level of CD14, TLR4 and MD-2 mRNA ($P < 0.05$) when compared to those in saline-distended jejunal tissues, those following jejunal distension alone and LPS jejunal injection alone, or those from the sham-operated controls (Figure 4). We then measured the systemic cytokine responses in the experimental NEC animals by ELISA. The serum TNF- α and IL-6 levels in the combined distension + LPS group were 3-4 times higher than those of the other single treatment groups (distension or LPS jejunal injection alone) and the sham-operated control rats (Figure 5A and B).

Therapeutic potential of CD14/CD18 blockage in NEC inflammatory response

We tested the therapeutic efficacy of CD14 or CD18 blockade in the established rat NEC model by using a CD14-neutralizing monoclonal antibody (M-20) as an antagonist, and a novel LPS-neutralizing peptide, CD18 β A peptide, which have been shown to neutralize the biological activity of endotoxin. Treatment with M-20

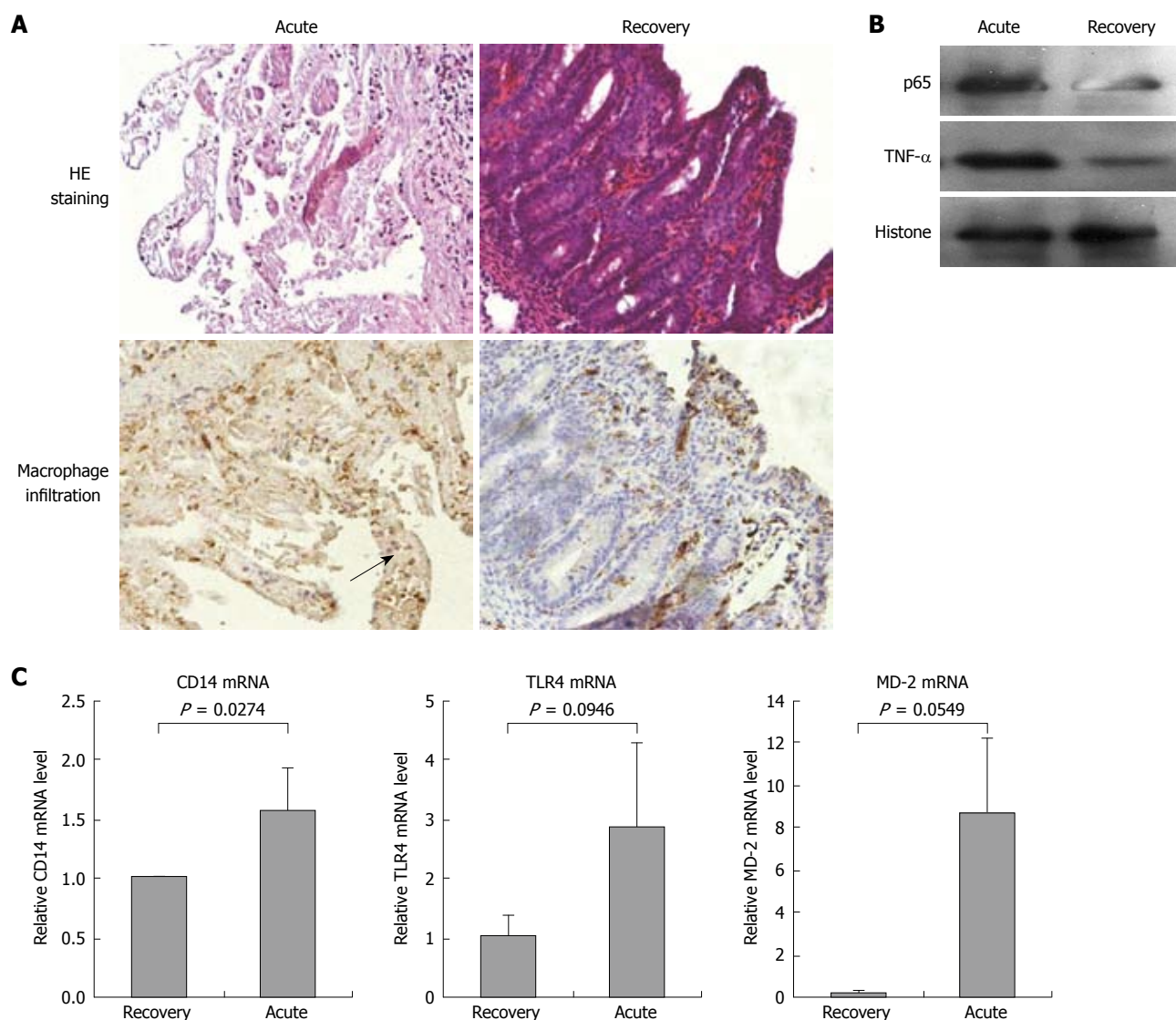


Figure 1 Pathological features of human necrotizing enterocolitis (NEC). A: Representative micrographs that show the microarchitecture of (upper panel), and the extent of macrophage infiltration (as marked by arrow) (lower panel) in jejunal tissues from NEC patients, which were collected in the acute phase and during recovery. Magnification $\times 200$; B: Immunoblotting showed overexpression of TNF- α and upregulated nuclear content of the p65 subunit of NF- κ B in jejunal tissues from NEC patients in the acute phase. Nuclear histone level was used as the loading control; C: Real-time PCR detected the mRNA transcript levels of CD14, TLR4 and MD-2 in jejunal tissues from NEC patients in the acute phase and during recovery. Statistical significance was determined by Student's *t* test, and all data are presented as the mean \pm SD of triplicate samples from two independent experiments.

(Figure 2E) or LPS-neutralizing peptide (Figure 2F) restored the jejunal microarchitecture to near normal (score 0), and attenuated the increased extent of macrophage infiltration in those loops that was distended with LPS-containing saline, and contained either an antibody against CD 14 (Figure 3E) or LPS-neutralizing peptide (Figure 3F). This was a sharp contrast to the jejunal distension that was caused by LPS-containing saline (Figures 2D and 3D), which caused marked histological changes such as villous sloughing and destruction. These histological changes were accompanied by concomitant changes in the expression levels of CD14, MD-2 and TLR4 mRNA (Figure 4), and the serum levels of TNF- α and IL-6 (Figure 5).

DISCUSSION

We investigated the role of innate immunity in the

pathogenesis of NEC by examining the LPS/CD14/TLR4-mediated inflammatory response in jejunal tissues from premature neonates with NEC. Using our recently established rat NEC model, we demonstrated that disease development requires intestinal bowel distension and bacterial LPS stimulation. In the human NEC tissues, increases in NF- κ B p65 and TNF- α and mRNA for CD14, TLR4 and MD-2 were found. All these signaling-cascade increases were also noted in the intestine of the rat NEC model, with a parallel increase in serum TNF α and IL-6. Histologically, changes in the LPS-distended rat bowel and human NEC tissue were similar. Furthermore, we showed in this model that the consequent pro-inflammatory reactions could be suppressed or modulated by blocking endotoxin activity using a monoclonal CD14 antibody, and a CD18 β A peptide that neutralizes LPS binding and inhibits leukocyte infiltration into inflamed tissues. From these

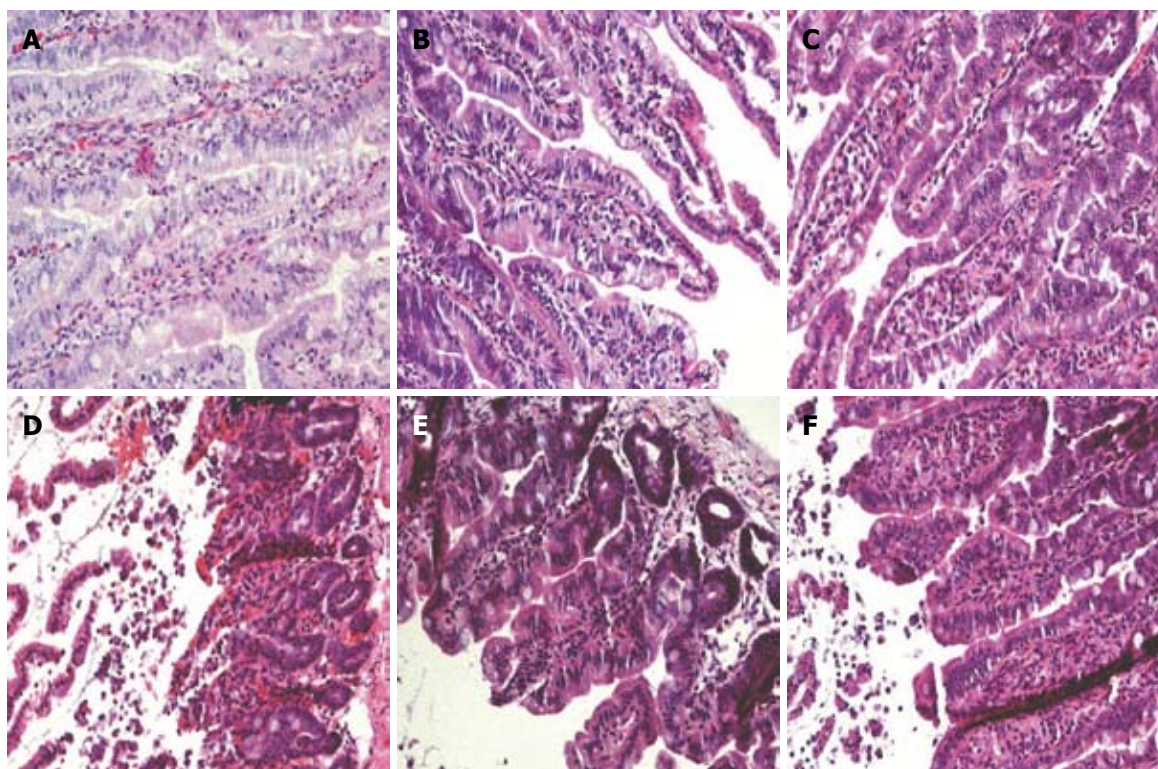


Figure 2 Histological features of rat jejunum. The severity of damage in the jejunum of rats from the different treatment groups was assessed histologically after HE staining. A: Sham-operated controls; B: LPS jejunal injection; C: Saline jejunal distension; D: Saline jejunal distension and LPS jejunal injection; E: Saline jejunal distension and LPS jejunal injection, and CD14 antibody treatment; F: Saline jejunal distension and LPS jejunal injection, and LPS-neutralizing peptide treatment. Only in (D) did the jejunal tissue show histological changes with a score of 2 for NEC (sloughing of villi and vascular congestion). Magnification $\times 200$.

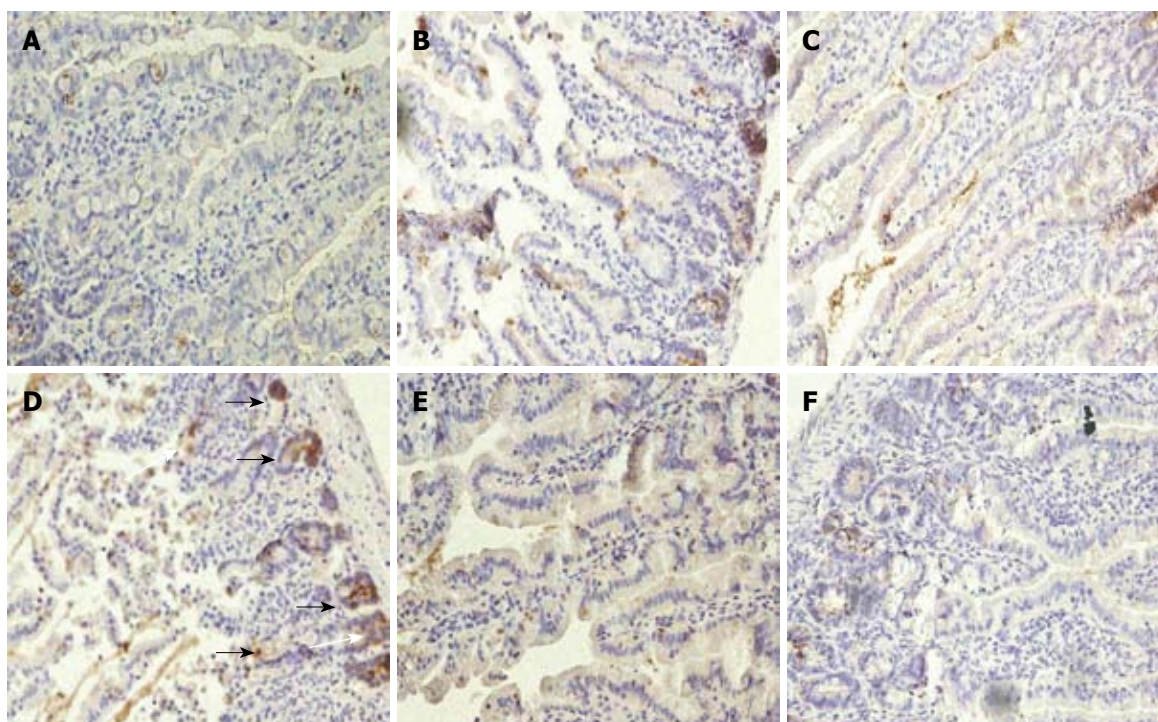


Figure 3 Macrophage infiltration into the rat jejunum. The extent of macrophage infiltration into the jejunum of rats from the different treatment groups was evaluated by immunohistochemistry using a macrophage staining reagent. Stained macrophages are marked by arrows. A: Sham-operated control; B: LPS jejunal injection; C: Saline jejunal distension; D: Saline jejunal distension and LPS jejunal injection; E: Saline jejunal distension and LPS jejunal injection, and CD14 antibody treatment; F: Saline jejunal distension and LPS jejunal injection, and LPS-neutralizing peptide treatment. Magnification $\times 200$.

results, we concluded that each molecule may have therapeutic potential for treating infants with NEC.

The essential role of the innate TLR4 immunity has been reported in a recent study in which TLR4-

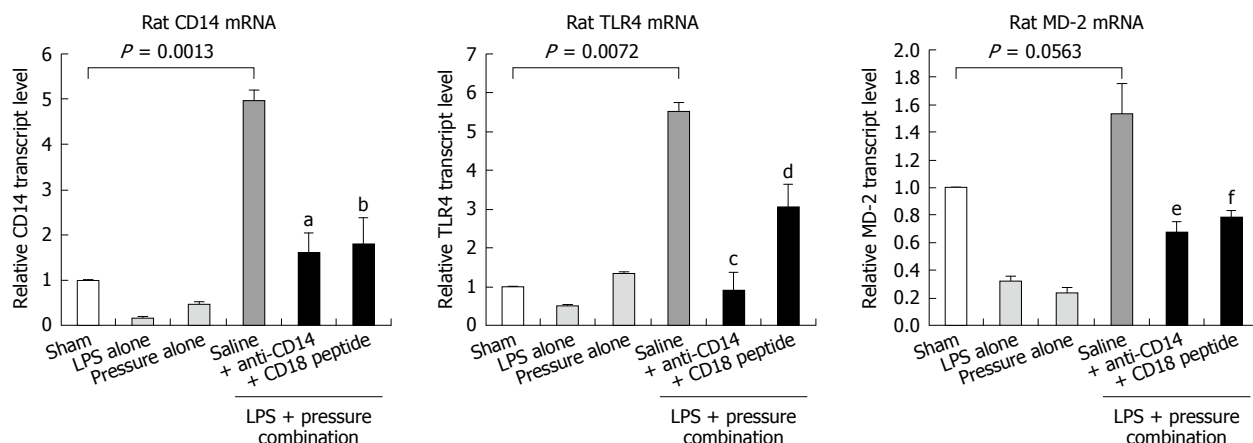


Figure 4 CD14, TLR4 and MD-2 mRNA expression in rat jejunum. Real-time PCR detected mRNA transcript levels for CD14, TLR4 and MD-2 in the jejunum of rats from the different treatment groups. Distension of jejunum with LPS-containing saline upregulated the expression of all studied transcripts, and these upregulated levels were attenuated by CD14 antibody and LPS-neutralizing peptide treatments. There were six rats in each experimental group. Data are displayed as the mean \pm SD, and analyzed statistically by one-way ANOVA. $^aP = 0.00126$; $^bP = 0.00498$; $^cP = 0.00838$; $^dP = 0.0127$; $^eP = 0.0151$; $^fP = 0.0187$.

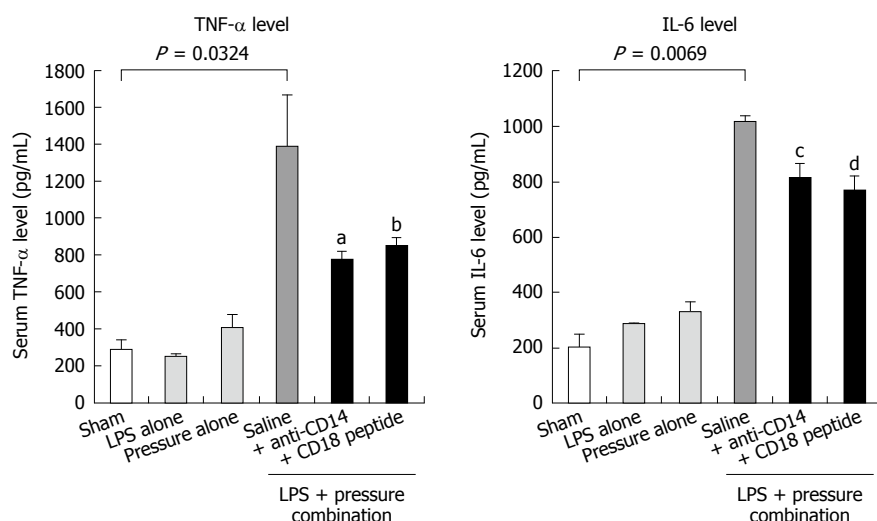


Figure 5 Serum TNF- α and IL-6 levels in NEC rats. Serum TNF- α and IL-6 levels in all NEC rats were measured by commercial ELISA kits. Distension of jejunum with LPS-containing saline significantly increased serum TNF- α and IL-6 levels, and these increases were suppressed when CD14 antibody and LPS-neutralizing peptide were added to the saline. There were six rats in each experimental group. Data are displayed as the mean \pm SD, and analyzed statistically by one-way ANOVA. $^aP = 0.0237$; $^bP = 0.0259$; $^cP = 0.0819$; $^dP = 0.0477$.

mutant C3H/HeJ mice were protected from developing NEC^[23]. The findings in our study support this essential role. We demonstrated that the activity of the TLR4 signaling pathway was upregulated in intestinal tissues from premature neonates with NEC. Given that TLR4 is the major receptor for bacterial LPS on immune and some epithelial cells, as well as endothelial cells, we examined whether activation of the TLR4 signaling pathway occurred in the intestine of rats with experimentally induced NEC. In this model, we injected or infused bacterial LPS into a jejunal segment because the jejunum, unlike the ileum and colon, is less likely to be contaminated by resident bacteria. By choosing this segment, we assumed that we would have better control over the bacterial LPS concentration in our investigation. We studied also the effect of bowel distension on the pathogenesis of NEC, because intestinal distension as a result of overfeeding is one of the many risk factors for NEC.

One-month-old rats were chosen for the study because, in our previous study^[17], the typical NEC histological pictures in the intestine could be seen when the correct distension pressure and bacterial

concentration were used. Further, these rats are more stable and can avoid changes caused by operative risks such as hypotension and hypothermia. NEC also occurs in full-term and older infants, and these infants have been found to have higher congenital heart diseases that also can cause hypoxia^[24].

Our data showed that bowel distension with saline or LPS injection alone could not induce significant inflammatory responses in rat jejunal segments. In contrast, bowel distension with saline that contained bacterial LPS induced substantial release of TNF- α and IL-6 into the serum, and increased the expressions of the LPS receptor and co-receptor mRNAs, and the extent of leukocyte infiltration into the jejunal mucosa. These findings suggest that bowel distension in overfed premature neonates is capable of potentiating LPS action on innate TLR4 immunity. The underlying mechanism by which bowel distension potentiates the action of LPS was not studied here. However, we believe that bowel distension injures the intestinal mucosa, and exposes the enterocytes to bacterial LPS. The hypoxic environment that is induced by bowel distension might also participate in this process by upregulating TNF- α

production from inflammatory macrophages^[24].

Perhaps more importantly, our results identified a monoclonal CD14 neutralizing antibody and an LPS-neutralizing peptide as potential therapeutic agents for use in NEC. We showed that both molecules could suppress the inflammatory response that was induced by bowel distension and bacterial LPS. This provides additional evidence for the critical role of innate TLR4 immunity in the pathogenesis of NEC. Moreover, our findings suggest that antagonism against members of the LPS signaling pathway could be beneficial therapeutically in the treatment of NEC. Given that the current management of premature neonates with NEC is still challenging, our novel molecules for treating NEC are of potential therapeutic importance.

In conclusion, our results suggest that bowel distension from overfeeding in premature neonates damages the intestinal mucosa. This resultant injury potentiates the action of bacterial LPS on enterocytes and resident immune cells, and triggers an immunological cascade that results in the development of NEC. In addition, we have shown that antagonistic molecules that target key members of this cascade can be used as potential therapeutic agents for treating infants with NEC.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Mr. Jensen To and Ms. Jana Wo of the Experimental Surgical Unit of The Hong Kong University, and Dr. Arie Bomzon for his critical review and professional editing of the manuscript.

COMMENTS

Background

Neonatal necrotizing enterocolitis (NEC) is a disease that affects mainly premature babies and its mortality and morbidity remain high. The pathogenesis of the disease is still not known and preventive measures have not been agreed upon. Its treatment is still difficult.

Research frontiers

Bacteria are required for the development of NEC. Since there are no bacteria *in utero*, there is no NEC in fetuses. At present, there is no single bacterium that has been identified to cause NEC. Many clinical studies have shown that premature babies with overfeeding or feed intolerance have a higher incidence of NEC. However, the mechanism by which feeding problems cause NEC is still unknown.

Innovations and breakthroughs

In many other studies, it has been shown that, in NEC tissue and patient serum, there is a marked increase in the pro-inflammatory cytokine profile, although again, the cause is unknown. In the present study, human NEC tissue showed increased levels of nuclear factor (NF)- κ B p65, tumor necrosis factor (TNF)- α , and CD14, toll-like receptor 4 (TLR4) and myeloid differentiation protein (MD)-2 mRNA. Bowel distension is common in overfed and feed-intolerant babies. In the present rat model used to mimic this condition, the intestine distended with lipopolysaccharide (LPS) showed increased mRNA for CD14, TLR4 and MD-2. Also, the rat serum showed raised TNF- α and IL6. Furthermore, the intestinal tissue in the rat after distension with LPS showed the typical features of NEC with villous sloughing and vascular congestion. These changes could be suppressed by blocking the endotoxin activity using a monoclonal CD14 antibody or a CD18 peptide that neutralized LPS binding and thus inhibited leukocyte infiltration into the inflamed tissue.

Applications

This study provides evidence that NEC can be caused by bowel distension with bacterial LPS, which is common in overfeeding and feed intolerance. The

process can be prevented by applying a carefully controlled feeding regimen to premature babies. Early detection and stoppage of feeding in the feed-intolerant babies can prevent the activation of the cytokine cascade pathway of the LPS and TLR4. In babies who have developed NEC, anti-CD14 antibody and CD18 neutralizing peptide can be a potential strategy to block the LPS activation pathway and prevention of leukocyte infiltration.

Terminology

TLRs are transmembrane molecules that help the immune system to recognize pathogens, and TLR4 is among the one that sensitizes immune cells to bacterial LPS. When stimulated by bacterial LPS, many intracellular signaling pathways are activated and lead to the generation of a cascade of pro-inflammatory cytokine production and release.

Peer review

It should be emphasized that this rat model of NEC resembles the phenotype of human NEC. Therefore, further studies may be needed to prove whether there is a correlation between the pathogenesis and possible treatment in the rat and human models.

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Emodin protects rat liver from CCl₄-induced fibrogenesis *via* inhibition of hepatic stellate cells activation

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Received: June 10, 2009 Revised: August 21, 2009

Accepted: August 28, 2009

Published online: October 14, 2009

Abstract

AIM: To investigate the role of emodin in protecting the liver against fibrogenesis caused by carbon tetrachloride (CCl₄) in rats and to further explore the underlying mechanisms.

METHODS: Rat models of experimental hepatic fibrosis were established by injection with CCl₄; the treated rats received emodin *via* oral administration at a dosage of 20 mg/kg twice a week at the same time. Rats injected with olive oil served as a normal group. Histopathological changes were observed by hematoxylin and eosin staining. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum and hepatic hydroxyproline content were assayed by biochemical analyses. The mRNA and protein relevant to hepatic stellate cell (HSC) activation in the liver were assessed using real-time reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, western blotting and enzyme-linked immunosorbent assay.

RESULTS: The degree of hepatic fibrosis increased markedly in the CCl₄ group compared to the normal group ($P < 0.01$), and decreased markedly in the emodin group compared to the CCl₄ group according to METAVIR scale ($P < 0.01$) compared with those in the normal control group (51.02 ± 10.64 IU/L and 132.28 ± 18.14 IU/L). The activities of serum ALT and AST were significantly higher in rats injected with CCl₄ (289.25 ± 68.84 IU/L and 423.89 ± 35.67 IU/L, both $P < 0.05$). The activities of serum ALT and AST were significantly reduced by administration of emodin (176.34 ± 47.29 IU/L and 226.1 ± 44.52 IU/L, both $P < 0.05$). Compared with the normal controls (54.53 ± 13.46 mg/g), hepatic hydroxyproline content was significantly higher in rats injected with CCl₄ (120.27 ± 28.47 mg/g, $P < 0.05$). Hepatic hydroxyproline content was significantly reduced in the rats treated with emodin at 20 mg/kg (71.25 ± 17.02 mg/g, $P < 0.05$). Emodin significantly protected the liver from injury by reducing serum AST and ALT activities and reducing hepatic hydroxyproline content. The mRNA levels of transforming growth factor- β 1 (TGF- β 1), Smad4 and α -SMA in liver tissues were significantly down-regulated in SD rats that received emodin treatment. Furthermore, significant down-regulation of serum TGF- β 1 protein levels and protein expression of Smad4 and α -SMA in liver tissues was also observed in the rats. Emodin inhibited HSC activation by reducing the abundance of TGF- β 1 and Smad4.

CONCLUSION: Emodin protects the rat liver from CCl₄-induced fibrogenesis by inhibiting HSC activation. Emodin might be a therapeutic antifibrotic agent for the treatment of hepatic fibrosis.

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Key words: Emodin; Hepatic fibrosis; Transforming growth factor- β 1; Smad4; Hepatic stellate cell; α -smooth muscle actin

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Dong MX, Jia Y, Zhang YB, Li CC, Geng YT, Zhou L, Li XY, Liu JC, Niu YC. Emodin protects rat liver from CCl₄-induced fibrogenesis *via* inhibition of hepatic stellate cells activation.

World J Gastroenterol 2009; 15(38): 4753-4762 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4753.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4753>

INTRODUCTION

Hepatic fibrosis occurs in advanced liver disease, where normal hepatic tissue is replaced with collagen-rich extracellular matrix (ECM) and, if left untreated, results in cirrhosis. Several studies have shown that hepatic fibrosis is a reversible disease, therefore an effective treatment would probably prevent or reverse the fibrotic process in the liver^[1,2]. Transforming growth factor β 1 (TGF- β 1) is one of the strongest profibrotic cytokines^[3,4], and TGF- β 1/Smad signaling is the cardinal signal transduction pathway involved in fibrosis which has been verified by several related studies^[5]. The down regulation of TGF- β 1 expression and modulation of TGF- β /Smad signaling may be effective in preventing liver fibrosis^[6].

In the last decade, advances in the understanding of genes promoting hepatic stellate cell (HSC) activation are impressive^[7]. However, there are few breakthroughs in therapeutic intervention of hepatic fibrogenesis. Efficient and well-tolerated antifibrotic drugs are lacking and current treatment of hepatic fibrosis is limited to withdrawal of the noxious agent^[8]. Therefore, research identifying innocuous antifibrotic agents is of high priority and urgently needed.

Emodin (1,3,8-trihydroxy-6-methylantraquinone), isolated from the rhizome of the Giant Knotweed Rhizome, has been used for centuries in Asia as a treatment for inflammation, gastrointestinal, pulmonary, and liver disorders. Emodin is regarded as the most active constituent in Giant Knotweed Rhizome and exerts many potent biological effects^[9,10], such as anticancer^[11], antimicrobial^[12], and anti-inflammatory effects^[13]. Several studies have revealed that emodin is efficacious in the management of hepatic fibrosis^[14,15]. However, the mechanisms underlying remain to be elucidated.

The current study evaluates the *in vivo* role of emodin in the protection of the liver from fibrogenesis caused by carbon tetrachloride (CCl₄) in a rat model and further explores the underlying mechanisms. We hypothesize that emodin might protect the liver from CCl₄-induced fibrogenesis by inhibiting activation of HSC *via* modulating TGF- β 1/Smad signaling pathways. Results in this study support our hypothesis and provide novel insight into the mechanisms of emodin in the protection of the liver.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Care and Use Committee of Qiqihar Medical University. A total of 50 pathogen-free male Sprague-Dawley (SD) rats (weight range: 200-240 g) were employed in the study. The animals were obtained from the Beijing Vital River

Experimental Animals Technology (Beijing, China), and were housed in sterile cages under laminar airflow hoods in a specific pathogen-free room with a 12 h light and 12 h dark schedule and fed autoclaved chow and water *ad libitum*. The animals were weighed every 7 d for the adjustment of the CCl₄ and emodin doses. Emodin were purchased from Xi'an Sino-Herb Bio-Technology CO., LTD (Purity: 98% by HPLC).

Establishment of a rat model with hepatic fibrogenesis caused by CCl₄

The rat model was established using the method originally described by Proctor *et al*^[16] and since used by many others^[17], with minor modifications. Fifty male SD rats were randomly divided into three groups: the normal control ($n = 10$) in which rats were not administered CCl₄ or emodin, but they were injected with olive oil and orally given sodium carboxymethylcellulose (CMC); the CCl₄ group ($n = 20$) in which rats were subcutaneously injected with CCl₄, without emodin treatment; the emodin group in which rats were injected with CCl₄ and treated with emodin at 20 mg/kg. Rats from the emodin group and the CCl₄ group were subcutaneously injected with a mixture of 40% CCl₄ (a mixture of pure CCl₄ and sterile olive oil) at 200 μ L/100 g body weight twice weekly for 12 wk. Emodin was dissolved in 0.5% sodium CMC and given once daily by gavage at 20 mg/kg. The rats in the normal group were similarly handled, including subcutaneous injections with the same volume of olive oil and oral administration of the same volume of CMC without emodin. At the end of the experiment, the survivors in the normal group, CCl₄ group and emodin group were 10/10, 9/20 and 11/20, respectively.

Forty-eight hours after the last CCl₄ injection, rats were sacrificed after being anesthetized by i.p. pentobarbital (50 mg/kg). A small portion of the liver was removed for hematoxylin and eosin (HE) staining and immunohistochemistry (IHC) studies by fixation with 10% formalin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen for extraction of total RNA and protein. Blood was collected directly from the rats when they were sacrificed. Serum was separated by centrifugation within 1 h of blood collection and stored at -20°C until analyzed.

Light microscopy

Midsections of the liver lobe a few mm thick were taken from each rat and processed for observation by light microscopy. The process involved fixing the tissue specimen in 10% neutral buffered formalin solution, preparing the block in paraffin, cutting into 5-6 μ m thick sections, and staining the sections with HE. The sections were scanned and analyzed by a pathologist who was blinded to the different treatments in the experiment.

The histological changes were measured on HE stained sections. Lobular inflammatory activity and severity of liver steatosis were determined according to the criteria of the Chinese Medical Association Committee of Fatty Liver Disease in 2006 and Nouchi *et al*^[18,19]. Steatosis was

graded on the basis of the extent of parenchyma involved as Grade 0, no hepatocytes were involved; Grade 1, < 30% of hepatocytes were involved; Grade 2, 30% to 50% of hepatocytes were involved; Grade 3, 51% to 75% of hepatocytes were involved; Grade 4, > 75% of hepatocytes were involved. Inflammation was graded as Grade 1, focal collections of mononuclear inflammatory cells; Grade 2, diffuse infiltrates of mononuclear inflammatory cells; Grade 3, focal collections of polymorphonuclear cells in addition to mononuclear cell infiltrates; and Grade 4, diffuse infiltrates of polymorphonuclear cells in the parenchymal area or lobular area. The stage of liver fibrosis was graded with the METAVIR scale^[20], which grades fibrosis on a five-point scale: Grade 0, no fibrosis; Grade 1, portal fibrosis without septa; Grade 2, portal fibrosis with a few septa; Grade 3, numerous septa without cirrhosis; and Grade 4, cirrhosis.

Biochemical parameters

Activities of alanine transaminase (ALT) and aspartate aminotransferase (AST) in serum were measured by routine laboratory methods using a 7170-automatic biochemistry analyzer (Tokyo, Japan).

Determination of the hepatic hydroxyproline content

The hydroxyproline kit was purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The content of hepatic hydroxyproline was determined by using the hydroxyproline kit following the protocol provided by the manufacturer. Results were expressed as micrograms of hydroxyproline per gram of hepatic tissue.

Enzyme-linked immunosorbent assay (ELISA)

The TGF- β 1 ELISA kit was obtained from Boster Biotechnology Co. Ltd. (Wuhan, China). The levels of TGF- β 1 in serum were determined by using the TGF- β 1 ELISA kit according to the manufacturer's protocol. In brief, 100 μ L of a serum sample was added to each well of the plate, followed by incubation for 2 h at 37°C. A Working Detector (100 μ L; Boster Biotechnology Co. Ltd) was loaded into each well, and the plate was incubated for an additional 1 h at room temperature (RT) before the addition of substrate solution (100 μ L; Boster Biotechnology Co. Ltd). The reaction was stopped by adding stop solution (1 drop; Boster Biotechnology Co. Ltd). The absorbance was read at 492 nm using a Microplate reader (LabSystems Multiskan Ascent 354, Finland). Calculation of the concentrations of TGF- β 1 was performed in a log-log linear regression according to the instructions in the protocol.

IHC analysis

Liver tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained for routine histology. The sections were incubated at 4°C overnight with primary antibody (Boster Biotechnology, Wuhan, China) in concentrations of 1:100 (Smad4) and 1:200 (α -SMA). As a secondary antibody, horseradish peroxidase-conjugated immunoglobulin G (Boster

Biotechnology), was used for 30 min at 37°C. After further washing with Tris-buffered saline, sections were incubated with complex/horseradish peroxidase (1:200 dilution) for 30 min at 37°C. Immunolocalization was performed by immersion in 0.05% 3,3'-diaminobenzidine tetrahydrochloride as chromagen. Slides were counter-stained with hematoxylin before dehydration and mounting. Incubation without the primary antibody was performed as a control for the background staining. Histological evaluation was performed by a pathologist who was blind to the pharmacological characteristics of the drugs.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from 100 mg of frozen liver tissues using RNAiso Reagent kit (Takara Biotechnology, Dalian, China) according to the protocol provided by the manufacturer. cDNA was synthesized with SYBR ExScript™ RT-PCR kit (Takara Biotechnology, Dalian, China) according to the protocol provided by the manufacturer. Reverse transcription was carried out as follows: 42°C for 15 min, 95°C for 2 min (one cycle). cDNA was stored at -20°C for PCR. Real-time PCR was performed in 50 μ L of reaction solution containing 2 \times SYBR Premix Ex Taq polymerase, deoxynucleoside triphosphates, ROX Reverence Dye and the corresponding primers. The cycles for PCR were as follows: 1 cycle of 95°C for 10 s, 40 cycles of 5 s at 95°C, 5 s at 60°C, 31 s at 60°C and a final 7 min at 72°C. Melting curve analysis was always included to validate the specificity of the PCR products. Serial cDNA dilution curves were produced to calculate the amplification efficiency for all genes. A graph of threshold cycle (Ct) versus log 10 relative copy number of the sample from a dilution series was produced. The slope of the curve was used to determine the amplification efficiency. Reactions were performed in an ABI7300 Real-time PCR system (Applied Biosystems, CA) and threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA). GAPDH was used as an internal control. mRNA -fold change relative to GAPDH was calculated with the comparative Ct method of $2^{-\Delta\Delta C_t}$ ^[21]. The following primers were used. 5'-GACAACTTTGGCATCGTGGA-3' (sense) and 5'-ATGCAGGGATGATGTTCTGG-3' (antisense) for the GAPDH gene; 5'-CCTGATGCTTCACTGTTCTGCAA-3' (sense) and 5'-CAACTGCACGGTTTCCGTTATTC-3' (antisense) for the Smad4 gene; 5'-TATAGCAACAATTCCTGGCG-3' (sense) and 5'-TGCTGTACAGGAGCAGTG-3' (antisense) for the TGF- β 1 gene; 5'-CCGAGATCTCACCGACTACC-3' (sense) and 5'-TCCAGAGCGACATAGCACAG-3' (antisense) for the α -SMA gene. mRNA levels were expressed as -fold changes after normalization with GAPDH. All tests were done in triplicate to ensure reproducibility.

Western blotting

Cytoplasm proteins were isolated from 120 mg of frozen liver tissues using a Cytoplasmic Protein

Extraction kit (Beyotime Biotechnology, Haimen, China) according to the protocol provided by the manufacturer. Protein concentrations were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer (Beyotime Biotechnology, Haimen, China). 100 μ L of supernatant was added to an equal volume of $2 \times$ SDS sample buffer and boiled for 5 min at 100°C. The samples were then stored at -80°C until analyzed. The electrophoretic mobility of the proteins analyzed in this study was determined by SDS-polyacrylamide gel electrophoresis using 15% acrylamide concentrations. After electrophoresis, the proteins were transferred electrophoretically to a nitrocellulose filter membrane that was then blocked for 4 h in a solution of 8% nonfat dry milk in Tris-buffered saline containing 0.1% tween (pH 7.6) at RT. The membrane was then incubated overnight at 4°C with Smad4 antibody and GAPDH antibody which are represented on Western blotting by two distinct bands at 65 and 36 kDa. Bands were washed four times, after which they were incubated with Horseradish Peroxidase Labeled Anti-Mouse IgG (Medical Biological Laboratory, Nagoya, Japan) for 2 h and again washed four times. The blots were developed using an ECL Western blotting kit as recommended by the manufacturer. GAPDH was probed as an internal control. GAPDH was used to confirm that an equal amount of protein was loaded in each lane. Band intensities were determined using an AlphaImager™ 2200 using the SpotDenso function of AlphaEaseFC™ Software version 3.1.2 (Witec, Littau, Switzerland).

Statistical analysis

All determinations were repeated three times, and results are expressed as the mean \pm SD. ANOVA was used to evaluate the difference among multiple groups followed by a post hoc test (Student-Newman-Keuls) for quantitative data, and RIDIT test was used for statistical analysis of qualitative data. The data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

RESULTS

Emodin protected the liver against CCl₄-induced injury and suppressed hepatic fibrogenesis in the rat model

The effects of emodin on the protection of the liver from injury and fibrogenesis were initially evaluated by histological analyses. Representative views of liver sections are shown in Figure 1A. As shown in tissue sections stained with HE, compared with sections from livers in the vehicle controls (normal group), CCl₄ caused prominent hepatic steatosis, necrosis, and formation of regenerative nodules and fibrotic septa between the nodules (CCl₄ group). Oral administration of emodin daily for 12 wk improved the state of steatosis with a significant reduction in the number of macro- and microvesicular steatosis lesions, and it apparently suppressed hepatic fibrogenesis by reducing the thickness of bridging fibrotic septa (emodin group).

Table 1 Pathological score of CCl₄-induced liver fibrosis in rats

Group	n	Degree of pathological					U
		0	1	2	3	4	
Steatosis							
Normal control	10	10	0	0	0	0	3.89 ^b
CCl ₄	9	0	0	1	1	7	
CCl ₄ + emodin	11	0	1	4	5	1	2.77 ^b
Inflammation							
Normal control	10	10	0	0	0	-	4.21 ^b
CCl ₄	9	1	1	3	4	-	
CCl ₄ + emodin	11	3	7	1	0	-	2.03 ^a
Fibrosis							
Normal control	10	10	0	0	0	0	3.89 ^b
CCl ₄	9	0	0	0	5	4	
CCl ₄ + emodin	11	0	3	4	3	1	2.81 ^b

A piece of liver tissue from rats in each group was fixed with formalin, and then it was embedded in paraffin. Thin sections were cut and stained with HE. U represents the RIDIT value of the two groups, $P < 0.05$ indicates $U > 1.96$, $P < 0.01$ indicates $U > 2.58$. ^a $P < 0.05$ vs CCl₄ group; ^b $P < 0.01$ vs CCl₄ group.

According to METAVIR scale, the degree of hepatic fibrosis increased markedly in the CCl₄ group compared to the normal group, and decreased markedly in the emodin group compared to the CCl₄ group ($P < 0.01$, Table 1). Taken together, emodin reduced hepatic fibrogenesis caused by chronic CCl₄ intoxication.

Emodin reduced the content of hepatic hydroxyproline in the CCl₄ rat model

The efficacy of treatment with emodin on protection of the liver from fibrogenesis was further evaluated by using a quantitative method to determine the content of hepatic hydroxyproline in the rat model. Compared with the normal controls (54.53 ± 13.46 mg/g), the hepatic hydroxyproline content was significantly higher in rats injected with CCl₄ (120.27 ± 28.47 mg/g, $P < 0.05$). The hepatic hydroxyproline content was significantly reduced in rats treated with emodin at 20 mg/kg (71.25 ± 17.02 mg/g, $P < 0.05$).

Emodin suppresses serum activities of ALT and AST in the CCl₄ rat model

Biochemical analyses of serum enzymes were performed to verify the role of emodin in the protection of the liver from injury. As shown in Figure 2, compared with those in the normal controls (51.02 ± 10.64 IU/L and 132.28 ± 18.14 IU/L), the activities of serum ALT and AST were significantly higher in rats injected with CCl₄ (289.25 ± 68.84 IU/L and 423.89 ± 35.67 IU/L). The activities of serum ALT and AST were significantly reduced by administration of emodin (176.34 ± 47.29 IU/L and 226.1 ± 44.52 IU/L). These results demonstrated that emodin protected the liver against CCl₄-induced injury.

Emodin reduces HSC activation in the liver in the CCl₄ rat model

IHC and real-time PCR experiments were performed to further evaluate the impact of emodin on regulating

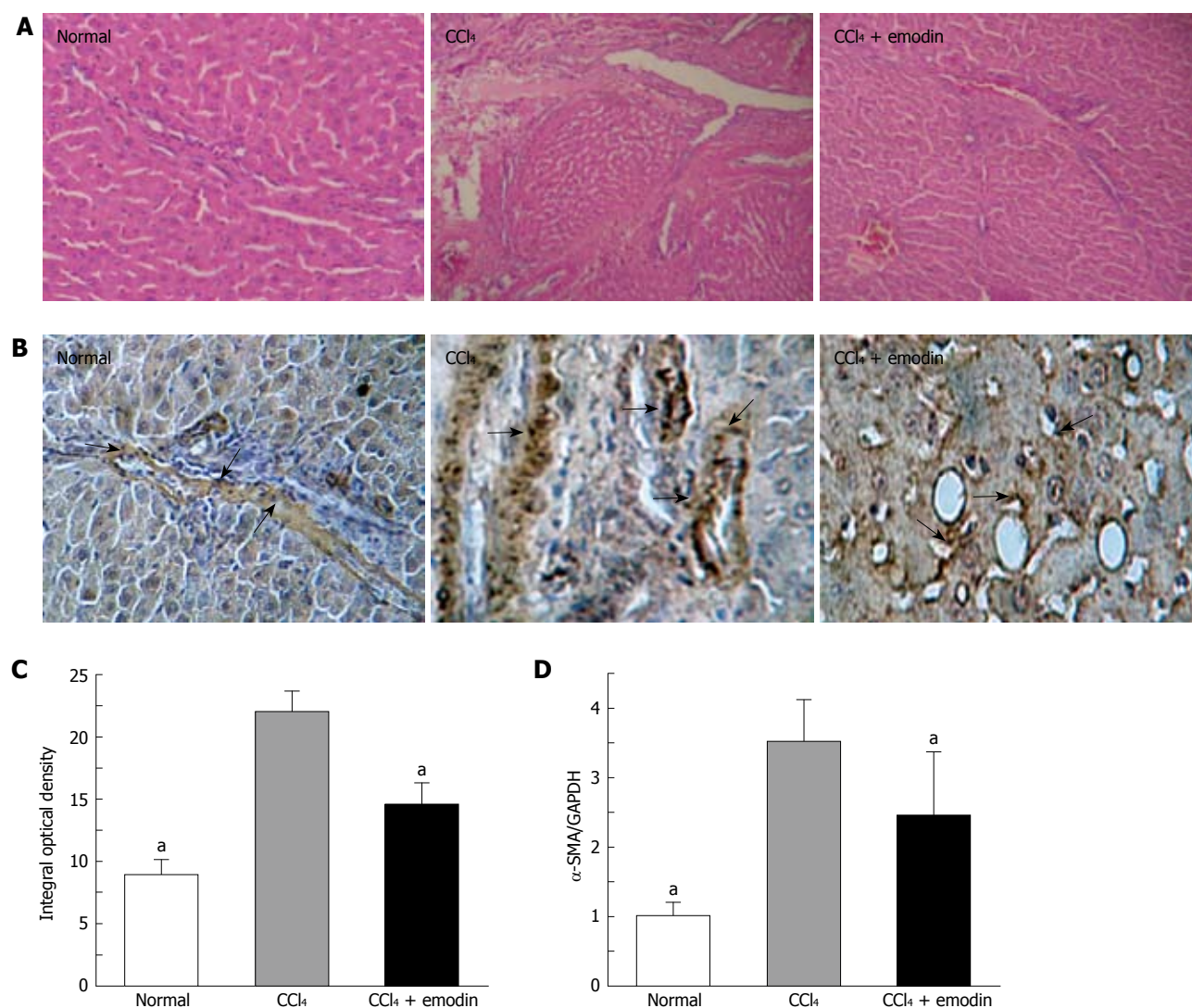


Figure 1 Emodin protects the liver from CCl₄-induced fibrogenesis in rats via inhibition of hepatic stellate cell activation. A piece of liver tissue from each treated rat ($n = 6$ per group) was fixed with formalin, and then it was embedded in paraffin. A: Thin sections were cut and stained with HE; B: α -SMA proteins were stained by immunohistochemistry in liver tissue. Representative views from each group ($n = 6$ per group) are presented (original magnification, $\times 400$). Arrows indicated an area positively labeled with α -SMA; C: Semi-quantification of α -SMA staining. Values are expressed as mean \pm SD, $n = 6$ per group. $^aP < 0.05$ vs CCl₄ group. Total RNA extracted from rat liver tissues was used to synthesize cDNA; D: The α -SMA mRNA was detected by real-time PCR, and mRNA levels are expressed as -fold differences relative to normal rat after normalization to the housekeeping gene's GAPDH mRNA. Values are expressed as mean \pm SD, $n = 5$ per group. $^aP < 0.05$ vs CCl₄ group.

the expression of α -SMA, the marker of activated HSC. Liver sections from each group were immunolabeled with antibodies against α -SMA. As shown in Figure 1B, as expected, few cells in the liver sections from the normal group were recognized by antibodies against α -SMA, suggesting few activated HSC in the normal livers in the vehicle control rats. Administration of CCl₄ caused a significant increase in the number of cells recognized by antibodies against α -SMA. Emodin treatment significantly reduced the number of cells labeled with α -SMA antibodies, suggesting that emodin might suppress HSC activation in the rat model. The comparative Ct method of $2^{-\Delta\Delta C_t}$ and IHC evaluation result showed that protein and mRNA levels of α -SMA in liver tissues from normal control rats were 8.88 ± 1.26 and 1.01 ± 0.19 , respectively while those in the CCl₄ group were 21.97 ± 1.68 and 3.52 ± 0.60 , respectively. Treatment of rats with emodin during CCl₄ exposure largely increased expression of α -SMA and

resulted in protein and mRNA levels of 14.61 ± 1.67 and 2.46 ± 0.91 , respectively (Figure 1C and D).

Emodin reduces the concentration of TGF- β 1 in serum and mRNA levels in liver tissues

TGF- β 1 is the major profibrogenic factor during hepatic fibrogenesis. We examined the effect of emodin on the concentration of TGF- β 1 in serum and mRNA levels in liver tissues of the rat model by ELISA and real-time PCR. As shown in Figure 3, compared with those in the normal group (84.89 ± 27.14 pg/mL and 1.01 ± 0.16 , respectively), the levels of TGF- β 1 in serum (Figure 3A) and mRNA levels of TGF- β 1 in liver tissues (Figure 3B) were dramatically increased in the CCl₄ group (313.40 ± 57.75 pg/mL and 3.89 ± 1.00 , respectively, both $P < 0.05$ vs normal group). The levels of TGF- β 1 in serum and mRNA levels of TGF- β 1 in liver tissues were significantly reduced in the emodin group (151 ± 47.64 pg/mL and

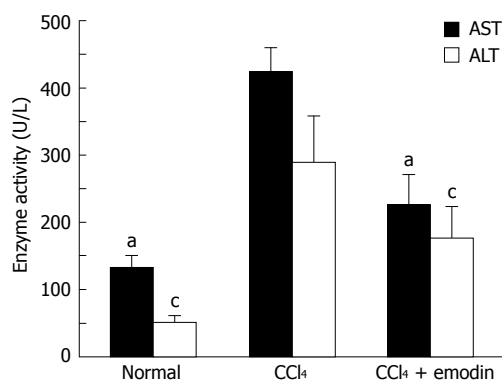


Figure 2 Emodin decreases the levels of serum aspartate amino-transferase (AST) and alanine aminotransferase (ALT) activities. Serum samples were obtained from each treated rat through heart puncture when sacrificed. After coagulation, serum AST and ALT activities were determined by routine laboratory methods. Values are expressed as mean \pm SD, $n = 6$ per group. AST: ^a $P < 0.05$ vs CCl₄ group; ALT: ^c $P < 0.05$ vs CCl₄ group.

2.16 \pm 0.73, respectively, both $P < 0.05$ vs CCl₄ group). Although these were still higher than those of the normal group, these data indicated that emodin significantly reduced the levels of TGF- β 1 in serum and mRNA levels in liver tissues in the rat model, which might result in the inhibition of HSC activation stimulated by CCl₄.

Emodin down-regulates the protein and mRNA levels of Smad4 in liver tissues of the CCl₄ rat model

Because TGF- β 1 signals within the cell through Smad is involved in fibrosis, the effects of emodin on mRNA and protein levels of Smad4 in liver tissues were demonstrated by real-time PCR (Figure 4C), Western blotting (Figure 4D and E), and IHC analyses (Figure 4A and B). Experiments revealed that exposure of rats to CCl₄ significantly increased mRNA and protein levels of Smad4 in liver tissues from 1.00 \pm 0.13, 0.54 \pm 0.04 and 5.78 \pm 1.05, respectively, in the normal group to 4.63 \pm 0.86, 13.44 \pm 0.64 and 23.95 \pm 3.23, respectively, in the CCl₄ group. In contrast, protein and mRNA levels of Smad4 in liver tissues from rats treated with emodin during CCl₄ exposure were attenuated and were 2.94 \pm 0.74, 9.25 \pm 0.84 and 17.00 \pm 1.88, respectively. Treatment of rats with emodin during CCl₄ exposure blunted the increase in protein and mRNA levels of Smad4 significantly.

DISCUSSION

In the present study, we confirmed that emodin protects the rat liver from CCl₄-induced injury and fibrogenesis. The mechanism for this protective effect may relate to the fact that emodin efficiently inhibits HSC activation *in vivo*.

Hepatic fibrosis, which may lead to cirrhosis, is associated with most chronic liver diseases^[22]. Hepatic fibrosis is thought to be a reversible disease, however, there is no satisfactory method in clinical practice to reverse the pathological process yet^[23]. Several drugs, including antisense TGF- β 1 receptors, cytokines^[24], antioxidants, chemical drugs^[25], soluble type II receptor of TGF- β 1, and TGF- β 1 antibodies^[26] have been used in research work to block experimental hepatic fibrosis, but

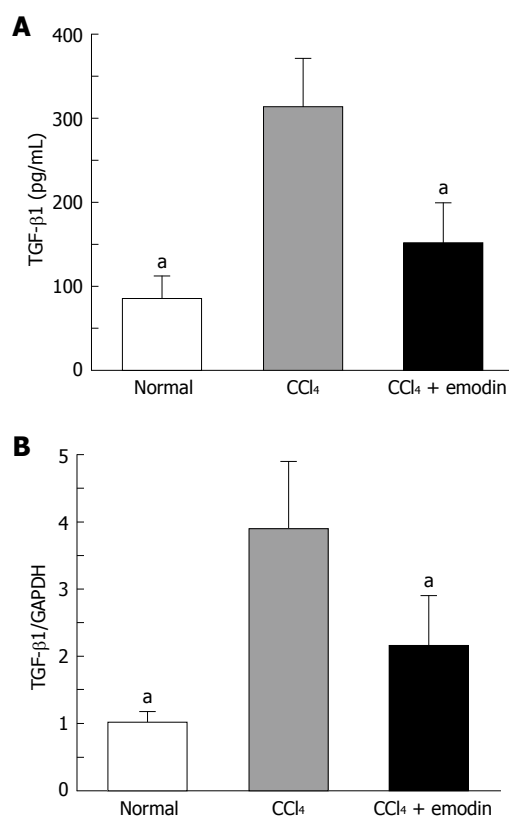


Figure 3 Emodin decreases the levels of serum transforming growth factor- β 1 (TGF- β 1) and mRNA levels in liver tissues in the rat model. Serum and liver tissue were obtained from each treated rat. A: Levels of serum TGF- β 1 were determined by ELISA; B: Total RNA extracted from rat liver tissues was used to synthesize cDNA. The mRNA level of TGF- β 1 in liver tissues was detected by real-time PCR, and mRNA levels were expressed as -fold differences relative to normal rat after normalization to the housekeeping gene's GAPDH mRNA. Values are expressed as mean \pm SD, $n = 5$ per group. ^a $P < 0.05$ vs CCl₄ group.

their effects were not as prosperous as we had expected. Some traditional Chinese drugs have been found effective in preventing fibrogenesis and other causes of chronic liver injury^[27,28], and this helps to develop a more hopeful future in controlling liver fibrosis and cirrhosis. Emodin is a main active monomer isolated from Giant Knotweed Rhizome, which is widely used in traditional Chinese herb treatment of liver cirrhosis^[29]. It is easy to extract, isolate and identify emodin, so it shows excellent prospects in the development of some new drugs for treating hepatic fibrosis.

CCl₄, a highly toxic chemical agent, causes hepatic injury including hepatocytic necrosis, steatosis, and inflammation. Research for establishing a model of liver fibrosis with CCl₄ began in 1936. Since then many methods to establish a model of liver fibrosis have been tried^[30]. Among them, hepatic fibrosis caused by CCl₄ has been extensively used in experimental models in rats because hepatic responses in rats to chronic CCl₄ stimulation are shown to be superficially similar to human cirrhosis^[31]. Hepatocyte damage is the initial factor of hepatic fibrogenesis and activities of ALT and AST in serum are the most commonly used biochemical markers of liver injuries^[32]. Hydroxyproline is an amino acid found

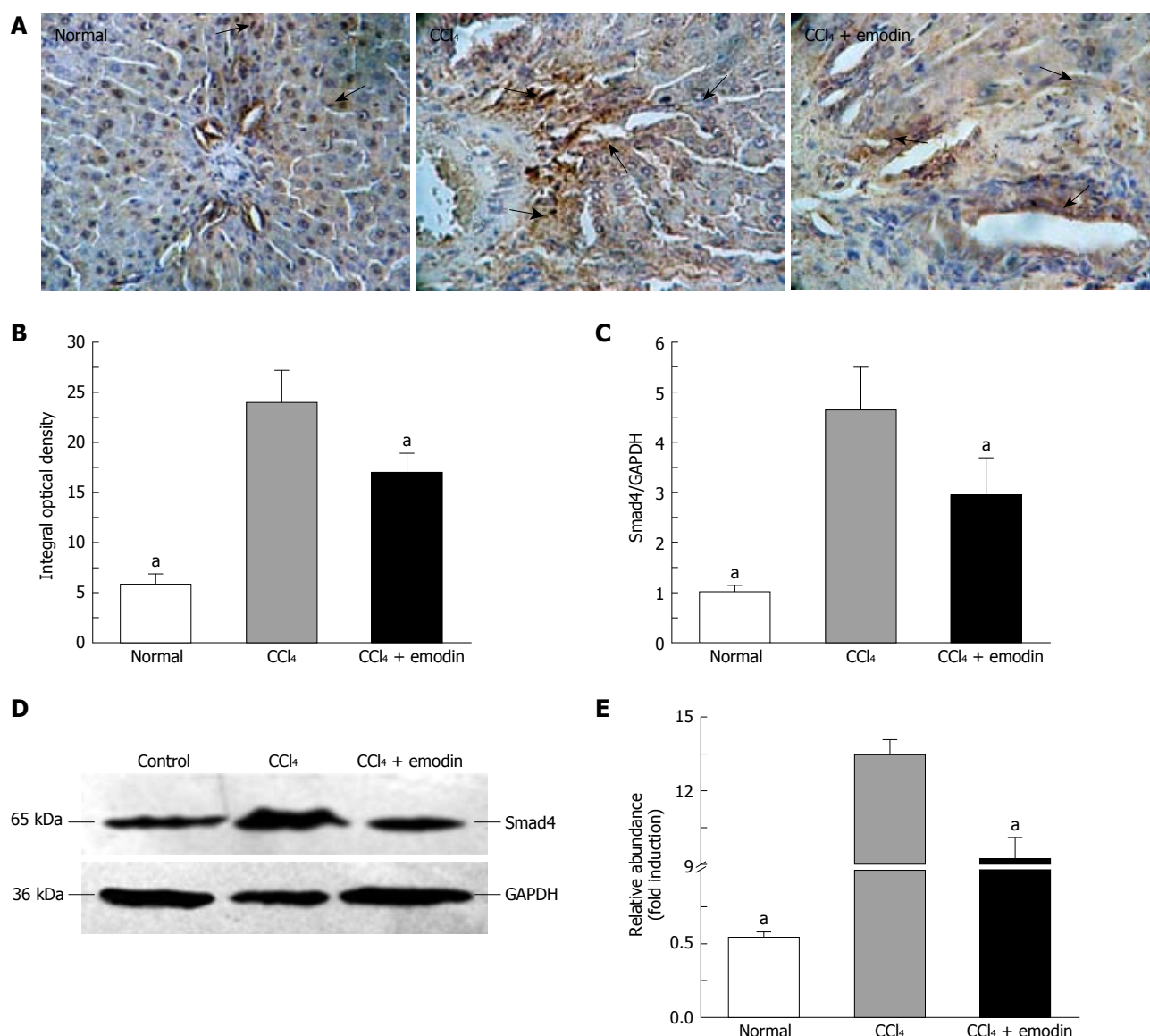


Figure 4 Emodin inhibits expression of protein and mRNA levels of Smad4 in liver tissues in the rat model. A piece of liver tissue was collected from each treated rat. A: Smad4 proteins in liver tissue were stained by IHC. Representative views from each group ($n = 6$ per group) are presented (original magnification, $\times 400$). Arrows indicate an area positively labeled with Smad4; B: Semi-quantification of Smad4 staining. Values are expressed as mean \pm SD, $n = 6$ per group. $^aP < 0.05$ vs CCl₄ group; C: Real-time PCR analyses of the mRNA levels of Smad4. Values are presented as means \pm SD, $n = 5$ per group. GAPDH was used as an invariant internal control for calculating mRNA-fold changes; D, E: Western blotting analyses of the protein abundance of Smad4. GAPDH was used as an invariant control for equal loading. $^aP < 0.05$ vs CCl₄ group.

almost exclusively in collagens. Determination of the content of hydroxyproline in liver tissue is regarded as a good method to quantify fibrosis and to evaluate the effectiveness of new potentially antifibrotic agents. In this study, the method of subcutaneously injecting CCl₄ was used to establish the model of liver fibrosis. Histological analysis showed CCl₄ caused prominent hepatic steatosis, necrosis, and formation of regenerative nodules and fibrotic septa between the nodules. Biochemical assay showed serum ALT activities, serum AST activities, and content of hepatic hydroxyproline were markedly increased in rats injected with CCl₄ for 12 wk, which are consistent with the histological observations.

Our results suggest that oral administration of emodin daily for 12 wk improved the state of steatosis with a significant reduction in the number of macro- and

microvesicular steatosis, and it also apparently suppressed hepatic fibrogenesis by reducing the thickness of bridging fibrotic septa. Emodin could decrease the scores of hepatic fibrosis grading, inhibit the ALT and AST activities in serum and reduced the content of hepatic hydroxyproline. All results confirm that emodin protected the liver from injury and fibrogenesis caused by CCl₄ in the rat model.

Chronic liver injury may lead to development of fibrosis, a process in which HSC play a major role. As a result of liver injury, HSC, which in the healthy organ store vitamin A, undergo a process of activation that is mediated by the concerted action of resident hepatic cell types such as Kupffer cells, liver endothelial cells, and hepatocytes. The phenotype of activated HSC is characterized by α -smooth muscle actin (α -SMA)

expression^[33]. α -SMA expression in the liver tissues is an indicator of hepatic stellate cell activation, which is recognized as being critical in liver fibrogenesis. Thus inhibition of the accumulation of activated HSCs is an important therapeutic strategy^[34]. Our results showed the levels of α -SMA in rat liver tissues increased significantly after CCl₄ administration for 12 wk. Emodin reduced α -SMA expression at mRNA and protein levels.

Inflammation is commonly associated with hepatic fibrogenesis during chronic liver diseases^[35]. CCl₄ is metabolized in the liver by cytochrome P450 into the free radical CCl₃^[36]. The free radical attacks hepatocytes and causes necrosis of parenchymal cells, which promotes inflammatory responses in the liver^[37]. Results in this study indicated that emodin suppressed inflammation caused by CCl₄, which might lead to the protection of the liver from injury. It is now widely accepted that the pro-inflammatory cytokine TGF- β 1 is a major cytokine in the regulation of the production, degradation, and accumulation of ECM^[38], and it has been suggested that overexpression of TGF- β 1 for a prolonged period of time after tissue damage may induce a fibroproliferative response and deposition of ECM, resulting in fibrosis in vital organs^[39]. Many studies have detected the presence of TGF- β 1, in the form of either protein or message, in the fibrotic tissues of animal models or human samples^[40]. Partial inhibition of the accumulation of ECM using either anti-TGF- β 1 serum or a TGF- β 1-binding protein has been reported in fibrosis models^[41]. Our results showed that TGF- β 1 mRNA levels and serum TGF- β 1 protein levels in normal rat were low. After injection of CCl₄ for 12 wk, mRNA and protein levels of TGF- β 1 increased significantly. Emodin down-regulated mRNA levels of TGF- β 1 expression in liver tissue. Furthermore, serum TGF- β 1 levels in the model rats were also significantly down-regulated by emodin treatment in a manner similar to hepatic fibrosis attenuation. These findings imply that emodin might attenuate hepatic fibrosis through down-regulation of TGF- β 1 expression *in vivo*.

Smad4 is well known to function as one of the downstream effectors of TGF- β 1, and it mediates TGF- β 1-induced collagen synthesis^[42]. Smads are intracellular signal transductive molecules of the TGF- β super family. According to differences in structure and function, nine Smads have been reported and classified into three groups. Smads 2 and 3 are named R-Smads in the pathway and Smad4 Co-Smads for all these pathways. Smads 6, 7, 8 are inhibitory factors of these Smads. When TGF- β 1 binds to its receptor, Smad 2/3 is phosphorylated and binds with Smad4 and together they move into the nucleus for translation and expression of the target gene^[43,44]. Smad signal transduction pathways are thought to play a crucial role in the process of liver damage and recovery, as well as liver fibrosis. These transcriptional responses appear to be mediated predominantly through Smad4. The widely held conclusion that Smad4 occupies a central role in transduction of TGF- β 1 signals comes from multiple lines of biochemical and genetic evidence^[45]. In reconstitution experiments, cell lines that lack Smad4

fail to respond to TGF- β 1 signals, transfection of wild-type Smad4 restores the signaling capabilities of these cells^[46]. Our study showed that both mRNA and protein expressions of Smad4 were remarkably up-regulated in fibrotic rats. We also observed down-regulation of Smad4 expression in emodin-treated fibrotic rats, suggesting that emodin attenuate hepatic fibrosis by regulating TGF- β 1/smud signaling.

In conclusion, the data presented herein provide evidence that emodin is active as an antifibrogenic drug able to reduce the biological effects of TGF- β 1 in ongoing fibrogenesis. Giant Knotweed Rhizome, a traditional Chinese herbal medicine, is widely used in clinical practice for treating cirrhosis. Emodin, the main active monomer isolated from Giant Knotweed Rhizome, may be an attractive therapeutic agent for the treatment of fibrotic liver diseases.

COMMENTS

Background

In the last decade, advances in the understanding of genes promoting hepatic stellate cell (HSC) activation are impressive. However, there are few breakthroughs in therapeutic intervention of hepatic fibrogenesis. Efficient and well-tolerated antifibrotic drugs are lacking and current treatment of hepatic fibrosis is limited to withdrawal of the noxious agent. Research identifying innocuous antifibrotic agents is of high priority and urgently needed.

Research frontiers

Emodin is efficacious in the management of hepatic fibrosis. However, the mechanisms underlying its effects remain to be elucidated. The current study evaluates the *in vivo* role of emodin in the protection of the liver from fibrogenesis caused by carbon tetrachloride (CCl₄) in a rat model and further explores the underlying mechanisms.

Innovations and breakthroughs

To the best of the authors' knowledge, this is the first study to report that emodin protects the liver from CCl₄-induced fibrogenesis by inhibiting activation of HSC *via* modulating transforming growth factor- β 1 (TGF- β 1)/Smad signaling pathways. Results in this study provide novel insight into the mechanisms of emodin in the protection of the liver.

Applications

By evaluating the role of emodin in protecting the liver against fibrogenesis caused by carbon tetrachloride (CCl₄) in rats *via* inhibition of hepatic stellate cells activation, emodin might be a therapeutic antifibrotic agent for the treatment of hepatic fibrosis.

Terminology

Smad4 is a protein which in humans is encoded by the *SMAD4* gene. SMAD4 is a 552 amino acid protein involved in cell signaling. It is the only known mammalian coSmad. It is a homolog of the *Drosophila* protein: "Mothers against decapentaplegic".

Peer review

This study examines the effects of emodin on CCl₄-induced liver fibrosis. The authors show reduced fibrosis, decreased stellate cell smooth muscle actin expression and decreased TGF- β expression. The study has been suitably designed and clearly reported.

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S- Editor Li LF L- Editor O'Neill M E- Editor Lin YP

Effects of metoclopramide on gastric motility measured by short-term bio-impedance

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Supported by A grant from CONACYT (J50182), The University of Guanajuato (DINPO 00098-08) and the Dean's Undergraduate Research Fund from The College of Arts and Science, New York University

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Received: June 6, 2009 Revised: August 26, 2009

Accepted: September 1, 2009

Published online: October 14, 2009

Abstract

AIM: To analyze the accuracy of short-term bio-impedance as a means of measuring gastric motility.

METHODS: We evaluated differences in the short-term electrical bio-impedance signal from the gastric region in the following conditions: (1) fasting state, (2) after the administration of metoclopramide (a drug that induces an increase in gastric motility) and (3) after food ingestion in 23 healthy volunteers. We record-

ed the real component of the electrical impedance signal from the gastric region for 1000 s. We performed a Fast Fourier Transform (FFT) on this data and then compared the signal among the fasting, medicated, and postprandial conditions using the median of the area under the curve, the relative area under the curve and the main peak activity.

RESULTS: The median of the area under the curve of the frequency range in the region between 2-8 cycles per minute (cpm) decreased from 4.7 cpm in the fasting condition to 4.0 cpm in the medicated state ($t = 3.32$, $P = 0.004$). This concurred with the decrease seen in the relative area under the FFT curve in the region from 4 to 8 cpm from 38.3% to 26.6% ($t = 2.81$, $P = 0.012$) and the increase in area in the region from 2 to 4 cpm from 22.4% to 27.7%, respectively ($t = -2.5$, $P = 0.022$). Finally the main peak position also decreased in the region from 2 to 8 cpm. Main peak activity in the fasting state was 4.72 cpm and declined to 3.45 cpm in the medicated state ($t = 2.47$, $P = 0.025$). There was a decrease from the fasting state to the postprandial state at 3.02 cpm ($t = 4.0$, $P = 0.0013$).

CONCLUSION: Short-term electrical bio-impedance can assess gastric motility changes in individuals experiencing gastric stress by analyzing the area medians and relative areas under the FFT curve.

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Key words: Bio-impedance; Fast Fourier Transform; Gastric motility; Metoclopramide; Postprandial

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Huerta-Franco MR, Vargas-Luna M, Capaccione KM, Yañez-Roldán E, Hernández-Ledezma U, Morales-Mata I, Córdova-Fraga T. Effects of metoclopramide on gastric motility measured by short-term bio-impedance. *World J Gastroenterol* 2009; 15(38): 4763-4769 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4763.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4763>

INTRODUCTION

Numerous studies have demonstrated that gastric motility occurs at a rate of three cycles, or peristaltic waves, per minute. This consistent rate is due to myoelectrical activity, which produces ring contractions through the distal gastric wall. These peristaltic waves facilitate food digestion and gastric emptying^[1-3]. In various studies, gastric emptying has been used to assess many diseases using several different measurement techniques and methods of data analysis^[4-8].

Scintigraphy has become the gold standard technique for the evaluation of gastric activity despite the fact that it measures gastric emptying as the main consequence of gastric motility^[3,4,9]. Also for the evaluation of the gastric motility, there are invasive methods such as manometry, which uses a pressure probe device in a specific region of the gastric tract to directly measure the amplitude and frequency of the gastric movements^[10-12]. Electrogastrography could be another invasive method, if electrodes are introduced into the gastro-intestinal tract in order to record the myoelectrical activity in a specific area of the tract^[13]. The bio-impedance technique can also be invasive, for example, when used to measure intraluminal impedance in esophageal function studies^[14,15]. Non-invasive options include electrogastrography if recordings are performed through the skin using cutaneous electrodes^[16-23], and electrical bio-impedance in the gastric region when measured using cutaneous electrodes^[24-26]. As opposed to gastric electrical stimulation^[27,28], the bio-impedance technique uses a low current and voltage to obtain passive gastric impedance characteristics. In these two cases, uncertainties arise in the interpretation of the signal because cutaneous recording leads to the simultaneous acquisition of signals at multiple frequencies within the gastrointestinal (GI) system. The main challenge of this technique is the decomposition of the data to isolate the frequencies originating in the gastric tract^[16,25,29,30].

In looking for a balance between accuracy, reproducibility and patient comfort, electrogastrography and electrical bio-impedance are the most suitable techniques for the evaluation of gastric motility^[31], despite the fact that occasionally electrical activity does not correlate directly with mechanical movements^[11]. In the context of selecting one of these two techniques for gastric motility assessment, electrical bio-impedance has the advantage of being directly sensitive to movements and therefore a better option than electrogastrography for gastric motility evaluation.

In the evaluation of gastric motility, bio-impedance techniques have been shown to be sensitive to material changes, including internal conformational changes (bowel movements)^[26,32] and conduction properties (stomach acidity or properties of ingested food)^[33]. This method has been used in gastric emptying studies for long-term measurements. Measurements are evaluated by looking for global impedance changes, which are changes over long time periods and ignoring impedance oscillations, short-term faster oscillating changes, of the gastric region^[24].

Recently, this long-term assessment method has been proposed for the evaluation of gastric secretions because it is highly sensitive to the conductive properties of ingested food and internal fluid secretions, and therefore may be a useful method for such evaluations^[34].

For gastric motility, bio-impedance evaluation produces results consistent with those of other gastric motility methods, mainly the position of the main peak activity around three cycles per minute (cpm)^[24,25]. However, the sensitivity to other gastrointestinal regions and the overlapping of movements from those regions produce signals in other frequency ranges that are neither due to bradygastria or tachygastria but are associated with characteristic intestinal or colon frequencies^[1].

There are several benefits to the bio-impedance technique: it directly measures motility, it is non-invasive, and its use for interval measurements (short term bio-impedance) can accurately reconstruct a long period of measurement. The first benefit emphasizes accuracy while the latter two maximize patient comfort. Additionally, this approach is less expensive than other techniques and so the development of this method for clinical purposes can benefit a wider range of people worldwide.

In a previous study, we evaluated normal subjects in both the fasting and postprandial states using short-term electrical bio-impedance^[32]. We compared our data with those obtained from the concurrent use of long-term bio-impedance, and found consistent results. Thus, we concluded that short-term electrical bio-impedance is an accurate evaluation method in healthy volunteers. In particular, the position of the main activity around 3 cpm was strong support for the usefulness of this method in healthy volunteers because of its unequivocal agreement with several other methods of gastric motility assessment. However, for this technique to attain clinical relevance, bio-impedance must be shown to be equivalent to validated methods in subjects experiencing gastric stress, which manifests itself through small changes in peristaltic wave frequency. Thus, we must evaluate if short-term electrical bio-impedance (5-15 min) has sufficient sensitivity to record these changes.

In this study, we use the drug metoclopramide to induce gastric stress in healthy volunteers, mimicking a state of gastric stress that would warrant clinical evaluation. By comparing the data from the area under the Fast Fourier Transform (FFT) spectra curves to those obtained from the main peak of the spectra, we assessed the potential of electrical bio-impedance as a method for the evaluation of gastric motility in systems experiencing gastric stress.

MATERIALS AND METHODS

Subjects

For this study we recruited twenty three volunteers. Each met the following criteria: 18-30 years of age, no prior gastrointestinal disease, no prior disease that may affect the GI system function (including diabetes, Parkinson's disease, amyloidosis, myotonic dystrophy, polymyositis,

HIV infection or cytomegalovirus infection), and they must not have been taking medicine that could interfere with gastric activity. Additionally, participants must not have had significant weight loss within the 3 mo prior to the study, and must not have been overweight ($\text{BMI} \geq 25 \text{ kg/m}^2$). In addition to these physical criteria, subjects must have been free from psychological problems such as anxiety, stress, depression and mental disease, because these conditions could potentially affect gastric activity through endocrine and central nervous system modulation. Also, subjects were free from any other endocrine disorders and had no food allergies that interfered with the food administered in this trial.

All women who participated in this study underwent evaluation within the first 10 d (the early follicular phase) of their menstrual cycle. All subjects who participated in this study signed a consent form approved by the Human Ethics Committee of the University of Guanajuato. The study was conducted according to the Declaration of Helsinki^[35].

Metoclopramide administered orally

Metoclopramide [IUPAC name: 4-amino-5-chloro-N-(2-diethylamino ethyl)-2-methoxybenzamide] is a dopamine receptor antagonist with both antiemetic and prokinetic properties. The antiemetic property treats nausea and vomiting while the prokinetic properties enhance the peristaltic waves of the stomach and intestine, increasing the rate of absorption and thus the rate of passage through the stomach. Therefore, the administration of metoclopramide in healthy volunteers speeds gastric motility and subsequently the rate of gastric emptying in a measurable way. In healthy patients, metoclopramide is absorbed rapidly and almost completely from the GI tract following oral administration. The pharmacological action of orally administered metoclopramide begins 30–60 min after the subject takes the drug and persists for 1–2 h^[36]. Therefore, after performing our baseline recordings in the fasting individual, metoclopramide was administered and we took bio-impedance measurements after a 60 min resting period. This ensured that the subjects were experiencing the maximal pharmacological effects of metoclopramide while the bio-impedance measurements were taken.

Clinical evaluation

Before beginning the experiment, a clinical history was taken from each of the subjects. This evaluation included the following sections: (1) General Information: name, age, gender, occupation; (2) Pathological Antecedents: clinical history of disease related to the GI tract; (3) Physiological Information: anthropometrical measurements and body mass index; (4) Life-style variables: exercise, sleep habits, substance use (smoking, alcohol, medication); (5) Observations: additional information gained from the physical examination; (6) Gynecologic-Obstetric Characteristics: for all women, we collected data regarding the onset of menarche, last menstrual period, and history of gynecological problems.

Experiment

Before participation, each subject fasted for 8 h immediately prior to the experiment. In addition, they abstained from smoking, alcohol consumption, strenuous exercise, and substances containing caffeine for 24 h before testing.

A basal bio-impedance measurement of each individual in the fasting state was acquired during 16.7 min (1000 s) and served as a baseline for comparison with the experimental condition (metoclopramide). Patients subsequently ingested a 20 mg dose of metoclopramide with 150 mL of water. We waited 1 h before taking the second bio-impedance measurement so that patients would experience the peak pharmacological effects of metoclopramide during the test period. After this, another 1000 s bio-impedance measurement was recorded. We performed a third bio-impedance recording after the administration of a test meal that consisted of one cereal bar containing 124 calories (cal): 6.8 cal (1.7 g) of protein, 44.1 cal (4.9 g) of fat, and 73.1 cal (18.3 g) of carbohydrates and a fat free Yoghurt Drink containing 83 cal: 32 cal (7.8 g) of protein, zero cal (0 g) of fat, and 51 cal (12.5 g) of carbohydrates. In total, the subjects consumed 207 cal, of which 38.8 cal (9.5 g) were protein, 44.1 cal (4.9 g) were fat, and 124 cal (30.8 g) were carbohydrates.

To control the consistency of the ingested food, we divided the bars into four equal pieces and instructed the subjects to chew each piece 10 times and then immediately swallow. They were to repeat this with all four pieces of the cereal bar and when finished, immediately drink the yoghurt.

Approximately 5 min after food ingestion, subjects were measured again using the bio-impedance technique to assess gastric motility. There are two factors that affect the bio-impedance signal: the first is the dielectric properties of the food ingested, where fats lead to deadening of the signal. In our experiment, we controlled for this by administering a test meal low in fats. The second factor affecting the signal is noise produced by involuntary movements of the subjects, such as yawning, coughing and heavy breathing. Here, the use of short-term bio-impedance mitigated these effects by reducing the consecutive testing time for subjects.

For this experiment, we used a configuration of three electrodes as shown in Figure 1. This configuration consisted of two electrodes placed on the abdomen and a ground electrode placed on the back. The first (excitation) electrode was placed using the midline as a marker, and then by following the ribcage to the level just above and to the left of the umbilicus. The second electrode was placed approximately five centimeters from the first at a 45 degree angle up and to the left of this (recording) electrode. For the ground electrode, the vertebral column was used as a reference and the electrode was placed at approximately the average height of the corresponding abdominal electrodes.

All measurements were obtained with a SOLARTRON 1260 impedance analyzer in conjunction with a SOLARTRON 1294 biological sample interface. Disposable

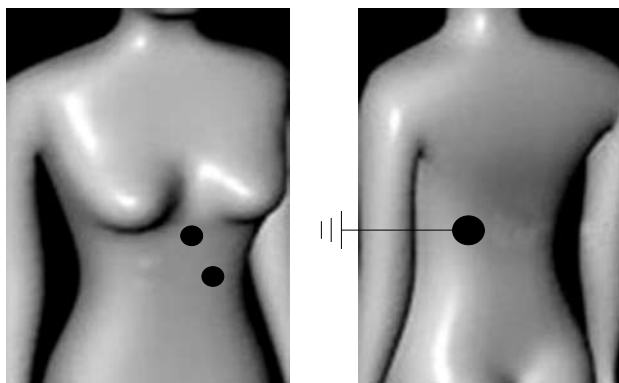


Figure 1 The figure shows the three electrode configuration, with the ground electrode placed on the back.

3M “Red Dot” monitoring electrodes were used in each patient. A BI stimulus frequency of 1 V AC was applied at 50 kHz. During the experiment, data were recorded four times per second for 1000 consecutive seconds.

Statistical analysis

Before conducting the data analysis, we preliminarily assessed the results of the anthropometrical, lifestyle, and clinical variables to ensure that our group was homogeneous in terms of these variables and that there were no unusual fluctuations in our sample.

To analyze the information from each individual over time, we used a Butterworth filter in the framework of a FFT analysis for the frequency range of 1-12/min (or cpm) (0.017-0.2 Hz). We particularly noted the data in the region between 2-4 cpm (0.033-0.066 Hz) and between 4 and 8 cpm (0.066-0.132 Hz). These analyses were carried out using MatLab 6.5 and Origin 6.0.

The data recordings were cleaned of noisy periods, sudden fluctuations and data drifting, thus the remaining data periods for analysis were in the range of 5-10 min; we discarded recordings of less than 5 min.

The frequency range was divided in four regions: the first region (R1) spans 1-2 cpm, and is defined as a very low frequency range and was therefore not considered to be an important region in this work. The second region (R2), spans the frequencies from 2 to 4 cpm and has generally been recognized as the main frequency range in the literature regarding gastric studies^[1,24,25,32]. The third region (R3) spans 4-8 cpm, and contains the frequencies where movements from the intestines are important^[1]. The fourth region (R4) spans 8-12 cpm and corresponded with the respiration frequency range of the subjects. This region may contain important data regarding the gastrointestinal system, but because respiration generates significant signal noise, we were not able to use it. Other, more expansive regions were defined as region R5, spanning 2-8 cpm and region R6, spanning 2-12 cpm (Figure 2).

For the statistical analysis, we divided the FFT signal from 1-12 cpm into the regions described above and we used both the area under the FFT curve and the median of this area (the frequency which divides the area of the frequency region into two equal parts) and the position

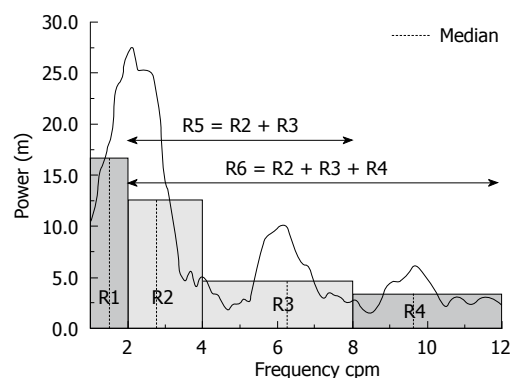


Figure 2 The figure demonstrates the definition of the regions (R1 to R6) considered in this study.

of the main peak. Together, these measures described the characteristics of the entire frequency range from 2-8 cpm. This range was used to compare the relative activities within the high and low frequency ranges. We disregarded the activity due to respiratory movements, which contributed data at approximately 8-12 cpm.

To test the accuracy of our results, we also analyzed the position of the highest activity in the range of 2-4 cpm, which is the frequency range typically used in the assessment of gastric activity.

For data analysis, we used a MatLab platform to analyze the spectra. *T*-tests for dependent samples were performed to assess differences between fasting, medicated and post-prandial states using the parameters mentioned. Previously, the use of bio-impedance has been limited because of the complexity of the signal acquired from the impedance measurements. Therefore, the only parameter used was the average magnitude of resistance of the raw signal and its change over time. Our technique refines this previous method by parsing more parameters that can be independently evaluated and statistically analyzed. All statistical analyses were performed using Statistica (Stat-Soft, Inc, Tulsa OK, USA), and the threshold for significance was standardized at $P = 0.05$.

RESULTS

As mentioned above, three parameters were considered in this study: (1) The median of the area under the FFT curve for each region, (2) The ratio of the area of each region to the total area under the curve of the range from 1 to 12 cpm; and (3) the position of the highest peak in each region. The three parameters for each region were obtained from smoothed versions of the FFT curves. We took the average of all individuals evaluated in this study and used these data for further comparison.

Based on the three parameters used, we found the following.

Medians

In R2, the median changed from 2.8 cpm in the fasting state to 2.75 cpm in the medicated state. However, this change was not statistically significant ($P = 0.46$). In R3,

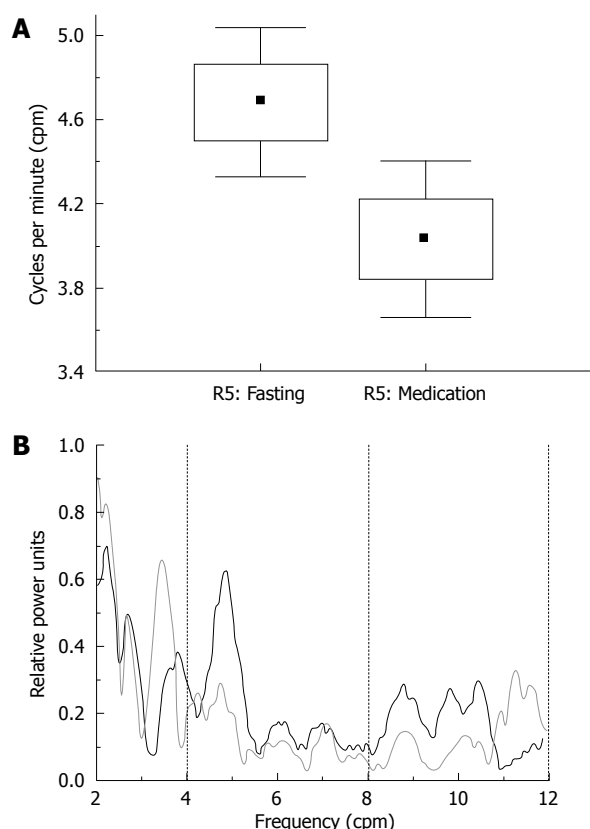


Figure 3 Changes in the median of the area under the region R5, from 2 to 8 cpm. A: Median change for the average of all individuals between the fasting and medicated states for R5 (from 2 to 8 cpm) $t = 3.32$, $P = 0.004$; B: This graph is an example of the change of R5 in an individual recording: fasting in black and medication in grey.

this parameter changed from 5.75 cpm in the fasting state to 5.9 cpm in the medicated state, however, this change was also not statistically significant ($P = 0.2$). As shown in Figure 3, R5 (R2 + R3) displayed a significant decrease in the median from 4.7 cpm in the fasting condition to 4.0 cpm in the medicated state ($t = 3.32$, $P = 0.004$). We did not find a statistically significant change in the median value in any region between the medicated and post-prandial states in the regions analyzed in this study. However, in the instances where the changes were not statistically significant, these changes showed a similar trend to those indicated in previous studies^[32].

Areas

The change observed in the median of R5 may be understood as the combination of the changes seen in the area under the FFT curve of R2 and R3. For R2, when we compare the ratios of the regional area to the total area between the fasting and medicated states, we observed an increase from 22.4% to 27.7%, respectively ($t = -2.5$, $P = 0.022$). The change was in the opposite direction for R3, with a decrease from 38.3% to 26.6% ($t = 2.81$, $P = 0.012$) for the fasting and medicated states, respectively. Figure 4A and B show these changes for R2 and R3, respectively.

In contrast to our findings regarding the changes in the median of the area under the FFT curve for R5, there were no significant differences in the area of R5

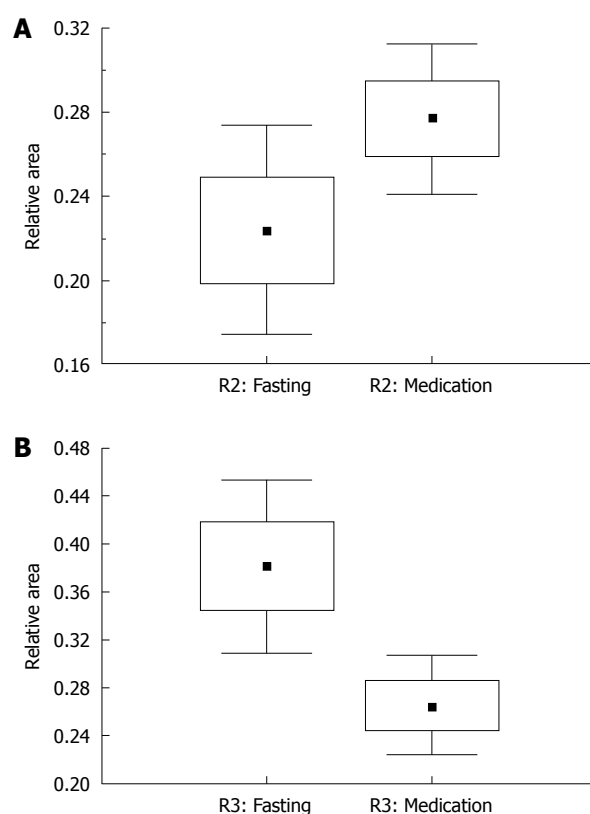


Figure 4 Average change in the median for R2 (A: $t = -2.5$, $P = 0.022$) and R3 (B: $t = 2.81$, $P = 0.012$) between the fasting and medication conditions.

between the post-prandial state and either the fasting or medicated state.

Peaks

Previously, the main parameter considered in the evaluation of gastric motility was the main peak in R2 (from 2 to 4 cpm) of the frequency domain and its shift across states. As discussed above as well as in previous works, the large overlap of activity in the abdominal cavity makes determination of the main peak difficult in certain frequency regions. When the main peak activity of R5 was analyzed, we found differences in the position of the highest peak which were statistically significant. Main peak activity in the fasting state was 4.72 cpm and declined to 3.45 cpm in the medicated state ($t = 2.47$, $P = 0.025$), as shown in Figure 5. There was also a decrease from the fasting state to the post-prandial state at 3.02 cpm ($t = 4.0$, $P = 0.0013$) but this change was not statistically significant with respect to the medicated state ($P = 0.28$).

DISCUSSION

These results demonstrate that global changes in the gastrointestinal tract can be measured using short-term electrical bio-impedance, giving useful information on gastric motility.

The typical parameter for gastric motility evaluation is the position of the main peak in the frequency region between 2-4 cpm and should be reconsidered in the context of global behavior of the combined frequency

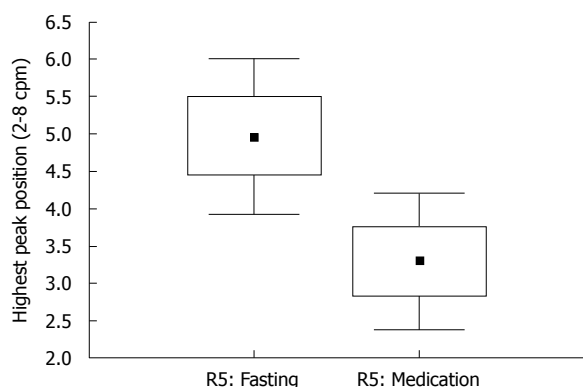


Figure 5 Change in average main peak position between the fasting and medicated state in R5. *T* test for dependent samples, $t = 2.47$, $P = 0.025$.

ranges. The results described in our previous work were confirmed with the accurate measurement of gastric motility in the fasting state^[32]. In addition, this study demonstrated that the bio-impedance technique is also valid for systems experiencing gastric stress, as induced by metoclopramide. In this study, we demonstrated that there was a decrease in the median of the area under the FFT curve over time in the region from 2 to 8 cpm, which was a consequence of the change in the relative areas under the curves in the regions from 2 to 4 cpm (increase) and from 4 to 8 cpm (decrease). The position of the main peak can also be used as a parameter for gastric motility assessment, and in this case the main peak would be representative of the entire 2-8 cpm region.

When analyzing the lack of significant change in the main peak position in R5 between the medicated and post-prandial states, we can understand this physiologically as a result of the continued effects of metoclopramide during the post-prandial state. Peak effects of metoclopramide occur at 60 min after administration, but can persist for up to 2.2 h^[36]. Food administration and subsequent testing were well within this period, likely accounting for the lack of significant differences between the two states. The increased smooth muscle activity induced by metoclopramide administration persisted throughout the post-prandial state, causing similar bio-impedance activity in both states; this resulted in a lack of significant changes in gastric motility.

This study should be followed up with comparative studies between electrical bio-impedance and electrogastrigraphy and other standard techniques to ensure the equivalence of this technique to other proven methods and the overall validity of electrical bio-impedance for gastric motility assessment so that it can be used in a clinical context. Frequent and easy monitoring of gastric motility in subjects with problems that involve gastric motility may be an important clinical application of this technique, for example in the clinical diagnosis, and evaluation of gastroparesis. Other applications may include pediatrics, because this technique allows easy clinical evaluation. Extended monitoring of patients experiencing gastric distress may lead to more accurate assessment of the pathological antecedents of the chief complaint. This technique could also be used in the evaluation of the

effects of diverse drugs which act in the gastric motility.

The results of this study demonstrate that electrical bio-impedance can be used as an accurate and valuable clinical technique for gastric motility evaluation in individuals experiencing gastric stress.

COMMENTS

Background

Gastric motility assessment is an important diagnostic technique for a variety of diseases of the gastrointestinal tract. For the evaluation of gastric motility, there are invasive methods such as scintigraphy and manometry. Electrogastrography is an invasive method, if electrodes are introduced into the gastro-intestinal tract. Non-invasive options include electrogastrigraphy, when recordings are performed through the skin using cutaneous electrodes, and electrical bio-impedance in the gastric region when cutaneous electrodes are applied. In these latter two cases, uncertainties arise in the interpretation of the signal because the cutaneous recording leads to the simultaneous acquisition of signals at multiple frequencies within the gastrointestinal system. The main challenge of this technique is the decomposition of the data to isolate the frequencies originating in the gastric tract. Presently, research is underway to develop accurate gastric motility assessment tools which optimize patient comfort and cost.

Research frontiers

Electrical bio-impedance is a non-invasive technique that can measure gastric motility. Previously, assessment has been over long time intervals in gastric emptying studies that required immobilization of the patient for the duration of the test. Movement compromises results and the overlap of internal movements from the gastric region produce signals in other frequency ranges. Other alternatives to analyze bio-impedance data from the gastric region have been proposed in the literature, for example, discrete wavelet transform.

Innovations and breakthroughs

Here, the authors demonstrate that short-term bio-impedance can be used to assess gastric motility in healthy volunteers with gastric systems under stress. The areas and the median of the areas of specific regions under the Fast Fourier Transform (FFT) spectra were analyzed, as an alternative, to accurately depict gastric motility. Stress induced by metoclopramide mirrors a disease state by enhancing the peristaltic waves of the stomach and intestines. Thus, this work supports the clinical application of the bio-impedance technique.

Applications

In future studies, the authors hope to test short-term bio-impedance in parallel with other, previously validated methods for gastric motility assessment. If studies prove these methods to be equivalent, short-term bio-impedance could be used clinically to optimize gastric motility assessment with regard to both cost and patient comfort.

Terminology

Bio-impedance is a term used to describe the response or resistance of a living organism to an externally applied electric current. It can be described by the resistance or the conductivity. Short-term bio-impedance is a term used for short time recordings of bio-impedance signal. FFT is a mathematical technique to transform data from time domain to frequency domain. It gives information about the main variation frequencies for the data. Metoclopramide, is a dopamine receptor antagonist with both antiemetic and prokinetic properties. The antiemetic property treats nausea and vomiting while the prokinetic properties enhance the peristaltic waves of the stomach and intestine, increasing the rate of absorption and thus the rate of passage through the stomach.

Peer review

The manuscript entitled "Effects of metoclopramide on gastric motility measured by short-term bio-impedance" is an interesting report. They found that electrical bio-impedance can assess changes in gastric motility in individuals experiencing gastric stress. The manuscript add important information to the current knowledge.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM

BRIEF ARTICLES

Carotid lesions in outpatients with nonalcoholic fatty liver disease

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Author contributions: Ramilli S, Pretolani S and Arienti V designed the study; Ramilli S performed carotid ultrasound assessments; Pacelli B performed statistical analysis of the data; Ramilli S, Pretolani S, Pacelli B and Muscari A wrote the paper; Muscari A and Arienti V critically revised the manuscript.

Supported by (in part) A grant from Gruppo Italiano di Ultrasonologia in Medicina Interna (GIUMI)

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Received: August 7, 2009 Revised: August 24, 2009

Accepted: August 31, 2009

Published online: October 14, 2009

CONCLUSION: An incidental finding of hepatic steatosis may suggest the presence of silent carotid atherosclerotic lesions.

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Key words: Hepatic steatosis; Nonalcoholic fatty liver disease; Metabolic syndrome; Carotid atherosclerosis; Plaque; Intima-media thickness

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Ramilli S, Pretolani S, Muscari A, Pacelli B, Arienti V. Carotid lesions in outpatients with nonalcoholic fatty liver disease. *World J Gastroenterol* 2009; 15(38): 4770-4774 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4770.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4770>

Abstract

AIM: To ascertain whether carotid lesions are more prevalent in outpatients with incidental findings of nonalcoholic fatty liver disease (NAFLD) at abdominal ultrasound (US).

METHODS: One hundred and fifty-four consecutive outpatients (age range 24-90 years, both sexes) referred by general practitioners for abdominal US, and drinking less than 20 g alcohol/day, underwent carotid US for an assessment of carotid intima-media thickness (c-IMT) and carotid plaque prevalence. Hepatic steatosis, visceral fat thickness and subcutaneous fat thickness were also assessed at ultrasonography.

RESULTS: Higher c-IMT values were found in the presence of NAFLD (90 patients), even after adjustment for indices of general and abdominal obesity and for the principal cardiovascular risk factors (0.84 ± 0.10 mm *vs* 0.71 ± 0.10 mm, $P < 0.001$). The prevalence of carotid plaques was 57.8% in the patients with NAFLD *vs* 37.5% in the patients without this condition ($P = 0.02$). The adjusted relative risk of having carotid plaques for patients with NAFLD was 1.85 (95% CI: 1.33-2.57, $P < 0.001$).

INTRODUCTION

Hepatic steatosis is a feature of the metabolic syndrome^[1,2], a condition associated with a high cardiovascular risk and, in particular, an increased prevalence of carotid lesions^[3]. In the last few years several studies have assessed the association between hepatic steatosis and carotid atherosclerosis. Some case-controlled and cross-sectional studies^[4-8] showed a relationship between nonalcoholic fatty liver disease (NAFLD) and carotid intima-media thickness (c-IMT), an early independent predictor of cardiovascular events^[9-11]. In addition, other studies also showed a relationship with the presence of more advanced atherosclerotic lesions in the carotid arteries^[12-14]. However, the only study performed so far in the general population (4222 subjects) did not find any independent association between hepatic steatosis and c-IMT, showing an association with plaque prevalence only^[15]. The lack of concordance of these results may be related to non-standardized measurement methods and to the use of low frequency linear ultrasound (US) probes.

A possible relationship between hepatic steatosis and carotid lesions might have important practical consequences, considering the frequent incidental finding of hepatic steatosis in subjects undergoing abdominal US for any reason. In these subjects, an US

assessment of carotid arteries might also be advisable.

Thus, we have prospectively examined a random group of consecutive outpatients undergoing abdominal US to establish whether those with NAFLD had an increased prevalence of early or advanced carotid lesions. The study was performed following the Mannheim Consensus^[16], which proposed a standardized method for c-IMT measurement.

MATERIALS AND METHODS

Patients

From December 2006 to February 2007, we performed a cross-sectional study on 186 consecutive Caucasian outpatients, referred to our centre by general practitioners to perform an abdominal US assessment.

Body weight was measured in light clothing, without shoes, to the nearest 0.5 kg. Height was measured at the nearest 0.5 cm. Body mass index (BMI) was calculated as kilograms divided by square meters. Hypertension was diagnosed for values > 140/90 mmHg in non-diabetics and > 130/80 mmHg in diabetics, or when subjects were taking anti-hypertensive drugs^[17].

The design of the study did not allow any biochemical measurements, since many US assessments were performed in the late morning and some patients were not fasting. However, demographic characteristics (sex and age), some cardiovascular risk factors (smoking, history of diabetes and/or dyslipidemia) and possible causes of steatosis [alcohol consumption, anti-retroviral drugs, tamoxifen, amiodarone, corticosteroids, jejunal-ileal bypass, inflammatory bowel diseases, hepatitis C virus (HCV), and Human Immunodeficiency virus (HIV)] were collected in a structured questionnaire. The presence of dyslipidemia or diabetes was assumed if subjects were taking lipid-lowering/antidiabetic drugs, or positively answered the questions: “do you know you have high cholesterol or triglyceride values?” and “do you know you have diabetes?”. To quantify the number of cigarettes smoked, the total pack-years^[18,19] was calculated: (number of cigarettes smoked daily/20) x years of smoking. Smoking was categorized into the 3 following levels: never smoked, up to 20 pack-years and more than 20 pack-years. The cut-off value of 20 corresponded to the 75th percentile of pack-years distribution excluding non-smokers.

Thirty-two patients (17%) were excluded due to significant alcohol consumption (> 20 g/d, average weekly consumption). No other exclusion criteria were utilized. Thus, our study group consisted of 154 patients (75 male, mean age 59.6 ± 14.2 years, range 24-90 years) who underwent an abdominal US assessment for the following reasons: abdominal pain (44.2%), biliary/kidney stones (5.2%), follow-up of benign cysts of the liver (24.7%), kidney (15.6%) and ovary (3.2%), and for colorectal cancer (5.8%) and hepatocellular carcinoma (1.3%) surveillance.

Ultrasound evaluation

All subjects underwent abdominal and carotid US in

order to assess hepatic steatosis, visceral fat thickness (VFT), subcutaneous fat thickness (SFT) and c-IMT, and to ascertain the presence of carotid plaques. We used a Technos MP (Esaote, Italy), with convex probes (2.5-5 MHz) to scan the liver, and high frequency linear probes (7.5-13 MHz) to scan carotid arteries. All investigations were performed by two experienced operators (for abdominal and carotid US), blinded to each other regarding the respective US measurements and unaware of patients' clinical data.

Following the AGA classification of NAFLD^[20], steatosis was defined as the presence of diffuse hyperechoic echotexture, bright liver^[21], increased liver echotexture compared with the kidneys, vascular blurring and deep attenuation of the ultrasonic beam.

VFT and SFT were measured with the probe located 1 cm above the umbilicus, and defined as the distance between the linea alba and the anterior wall of the aorta, and as the distance between the skin and the linea alba, respectively^[22,23]. VFT and SFT US measurements correlate with visceral and subcutaneous fat areas as measured by CT scan^[24].

All measurements concerning c-IMT and plaques were performed according to the Mannheim Consensus^[16]. Longitudinal images of both the left and right side at the level of the common carotid artery, bulb and internal carotid were obtained in each patient. The arterial wall was assessed with a high frequency linear probe with appropriate focus, frame rate and gain setting to obtain a symmetrical brightness on the near and far wall.

c-IMT was measured in the far wall of the common carotid artery, along a 15 mm section free of plaque from the bifurcation, as the distance between the leading edges of the lumen-intima and media-adventitia interfaces. Maximum rather than mean values of c-IMT were considered, and edge detection was performed manually. c-IMT measurements from the left and right side were averaged. Previous studies have shown that “normal IMT values” vary according to age and atherosclerosis-related risk factors, ranging from 0.60-0.75 mm (30-49 years) to 0.79-0.86 mm (70-79 years)^[16]. Thus, values > 0.90 mm should be considered as increased in all cases.

A plaque was defined as a focal structure encroaching into the arterial lumen by at least 0.5 mm or 50% of the surrounding IMT value, or having a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface. The presence of plaques was evaluated in a 30 mm-long segment both in the left and right common carotid, internal carotid and bulb. Quality controls (phantom scans and proper US calibration) were performed monthly.

Statistical analysis

Analyses were performed using SPSS (14.0, Chicago, IL, USA) and Stata (8.0, StataCorp LP, Tx, USA) software. Quantitative characteristics were expressed as mean ± SD, or *n* (%). The study sample was divided into two groups according to the presence or absence of hepatic steatosis. Comparisons between groups were made by analysis of variance (ANOVA) and Kruskal Wallis' test for continuous

data, and using the χ^2 test with Yates correction for nominal data. ANOVA and Spearman's test were used to investigate the associations between c-IMT and demographic, clinical, anthropometrical and US characteristics. Multivariable analysis was performed with respect to c-IMT by means of analysis of covariance (ANCOVA). The mean scores of c-IMT, adjusted for the main risk factors (age, sex, BMI, diabetes, dyslipidemia, hypertension and smoking) were estimated in subjects with and without steatosis. To determine whether the adjusted mean scores of the two groups were significantly different, we tested the hypothesis that the steatosis coefficient was null using a multiple-partial F test. The assumption of parallelism was assessed by comparing a fitted model and expanded model with interaction terms with a multiple-partial F test. Adjusted R^2 was used to estimate the percentage of c-IMT variability explained by the model.

The adjusted relative risk of the presence of plaques associated with hepatic steatosis was assessed as prevalence ratio by the Cox regression model with equal time of follow-up assigned to all individuals and robust variance estimates, so that the calculated hazard ratio equaled the correct prevalence ratio^[25]. This method is preferred in cross-sectional studies with frequent outcomes, such as in this case, to avoid the overestimation of prevalence ratios obtained by odds ratios^[25]. Two-tailed tests were performed throughout and a P value < 0.05 was considered statistically significant.

RESULTS

Table 1 reports the clinical, anthropometrical and US characteristics of the population sample. No patients had chronic liver disease (except for two HCV-positive subjects), HIV-positivity, inflammatory bowel disease, abdominal surgery or received parenteral nutrition. None had taken drugs that could lead to hepatic steatosis.

Significantly higher BMI, VFT and SFT values were found in the subjects with NAFLD, who also reported a higher prevalence of dyslipidemia. Age, sex and smoking did not differ between the two groups (Table 2).

c-IMT was significantly associated with all cardiovascular risk factors, except for sex and SFT (Table 3). In univariate analysis c-IMT was also associated with NAFLD (Table 4).

In multivariable analysis, after adjustment for age, sex, BMI, smoking, hypertension, dyslipidemia and diabetes, c-IMT was still greater in the NAFLD group. The full regression model explained 46.4% of the variability of c-IMT.

The prevalence of plaques in the subjects with NAFLD was 52/90 (57.8%) *vs* 24/64 (37.5%) in those without ($P = 0.02$), which corresponded to a relative risk of 1.54; this risk even increased (1.85, $P < 0.001$) after adjustment for confounding factors (Table 4).

DISCUSSION

This study has shown that an incidental finding of NAFLD is associated with c-IMT and predicts the

Table 1 Clinical, anthropometrical and ultrasound characteristics of the study sample ($n = 154$)

Variable	Value
Age (yr)	59.6 ± 14.2
Gender (male)	75 (48.7)
BMI (kg/m ²)	26.1 ± 4.3
Diabetes	15 (9.7)
Hypertension	61 (39.6)
Dyslipidemia	53 (34.4)
Smoking	
Never	82 (53.3)
1-20 pack-years ¹	56 (36.4)
> 20 pack-years ¹	16 (10.4)
SFT (mm)	19.1 ± 6.3
VFT (mm)	51.6 ± 21.7
NAFLD	90 (58.4)
c-IMT (mm)	0.80 ± 0.18
Carotid plaque	76 (49.4)

Data are mean ± SD, or n (%). ¹Both current and former smokers. BMI: Body mass index; Pack-years: Packs of cigarettes smoked daily × years of smoking; SFT: Subcutaneous fat thickness; VFT: Visceral fat thickness; NAFLD: Nonalcoholic fatty liver disease; c-IMT: Common carotid intima-media thickness.

Table 2 Univariate associations of NAFLD with cardiovascular risk factors

Variable	No NAFLD ($n = 64$)	NAFLD ($n = 90$)	P value ¹
Age (yr)	60.1 ± 15.6	59.3 ± 13.2	0.72
Gender (male)	29 (45.3)	46 (51.1)	0.59
BMI (kg/m ²)	24.5 ± 3.4	27.2 ± 4.5	< 0.001
Diabetes	3 (4.7)	12 (13.3)	0.13
Hypertension	22 (34.4)	39 (43.3)	0.34
Dyslipidemia	16 (25.0)	37 (41.1)	0.05
Smoking			
Never	34 (53.1)	48 (53.3)	0.93
1-20 pack-years	24 (37.5)	32 (35.6)	
> 20 pack-years	6 (9.4)	10 (11.1)	
SFT (mm)	17.6 ± 5.2	20.2 ± 6.9	0.01
VFT (mm)	43.9 ± 16.7	57.0 ± 23.4	< 0.001

Data are mean ± SD, or n (%). ¹ χ^2 test (nominal data); ANOVA or Kruskal Wallis when appropriate (continuous data).

presence of carotid plaques in outpatients undergoing abdominal US assessment.

An association between hepatic steatosis and c-IMT has already been reported in some previous studies^[4-8,12-15], and even in children^[26]. In 85 healthy, non-obese, male volunteers, Targher *et al*^[4] found a significant increase in c-IMT in the presence of non-alcoholic hepatic steatosis, and both conditions seemed to be due to visceral fat accumulation. In our sample, hepatic steatosis was independently associated with c-IMT, while VFT was associated with c-IMT in univariate analysis, but not in multivariable analysis. This may be explained by differences in sample composition and statistical analysis. Other studies that found an association between visceral obesity and c-IMT did not assess hepatic steatosis^[24,27,28]. One study reported that the association between NAFLD and c-IMT concerned only the patients with metabolic syndrome^[8]. On the other hand, two studies showed that the same relationship is absent,

Table 3 Univariate associations of c-IMT with cardiovascular risk factors

Variable	c-IMT		P value ¹
	mean \pm SD (mm)	Rho	
Gender			0.27
Male	0.81 \pm 0.19		
Female	0.78 \pm 0.17		
Age (yr)		0.45	< 0.001
BMI (kg/m ²)		0.3	< 0.001
< 25	0.76 \pm 0.18		0.02
25-30	0.82 \pm 0.14		
> 30	0.86 \pm 0.22		
Hypertension			< 0.001
Yes	0.88 \pm 0.17		
No	0.74 \pm 0.17		
Dyslipidemia			0.006
Yes	0.85 \pm 0.18		
No	0.77 \pm 0.17		
Diabetes			0.001
Yes	0.95 \pm 0.21		
No	0.78 \pm 0.17		
Smoking			0.03
Never	0.79 \pm 0.18		
1-20 pack-years	0.78 \pm 0.18		
> 20 pack-years	0.91 \pm 0.19		
VFT (mm)		0.21	0.01
SFT (mm)		0.08	0.32

Rho: Spearman's correlation coefficient between continuous variables and c-IMT; ¹ANOVA (nominal data) or Spearman's Rho (continuous data).

or present but largely explained by insulin resistance, in type 2 diabetic patients^[29,30]. Finally, in the only large cross-sectional study to date, Volzke *et al.*^[15] described an independent association of hepatic steatosis with carotid plaques, but not with c-IMT. The discordance with our results might be due to the fact that these authors used low frequency (5 MHz) US probes, which are known to provide less accurate c-IMT measurements^[16].

In the present study, with high frequency probes and a standardized protocol to measure c-IMT, we found that NAFLD was independently associated with both c-IMT and the presence of carotid plaques. Defining the role played by NAFLD in the formation of initial or advanced carotid lesions is beyond the scope of this study, which was only designed to ascertain whether an incidental finding of NAFLD in outpatients may suggest the search for carotid lesions. The cross-sectional design, together with the impossibility of measuring metabolic variables such as serum lipids, glucose and insulin, and to exclude with certainty the presence of HCV or HIV infection, are the main limitations of this study, and impede any assessment of causality. The lack of metabolic measurements, together with the low frequencies, may also explain why the associations of NAFLD with diabetes and dyslipidemia were borderline or not significant.

In agreement with Targher *et al.*^[4] it seems likely that abdominal obesity may be the common antecedent of both NAFLD and carotid atherosclerosis, with the metabolic syndrome as an intermediate. However, we previously showed that endothelial dysfunction was more prevalent in patients with NAFLD than in controls matched for age and sex and with similar features of the

Table 4 Unadjusted and adjusted associations of NAFLD with c-IMT and presence of plaque

Variable	No NAFLD (n = 64)	NAFLD (n = 90)	P value ¹
c-IMT (mm)	0.71 \pm 0.15	0.86 \pm 0.18	< 0.001
c-IMT adjusted (mm)	0.72 \pm 0.10	0.84 \pm 0.10	< 0.001
Carotid plaque [RR (95% CI)]	1	1.54 (1.07-2.22)	0.02
Carotid plaque adjusted [RR (95% CI)]	1	1.85 (1.33-2.57)	< 0.001

¹ANCOVA (c-IMT) and Cox regression with constant risk period and robust variance estimate (RR of carotid plaque). Adjustments for age, sex, BMI, smoking, hypertension, dyslipidemia and diabetes. RR: Relative risk (prevalence ratio); CI: Confidence interval.

metabolic syndrome^[31]. Moreover, in the present study the association between NAFLD and carotid lesions was independent from indicators of general and abdominal obesity, such as BMI, SFT and VFT. It thus seems possible that NAFLD may identify a subgroup of metabolic syndrome patients at higher cardiovascular risk.

In conclusion, hepatic steatosis is a marker of increased c-IMT and of the presence of carotid plaques in outpatients undergoing abdominal US. Any incidental US finding of hepatic steatosis should prompt medical practitioners not only to assess the metabolic risk, but also to consider the search for silent carotid lesions.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is often caused by abdominal obesity, which is also one of the main causes of insulin resistance and metabolic syndrome. The latter, in turn, is an important cardiovascular risk factor, and has been found to be associated with the presence of carotid atherosclerotic lesions. It is therefore understandable that an association may exist between NAFLD and carotid lesions.

Research frontiers

Although the association between NAFLD and carotid lesions is plausible and demonstrated, its practical implications have not been fully understood. This study highlights the possible relevance of these implications.

Innovations and breakthroughs

The association between NAFLD and early or advanced carotid lesions is not new, but in this study this association has been demonstrated for the first time in a random group of outpatients undergoing abdominal ultrasound. It is indeed in this type of patients that, not infrequently, a previously unknown hepatic steatosis is found. In the same patients an ultrasound assessment of the carotid arteries might prove particularly useful.

Applications

This study suggests that an incidental finding of hepatic steatosis may represent a new indication for performing an ultrasound assessment of the supra-aortic branches to search for silent arterial lesions.

Terminology

NAFLD is a general term including all cases of hepatic steatosis, with or without inflammation (steatohepatitis), which are not caused by alcohol abuse. It is often found associated with abdominal obesity and metabolic syndrome. Intima-media thickness (IMT) is the thickness of the two internal layers of the arterial wall, and is usually measured by ultrasound at the level of the common/internal carotid arteries. Its increase corresponds to the initial phase of the atherosclerotic process, and is associated with an increased cardiovascular risk.

Peer review

Although not novel, this is a well-conducted study that is reported quite concisely. The strengths over prior similar studies are the thorough design and the measurement of carotid intima-media thickness using the new Mannheim

consensus criteria. Moreover patients were enrolled consecutively, and pertinent demographic and clinical data were collected at the time of imaging, which was performed in a blinded fashion by experienced operators using high quality equipment.

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Duodenal biopsy may be avoided when high transglutaminase antibody titers are present

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Supported by A grant of the Instituto de Salud Carlos III (FIS ref. PI070611) and by a grant of the Junta de Castilla y León, Consejería de Sanidad (Ref 318/A/08)

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Received: July 3, 2009 **Revised:** August 4, 2009

Accepted: August 11, 2009

Published online: October 14, 2009

Abstract

AIM: To evaluate the predictive value of tissue transglutaminase (tTG) antibodies for villous atrophy in adult and pediatric populations to determine if duodenal biopsy can be avoided.

METHODS: A total of 324 patients with celiac disease (CD; 97 children and 227 adults) were recruited prospectively at two tertiary centers. Human IgA class anti-tTG antibody measurement and upper

gastrointestinal endoscopy were performed at diagnosis. A second biopsy was performed in 40 asymptomatic adults on a gluten-free diet (GFD) and with normal tTG levels.

RESULTS: Adults showed less severe histopathology (26% vs 63%, $P < 0.0001$) and lower tTG antibody titers than children. Levels of tTG antibody correlated with Marsh type in both populations ($r = 0.661$, $P < 0.0001$). Multiple logistic regression revealed that only tTG antibody was an independent predictor for Marsh type 3 lesions, but clinical presentation type and age were not. A cut-off point of 30 U tTG antibody yielded the highest area under the receiver operating characteristic curve (0.854). Based on the predictive value of this cut-off point, up to 95% of children and 53% of adults would be correctly diagnosed without biopsy. Despite GFDs and decreased tTG antibody levels, 25% of the adults did not recover from villous atrophy during the second year after diagnosis.

CONCLUSION: Strongly positive tTG antibody titers might be sufficient for CD diagnosis in children. However, duodenal biopsy cannot be avoided in adults because disease presentation and monitoring are different.

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Key words: Biopsy; Celiac disease; Diagnosis; Duodenum; Transglutaminases

Peer reviewer: Nikolaus Gassler, Professor, Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Vivas S, Ruiz de Morales JG, Riestra S, Arias L, Fuentes D, Alvarez N, Calleja S, Hernando M, Herrero B, Casqueiro J, Rodrigo L. Duodenal biopsy may be avoided when high transglutaminase antibody titers are present. *World J Gastroenterol* 2009; 15(38): 4775-4780 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4775.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4775>

INTRODUCTION

Serological assessment is the first step in celiac disease (CD)

diagnosis, and wide availability of serological antibodies allows for easy CD testing^[1]. IgA anti-endomysial antibodies (EMAs) are used as the “gold standard” for CD screening because of their high sensitivity and specificity. A high correlation between EMA titer and duodenal histopathology has also been reported^[2-4]. More recently, human anti-tissue transglutaminase (tTG) antibodies have also shown to be correlated with mucosal damage and are used widely in CD screening. However, neither antibody is detected in patients with minor mucosal changes (Marsh types I - II and IIIa) and IgA deficiency may be ruled out when using IgA type antibodies.

In the clinical setting, a patient with positive serological results requires duodenal biopsy to confirm CD diagnosis. However, a definitive diagnosis is only made when a response to gluten-free diet (GFD) is present^[5]. Furthermore, duodenal biopsy has several pitfalls: (1) at least four forced biopsies are needed to achieve good readability; (2) poorly oriented or inadequate biopsies may not be useful for diagnosis; and (3) it is an invasive procedure, both in children and adults. In the last few years, a more prominent role for a definitive diagnosis based solely on serological assays has been proposed. In pediatric populations, strongly positive tTG antibody results (≥ 100 U) showed a high specificity for Marsh type 3a or greater changes^[6,7]. This predictive value of high tTG antibody titers has also been reported in a retrospective cohort of adult and pediatric CD patients^[8]. Based on these studies, some authors have proposed to start a GFD for those patients with high tTG antibody levels and to perform a duodenal biopsy only when the patient's symptoms do not improve after a GFD.

The primary objective of the present work was to analyze the predictive value of a finding of high tTG antibody titers for the presence of duodenal atrophy at the time of diagnosis in adult and pediatric CD patients. In addition, the possibility that avoiding duodenal biopsy in these 2 groups of CD patients was explored.

MATERIALS AND METHODS

Ethical considerations

The study was approved by the Research and Ethical Committees of the participating hospitals.

Patients

Adult and pediatric CD patients were recorded prospectively from 2000 to 2008 at two tertiary centers in the North of Spain: Hospital Universitario Central de Asturias and Hospital de León. Pediatric and adult gastroenterology units at both centers have specialty CD clinics. Patients were referred from primary care settings or from other medical specialties for diagnosis and follow-up. Subjects were referred for evaluation of clinical complaints suggestive of CD, had positive family history or belonged to some high-risk group for CD.

None of the patients included had a previous diagnosis of CD before they attended at our clinic and were on a free diet (gluten containing diet). They were informed regarding the suspicion of illness and gave

informed consent to perform the complementary studies. The pediatric population included children ≤ 14 years and adults were ≥ 15 years old. A life time of 14 years was selected to discriminate children from adults because pediatric patients were below this age in our hospitals. The elapsed time between serological assay and duodenal biopsy was always < 8 wk.

The clinical spectrum was divided into two categories according to the main symptoms that led to diagnosis: (1) typical or classical, clinical malabsorption, chronic diarrhea or failure to thrive (children ≤ 2 years); and (2) atypical or oligosymptomatic, abdominal pain, iron deficiency anemia, chronic hypertransaminasemia, growth failure (children ≥ 3 years) or screening of risk groups or familial study.

Serology and human leukocyte antigen (HLA) genotype

Quantitative detection of human IgA class tTG antibody used the same commercial kits (Phadia Diagnostics, Uppsala, Sweden) in both centers. The manufacturer reference ranges for positive results were values > 10 U. From our experience, a cut-off value of 4 U showed a diagnostic value similar to IgA anti-endomysial antibodies^[9,10]. A cut-off level > 30 U tTG antibody was considered strongly positive for additional evaluations. IgA level was determined together with tTG antibodies and IgA deficiency patients were not included.

Adult CD patients were typed for HLA-DQ2 (DQA1*0501 and DQB1*0201 alleles) and DQ8 (DQA1*03 and DQB1*0302 alleles) by polymerase chain reaction. HLA genotype was performed only when duodenal biopsy showed inflammatory lesions (Marsh 1 and 2), in order to confirm CD diagnosis.

Histopathology

Biopsies from the distal duodenum (minimum of four forceps biopsies) were obtained by upper gastrointestinal endoscopy. Two pathologists experienced with CD diagnosis reviewed the histopathological specimens in each center and classified them according to Marsh's criteria as modified by Oberhuber *et al*^[11]. Type 3 specimens (any degree of villous atrophy) were considered characteristic of CD. Marsh 1 and 2 lesions were considered nonspecific, but consistent with CD diagnosis if serology was positive. When serology was negative, HLA-DQ was typed and symptoms evaluated with a GFD. Only those cases that were positive for HLA-DQ2 and DQ8, together with the disappearance of symptoms were included as definitive CD cases. Those cases with Marsh 3 lesions and negative serology were evaluated for an alternative diagnosis that could explain the histological abnormalities.

Follow-up

A second biopsy was performed in a selected cohort of 40 patients at Hospital de Leon to evaluate duodenal atrophy recovery. All of them showed, at the beginning, Marsh 3 lesions, and the second biopsy was performed during the second year after diagnosis, in patients taking a GFD, who were asymptomatic and with normal tTG antibody level.

Statistical analysis

Data were analyzed using SPSS version 13.0. Categorical

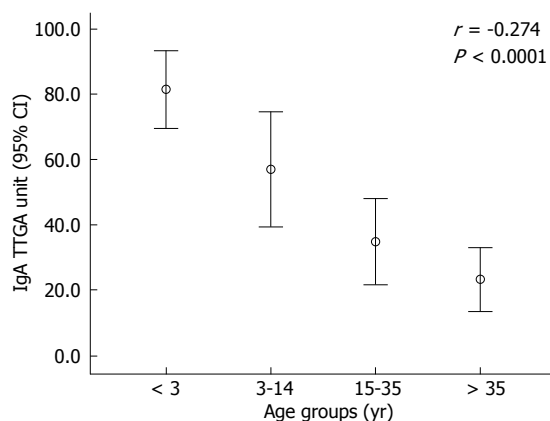


Figure 1 Serum tTG antibody level vs patient age. An inverse relationship was observed for the levels of serum tTG antibody with increasing patient age.

variables were expressed as numbers and percentages and quantitative variables as mean \pm SD. Categorical variables were analyzed by cross-tabulations using a χ^2 test with a continuity correction test when necessary. Differences between groups for quantitative variables were assessed by Student's *t* test or ANOVA. A non-parametric Mann-Whitney *U* test was used when the groups values deviated from a normal curve. Associations between quantitative variables were assessed by Pearson correlation test or Spearman rank correlation test. $P < 0.05$ was selected to reject the null hypothesis by two-tailed tests. Multivariate logistic regression was used to determine independent associations between histopathological and serological or clinical data. Analysis of receiver operating characteristics (ROC) curve was used to evaluate cut-off points for tTG antibodies as a predictor of Marsh scores.

RESULTS

Patient characteristics

A total of 324 patients who fulfilled the established CD diagnostic criteria comprised the study population. The pediatric population included 97 children (mean age: 4.5 years; range: 1-14 years) and 227 adult CD subjects (mean age: 39 years; range: 15-80 years). Female/male ratio was 1.7 for children and 2.6 for adults ($P = 0.06$).

A typical CD presentation was observed for 64/97 (66%) children *vs* 82/227 (36%) adults ($P < 0.0001$). Age-related differences in tTG antibody titers and histopathology were found. An inverse relationship of tTG antibody titers at diagnosis with increasing patient age was found (Figure 1). Higher levels were seen in children aged ≤ 2 years and lower titers in adults > 35 years. A trend towards less severe histopathology with increasing age at diagnosis was observed (Figure 2). Marked villous atrophy (Marsh 3b and 3c) was present in 63% of children *vs* 26% of adults ($P < 0.0001$).

Human recombinant IgA tTG antibodies and Marsh type

The levels of tTG antibody were correlated significantly with Marsh types in the entire population (Figure 3) ($r = 0.661$, $P < 0.0001$), and separately for the pediatric ($r = 0.633$, $P < 0.001$) and adult ($r = 0.574$, $P < 0.0001$) groups.

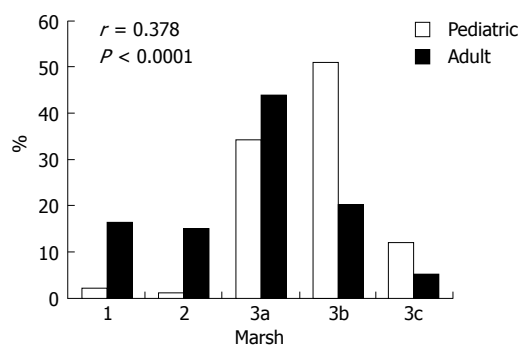


Figure 2 Histopathological differences between children and adults according to Marsh classification.

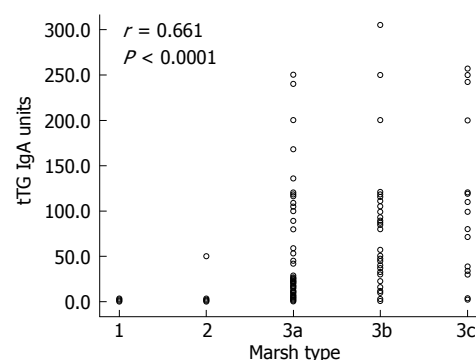


Figure 3 Serum tTG antibody levels vs Marsh classification. tTG IgA was significantly correlated with Marsh type.

Mean tTG antibody levels showed a progressive increase that was associated with higher Marsh types. Seventy-three patients showed Marsh types 1 and 2 (three were children and the remaining 70 were adults). In the pediatric group, only one Marsh type 2 patient showed tTG antibody titer < 30 U. Negative tTG antibody results were found for 46/73 (63%) Marsh types 1 and 2 CD subjects (all were adults). Twelve of 132 (9%) Marsh 3a CD patients had negative tTG antibody results (all were also adults). In contrast, none of the Marsh 3b and 3c patients had negative serology results. A definitive CD diagnosis was confirmed in this subgroup with minor mucosal changes and normal tTG antibody levels on the basis of clinical response to GFD, follow-up, and HLA-DQ2 or DQ8 compatibility.

Strongly positive tTG antibody titers (> 30 U) were present in 102 of 132 (77%) Marsh 3a patients, 79/95 (83%) Marsh 3b patients, and 24/24 (100%) Marsh 3c patients. Multiple logistic regression analysis showed that only the tTG antibody titer was an independent predictor for Marsh 3 lesions, but the clinical presentation type and patient age were not. As shown in Figure 4, at the cut-off point of ≥ 30 U tTG antibody, ROC curve analysis provided the highest area under the curve. Increasing this limit may increase the specificity and positive predictive value, but may decrease the area under the curve and sensitivity.

Duodenal biopsies can be avoided when strongly positive tTG antibody titer is found

If we had considered a cut-off point of 30 U tTG

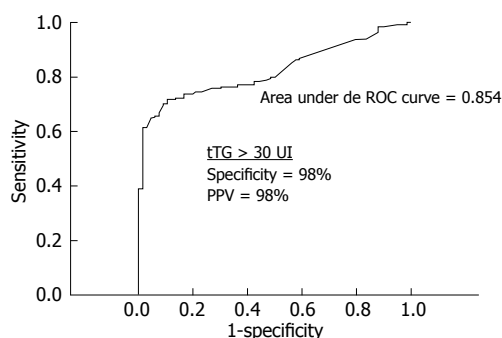


Figure 4 ROC showing the maximum area under the curve for Marsh type 3 histology at cut-off point of 30 U tTG antibody.

Table 1 Biopsies that would be avoided by considering a cut-off point of 30 U tTG antibody in each Marsh type and age group (%)

	≤ 2 yr	3-14 yr	15-35 yr	> 35 yr
Marsh 1	0	0/2	0/9 (0)	0/27 (0)
Marsh 2	0	1/1 (100)	0/19 (0)	0/15 (0)
Marsh 3a	9/10 (90)	22/24 (92)	29/43 (67)	41/57 (72)
Marsh 3b	28/28 (100)	20/21 (95)	22/24 (92)	15/20 (75)
Marsh 3c	10/10 (100)	1/1 (100)	7/7 (100)	6/6 (100)
No. of biopsies avoided	47/48 (98)	44/49 (90)	58/102 (57)	62/125 (50)

antibody to predict atrophy (Marsh 3), we would have avoided 212/324 (65%) biopsies. However, if we had considered children and adults separately, 95% of children, but only 53% adults with true atrophy, would have avoided biopsy (Table 1). Furthermore, in 40 adults with CD with an initial biopsy of Marsh 3 type, a second biopsy was performed during the second year after diagnosis. Ten (25%) showed persistence of villous atrophy despite a correct GFD, being symptom free, and normalization of tTG antibody levels (Figure 5). One patient developed refractory type II CD after an initial biopsy compatible with Marsh type 3c^[12].

DISCUSSION

A positive correlation between tTG antibody serum level and duodenal histopathology has been described previously for pediatric and adult CD populations^[6,8,13]. Although some of these early studies employed guinea pig rather than human tTG as an antigen^[13,14], the higher sensitivity and specificity have rendered the latter as the worldwide standard.

The pathogenesis of gluten-induced small-intestinal changes and atrophy is not understood fully^[15]. It is considered to be T-cell mediated. However, recent *in vitro* studies^[16,17], and a study that reported anti-tTG2 IgA deposits in a morphologically normal jejunum before systemic detection of tTG antibody predicted overt CD with atrophy^[18], suggest a pathogenic role for antibodies.

The study population in the present study was selected on the basis of a prospective clinical CD diagnosis. However, previous similarly designed studies have used retrospective laboratory results to select positive tTG

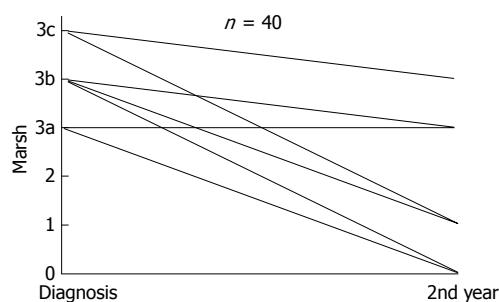


Figure 5 Follow-up of adult patients with an initial Marsh type 3 biopsy. Repeated biopsy showed persistent atrophy in 25% of the cases.

levels^[6,8]. It is more likely that the results presented in our study are more easily generalized to other populations than those based on retrospective laboratory results. Furthermore, we also describe differences between adult and pediatric populations that were not analyzed in previous studies. These differences confirm our previously reported results in a single center CD population^[19]. The higher tTG antibody titers and the more severe Marsh grades that were observed consistently in the pediatric population are two important points to consider when looking for correlations and predictions. Although tTG antibody levels were correlated with duodenal histopathology in the adult and pediatric populations, the higher percentage of Marsh type 3 lesions observed in children makes a high antibody titer especially interesting for CD prediction in children.

The choice of an upper cut-off limit of tTG antibody to predict accurately CD or Marsh type 3 lesions may depend on the commercial kit used for tTG IgA ELISA. On the basis of previous experience^[9,10], we considered 30 U as the cut-off that resulted in the highest predictive value. The same cut-off value has also been adopted by other authors who have used the same commercial kit^[20]. This value showed the highest area under the ROC curve for Marsh type 3 histology in the entire CD population. Other series have considered a cut-off value > 100 U tTG antibody for a better Marsh type 3 prediction^[6-8]. The cut-off should probably be standardized in each laboratory based on experience with different kits.

Our series established CD diagnosis based on clinical judgment and expertise. This accounts for the high percentage of Marsh type 1 and 2 adult patients that were included, most of whom showed negative serology or low tTG antibody levels. North American Society for Pediatric Gastroenterology, Hepatology and Nutrition and European Society for Pediatric Gastroenterology, Hepatology and Nutrition guidelines indicate that Marsh type 1 or 2 changes are less specific or, perhaps, unlikely to be included in CD^[21]. However, in adult CD populations, atypical presentations with milder symptomatology and minor histopathology changes are described more frequently^[19,22]. There is evidence that CD patients who show only increased intraepithelial lymphocyte numbers, without mucosal atrophy, may show clinical features similar to those with Marsh type 3 lesions, and can also develop nutritional deficiencies and malabsorption^[23,24].

Any effort aimed at recognizing the presence of latent or occult minimally symptomatic CD would be the key reason to look for subtle abnormalities behind the symptoms^[25-27].

The primary reason to continue using biopsy as the definitive step in CD diagnosis is the possibility of false-positive serological results. All the cases in our series were diagnosed finally with CD, therefore, this possibility could not be explored. For some other series, a high positive tTG antibody result has not always been associated with a final CD diagnosis^[28]. However, these reports may be of questionable value to us for several reasons: (1) they were small series that lacked a prospective follow-up of tTG positive patients; (2) false-negative duodenal biopsies may be observed in CD (biopsy is not correctly performed, pathologist does not have experience with CD, or patchy histopathology lesions may be present); and (3) guinea pig tTG antibody, which lacks specificity, was used in some of the studies. Although tTG antibody positivity may appear in other gastrointestinal and liver inflammatory disorders, to date, strong positive results have not been described for such conditions^[29-32]. In addition, many of these patients may have coexisting CD^[33-35].

Based on the observed high predictive value of high tTG antibody titers in our series, 65% of the biopsies may not have been necessary. An important clinical difference was observed between adult and pediatric populations, in that only 53% of adults *vs* 95% of children would have properly avoided biopsy. The results for the adult population are concordant with a similar recent study^[20]. In 25% of our adult patients, mucosal recovery was not achieved in the second year after diagnosis on a GFD, despite normalization of serology and symptomatology. In fact, a slow mucosal recovery and persistent villous atrophy on a strict GFD in adult CD have been reported previously^[36-38]. Although asymptomatic, these patients without mucosal recovery may be at risk for subsequent severe complications^[36,39]. We believe that a follow-up biopsy may be important for detecting these individuals, and thus, should be mandatory. From our experience, this follow-up can be important for the correct identification of refractory CD.

Although we did not perform follow-up biopsies in children, the available results coincide with the idea that most pediatric CD patients recover from villous atrophy shortly after starting a GFD^[38,39]. Thus, for children it would be less important to perform an initial biopsy, as histological follow-up appears not to be necessary.

Overall, our results confirm the high diagnostic accuracy and predictive value of serological tests. We suggest that because of the high predictive value of tTG antibody for mucosal atrophy, duodenal biopsy may not always be necessary. In children, CD diagnosis may only require clinical and serological features, thus avoiding an invasive procedure, and starting an earlier GFD. In contrast, for adults, CD presentation and monitoring are different, thus rendering necessary a histopathological confirmation in all the cases at diagnosis, and in some selected cases at follow-up on a GFD. Future CD guidelines may take into account these age-related differences.

COMMENTS

Background

Duodenal biopsy remains the gold standard for celiac disease (CD) diagnosis. However, it has several pitfalls and requires an invasive procedure in children. In the past few years, a more prominent role for a definitive diagnosis based solely on serology has been proposed. The predictive value of high levels of anti-tissue transglutaminase (tTG) antibodies has also been reported in retrospective CD cohorts. Based on these studies, some authors have proposed to start a gluten-free diet (GFD) for those patients with high tTG antibody levels, without duodenal biopsy.

Research frontiers

There is no agreement to start a GFD without biopsy to confirm mucosal atrophy. There are age-related differences in CD diagnosis that may be taken into account to evaluate the predictive value of tTG antibody for mucosal atrophy.

Innovations and breakthroughs

This study revealed that tTG antibody is correlated with Marsh types, and is an independent predictor for Marsh type 3 lesions in children and adults. However, adults with CD showed less severe atrophy and lower tTG antibody titers than children. Moreover, a significant proportion of adult patients did not recover from atrophy during follow-up. These age-related differences have not been defined clearly in previous studies, and they are associated with the predictive value of tTG antibody for duodenal atrophy.

Applications

These results suggest that high tTG antibody titers may be sufficient for CD diagnosis in children, but a biopsy may be necessary to diagnose and monitor adults patients. Starting a GFD without a confirmatory biopsy may be possible in selected cases.

Terminology

CD is characterized by intolerance to ingested gluten in susceptible individuals. Immunologically mediated inflammation of the small intestine mucosa and the consequent atrophy is now the main point for diagnosis. Serological testing is the first step for diagnosis and tTG antibody is available widely as a screening method.

Peer review

In this interesting study, the authors investigated the predictive value of tTG antibody (cut-off value 30 U) for establishment of villous atrophy in children and adults suffering from CD. The serum parameter was correlated with histopathological findings in duodenal biopsies. The authors demonstrated that disease presentation and monitoring are different between children and adults. The increase in tTG antibody has a better predictive value in children. In conclusion, the authors recommend histopathological confirmation of tTG antibody serum levels in all adults, whereas in children with > 30 U tTG antibody, histopathological confirmation can be omitted.

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Portal hypertensive colopathy is associated with portal hypertension severity in cirrhotic patients

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Received: July 7, 2009 Revised: August 8, 2009

Accepted: August 15, 2009

Published online: October 14, 2009

Abstract

AIM: To assess the prevalence of portal hypertension (PH) related colorectal lesions in liver transplant candidates, and to evaluate its association with the severity of PH.

METHODS: Between October 2004 and December 2005, colonoscopy was performed in 92 cirrhotic liver transplant candidates. We described the lesions resulting from colorectal PH and their association with the grade of PH in 77 patients who underwent measurement of hepatic venous pressure gradient (HVPG).

RESULTS: Mean age was 55 years and 80.7% of patients were men. The main etiology of cirrhosis was

alcoholism (45.5%). Portal hypertensive colopathy (PHC) was found in 23.9%, colonic varices in 7.6% and polyps in 38% of patients (adenomatous type 65.2%). One asymptomatic patient had a well-differentiated adenocarcinoma. The manifestations of colorectal PH were not associated with the etiology of liver disease or with the Child-Pugh grade. Ninety percent of patients with colopathy presented with gastroesophageal varices (GEV), and 27.5% of patients with GEV presented with colopathy ($P = 0.12$). A relationship between higher values of HVPG and presence of colopathy was observed (19.9 ± 6.2 mmHg vs 16.8 ± 5.4 mmHg, $P = 0.045$), but not with the grade of colopathy ($P = 0.13$). Preneoplastic polyps and neoplasm ($P = 0.02$) and spontaneous bacterial peritonitis ($P = 0.006$) were more prevalent in patients with colopathy. We did not observe any association between previous β -blocker therapy and the presence of colorectal portal hypertensive vasculopathy.

CONCLUSION: PHC is common in cirrhotic liver transplant candidates and is associated with higher portal pressure.

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Key words: Liver cirrhosis; Portal hypertension; Polyps; Colopathy; Liver transplantation

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Diaz-Sanchez A, Nuñez-Martinez O, Gonzalez-Asanza C, Matilla A, Merino B, Rincon D, Beceiro I, Catalina MV, Salcedo M, Bañares R, Clemente G. Portal hypertensive colopathy is associated with portal hypertension severity in cirrhotic patients. *World J Gastroenterol* 2009; 15(38): 4781-4787 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4781.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4781>

INTRODUCTION

Orthotopic liver transplantation (OLT) is an effective therapeutic approach to chronic end-stage and acute liver

diseases^[1]. Portal hypertension (PH)-related lesions in the gastrointestinal tract of cirrhotic patients are frequent. In the evaluation of liver transplant candidates these lesions must be taken into account so that appropriate treatment can be administered before OLT. Although gastroscopy is a well established technique for the detection of upper PH-related lesions in liver transplant candidates, colonoscopy is not performed routinely in all centers.

In OLT candidates, it is important to identify and prevent the risk of bleeding from gastroesophageal varices (GEV) and portal hypertensive gastropathy. Furthermore, although colorectal lesions are a source of acute and chronic bleeding, they have received little attention in the literature. Colonoscopy allows us to evaluate the presence of PH-related lesions such as portal hypertensive colopathy (PHC), colorectal varices and hemorrhoids^[2,3]. The variability of the results of previous studies does not allow us to define with any certainty the prevalence of these lesions. Thus prevalence ranges from 3%^[4] to 84%^[5] for PHC, and from 3.6%^[6] to 89%^[7] for colorectal varices and from 22%^[8] to 79%^[9] for hemorrhoids.

The association between portal hypertensive vasculopathy and the severity of PH has been evaluated elsewhere. Four studies have evaluated this association by measuring indirect parameters of PH^[10-13], while four other authors have evaluated it using the gold-standard measurement of PH grade, that is, the hepatic venous pressure gradient (HVPG). Nevertheless the results are contradictory^[9,14-16].

On the other hand, recent studies have demonstrated an increase in the risk of adenomatous polyps and neoplasm in liver transplant recipients, so the detection of these lesions prior to OLT is essential^[17]. The presence of these lesions has been reported to be as high as 42%. The question is that whether colorectal PH-related lesions influence the development of polyps is unknown.

The objectives of the present study were to define the prevalence of lower gastrointestinal abnormalities detected as a result of an endoscopic screening of cirrhotic patients being evaluated for liver transplantation, and to relate the occurrence of these lesions to the degree of PH measured using the HVPG, and the severity of liver disease.

MATERIALS AND METHODS

Patients

The study was designed and performed according to the principles of the Declaration of Helsinki, and informed consent was obtained from each patient. The protocol was reviewed and approved by the Hospital Ethics Committee.

Between October 2004 and December 2005, one hundred and forty-nine patients were considered for OLT due to acute or chronic decompensated liver

disease. One hundred and twenty-one patients had liver cirrhosis. Of these, 92 were evaluated by colonoscopy as a part of pre-OLT screening for colorectal cancer or preneoplastic manifestations. The selection criteria for colonoscopy were age over 50 years and/or clinical symptoms such as abdominal pain or rectal bleeding, or family history of colorectal cancer. Diagnosis of cirrhosis was based on histology assessment or a combination of clinical, laboratory and ultrasonography findings. The severity of cirrhosis was classified according to the Child-Pugh classification. Four patients were excluded due to the presence of an implanted permeable transjugular intrahepatic portosystemic shunt before evaluation for OLT. Therefore the final cohort consisted of 88 patients. Patients with previous abdominal or pelvic surgery, hemorrhoidectomy or a previous history of malignancy (except primary liver cancer) and those who refused to undergo colonoscopic examination were not included in the study. Of the 88 patients evaluated, 50 (56.8%) were receiving non-selective β -blockers as primary or secondary prophylaxis for PH bleeding, and 12 (13.6%) had received previous endoscopic band ligation (EBL).

Information on epidemiological features, etiology of cirrhosis, Child-Pugh grade, blood test results, presence of hepatocellular carcinoma, clinical manifestations of PH [hepatic encephalopathy, ascites, spontaneous bacterial peritonitis (SBP), and variceal bleeding], and upper PH-related endoscopic findings (GEV, portal hypertensive gastropathy and duodenopathy) were analyzed. In addition, the severity of PH was quantified by measuring portal pressure.

Endoscopic examinations

An upper gastrointestinal endoscopy was performed in all patients to evaluate the presence of GEV, portal hypertensive gastropathy and duodenopathy. All colonoscopies were performed by well-experienced endoscopists who were unaware of the diagnosis and the severity of liver disease using a Pentax EC-3840LK model videoscope (Hoya Corporation, Tokyo, Japan). After an overnight fast, patients were administered a polyethylene glycol electrolyte lavage solution (Solución Evacuante Bohm®; Laboratorios Bohm S.A., Madrid, Spain) as a bowel-cleansing regimen. Colorectal portal hypertensive vasculopathy lesions included internal or mixed hemorrhoids, colonic varices and PHC. Hemorrhoids were described as vascular swellings affecting the internal and external arteriovenous vascular plexuses of the anal canal and were classified as external, internal or mixed (Figure 1A). Colonic varices were bluish, dilated, tortuous submucosal veins extending from the anal canal above the level of the hemorrhoids into the colon (Figure 1B). According to previous reports^[14], PHC was considered to be present if there were nonspecific inflammatory changes, resembling the lesions found in subjects with inflammatory chronic colitis and defined as granularity, diffuse hyperemia,

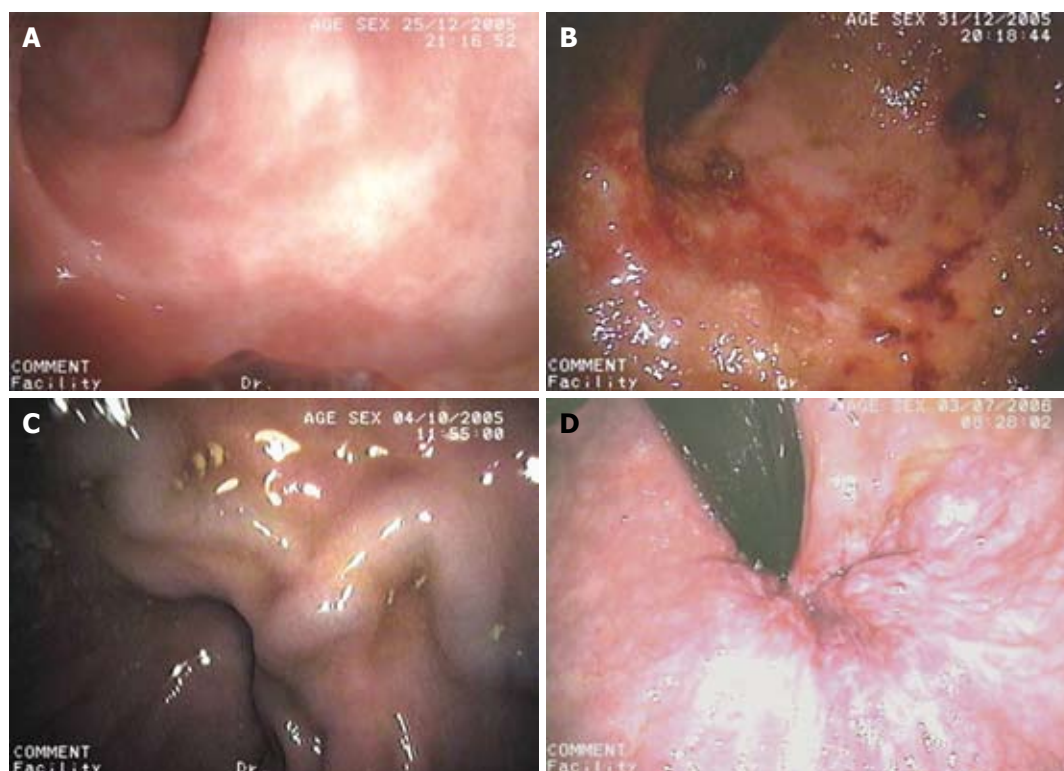


Figure 1 Endoscopic portal hypertension-related lesions. A: Mild portal hypertensive colopathy; B: Severe portal hypertensive colopathy; C: Colonic varix; D: Hemorrhoids.

edema and friability in mild grades (Figure 1C), and lesions such as vascular ectasias, angiodysplasia, arterial spider, and diffuse cherry red spots, in severe grades (Figure 1D). Arterial spider-like lesions are defined as the presence of a central arteriole, which blanches with pressure for a forceps biopsy and from which numerous small vessels radiate. The angiodysplasia-like lesions are defined by an irregular margin with a fern-like pattern and sometimes a pale halo around them, whereas the cherry red spots-like lesions are defined by the presence of a red spot in the colonic mucosa, similar to that seen in the gastric mucosa in patients with portal hypertensive gastropathy. Polyps and neoplasms were also described. Polyps were classified as hyperplastic or adenomatous.

Hemodynamic measurements

A splanchnic hemodynamic study with portal pressure measurement was performed in 77 of 88 patients. The time between the colonoscopy and the hemodynamic study was no more than 1 mo in all cases. The hemodynamic study was not performed in 11 patients because of previous clinical, laboratory or imaging findings that contraindicated the OLT. In all cases if the patient had significant ascites, a total paracentesis was performed before the study to avoid interference in the measurement of HVP, to diminish complications, and to make catheterization easier. After an overnight fast, the patient was prepared for the study in the supine position. Under local anesthesia, a vascular introducer sheath (Medikit Co. Ltd., Tokyo, Japan) was in-

serted into the right internal jugular vein. Then, under fluoroscopy, a 7F balloon catheter (Cordis Corporation, Miami, Florida, USA) was placed in the right hepatic vein to measure free and wedged hepatic venous pressure (FHVP and WHVP) as described previously^[18]. The wedged position was confirmed by the absence of reflux after injection of 2 mL of contrast medium, and FHVP was measured in the hepatic vein with the tip of the catheter just beyond the junction with the inferior cava vein. The HVP was calculated as WHVP minus FHVP. PH was defined as the presence of an HVP value > 5 mmHg. All hemodynamic measurements were performed using a previously calibrated strain-gauge transducer and recorded at least in duplicate. Two independent investigators who were unaware of the diagnosis, evaluated the tracings from the hemodynamic studies.

Statistical analysis

Quantitative variables were expressed as the mean \pm SD or median (range) if parametric or non-parametric, respectively, and qualitative variables were expressed as frequencies. Categorical and continuous variables were compared using the χ^2 and the Student's *t* test or the Mann-Whitney *U* test respectively, when appropriate. One-way analysis of variance (ANOVA) with polynomial contrasts was applied. A two-tailed 0.05 significance level was used in all statistical tests. Statistical analysis was performed using the Statistical Program for the Social Sciences version 13.0 (SPSS® 13.0; SPSS Inc, Chicago, Illinois, USA).

Table 1 Clinical characteristics and hemodynamic data (mean \pm SD, $n = 88$) n (%)

Sex (M/F)	71 (80.7)/17 (19.3)
Etiology	
Excessive alcohol consumption	40 (45.5)
HCV	20 (22.7)
HBV	9 (10.2)
HCV and excessive alcohol consumption	8 (9.1)
Other	11 (12.5)
Child-Pugh grade	
A	17 (19.3)
B and C	71 (80.7)
Hepatocellular carcinoma	30 (34.1)
Ascites	63 (71.6)
SBP	22 (23.9)
Hepatic encephalopathy	30 (34.1)
Previous variceal bleeding	16 (18.2)
Age (yr)	55 \pm 7
Hemoglobin (g/dL)	11.8 \pm 2.4
Platelets (cells/ μ L)	87 443 \pm 51 720
Leukocytes (cells/ μ L)	6286 \pm 8932
INR	1.58 \pm 0.53
Total bilirubin (mg/dL)	4.2 \pm 4.4
Serum albumin (g/dL)	3.2 \pm 0.6
α -fetoprotein (ng/mL)	65.1 \pm 299.3
CEA (ng/mL)	5.4 \pm 10.6
HVPG (mmHg, $n = 77$)	17.5 \pm 5.8

HCV: Hepatitis C virus; HBV: Hepatitis B virus; SBP: Spontaneous bacterial peritonitis; CEA: Carcino-embryonic antigen; HVPG: Hepatic venous pressure gradient.

RESULTS

Clinical characteristics and endoscopic findings

The clinical characteristics and hemodynamic data of the study population are shown in Table 1. Men comprised 80.7% of the sample and the median age was 55 (29-69) years. The main etiologies of cirrhosis were alcohol consumption (45.5%) and hepatitis C virus infection (31.8%). Most patients were Child-Pugh grade B-C (80.7%) and the HVPG tended to be high in relation to the severity of cirrhosis (Child-Pugh A 15.3 ± 6.6 mmHg *vs* Child-Pugh B-C 18.2 ± 5.4 mmHg, $P = 0.07$).

The colonoscopy revealed no lesions in 20.7% of the patients. The endoscopic PH-related lesions of the 88 patients evaluated are shown in Table 2. PHC was discovered in 23.9% of patients, colonic varices in 8% and internal or mixed hemorrhoids in 52.3%. No differences were observed between the colorectal manifestations of PH and the etiology of liver cirrhosis, Child-Pugh grade, history of ascites, hepatic encephalopathy, hepatocellular carcinoma or levels of serum albumin, total bilirubin, alanine aminotransferase, carcinoembryonic antigen, international normalized ratio or platelet count. Instead, we found a significant association between a history of SBP and PHC (45.5% *vs* 16.7%, $P = 0.006$).

Association between upper and lower gastrointestinal PH-related lesions

Table 3 compares clinical and endoscopic findings between the PHC and non-PHC groups. As for the lower and upper gastrointestinal tract, we found no association

Table 2 Portal hypertension-related endoscopic variables

	n (%)
Gastroesophageal varices	69 (78.4)
Portal hypertensive gastropathy	40 (48.2)
Portal hypertensive duodenopathy	11 (13.3)
Portal hypertensive colopathy	21 (23.9)
Mild	13 (61.9)
Severe	8 (38.1)
Colonic varices	7 (8)
Internal or mixed hemorrhoids	46 (52.3)

Table 3 Comparison of clinical and endoscopic findings between PHC and non-PHC groups (mean \pm SD) n (%)

	PHC group ($n = 21$)	Non-PHC group ($n = 67$)
Age (yr)	54.6 \pm 7.4	55.2 \pm 7
Hemoglobin (g/dL)	11.8 \pm 2.6	11.8 \pm 2.4
HVPG (mmHg, $n = 77$)	19.9 \pm 6.3	16.8 \pm 5.5 ^a
Sex		
Male	18 (85.7)	53 (79.1)
Female	3 (14.3)	14 (20.9)
Child-Pugh grade		
A	4 (19)	13 (19.4)
B and C	17 (81)	54 (80.6)
Excessive alcohol consumption	13 (61.9)	35 (52.2)
SBP	10 (47.6)	12 (17.9) ^a
Gastroesophageal varices	19 (90.5)	50 (74.6)
PH gastropathy	11 (55.0)	29 (46.0)
PH duodenopathy	2 (10.0)	9 (14.3)
Adenoma/adenocarcinoma	13 (61.9)	21 (31.3) ^a

^a $P < 0.05$ (ANOVA) *vs* PHC group. PHC: Portal hypertensive colopathy; PH: Portal hypertension.

between PHC and portal hypertensive gastropathy or duodenopathy. Of the patients with PHC, 90.5% had GEV. On the other hand, colonoscopy revealed that 27.5% of patients with GEV had PHC compared with 10.5% of patients without GEV ($P = 0.12$). We did not find an association between colonic varices and GEV.

Clinical implication of the presence of gastrointestinal PH-related lesions

Anemia is one of the most important consequences of chronic bleeding from PH-related lesions. We did not observe differences in hemoglobin values between patients with and without PHC (11.8 ± 2.6 g/dL *vs* 11.8 ± 2.4 g/dL, $P = 0.93$). There were also no differences in PHC grade (11.8 ± 2.6 g/dL *vs* 11.7 ± 2.7 g/dL, $P = 0.94$), although an association was found between the presence of anemia and portal hypertensive gastropathy (11.2 ± 2.4 g/dL *vs* 12.4 ± 2.3 g/dL, $P = 0.022$).

Relation between HVPG and colorectal PH-related lesions

In the whole cohort, a greater mean HVPG was found in patients with PHC than in those without (19.9 ± 6.3 mmHg *vs* 16.8 ± 5.5 mmHg, respectively, $P = 0.045$) (Figure 2), but it was not associated with PHC grade (mild PHC 19.8 ± 7.3 mmHg *vs* severe PHC $20.2 \pm$

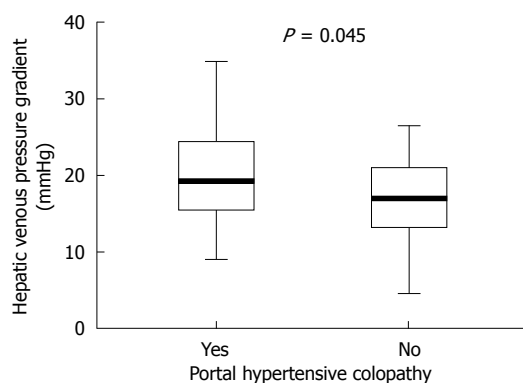


Figure 2 Comparison of HVP between PHC and non-PHC groups.

4.1 mmHg, $P = 0.91$). When we analyzed only the patients who had not previously been treated with non-selective β -blockers we found a higher association between the presence of PHC and HVP (22.9 \pm 6.7 mmHg *vs* 16.0 \pm 5.2 mmHg, $P = 0.007$).

HVP did not differ significantly between patients with or without colonic varices (17.8 \pm 3.2 mmHg *vs* 17.5 \pm 5.9 mmHg, respectively, $P = 0.91$) or with or without hemorrhoids (17.9 \pm 5.7 mmHg *vs* 17.1 \pm 5.8 mmHg, respectively, $P = 0.54$).

Influence of treatment of PH in the development of colorectal PH-related lesions

We did not find a significant association between treatment with non-selective β -blockers and the presence of PHC (24% *vs* 23.7%, $P = 0.97$) or colonic varices (10% *vs* 5.3%, $P = 0.69$). We could not demonstrate an influence of previous EBL in the development of these lesions.

Relation between PHC and colonic polyps and neoplasia

The prevalence of colonic polyps in our patients was 39.7% (35/88). Sixty-five percent of patients with polyps had adenomas and 30.4% had hyperplastic polyps. Six patients (40%) with adenomatous polyps presented with mild dysplasia. One asymptomatic patient (1.1%) had a well-differentiated adenocarcinoma. When we analyzed the association between PHC and polyps and/or adenocarcinoma we found that 42.9% of PHC patients had preneoplastic lesions or neoplasms compared with 10.4% of non-PHC patients ($P = 0.02$) (Table 3).

DISCUSSION

In the present study we suggest that colorectal endoscopic evaluation of OLT candidates can reveal findings that are directly related to the PH grade. It is reasonable to think that PH causes hemodynamic changes not only in the upper gastrointestinal tract but also in other areas, especially in the colon, a condition that is now receiving increasing attention in the literature. In our study, the prevalence of PHC was 23.9%, similar to that found previously^[6], although the prevalence is highly variable. These discrepancies may be due to the lack of

consensus on the endoscopic appearance of PHC, unlike portal hypertensive gastropathy, in which there does appear to be a certain degree of consensus. In our study we have chosen a simple classification of PHC: mild and severe grades. The reason for these categories is the higher risk of chronic or acute bleeding for severe grade lesions and their importance in clinical management and therapeutic options.

Several studies have demonstrated the relationship between PHC and indirect measurements of PH, for example low platelet counts^[10], the presence of portal hypertensive gastropathy^[11,13] or large esophageal varices^[11,12]. In our study we found an association between PHC and GEV, although it was not statistically significant.

Our main objective was to assess the association between PH vasculopathy and the severity of PH. We found a clear association between PHC and higher values of HVP, with a higher PH grade in the PHC group. This difference was maintained in patients who were not treated with non-selective β -blockers, avoiding possible bias from treatment influence. Few studies have compared this association with a hemodynamic measurement of PH. Sugano *et al.*^[15] and Yamakado *et al.*^[16] also demonstrated, as we did, an association between PHC and higher values of HVP. Unlike Sugano *et al.*^[15], we found that the presence of higher HVP values was related to the existence of PHC but not to PHC grade. Chen *et al.*^[14] also studied the relationship between colorectal portal hypertensive vasculopathy and PH severity, although they did not find a relationship between them.

We found no association between colorectal manifestations of PH and etiology of liver disease, Child-Pugh grade or previous history of hepatic decompensation, results which are consistent with those of some authors^[9,15]. Furthermore, like other authors, we were unable to demonstrate a relationship between HVP and colonic varices^[14,15]. However, we did find a statistical association between a history of previous SBP and the presence of PHC. In this sense, the main pathogenic mechanism of SBP, that is bacterial translocation, may be favoured by increased intestinal permeability at this site due to mucosal damage and vasodilatation in PHC.

The clinical implications of the presence of PH lesions and the therapeutic approach to chronic or acute bleeding have not been fully evaluated. Although the cross-sectional design of our study does not allow us to analyze adequately the effect of β -blockers on the outcome of these lesions, we observed no relationship between these drugs and PHC or colonic varices. In this sense, only two studies have evaluated the influence of β -blockers and nitroglycerin on the outcome of PHC, demonstrating that PHC improves after treatment with these drugs^[11,15]. Although there are no evidence at the moment that these treatments improve anemia or recurrent bleeding from PHC, the lesser presence and grade of these lesions should improve the clinical status of the patients, so the classification of PHC used in

our study is adequate, which is consistent with overall thinking in daily clinical practice.

A remarkable association was observed between preneoplastic lesions, neoplasms and PHC. In our study, the prevalence of polyps was 38%. Many of these were adenomatous (65.2%) and one adenocarcinoma was discovered. This association has not been previously described, suggesting that involvement of the colonic mucosal microvasculature in PHC could stimulate the proliferation of polyps, although the pathologic mechanisms are unknown and any explanation at this time would be highly speculative.

Although the best indicator of PH grade is the direct measurement of portal pressure by portal vein catheterisation, the difficulty and the morbidity of the procedure impede the habitual performance of this technique, particularly in patients with cirrhosis with altered coagulation factors and risk of complication. It is well documented that WHVP correlates well with direct portal pressure measurement and the agreement is sufficiently good to use it as a surrogate measurement of PH^[19], and it has been demonstrated in patients with hepatitis C^[20] and hepatitis B-related cirrhosis^[21]. In our study all the patients were cirrhotic and nearly 90% had viral or alcoholic etiology. In spite of this, the HVP is less accurate in liver diseases with a presinusoidal component and in our study this situation may occur only in a few patients.

Finally, our sample size was probably too small to obtain sufficient statistical power to extrapolate the results, although we do report the largest number of patients to date. Likewise, the same team of gastroenterologists were responsible for the endoscopic procedures and reporting and for reviewing the photographs taken during the explorations, thus ensuring the reliability of our data. However, the cross-sectional design of the study prevents us from evaluating the influence of β -blockers on the outcome of the lesions. Future prospective studies must take this into account if they are to improve the quality of life of these patients.

In conclusion, we demonstrated a high prevalence of colorectal hypertensive vasculopathy lesions in this cohort of patients, and a higher severity of PH with increased HVP values in patients with PHC, although we were unable to show an effect on PHC grade. Previous treatment with β -blockers does not appear to affect the outcome of PHC.

ACKNOWLEDGMENTS

We are indebted to Mr. Tom O'Boyle for his expert management of the manuscript.

COMMENTS

Background

Preoperative liver transplantation evaluation is performed to exclude contraindications and end-stage liver disease must be completely studied. Colonoscopy allows researchers to evaluate the presence of portal hyperten-

sion (PH)-related lesions such as portal hypertensive colopathy (PHC), colorectal varices and hemorrhoids. PHC is an area of increasing interest but little is known about its relation with the severity of PH. The measurement of the hepatic venous pressure gradient (HVPG) is the gold standard of PH grade.

Research frontiers

The relation between PHC and the severity of PH is not well established. In this study, the authors demonstrate that a higher value of HVPG is associated with the presence of PHC but not with other manifestations of PH-related colorectal lesions.

Innovations and breakthroughs

Previous reports remark on the association between PHC and the severity of PH with indirect measurements, and some studies with the gold standard of the HVPG, with contradictory results. The present study is the biggest cohort of patients in which HVPG measurement is performed to demonstrate this association.

Applications

Their study demonstrates that this association reflects a broad spectrum of lesions covering the whole gastrointestinal tract related with PH, so this supposes an advance in the knowledge of these lesions. Treatment of PH-related colorectal lesions is not well established and it must be elucidated.

Peer review

The authors exposed cirrhotic candidates for liver transplantation in which colonoscopy was performed, searching the association between PH-related colorectal lesions and the severity of PH measured by HVPG. The article is well written and the contents are credible.

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S- Editor Li LF L- Editor O'Neill M E- Editor Zheng XM



BRIEF ARTICLES

Effects of contrast media on the hepato-pancreato-biliary system

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Received: June 16, 2009 Revised: August 4, 2009

Accepted: August 11, 2009

Published online: October 14, 2009

Kupffer's cell hyperplasia were higher ($P < 0.05$) in the study groups than the control group. However, there were no significant differences ($P > 0.05$) between HOCM (2, 2p) and iso-osmolar CM (3, 3p) groups. Bile duct proliferation and regeneration in the Urographin® groups (2, 2p) were significantly higher ($P < 0.05$) than the Visipaque® groups (3, 3p) or the control groups (1, 1p). Although CM caused minor damage to the pancreas, there were no statistically significant differences ($P > 0.05$) between the groups. Application of the CM with pressure did not cause additional damage to the HPB system.

CONCLUSION: Iso-osmolar, non-ionic CM could be more reliable than the ionic HOCM, whereas the application of pressure during the CM application had no effect on the HPB system.

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Abstract

AIM: To determine the effects of high osmolarity contrast media (HOCM) and iso-osmolar contrast media (CM) application, with or without pressure, on hepato-pancreato-biliary (HPB) system.

METHODS: Sixty rats were divided into six equal groups as follows: Group 1: (0.9% NaCl, control), Group 2: (diatrizoate meglumine Na, ionic HOCM, Urographin®), Group 3: (iodixanol, iso-osmolar non-ionic CM, Visipaque®); each of which was applied without pressure, whereas the animals of the remaining three groups (1p, 2p, 3p) were subjected to the same CM with pressure. We performed a duodenal puncture and introduced a catheter into the ampulla. After the catheterization, 0.2 mL CM or 0.9% NaCl was injected with or without pressure. Blood samples were taken for biochemical evaluations. The histopathological examinations of liver, common bile duct, and pancreas were performed.

RESULTS: There were no significant differences between the six groups for blood amylase, alanine aminotransferases, aspartate aminotransferases, bilirubin levels ($P > 0.05$). Alkaline phosphatase and γ glutamyl transaminase levels were higher ($P < 0.05$) in the Urographin® groups (2, 2p) than the Visipaque® groups (3, 3p), or control groups (1, 1p). Hepatocyte necrosis, portal area inflammation, and

Key words: Contrast media; Liver; Pancreas; Biliary tract; Pressure

Peer reviewer: Zvi Fireman, MD, Associate Professor of Medicine, Head, Gastroenterology Department, Hillel Yaffe Med Ctr, POB 169, 38100, Hadera, Israel

Topcu O, Kurt A, Nadir I, Arici S, Koyuncu A, Aydin C. Effects of contrast media on the hepato-pancreato-biliary system. *World J Gastroenterol* 2009; 15(38): 4788-4793 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4788.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4788>

INTRODUCTION

Contrast media (CM) are commonly used in diagnostic imaging methods such as, computerized tomography, magnetic resonance, angiography, cardiac catheterization along, and imaging of the hepato-pancreato-biliary (HPB) system using endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic cholangiography, and intra- and post-operative cholangiography^[1].

ERCP is both a diagnostic and therapeutic procedure that has been used for 35 years. Complications of diagnostic ERCP include acute pancreatitis, cholangitis, and cholecystitis^[2-4]. Pancreatitis is a major cause of

the morbidity and the mortality related to ERCP^[2-6]. Hyperamylasemia (biochemical pancreatitis) and clinical pancreatitis are seen 20%-70% and 1.8%-6.4% of cases, respectively^[7-9]. It is caused by technical reasons such as the type of CM, frequency of application, fast and pressured injection of the CM, trauma of the sphincterotomy, and the type of diathermy^[5,10-12]. It is also affected by previous history of pancreatitis, the degree of experience of the endoscopist, and the amount of the injection^[2-5]. Intra- and extra-hepatic bile ducts, along with the pancreatic duct, are imaged during ERCP. Acute cholangitis is another serious complication of ERCP^[3-5]. Most studies on the adverse effects of CM used in ERCP have focused primarily on post-ERCP pancreatitis (PEP). However, the biliary epithelial cells are also exposed to CM during ERCP. Inflammation of the biliary tree, including acute cholangitis and cholecystitis, is another important complication of diagnostic ERCP^[3-5].

CM can be classified in two groups depending on their ionic properties (ionic or non-ionic) and three types according to their osmolality (high, low or iso-osmolar)^[13]. The effect of a CM on image quality during ERCP depends on its density, viscosity, and osmolality. The image quality appears to be similar when comparing high osmolality contrast media (HOCM) and low osmolality contrast media (LOCM)^[14]. The osmolality and the ionic nature of the CM are believed to be the major factors responsible for the adverse reactions^[13]. Systemic adverse reactions of CM used in ERCP could be characterized as idiosyncratic (anaphylactic; nasal congestion, laryngeal edema, and bronchospasm) or non-idiosyncratic (dose-dependent; renal tubular damage, vascular damage, cardiac depression, and arrhythmia)^[13,15]. The prevalence of CM reactions is lower with LOCM (1%-3%) than with HOCM (5%-12%). Fatal reactions are rarely seen, and there is no difference in mortality rates between the two types^[15-17]. An ideal contrast agent must be water soluble, biologically inert, of low viscosity, and should be thermally and chemically stable. It must also have lower or the same osmolality as human serum, be selectively excreted (*via* the kidney), and be safe and low cost^[18].

The osmolality of CM has been implicated as a contributing factor for the development of PEP. However, results of clinical trials are conflicting^[19]. One randomized crossover study^[20] and four randomized control trials^[10,21-23] have suggested a benefit from LOCM, while 10 others^[9,14,24-31] have not. Animal studies examining pancreatic duct epithelium damage due to CM have also been conflicting. In cats, Bub *et al.*^[32] demonstrated morphologic changes in the pancreatic duct epithelium shortly after injection with CM. Less damage was noted after injection with LOCM. However, Saari *et al.*^[33] demonstrated less acinar destruction with HOCM in pigs. Pfau *et al.*^[34] noted that there were no differences between HOCM and LOCM on pancreatic histology in a canine model.

Damage is caused by technical reasons, such as the type of CM, frequency of application, and the fast and

pressured injection of the CM. Haciahmetoglu *et al.*^[35] evaluated how intraductal pressure and the use of a contrast agent affect the development of pancreatitis after ERCP. The results of their study^[35] suggested a contrast agent should be administered under low pressure when it is needed.

In the present study, the effects of diatrizoate meglumine Na (ionic HOCM, Urographin®) and iodixanol (iso-osmolar, non-ionic CM, Visipaque®) application with or without pressure on the HPB system were determined.

MATERIALS AND METHODS

Ethics

This experimental study was performed in accordance with the guidelines for the care and use of laboratory animals established by the Ethics Committee of the Cumhuriyet University.

Animal studies

Sixty, three-month-old wistar male rats weighing 250 ± 50 g were used in the present study. The randomly selected animals were supplied by the Animal house of the Faculty of Medicine, Cumhuriyet University. Rats were randomly divided into six equal groups: Group 1: (control, 0.9% NaCl), Group 2: (diatrizoate meglumine Na, Urografin®, ionic HOCM), Group 3: (iodixanol, Visipaque®, iso-osmolar, non-ionic CM); CMs were used without pressure. The same CMs were applied with pressure in the remaining three groups (1p, 2p, 3p). Rats were anesthetized by an intramuscular injection of ketamine HCL 40 mg/kg body weight (Ketalar: Parke-Davis Eczacıbaşı, Istanbul, Turkey) and xylazin 5 mg/kg body weight (Rompum: Bayer Leverkusen, Germany). All animals were allowed to breath spontaneously during the experiments. After the abdomen was shaved and cleaned with povidone iodine, a 2 cm midline laparotomy was carried out, and the intestines were covered with sterile gauze pads soaked with isotonic saline at 37°C to minimize evaporation from the tissue. Body temperature was maintained between 36 and 38°C using a heating lamp. In addition, 5 mL Ringer's lactate solution was given subcutaneously to prevent dehydration of the animals during the experimental period. After making a midline abdominal incision, we performed a duodenal puncture by a sharp pointed lancet. We introduced a catheter (0.7 mm diameter) into the ampulla. After catheterization, 0.2 mL CM or 0.9% NaCl was injected with or without pressure. The catheter was then withdrawn. The duodenal puncture was closed by only one suture with an 8-0 polypropylene (prolene). There were no operative and post-operative mortalities. On the second day, blood samples were taken for biochemical assays, including blood amylases, alanine aminotransferases (ALT), aspartate aminotransferases (AST), bilirubin levels, alkaline phosphatase (ALP), and γ glutamyl transaminase (GGT). Animals were kept in separate cages for 15 d, during that time they were fed with rat chow *ad libitum* and tap water, and kept at room temperature (18-20°C). Fifteen days later, all rats were

Table 1 Histopathological changes in the liver of rats, extrahepatic biliary ducts, and pancreas¹

Histopathologic changes	Group						P value
	1	1p	2	2p	3	3p	
Liver of rats							
Portal area inflammation	1	2	10 ^a	10 ^a	10 ^a	10 ^a	< 0.05
Bile duct inflammation	0	0	0	0	0	0	> 0.05
Periductal fibrosis	0	0	0	0	0	0	> 0.05
Bile duct proliferation	0	0	8 ^a	10 ^a	3	4	< 0.05
Hepatocyte necrosis	0	1	10 ^a	10 ^a	10 ^a	10 ^a	< 0.05
Parenchymal necrosis	0	0	0	0	0	0	> 0.05
Kupffer's cell hyperplasia	1	2	10 ^a	10 ^a	10 ^a	10 ^a	< 0.05
Regeneration findings	1	2	10 ^a	8 ^a	2	1	< 0.05
Fibrosis	0	0	0	0	0	0	> 0.05
Extrahepatic biliary ducts							
Inflammation	2	2	2	3	2	3	> 0.05
Fibroblastic proliferation	0	0	4 ^a	5 ^a	0	0	< 0.05
Necrosis	0	0	0	0	0	0	> 0.05
Pancreas							
Hemorrhage	0	0	0	0	0	0	> 0.05
Necrosis	0	0	0	0	0	0	> 0.05
Inflammation	1	1	1	1	2	2	> 0.05

¹Numbers indicate the number of the animals in which histopathological changes have occurred; ^aIndicates the statistical significance.

killed (in a humanitarian way by cervical dislocation) under anesthesia, and a second laparotomy was performed. The liver, pancreas, and the extra hepatic biliary tracts were resected *en bloc* and fixed in 10% formalin solution. For microscopic examination, tissues were fixed in formalin, and embedded in paraffin.

Histopathological evaluation

Tissue sections from each block were stained with hematoxylin/eosin for histopathological evaluation. We performed histopathological examination of the liver to assess the following: (1) lesions of the lining of the portal and centrilobular areas; (2) lesions of the hepatocytes (focal necrosis, regeneration); (3) biliary duct proliferations; (4) Kupffer's cell hyperplasia; (5) periductal fibrosis; (6) sinusoidal lesions (distention, fibrosis); (7) parenchymal necrosis; and (8) biliary duct inflammation.

Histopathological examination of the common bile duct was performed in order to assess inflammation, fibroblastic proliferation, and necrosis. We examined the pancreas specimens histopathologically in order to assess inflammation, hemorrhage, and necrosis. The histopathological alterations were evaluated by one pathologist who was blinded as to which group the specimen belonged.

Statistical analysis

All data were analyzed using Kruskal-Wallis variance analysis, and Mann-Whitney *U* test. The SPSS computer program software (version 9.0; SAS Institute, Cary, NC, USA) was used. A value of $P < 0.05$ was accepted as the significance level.

RESULTS

Biochemical evaluations

There were no significant differences ($P > 0.05$) between

the six groups for blood amylase, ALT, AST, bilirubin levels. ALP and GGT levels were higher ($P < 0.05$) in the Urographin[®] groups (2, 2p) than in the Visipaque[®] groups (3, 3p), or the control groups (1, 1p). There were no significant differences ($P > 0.05$) between administrations with or without pressure.

Histopathological alterations

All histopathological findings for the liver are given in Table 1. In the liver, there were no significant differences ($P > 0.05$) between the groups for bile duct inflammation, periductal fibrosis, fibrosis, and parenchymal necrosis. Hepatocyte necrosis, portal area inflammation, and Kupffer's cell hyperplasia were higher ($P < 0.05$) in the study groups than in the control group. However, there were no significant differences ($P > 0.05$) between HOCM [Urographin[®], (2, 2p)] and iso-osmolar CM [Visipaque[®], (3, 3p)] groups. On the other hand, the above features were not affected by pressure when all the groups were compared. Bile duct proliferation (Figure 1A) and regeneration findings (Figure 1B) in the Urographin[®] groups (2, 2p) were significantly higher ($P < 0.05$) than the Visipaque[®] groups (3, 3p), or the control groups (1, 1p). There were no differences ($P > 0.05$) between any groups when CM applications were done with or without pressure (Table 1). Histopathological findings of extrahepatic biliary tract (EBT) are given in Table 1. Necrosis of the EBT was not seen in any of the groups. Inflammation of EBT was similar in each of the groups. Fibroblastic proliferation in the EBT for the Urographin[®] groups (2, 2p) were significantly higher ($P < 0.05$) than in the Visipaque[®] groups (3, 3p), or the control groups (1, 1p). There were no significant differences ($P > 0.05$) between administrations with or without pressure (Table 1).

Histopathological findings of the pancreas are given in Table 1. Necrosis and hemorrhage of the pancreas

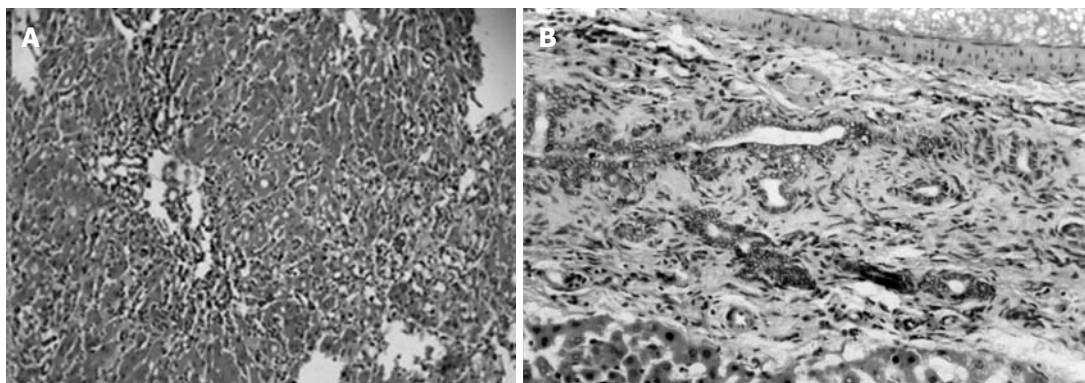


Figure 1 Proliferation (A) and regeneration findings (B) in the HOCM (Urographin®) group (HE, × 25).

were not seen in any of the groups. There were no significant differences ($P > 0.05$) between the groups for inflammation of the pancreas (Table 1).

DISCUSSION

The most feared ERCP complication is pancreatitis^[2-6,36]. It results in morbidity and occasional mortality. Osmolarity of the CM has been suggested to be a risk factor in the development of PEP. There are controversial data in current literature^[19]. O'Connor *et al.*^[22] noted that there was no difference in median increments of serum amylase at 6 h after ERCP, but there was a significant difference at 18 h after ERCP in the LOCM (iopamidol) group compared to the HOCM (meglumine diatrizoate) group. No patient, however, developed clinical pancreatitis. Banerjee *et al.*^[23] determined a significant difference between the abilities of HOCM and LOCM to cause pancreatitis. Their results proved that the incidence of pancreatitis is higher when HOCM is used^[23]. Barkin *et al.*^[10] determined the effects of LOCM (iohexol and ioxaglate) and HOCM (meglumine diatrizoate) agents in a prospective, double-blind, randomized study. The clinical symptoms of pancreatitis were lower in patients who received non-ionic LOCM than in those who received ionic HOCM^[10]. George *et al.*^[19] determined the incidence of PEP associated with HOCM and LOCM in their meta-analytical study. Clinical pancreatitis was evidenced by both elevation of pancreatic enzymes and pain. The results of their study indicated that there was no significant difference between HOCM and LOCM with respect to clinical pancreatitis^[1]. Although CM caused minimal adverse effects on the pancreatic tissue, there were no differences between the groups in the present study.

Bub *et al.*^[32] demonstrated morphologic changes in the pancreatic duct epithelium shortly after injection with CM in cats. Less damage was noted after injection with LOCM. Pfau *et al.*^[34] noted that there were no differences between HOCM and LOCM on pancreatic histology in a canine model. Saari *et al.*^[33] performed experimental pancreaticography on 25 piglets using three CM [diatrizoate meglumine Na (ionic HOCM)], meglumine

ioxaglate (ionic LOCM), and iohexol (non-ionic LOCM). They also injected the CM very slowly in order to avoid elevation of intraductal pressure and used a narrow cannula to enable free escape of pancreatic fluid and CM from the ductal system during injection, thus prevented overfilling. Minimal acinar destruction was seen on histological examination in all cases. This study showed that diatrizoate meglumine Na was rapidly emptying compared to the other CM; therefore the changes were least when diatrizoate was used. The emptying of CM from the ducts was significant. Saari *et al.*^[33] noted that rapid emptying might be an advantage in clinical ERCP. Hacıahmetoglu *et al.*^[35] evaluated how intraductal pressure and contrast agent affect the development of pancreatitis after ERCP. The results of their study suggested that the main mechanism for preventing pancreatitis after ERCP is to minimize trauma to the pancreatic canal, to cannulate the pancreas only when it is necessary, and to give contrast agent under low pressure when it is needed. In the present study, all of the CM were applied with and without pressure, but no difference ($P > 0.05$) was observed between them.

Mäkelä *et al.*^[27] determined no difference in the incidence of hyperamylasemia between diatrizoate meglumine (ionic HOCM) and iohexol (non-ionic LOCM) groups. The authors concluded that acute hyperamylasemia after ERCP was a complication of relatively minor importance, unlikely to be reduced by the use of LOCM^[27]. In the present study, there was no difference between the groups for the serum amylase measures.

Biliary epithelial cells are also exposed to CM after the ERCP procedure. Ju *et al.*^[37] performed a study to compare the cytotoxicity with gallbladder epithelial cells of ionic and non-ionic CM. They tested HOCM and LOCM for their effects on monolayer cell cultures of dog gallbladder epithelial cells. According to their results, HOCM were more cytotoxic than LOCM in gallbladder epithelial cells. In the present study, we observed that ionic HOCM were more destructive for the biliary tracts. In the literature, there are numerous studies on the effects of CM on pancreas, but fewer studies on the biliary tract^[37] and liver^[38]. In our study, we also evaluated biliary tract and liver. The findings revealed that HOCM

were more destructive than the iso-osmolar CM on the biliary tract and liver. These histopathological findings were also confirmed biochemically in the HOCM groups (group 2, 2p) by elevated ALP and GGT levels.

In conclusion, the findings of the present study have pointed out that iso-osmolar non-ionic CM could be more reliable than the ionic HOCM during the application of ERCP and diagnostic methods used for the imaging of the extra-hepatic biliary tracts. On the other hand, CM application with pressure might not cause additional damage to the HPB system.

COMMENTS

Background

Contrast media (CM) are commonly used in diagnostic imaging methods in visualizing the hepato-pancreato-biliary (HPB) system using Endoscopic retrograde cholangio pancreatography (ERCP), percutan transhepatic cholangiography, and intra- and post-operative cholangiography. Complications of diagnostic ERCP include acute pancreatitis, cholangitis, and cholecystitis.

Research frontiers

There are no experimental studies on effects of the iso-osmolar, non-ionic CM on the HPB system. Most studies on the adverse effects of CM used in ERCP have focused primarily on post-ERCP pancreatitis (PEP). However, the biliary epithelial cells are also exposed to CM during ERCP. Inflammation of the biliary tree, including acute cholangitis and cholecystitis, is another important complication of diagnostic ERCP. Therefore, the present study involved in the effects of high osmolarity contrast media (HOCM) and iso-osmolar CM on HPB system histopathologically and biochemically. In addition, the present study has also studied the effects of pressure during the injection of CM into the HPB system.

Innovations and breakthroughs

Although osmolarity of the CM has been suggested to be a risk factor in the development of PEP, there are controversial data in current literature. While there are several studies comparing the effects of low osmolarity contrast media and HOCM in the development of PEP, there are no studies on the comparison of the effects of non-ionic, iso-osmolar CM.

Applications

Findings of the present study have pointed out that iso-osmolar non-ionic CM could be more reliable than the ionic HOCM during the application of ERCP and diagnostic methods used for the imaging of the HPB system. On the other hand, CM application with pressure might not cause additional damage to the HPB system.

Peer review

The paper on the effect of the osmolarity of CM on the hepato-biliary tract is an interesting topic.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Zheng XM



BRIEF ARTICLES

Long-term treatment with proton pump inhibitor is associated with undesired weight gain

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Received: July 23, 2009 Revised: August 14, 2009

Accepted: August 21, 2009

Published online: October 14, 2009

with BW gain in patients with GERD. Reflux patients receiving PPI should be encouraged to manage BW through lifestyle modifications.

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Key words: Gastroesophageal reflux disease; Proton pump inhibitor; Body weight

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Abstract

AIM: To examine the effects of long-term proton pump inhibitor (PPI) therapy on body weight (BW) and body mass index (BMI) in patients with gastroesophageal reflux disease (GERD).

METHODS: The subjects were 52 patients with GERD and 58 sex- and age-matched healthy controls. GERD patients were treated with PPI for a mean of 2.2 years (range, 0.8-5.7 years), and also advised on lifestyle modifications (e.g. selective diet, weight management). BW, BMI and other parameters were measured at baseline and end of study.

RESULTS: Twenty-four GERD patients were treated daily with 10 mg omeprazole, 12 with 20 mg omeprazole, 8 with 10 mg rabeprazole, 5 with 15 mg lansoprazole, and 3 patients with 30 mg lansoprazole. At baseline, there were no differences in BW and BMI between reflux patients and controls. Patients with GERD showed increases in BW (baseline: 56.4 ± 10.4 kg, end: 58.6 ± 10.8 kg, mean \pm SD, $P < 0.0001$) and BMI (baseline: 23.1 ± 3.1 kg/m², end: 24.0 ± 3.1 kg/m², $P < 0.001$), but no such changes were noted in the control group. Mean BW increased by 3.5 kg (6.2% of baseline) in 37 (71%) reflux patients but decreased in only 6 (12%) patients during treatment.

CONCLUSION: Long-term PPI treatment was associated

with BW gain in patients with GERD. Reflux patients receiving PPI should be encouraged to manage BW through lifestyle modifications. *World J Gastroenterol* 2009; 15(38): 4794-4798 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4794.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4794>

INTRODUCTION

Gastroesophageal reflux disease (GERD) is the most common esophageal disorder, and frequently encountered in the primary care setting. It has been estimated that 15%-25% of persons experience reflux symptoms at least weekly, and 5%-12% suffer on a daily basis^[1].

The risk of reflux symptoms, erosive esophagitis, or esophageal adenocarcinoma increases with excessive weight and obesity^[2]. Accumulating evidence has confirmed the excellent efficacy and safety of proton pump inhibitor (PPI) therapy in patients with all grades of GERD, making these agents the mainstay of treatment. Consequently, PPIs comprise the largest outpatient pharmacy expenditure in the United States. Body weight loss is commonly recommended as part of a first-line therapeutic measure for GERD, although lifestyle modifications have been relegated to a minor role in the therapeutic regime due to the effectiveness and availability of PPIs as an acid-suppressive therapy^[3].

GERD is a chronic condition, necessitating continuous therapy for many patients to control symptoms and

prevent complications. Long-term therapeutic options include PPI therapy and surgical or endoscopic procedures^[4]. Recently, body weight loss after laparoscopic Nissen fundoplication was reported^[5]. There is an extensive literature database that addresses the efficacy and safety of long-term PPI therapy. However, the possible impact of changes in body weight or body mass index (BMI) in reflux patients while on long-term PPI therapy has not been examined. We present herein the first report elucidating the effect on nutritional parameters such as body weight and BMI in patients receiving long-term PPI therapy.

MATERIALS AND METHODS

Subjects

We evaluated 52 adult patients with GERD and 58 healthy controls. We selected patients undergoing daily maintenance therapy of PPI for at least 10 mo at the University Hospital of Occupational and Environmental Health and four Gastroenterology Clinics between June and November 2005. Patients who had prior fundoplication or poor compliance with medication were excluded. Patients with GERD had received advice on lifestyle modifications such as selective diet and weight management to accompany the PPI treatment.

The controls were sex- and age-matched subjects who visited the clinic for a yearly medical examination; they were free of reflux symptoms, and did not take PPIs or histamine receptor antagonists. They did not receive advice on lifestyle modifications. Informed consent was obtained from all subjects and the study was performed in accordance with the Declaration of Helsinki as revised in 1989.

Diagnosis of GERD

The diagnosis of GERD was made based only on the typical symptoms of GERD such as troublesome heartburn and/or acid regurgitation. Endoscopy at presentation was performed in patients with alarm symptoms such as dysphagia, odynophagia, bleeding, weight loss, and anemia that together suggested a complicated disease.

Treatment of GERD

Initial therapy was a standard dose of PPI (omeprazole 20 mg, rabeprazole 20 mg, or lansoprazole 30 mg) once daily for 8 wk followed by a daily maintenance half-dose therapy. The patients were followed-up at 4-wk intervals in the clinics to assess symptom recurrence. Patients found to have recurring symptoms of heartburn or acid regurgitation were placed back on their initial PPI dose. The patients were educated on lifestyle modifications by their physicians in addition to the PPI treatment. These instructions included avoidance of overeating, decreased fat intake, elevation of the head of the bed, cessation of smoking, avoiding recumbency for postprandial 3 h, and body weight control.

Nutritional parameters and blood pressure

Body weight, height, and blood pressure, as well as

Table 1 Baseline demographics and characteristics of reflux patients treated with long-term daily maintenance proton pump inhibitor therapy and healthy controls (mean \pm SD)

	Patients	Control	P
Number of subjects	52	58	-
Gender (male/female)	36/16	38/20	NS
Age (yr)	68.1 \pm 10.4	68.8 \pm 1.5	NS
Duration of observation (yr)			NS
PPI therapy	2.1 \pm 1.1	-	-
Medical checkup	-	2.0 \pm 0.4	-
Body weight (kg)	56.4 \pm 10.4	58.6 \pm 8.4	NS
Height (cm)	156.0 \pm 9.8	156.0 \pm 8.9	NS
Body mass index (kg/m ²)	23.1 \pm 3.1	24.1 \pm 2.7	NS
Blood pressure (mmHg)			-
Systolic	133 \pm 17	132 \pm 16	NS
Diastolic	75 \pm 11	75 \pm 9	NS
Serum total protein (g/dL)	7.2 \pm 0.3	7.2 \pm 0.3	NS
Serum total cholesterol (mg/dL)	210 \pm 39	210 \pm 25	NS
Serum triglyceride (mg/dL)	121 \pm 52	107 \pm 43	NS

PPI: Proton pump inhibitor.

fasting serum levels of total protein, total cholesterol, and triglycerides were determined at baseline and at the last visit. The BMI was calculated as body weight (kg)/[height (m)]². These parameters obtained within four weeks before the commencement of PPI therapy were defined as baseline data.

Statistical analysis

All results were expressed as mean \pm SD. Categorical outcome variables were analyzed with Fisher's exact test. For continuous variables, the Mann-Whitney *U*-test and Student's *t*-test were used where appropriate. A *P* value less than 0.05 denoted the presence of a statistically significant difference between the groups.

RESULTS

Characteristics and demographics of subjects

Table 1 details the characteristics of the 52 reflux patients and 58 healthy controls. There were no significant differences between the patient and control groups with regard to age, sex, duration of observation, body weight, body height, BMI, blood pressure, and serum values of total protein, total cholesterol, and triglycerides. *Helicobacter pylori* status, endoscopic findings, and PPI regimens of daily maintenance therapy in reflux patients are listed in Table 2.

Effect of long-term daily PPI maintenance therapy on nutritional parameters and blood pressure

No significant differences were found between patient and control groups with respect to changes in blood pressure or serum values of total protein, total cholesterol, and triglycerides. In contrast, patients treated with PPI experienced significantly greater increases from the baseline to the last visit in body weight (*P* < 0.0001) and BMI (*P* < 0.0005) than controls (Table 3).

The differences in body weight and BMI between the baseline and the last visit were analyzed separately

Table 2 Patient demographics

	Patients (n = 52)
<i>Helicobacter pylori</i> status	
Negative	9
Positive	11
ND	32
Endoscopic findings	
Normal	18
LA grade A	6
LA grade B	13
LA grade C	5
LA grade D	3
ND	7
Number of patients according to PPI regimens	
Omeprazole 10 mg once daily	24
Omeprazole 20 mg once daily	12
Rabeprazole 10 mg once daily	8
Lansoprazole 15 mg once daily	5
Lansoprazole 30 mg once daily	3

LA: Los Angeles classification; ND: Not determined.

for both groups (Table 3). Body weight ($P < 0.0001$) and BMI ($P < 0.0001$) significantly increased at the last visit in reflux patients. In contrast, there was no significant difference in body weight or BMI at the last visit in controls.

Categorical changes in body weight at the last visit compared to the baseline values are shown in Figure 1. Most of the control group (91%) remained stable, defined by a change of no more than 5% compared to baseline weight; however, only 60% of the PPI group remained stable. In addition, 36% of these patients had an increase in body weight above baseline of more than 5%, compared with 4% of the control group ($P < 0.0001$).

DISCUSSION

This study demonstrated for the first time that long-term PPI treatment is associated with undesirable body weight gain in patients with GERD, despite lifestyle modification recommendations by their physicians. Heartburn is the classical symptom of GERD, with patients generally reporting a burning feeling, rising from the stomach and radiating toward the neck and throat. It usually occurs postprandially, particularly after large meals or the consumption of fats. Untreated patients suffering from reflux symptoms find it difficult to have large meals, because this generally aggravates their symptoms. Untreated patients may therefore reduce their meal sizes and intake of fats intentionally or unintentionally. It is conceivable, therefore, that the resolution of reflux symptoms by PPI treatment leads to a higher food intake resulting in body weight gain.

Laparoscopic Nissen fundoplication has evolved as a gold standard in antireflux surgery. This surgical therapy induces a significant and persistent reduction in body weight, possibly due to postoperative dysphagia or delayed gastric emptying^[5]. In contrast, the option of long-term PPI therapy was associated with a significant

Table 3 Mean changes in nutritional parameters and blood pressure at the last visit compared to the baseline

	Patients (n = 52)	Control (n = 58)	P
Body weight (kg)	+2.2 ^b	-0.1 ^a	< 0.0001
Body mass index (kg/m ²)	+0.92 ^b	+0.15 ^a	0.0005
Blood pressure (mmHg)			-
Systolic	+2.7	+1.9	NS
Diastolic	-0.6	-1.9	NS
Serum total protein (g/dL)	+0.014	+0.019	NS
Serum total cholesterol (mg/dL)	-9.1	-17.1	NS
Serum Triglyceride (mg/dL)	+3.1	+3.8	NS

^aNS vs baseline; ^b $P < 0.0001$ vs baseline; NS: Not significant.

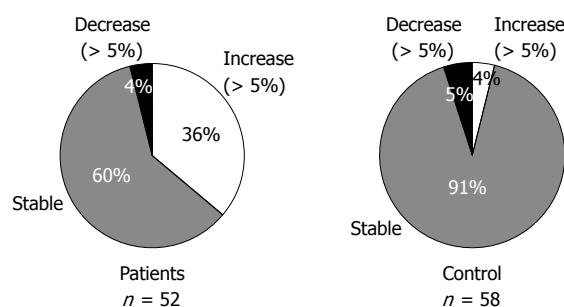


Figure 1 Categorical change in body weight at the last visit compared to the baseline reading. Most of the control group (91%) remained stable, within a 5% change, compared to weight at the baseline. However, only 60% of the PPI group remained stable. In addition, compared with 4% of the control group, 36% of patients had a more than 5% increase above the baseline in body weight ($P < 0.0001$). n: Number of patients or control subjects.

body weight gain in the present study. Omeprazole and other PPIs delay gastric emptying^[6-9], which induces postprandial fullness, dyspeptic symptoms, gastrointestinal bacterial overgrowth, and subsequent weight loss^[10,11]. Our results have clearly demonstrated that long-term PPI therapy contributed significantly to body weight changes in patients with GERD by relieving the adverse symptoms rather than altering the state of gastric emptying.

Numerous circulating peptides influence appetite. Ghrelin is produced in the stomach and acts as a meal initiator. A recent report revealed that long-term PPI therapy did not change the serum ghrelin level^[12]. Another peptide, leptin, is produced in the stomach and acts as an enteric signal involved in energy homeostasis. Change of this peptide associated with PPI therapy has not been reported.

A practice guideline for patients with GERD recommends the use of lifestyle modifications such as elevation of the bed head, a decreased intake of fat, chocolate, alcohol, peppermint, coffee, onions and garlic, cessation of smoking, and avoiding recumbency for three hours postprandially, in addition to taking antireflux medications^[13]. However, the positive advantage of such lifestyle modifications on the patient's condition is not well substantiated. Among these lifestyle interventions, elevation of the bed head, left lateral decubitus positioning, and weight loss are associated

with improvement in reflux symptoms in case-control studies^[14,15]. These modifications alone, however, are unlikely to control symptoms in the majority of patients^[13]. Our results support the finding that lifestyle modifications are an essential component of the treatment for GERD and the prevention of weight gain during PPI treatment.

There is a growing body of literature regarding the association between BMI and GERD^[2,16-24]. A recent large meta-analysis of previous studies demonstrated a strong positive relationship between BMI and reflux symptoms^[2]. In addition, moderate weight gains, even among normal-weight persons, resulted in the development or exacerbation of symptoms in GERD patients^[16]. In the present study, the patients significantly increased their body weight during PPI therapy. Appropriate management of body weight during PPI treatment should reduce the duration of PPI use or PPI dosage.

Excessive weight is associated with an increased risk of coronary heart disease, hypertension, angina, stroke, and diabetes, and constitutes an important cardiovascular health burden^[25]. Body weight gain associated with lifetime GERD treatment may induce further medical costs in addition to the PPI therapy. Unfortunately, potentially effective diet modifications are often underestimated in the presence of various PPI regimens. Healthcare providers still recommend lifestyle changes in a moderate percentage of GERD patients^[26], and while PPIs have become of pivotal importance for the initial and maintenance treatment of GERD, repeated lifestyle modification recommendations are required.

In conclusion, we elucidated in the present study the impact of long-term PPI therapy on body weight. Undesired body weight gain was observed in GERD patients on long-term PPI treatment. Reflux patients treated with a daily maintenance therapy of PPI should be strongly encouraged to manage their body weight through lifestyle modifications such as proper diet and avoidance of overeating. This measure may reduce the overall medical costs associated with obesity-related illness as well as GERD. Lifestyle modification must therefore remain the backbone of treatment for all patients with GERD, even in the PPI era.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is the most common esophageal disorder, and frequently encountered in the primary care setting. The risk of reflux symptoms and erosive esophagitis increases with excessive weight and obesity.

Research frontiers

Many studies have confirmed the efficacy and safety of proton pump inhibitor (PPI) therapy in patients with GERD, making these agents the mainstay of treatment.

Innovations and breakthroughs

This study demonstrated for the first time that long-term PPI treatment is associated with undesirable body weight gain in reflux patients, despite lifestyle modification recommendations by their physicians.

Applications

Reflux patients treated with a daily maintenance therapy of PPI should be strongly encouraged to manage their body weight through lifestyle modifications such as proper diet and avoidance of overeating.

Terminology

GERD is a common esophageal disorder which is becoming increasingly prevalent in the population in parallel with similar rises in the frequency of metabolic disorders.

Peer review

This study showed that long-term PPI treatment is associated with body weight gain and that lifestyle modification must therefore remain the backbone of treatment for patients with GERD. This report would impact on the ongoing treatment of GERD.

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S- Editor Tian L L- Editor Logan S E- Editor Zheng XM



Effect of *Lonicerae Flos* extracts on reflux esophagitis with antioxidant activity

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Received: July 20, 2009 Revised: August 18, 2009

Accepted: August 25, 2009

Published online: October 14, 2009

Abstract

AIM: To observe the effects of traditional anti-inflammatory medicine *Lonicerae Flos* (LF) on rat reflux esophagitis (RE) induced by pylorus and forestomach ligation compared with the well-known proton antioxidant, α -tocopherol.

METHODS: Rats were pretreated with three different dosages of LF (500, 250 and 125 mg/kg) orally, once a day for 14 d before pylorus and forestomach ligation. Nine hours after pylorus and forestomach ligation, changes to the stomach and esophagus lesion areas, gastric volumes, acid and pepsin outputs, antioxidant effects, esophageal lipid peroxidation, superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), myeloperoxidase and glutathione (GSH) levels, and collagen contents (marker of flexibility) were observed on the esophageal and fundic histopathology. The results were compared with an α -tocopherol (once orally, 1 h before operation, 30 mg/kg) treated group in which the effects on RE were already confirmed.

RESULTS: Pylorus and forestomach ligations caused marked increases of gross esophageal and gastric mucosa lesion areas, which corresponded with histopathological changes. In addition, increases of esophageal lipid peroxidation, decreases of SOD, CAT, and GSH-free radical scavengers, increases of collagen were observed. However, these pylorus and forestomach ligation induced RE were dose-dependently inhibited by treatment of 500, 250 and 125 mg/kg of LF extract, mediated by antioxidant effects. RE at 250 mg/kg showed similar effects α -tocopherol.

CONCLUSION: The results suggest that antioxidant effects of LF could attenuate the severity of RE and prevent the esophageal mucosal damage, and validate its therapeutic use in esophageal reflux disease.

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Key words: Reflux esophagitis; Tocopherol; *Lonicerae Flos*; Antioxidant; Myeloperoxidase; Pylorus and forestomach ligations

Peer reviewer: Tomohiko Shimatani, Assistant Professor, Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

Ku SK, Seo BI, Park JH, Park GY, Seo YB, Kim JS, Lee HS, Roh SS. Effect of *Lonicerae Flos* extracts on reflux esophagitis with antioxidant activity. *World J Gastroenterol* 2009; 15(38): 4799-4805 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4799.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4799>

INTRODUCTION

Lonicerae Flos (LF), also called Jinyinhua, is a widely used herb prescribed in many Chinese formulas. It has latent-heat-clearing, antipyretic, detoxicant, and antiinflammatory actions^[1].

It has been prescribed to treat fever due to common cold, febrile disease, dysentery, carbuncles, and virulent swellings, in Chinese medicine. Many previous reports have shown that LF is an effective antioxidant^[2,3]. Chlorogenic acid, one of the major components in LF, has been widely adopted to control the quality of LF, owing to its high content and antibiotic property. Chlorogenic acid was

revealed as having an effective activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing antioxidant power assay. In addition, 14 compounds in LF were found to possess a potential antioxidant activity. They were identified as chlorogenic acid, 1-O-caffeoylquinic acid (CQA), caffeic acid, 4-O-CQA, rutin, isoquercitrin, luteolin-7-O-glucoside, lonicerin, 4,5-O-dicaffeoylquinic acid, 3,5-O-diCQA, 1,3-O-diCQA, 3,4-O-diCQA, 1,4-O-diCQA, and luteolin^[4] and the antioxidant capacity of LF extracts in water, methanol and 70% ethanol to scavenge DPPH radical and reduce Fe^{3+} to Fe^{2+} was evaluated^[1].

Esophageal reflux disease has gained clinical and surgical importance over the past decade. It is one of the most common complaints, affecting approximately 10% of the population^[5].

More recent investigations confirmed that the pathogenesis of esophageal reflux seems to be multifactorial and the gastric contents refluxing into the esophagus contain complex components^[6,7].

Acid secreted from gastric parietal cells is a potentially damaging factor in the gastric lumen, and oxidative stress also plays an important role in depletion of the adherent mucus layer and damage the esophageal mucosa from the mechanical forces associated with digestion. Reflux of caustic gastric contents, reactive oxygen species such as superoxide radical and hydroxyl radical, and release of lysosomal enzymes, is known to directly or indirectly cause symptoms such as heartburn and nausea^[8,9].

Therapy that merely suppresses gastric acid secretion does not improve the function of the lower esophageal sphincter and oxidative stress is an added important factor in the pathogenesis of reflux esophagitis (RE) in rats^[8].

LF has oxidant and antiinflammatory activities^[1-4,8-10]; therefore, in the present study, we determined if an extract of LF could be useful for treating RE in rats, by detecting mucosal damages in an RE rat model.

MATERIALS AND METHODS

Animals

Ninety-six female SD rats (6-wk old upon receipt, SLC, Japan) were used after acclimatization for 7 d. The animals were allocated five per polycarbonate cage in a temperature (20-25°C) and humidity (40%-45%) controlled room. The light/dark cycle was 12 h/12 h, and feed and water were supplied ad libitum. The experimental protocols were carried out in accordance with internationally accepted principles for laboratory animal use and care, as stated in the US guidelines.

Preparation of drugs

A sample of LF was purchased from Omniherb Co. (Daegu, South Korea) in 2008. The plant was identified by Professor Seongsoo Roh and a reference specimen (Dipsacales) was deposited in our laboratory. Plant material (200 g) was extracted three times with distilled water. The extract was filtered and evaporated on a rotary evaporator (Buchi, Switzerland), dried in a freeze drier (Eyela FDU-540, Tokyo, Japan). The yield (w/w)

of the extract was about 12%. α -tocopherol was purchased from Sigma (MO, USA).

Induction of RE and treatment

RE was induced in fasted rats after 24 h under 25 mg/kg of Zoletile mixture (Vibrac, France). After anesthesia, the abdomen of the animal was opened by a median incision of about 2 cm, the transitional region between the forestomach and corpus was ligated with a silk thread (2-0), and the contiguous pylorus portion was ligated. A longitudinal cardiomyotomy of about 1 cm length across the gastro-esophageal junction was performed to enhance reflux from the stomach contents into the esophageal body. The incised regions were immediately sutured and the animals were kept in a recovery chamber before being returned to their home cages. 500, 250, and 125 mg/kg of LF were orally administered, once a day for 14 d before the operation, using distilled water as vehicle. α -tocopherol [diluted in corn oil (Sigma, MO, USA)] was administered once orally at doses of 25 mg/kg, 1h before ligation. In sham and vehicle control rats, only distilled water was orally administered, once a day, for 14 d.

Esophageal lesion scores

After nine hours, the animals were sacrificed and the esophagus and stomach were removed. The organs were opened along the greater curvature of the stomach, and the esophagus was longitudinally dissected out. The tissues were washed with physiological saline and examined for ulceration under a dissecting microscope (Nikon, Japan) according to a method described by Nagahama *et al*^[11]. Photographs were taken of specified areas of damage and the width of the damaged esophagitis area (mm^2) was determined and named the lesion score.

Tissue glutathione (GSH) and malondialdehyde (MDA) assays

Stomach samples were homogenized in ice-cold 150 mmol/L KCl for determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation^[12]. Results were expressed as nmol MDA/g tissue. GSH was determined by a spectrophotometric method using Ellman's reagent^[13]. Results were expressed as μmol GSH/g tissue.

Tissue superoxide dismutase (SOD) activity

SOD was determined by the modified version from the method of Minami and Yoshikawa^[14]. Briefly, 15 μL of gastric homogenate were mixed with 450 μL of cold deionized water, 125 μL of chloroform, and 250 μL of ethanol. The mixture was then, centrifuged at 8000 g for 2 min at 4°C. 500 μL of the extracts were added to a reaction mixture containing 500 μL of 72.4 mmol/L triscacodylate buffer with 3.5 mmol/L diethylene pentaacetic acid (pH 8.2; Sigma, MO, USA), 100 μL of 16% Triton X-100, and 250 μL of 0.9 mmol/L nitroblue tetrazolium (Sigma, MO, USA). The reaction mixture was incubated for 5 min at 37°C before adding 10 μL of 9 mmol/L of pyrogallol (Sigma, MO, USA) dissolved in

10 mmol/L HCl. The reaction was incubated for exactly 5 min at 37°C, before being stopped by the addition of 300 µL of 2 mol/L formic buffer (pH 3.5) containing 16% Triton X-100. The absorbance was measured at 540 nm in a spectrophotometer. One unit of SOD enzymatic activity is equal to the amount of enzyme that diminishes the initial absorbance of nitroblue tetrazolium by 50%.

Tissue catalase (CAT) activity

CAT was determined according to the method of Rice Evans and Diplock^[15]. Homogenate of rat gastric mucosa was diluted with buffer, as described before, to obtain an adequate dilution of the enzyme. Then, 2 mL of the enzyme dilution were added to a cuvette and mixed with 1 mL of 30 mmol/L H₂O₂, and the absorbance at 240 nm was measured for 100 s. Initial absorbance of the reaction mixture should be around 0.5. The enzyme activity (U/g of tissue) is expressed as the first order constant that describes the decomposition of H₂O₂ at room temperature.

Tissue myeloperoxidase (MPO) activity

The tissue samples (about 0.2 g) were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50 mmol/L K₂HPO₄, pH 6.0; Sigma, MO, USA) containing hexadecyltrimethyl-ammonium bromide (HETAB; 0.5% w/v; Sigma, MO, USA). The homogenate was centrifuged at 12000 g for 10 min at 4°C, and the supernatant was discarded. The pellet was then re-homogenized with an equivalent volume of 50 mmol/L K₂HPO₄ containing 0.5% (w/v) HETAB and 10 mmol/L EDTA (Sigma, MO, USA). MPO activity was assessed by measuring the H₂O₂-dependent oxidation of o-dianisidine-2 HCl. One unit (U) of enzyme activity was defined as the amount of the MPO present/g tissue weight that caused a change in absorbance of 1.0/min at 460 nm and 37°C^[16].

Tissue collagen measurement

Tissue samples were fixed in 10% formalin in 0.1 mol/L phosphate buffer (pH 7.2) in paraffin and 15 µm thick sections were obtained. Collagen content was measured according to a method described by López-De León *et al*^[17]. The method is based on selective binding of the dyes Sirius Red (Sigma, MO, USA) and Fast Green FCF (Sigma, MO, USA) to collagen and non-collagenous components respectively. Both dyes were eluted readily and simultaneously by using 0.1 mol/L NaOH-methanol (1:1). Finally, the absorbances at 540 and 605 nm were used to determine the amount of collagen and protein respectively.

Histopathological studies

Nine hours after the operations of pylorus and fore-stomach ligation, the junction area from the esophagus to the cardia (about 5 cm) and a part of the fundus tissue were separated and fixed in 10% neutral buffered formalin, after paraffin embedding, 3 µm serial sections were prepared and stained with hematoxylin and eosin. Thickness of mucosa, submucosa in the esophagus, and full thickness of esophagus were measured in

each prepared specimens using a CCD image analyzer (DMI-300, DMI, South Korea) as mm/crossly trimmed tissues. The invasive percentages of lesions in the fundus and percentage of mucosal damage of the esophagus were enumerated as follows: Invasive percentages of lesions (%) = (Length of lesions on the crossly trimmed esophageal or fundic walls/total thickness of crossly trimmed esophageal walls) × 100; Mucosal damage protecting percentages (%) = (Length of lesions on the crossly trimmed esophageal mucosa/total length of crossly trimmed esophageal mucosa) × 100.

Statistical analysis

Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test, followed by least-significant differences (LSD) multi-comparison test to determine which pairs of group comparison were significantly different. In cases where significant deviations from variance homogeneity were observed using the Levene test, a non-parametric comparison test, the Kruskal-Wallis *H* test, was conducted. When a significant difference was observed in the Kruskal-Wallis *H* test, the Mann-Whitney U-Wilcoxon Rank Sum *W* test was conducted to determine the specific pairs of group comparison that are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 12.0K SPSS Inc., IL, USA).

RESULTS

Esophageal lesion scores

A significant ($P < 0.01$) increase of esophageal lesion scores was detected in RE control as compared with sham control. However, these increases of esophageal lesion scores were markedly decreased by treatment with all three dosages of LF and α-tocopherol, as compared with the RE control (Figure 1).

Histopathological changes

We examined hole sizes of mucosa on histological images, measured damaged sizes of the mucosa, and calculated esophagus damage protecting percentages. Lesions on the mucosa in the sham are not shown. Lesions in the RE control, α-tocopherol, and LF groups were significantly ($P < 0.01$) increased compared to the sham. However, lesions in the α-tocopherol and LF groups were significantly ($P < 0.05$ and $P < 0.01$) decreased compared to the RE control. Infiltrations of inflammatory cells in esophagus tissue of the α-tocopherol and LF groups were significantly ($P < 0.05$ and $P < 0.01$) decreased compared to the RE control. In addition, the hemorrhage depth in the stomach of the α-tocopherol and LF groups was decreased compared to the RE control. Thicknesses of esophagus tissue in the RE control, α-tocopherol and LF groups were increased compared to the sham, but the thicknesses of esophagus tissue of α-tocopherol and LF groups were significantly

Table 1 Changes on the esophageal and gastric histomorphometry in RE rats

Group	Damage protecting percentages (%)	Inflammatory cells infiltration in esophagus (%)	Thickness of mucosa in esophagus (μm)	Hemorrhage depth in stomach (mm)
Controls				
Sham	1.23 \pm 0.13	0.86 \pm 0.7	261.89 \pm 23.67	0.003 \pm 0.005
RE	12.72 \pm 3.81 ^b	94.80 \pm 4.07 ^b	20.81 \pm 46.53 ^b	0.963 \pm 0.343
α -tocopherol	29.15 \pm 10.23 ^{b,c}	72.53 \pm 1.17 ^{b,d}	57.12 \pm 43.83 ^b	0.19 \pm 0.11 ^{b,d}
LF extracts (mg/kg)				
125	24.86 \pm 4.8 ^{b,d}	78.92 \pm 10.42 ^{b,c}	138.56 \pm 48.8 ^{a,d}	0.13 \pm 0.08 ^{b,d}
250	26.56 \pm 8.8 ^{b,c}	68.31 \pm 10.79 ^{b,d}	99.75 \pm 85.6 ^b	0.18 \pm 0.08 ^{b,d}
500	46.41 \pm 17.77 ^{b,c}	56.11 \pm 4.05 ^{b,d}	118.64 \pm 80.38 ^{a,c}	0.25 \pm 0.18 ^{b,d}

Values are expressed mean \pm SD of five rats; ^a P < 0.05 and ^b P < 0.01 compared to sham control; ^c P < 0.05 and ^d P < 0.01 compared to RE control; RE: Reflux esophagitis; LF: Lonicerae Flos.

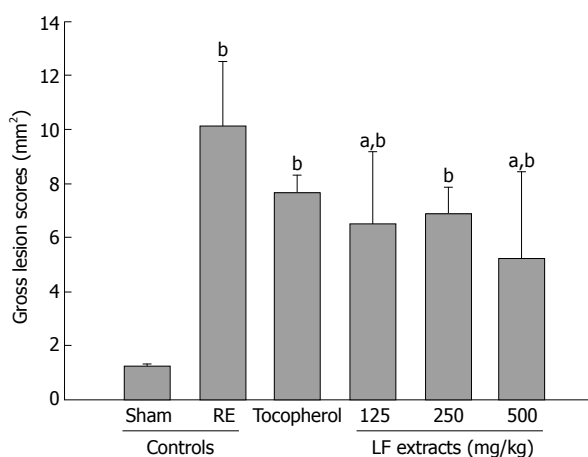
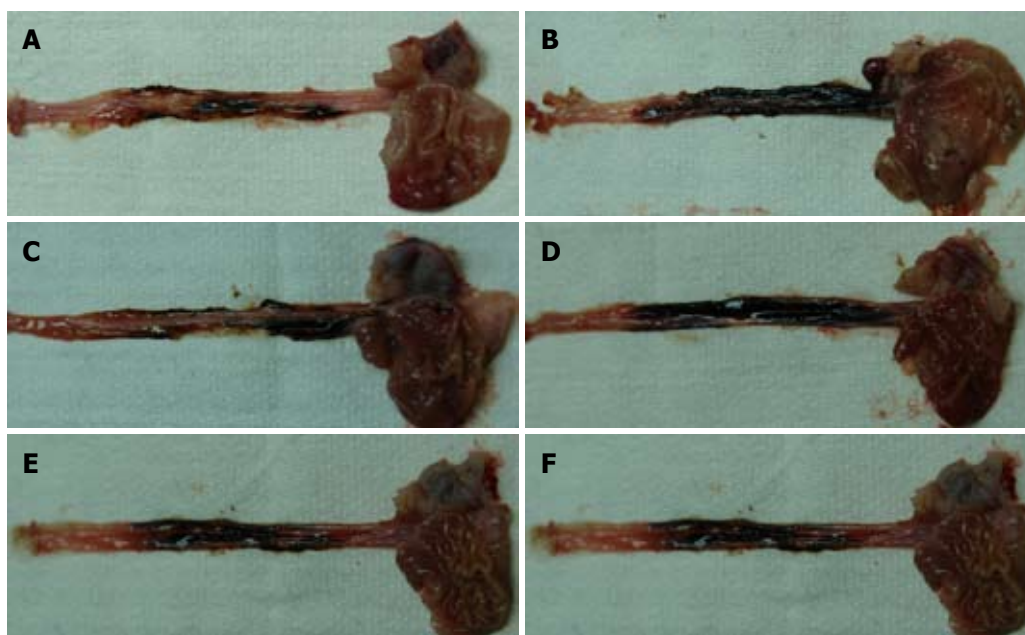


Figure 1 Changes on the gross lesion scores of Sham (A), RE (B) controls, α -tocopherol (C), LF 125 (D), 250 (E) and 500 mg/kg (F) treated rats. Sham: Normal rats had a laparotomy but not pylorus and forestomach ligation operation; RE: Rats had a pylorus and forestomach ligation operation not treated with drug; Tocopherol: Rats had a pylorus and forestomach ligation operation treated with α -tocopherol (30 mg/kg); and LF extract: Rats had a pylorus and forestomach ligation operation treated with extract of LF (respectively 125, 250 and 500 mg/kg). Values are expressed mean \pm SD of five rats; ^a P < 0.05 compared to RE control; ^b P < 0.01 compared to Sham control.

(P < 0.05 and P < 0.01) decreased compared to the RE control. Mucosal thicknesses in the RE control, α -tocopherol and LF groups were significantly (P < 0.05 and P < 0.01) decreased compared to the sham, but mucosal thickness of the α -tocopherol and LF groups

were significantly (P < 0.05 and P < 0.01) increased compared to the RE control (Table 1 and Figure 2).

Antioxidant effects

Malondialdehyde (MDA) content increased significantly in

Table 2 Changes on the stomach antioxidant systems in RE rats

Group	MDA (nmol/g tissue)	GSH (μ mol/g tissue)	SOD (U/g protein)	CAT (U/g tissue)
Controls				
Sham	10.11 \pm 1.21	1.61 \pm 0.29	64.83 \pm 6.80	4.95 \pm 0.69
RE	16.39 \pm 0.75 ^b	0.91 \pm 0.22 ^b	42.67 \pm 6.70 ^b	3.19 \pm 0.56 ^b
α -tocopherol	11.92 \pm 0.94 ^{a,c}	1.18 \pm 0.13 ^{b,c}	54.08 \pm 6.79 ^{b,d}	4.08 \pm 0.50 ^{a,c}
LF extracts (mg/kg)				
125	15.05 \pm 1.14 ^b	1.03 \pm 0.15 ^b	49.04 \pm 5.36 ^b	3.78 \pm 0.81 ^b
250	12.11 \pm 1.61 ^{b,d}	1.19 \pm 0.17 ^{b,c}	53.58 \pm 4.80 ^{b,d}	4.04 \pm 0.28 ^{a,c}
500	10.54 \pm 1.54 ^d	1.31 \pm 0.20 ^{b,d}	60.26 \pm 5.22 ^d	4.41 \pm 0.46 ^d

Values are expressed mean \pm SD of five rats; ^a P < 0.05 and ^b P < 0.01 compared to sham control; ^c P < 0.05 and ^d P < 0.01 compared to RE control; MDA: Malondialdehyde; GSH: Glutathione; SOD: Superoxide dismutase; CAT: Catalase.

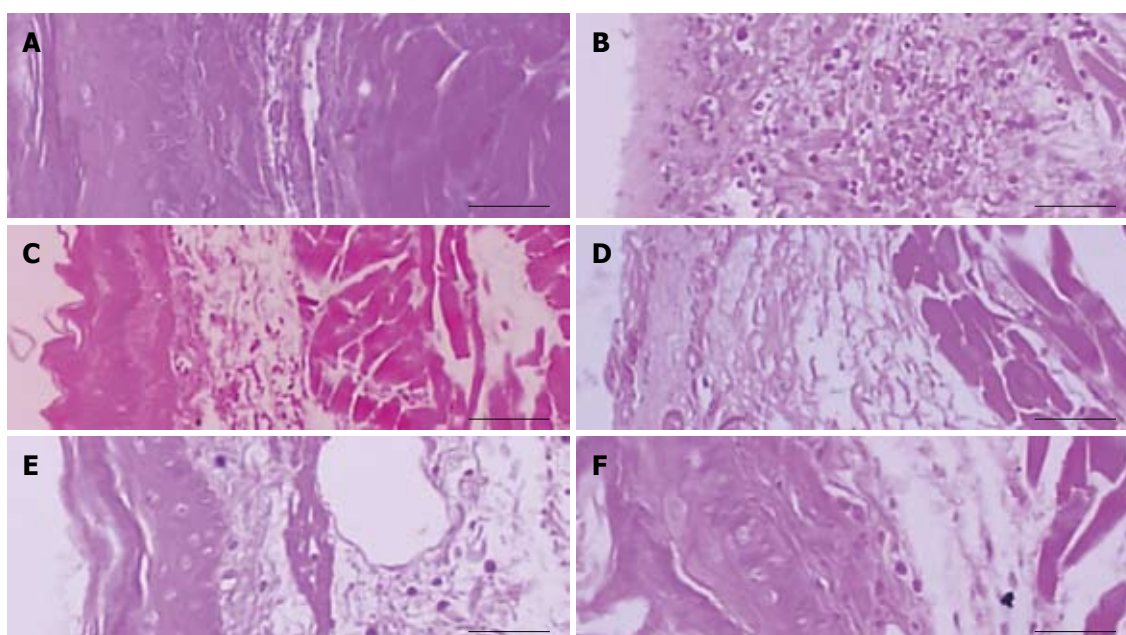


Figure 2 Changes of the histopathological profiles of esophagus of Sham (A), RE (B) controls, α -tocopherol (C), LF 125 (D), 250 (E) and 500 mg/kg (F) treated rats. Sham: Normal rats had a laparotomy not pylorus and forestomach ligation operation; RE: Rats had a pylorus and forestomach ligation operation not treated with drug; Tocopherol: Rats had a pylorus and forestomach ligation operation treated with α -tocopherol (30 mg/kg); and LF extract: Rats had a pylorus and forestomach ligation operation treated with extract of LF (respectively 125, 250 and 500 mg/kg). All HE stains; Scale bars: 80 μ m.

the gastric mucosa after the induction of RE. α -tocopherol and all three dosages of LF, significantly (P < 0.01) inhibited MDA production as compared with the RE control (Table 2). GSH levels increased with esophageal mucosal damage. Significant differences were found between rats with gastroesophageal reflux as compared to normal esophagus. Gastric GSH contents of all experiment group were significantly (P < 0.01) lower than in normal esophageal mucosa (Table 2). However, those of the α -tocopherol and LF groups were significantly (P < 0.05, P < 0.01) higher than in RE control esophageal mucosa (Table 2). SOD content showed a significant decrease in biopsies taken from RE control group with erosive esophagitis, when compared to that of sham rats. However, of the SOD levels in the α -tocopherol and LF groups were significantly (P < 0.05, P < 0.01) higher than in the RE control group (Table 2). Gastric catalase content decreased in the RE control group compared to sham rats. However, of the catalase activities of the α -tocopherol and LF groups were significantly (P < 0.05, P < 0.01) higher than in the RE control group (Table 2).

Myeloperoxidase activity

Accumulation of polymorphonucleocytes (PMNs) in the surgically induced reflux esophageal tissue is considered one of the primary contributory mechanisms to esophageal injury. The myeloperoxidase (MPO) activity in the esophageal tissue was measured as a marker for PMN accumulation (Figure 3A). The MPO activity was low in sham rats. However, the surgically induced esophagitis rats showed a large increase in esophageal MPO activity (P < 0.01). Treatment of esophagitis rats with α -tocopherol and LF significantly attenuated esophageal MPO activity in comparison with untreated rats (P < 0.05, P < 0.01), indicating that α -tocopherol and LF retarded the accumulation of PMNs in the RE model.

Collagen contents

The collagen contents rose significantly (P < 0.01) in the RE-induced experiment groups; however, α -tocopherol and LF significantly (P < 0.05, P < 0.01) attenuated the elevated levels of collagen in esophageal tissue compared to the RE control (Figure 3B).

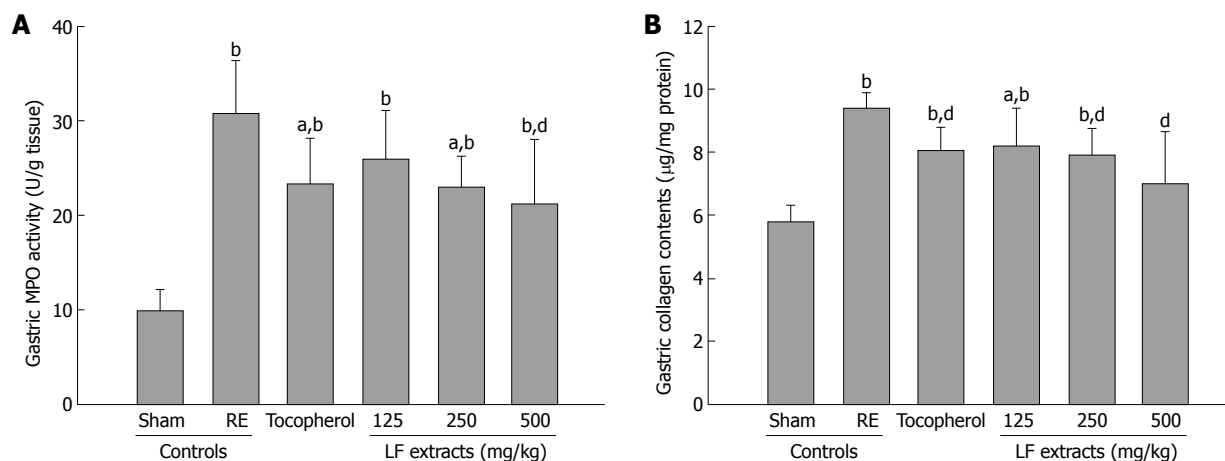


Figure 3 Changes on the gastric MPO activity (A) and gastric collagen contents (B) in RE rats. Values are expressed mean \pm SD of five rats. ^b $P < 0.01$ compared to Sham control; ^a $P < 0.05$ and ^d $P < 0.01$ compared to RE control.

DISCUSSION

Many researches have reported that LF had an antioxidant effect^[1,2,4,18], and the protection provided by the dose of α -tocopherol was chosen according to an earlier report^[19] in which 16 mg/kg prevented oxidative stress in gastric ulcers. α -tocopherol is the most potent antioxidant that can break the propagation of the free radical chain reaction in the lipid part of the biological membrane. Chandana and Madhavan^[20] reported that α -tocopherol has efficacy to esophagitis. Thus, we attempted to determine any anti-esophagitis effects of LF in a rat model of RE induced by pylorus and forestomach ligation. The RE control group showed induced esophageal inflammation, edema, and ulcer. Histology revealed increasing of thickness, damage to the mucosa, and hemorrhages in esophagus tissues. However, the experimental groups treated with α -tocopherol (30 mg/kg) and LF (at doses of 500, 250 and 125 mg/kg) showed decreased lesions of esophagitis and reduced edema, damage to the mucosa and hemorrhages in the stomach (Table 1 and Figure 1).

It has been suggested that lipid peroxidation, a sensitive marker of membrane damage caused by free radicals, is involved in reflux oesophagitis^[21]. Antioxidants constitute the foremost defense system for limiting the toxicity associated with free radicals. Under physiological conditions, oxygen radicals are part of normal regulatory circuits, and the cellular redox state is very sensitive to antioxidants^[22]. In recent years, there has been an upsurge in research on medicinal herbs and plant derived compounds, especially in combating oxidative stress and free radical damage^[23]. GSH, SOD, and catalase are important radical superoxide scavengers that provide major protection by participating in the cellular defense systems against oxidative damage^[8,9,24]. The gastric mucosa contains high levels of glutathione, which is important for maintenance of mucosal integrity, because depletion of GSH from the gastric mucosa by electrophilic compounds induces macroscopic mucosal ulceration^[25]. In the present study, marked increases in lipid peroxidation were detected in the gastric mucosa of the RE control with decreases

of endogenous antioxidants, GSH, SOD, and catalase, respectively (Table 2).

Many studies have focused on the role of neutrophil infiltration in the development of gastric mucosal injury^[26-28]. Besides their direct damaging effects on tissues, it is well established that oxygen metabolites play a role in the recruitment of neutrophils, preferentially PMNs, into injured tissues^[29]. Activated PMNs are also a potential source of oxygen metabolites^[30] and MPO an activating cytotoxic enzyme released from PMNs^[31]. In the present study, marked increases of gastric MPO activities were detected in the RE control compared with the intact control. We examined infiltration percentages of inflammatory cells in the esophagus and stomach. Inflammation cells infiltrations in esophageal tissue of α -tocopherol and LF-treated groups were decreased compared to the RE group ($P < 0.05$ and $P < 0.01$) (Table 1 and Figure 1). Figure 3A shows the changes to the levels of in gastric MPO.

The severity of RE impairs the strength and flexibility of collagen fibers. Collagen constitutes the major structural protein in the extracellular matrix, providing mechanical strength and structural integrity to the various connective tissues of the body^[32]. In the present study, gastric collagen contents were determined as a marker of oxidant-induced fibrosis^[31], and marked increases of gastric collagen contents were observed in the RE control as compared with the sham control (Figure 3B). Collagen contents rose significantly in the esophagitis-induced experiment groups; however, α -tocopherol and LF significantly attenuated the elevated levels of collagen in esophageal tissue as that of RE control (Figure 3B).

In conclusion, LF has an effective antioxidant activity and the clinical administration of LF could attenuate the severity of RE and prevent direct esophageal mucosal damage. The present study validates the potential therapeutic use of LF for gastroesophageal reflux disease.

COMMENTS

Background

Reflux esophagitis (RE) is occurs widely in the world and is generally treated

with antacids, prokinetics, histamine type 2 receptor antagonists, and proton pump inhibitors (PPI). Especially, antioxidants having antiinflammatory activity are useful for treating RE. *Lonicerae* Flos (LF) might be effective for RE-induced by pylorus and forestomach ligation through its antioxidant activity.

Research frontiers

The research field of this research is RE, which is one of the most common complaints, affecting approximately 10% of the population. An important focus of the authors' research is that effective antioxidants in Chinese medicine have potential for use in RE.

Innovations and breakthroughs

Omeprazol is a representative PPI drug that also has antioxidant activity. As alternatives, natural medicines having antioxidant effects and no side effects could be potential remedies. This article might lead a researcher of natural products to study drug treatments for RE.

Applications

By examining which components of LF are more effective against RE, a natural new drug could be manufactured. The ability to easily obtain LF from the environment will encourage patients with RE to take folk remedies as medicines to cure RE.

Terminology

In RE, the acid secreted from gastric parietal cells is a potentially damaging factor in the gastric lumen and oxidative stress also plays an important role in depletion of the adherent mucus layer. Damage to the esophageal mucosa from the mechanical forces associated with digestion might also occur. Reflux of caustic gastric contents, reactive oxygen species such as superoxide radicals and hydroxyl radical, and release of lysosomal enzymes can also cause damage.

Peer review

In this article, the authors determined that the antioxidant effects of LF could attenuate the severity of RE and prevent esophageal mucosal damage. α -tocopherol is a major antioxidant, a previous report explained that it was useful in RE of pylorus and forestomach ligation. LF is an antioxidant and anti-inflammatory agent, and has effective activity on RE in the pylorus and forestomach ligation rat model.

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BRIEF ARTICLES

Metastasis to the gallbladder: A single-center experience of 20 cases in South Korea

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Received: June 26, 2009 Revised: August 6, 2009

Accepted: August 13, 2009

Published online: October 14, 2009

common origin was the stomach. The median survival of MGB was 8.7 mo.

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Key words: Gallbladder; Neoplasms; Gastrointestinal neoplasms; Neoplasm metastasis; Biliary tract neoplasms

Peer reviewer: Curt Einarsson, Professor, Department of Medicine, Karolinska Institute, Karolinska University Hospital Huddinge, Dept of Gastroenterology and Hepatology, K 63, Huddinge SE-141 86, Sweden

Yoon WJ, Yoon YB, Kim YJ, Ryu JK, Kim YT. Metastasis to the gallbladder: A single-center experience of 20 cases in South Korea. *World J Gastroenterol* 2009; 15(38): 4806-4809 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4806.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4806>

Abstract

AIM: To evaluate the clinicopathologic characteristics of patients with metastases to the gallbladder (MGBs).

METHODS: We performed a single-center retrospective study of 20 patients with MGBs diagnosed pathologically from 1999 to 2007.

RESULTS: Among 417 gallbladder (GB) malignancies, 20 (4.8%) were MGBs. The primary malignancies originated from the stomach ($n = 8$), colorectum ($n = 3$), liver ($n = 2$), kidney ($n = 2$), skin ($n = 2$), extrahepatic bile duct ($n = 1$), uterine cervix ($n = 1$), and appendix ($n = 1$). Twelve patients were diagnosed metachronously, presenting with cholecystitis ($n = 4$), abdominal pain ($n = 2$), jaundice ($n = 1$), weight loss ($n = 1$), and serum CA 19-9 elevation ($n = 1$); five patients were asymptomatic. The median survival after the diagnosis of MGB was 8.7 mo. On Cox regression analysis, R0 resection was the only factor associated with a prolonged survival [hazard ratio (HR): 0.01, $P = 0.002$]; presentation with cholecystitis was associated with poor survival (HR: 463.27, $P = 0.006$).

CONCLUSION: MGBs accounted for 4.8% of all pathologically diagnosed GB malignancies. The most

INTRODUCTION

Metastases to the gallbladder (MGBs) are rare in clinical practice^[1]. Although malignant melanoma^[2] and renal cell carcinoma^[3] are reported to metastasize to the gallbladder (GB), these data are based on autopsy series of these tumors. Reports on MGBs arising from malignancies other than malignant melanoma and renal cell carcinoma are usually in the form of single case reports^[4,5]. There are few, if any, reports that describe MGBs from the perspective of the GB to this date.

This paper describes the clinicopathologic features of 20 patients with MGBs diagnosed over a period of 9 years at a single tertiary hospital.

MATERIALS AND METHODS

We reviewed the pathology reports of all GB malignancies diagnosed with pathological confirmation from January 1999 to December 2007 at Seoul National University Hospital. We evaluated the clinicopathologic characteristics of the patients with MGBs. Patients were excluded when direct invasion of the GB from the primary malignancy was confirmed on imaging or intraoperatively.

By reviewing the medical records, sex, the primary origin of the MGB, age at diagnosis of the primary malignancy, age at diagnosis of the MGB, presenting

Table 1 Origin and pathology of the primary malignancies

Site of origin	Pathology
Stomach (<i>n</i> = 8)	Adenocarcinoma (<i>n</i> = 7) Signet ring cell carcinoma (<i>n</i> = 1)
Colorectum (<i>n</i> = 3)	Adenocarcinoma (<i>n</i> = 3)
Liver (<i>n</i> = 2)	Hepatocellular carcinoma (<i>n</i> = 2)
Kidney (<i>n</i> = 2)	Renal cell carcinoma (<i>n</i> = 2)
Skin (<i>n</i> = 2)	Melanoma (<i>n</i> = 2)
Extrahepatic bile duct (<i>n</i> = 1)	Adenocarcinoma (<i>n</i> = 1)
Uterine cervix (<i>n</i> = 1)	Squamous cell carcinoma (<i>n</i> = 1)
Appendix (<i>n</i> = 1)	Mucinous adenocarcinoma (<i>n</i> = 1)

symptoms and signs at diagnosis of the MGB, time interval between the diagnoses of the primary malignancy and the MGB, involvement of organs other than the GB, diagnosis of secondary involvement of the GB before surgery, treatment after the diagnosis of the MGB, and survival after the diagnosis of the MGB were evaluated. Overall follow-up survival information was obtained by contacting the Resident Service Division of the Ministry of Public Administration and Security, Seoul, Korea, and by reviewing medical records. The endpoints of this study were patient death or June 30, 2008.

The median survival was estimated using the Kaplan-Meier method. Factors associated with prolonged survival were determined using the log-rank test. The factors including age at diagnosis of the MGB, sex, and factors associated with survival in the univariate analysis at $P < 0.20$ were included as covariates in the Cox regression analysis. Values are reported as the median. Two-sided P values of < 0.05 were considered statistically significant. All analyses were performed using SPSS for Windows Ver. 11.0 (SPSS Inc., Chicago, Ill., USA). This study was approved by the institutional review board at our hospital.

RESULTS

Overview of the patients

A total of 417 cases of GB malignancies were diagnosed with pathological confirmation. Among these, 20 cases (14 male and six female) were MGBs, accounting for 4.8% of the GB malignancies.

The median age at diagnosis of MGB was 65 years (range, 28-76 years). The median age at diagnosis of the primary malignancy was 62.5 years (range, 27-76 years). The primary malignancies originated from the stomach ($n = 8$), colorectum ($n = 3$), liver ($n = 2$), kidney ($n = 2$), skin ($n = 2$), extrahepatic bile duct ($n = 1$), uterine cervix ($n = 1$), and appendix ($n = 1$) (Table 1). Cancer involvement of organs other than the GB was present in 11 patients.

Eight MGBs were diagnosed synchronously. The primary origins were the stomach ($n = 3$), colorectum ($n = 3$), extrahepatic bile duct ($n = 1$), and liver ($n = 1$). Twelve MGBs were diagnosed metachronously with a median interval of 24.9 mo (range, 5-191 mo). The primary origin in these patients were the stomach ($n = 5$), kidney ($n = 2$), skin ($n = 2$), liver ($n = 1$), uterine

Table 2 Concurrent procedures at the time of cholecystectomy

Synchronously diagnosed	Metachronously diagnosed
Anterior resection and liver tumorectomy	Distal pancreatectomy (for pancreatic metastasis)
Low anterior resection and liver tumorectomy	Right hemicolectomy
Total colectomy and liver tumorectomy	Debulking surgery
Palliative common bile duct resection	
Palliative gastrojejunostomy	
Palliative total gastrectomy	
Palliative left lobectomy of the liver	
Palliative subtotal gastrectomy and palliative right hemicolectomy	

cervix ($n = 1$), and appendix ($n = 1$). Metachronously diagnosed patients presented with cholecystitis ($n = 4$), abdominal pain ($n = 2$), jaundice ($n = 1$), weight loss ($n = 1$), and elevation of serum CA 19-9 ($n = 1$); five patients were asymptomatic and the MGBs were detected during follow-up of the primary malignancy. In the four patients that presented with cholecystitis, only one patient had gallstones. Recurrence of the primary malignancy in the remaining primary origin was detected in one patient.

Treatment and prognosis

Treatment consisted of surgery ($n = 9$), surgery plus chemotherapy ($n = 8$), surgery plus chemoradiation ($n = 2$), and surgery plus transarterial chemoembolization ($n = 1$). All synchronously diagnosed patients underwent concurrent surgical procedures; three of 12 metachronously diagnosed patients underwent concurrent surgical procedures (Table 2). The preoperative radiological diagnosis of MGB was made in 9 (45%) patients. Complete surgical resection of all tumors present, i.e. R0 resection, was achieved in nine patients. There was a tendency to achieve R0 resection in patients with preoperative diagnosis of MGBs (66.7% in patients with preoperative diagnosis *vs* 27.3% in patients without preoperative diagnosis, $P = 0.078$).

The overall median survival after the diagnosis of MGB was 8.7 mo (Figure 1). On univariate analysis, R0 resection ($P < 0.001$), asymptomatic on diagnosis of MGB ($P = 0.023$), and preoperative diagnosis of MGB ($P = 0.007$) were associated with a prolonged survival; presentation with acute cholecystitis ($P < 0.001$) and age at diagnosis of MGB ≥ 70 years ($P = 0.009$) were associated with a poor survival (Table 3). On multivariate analysis, the only factor associated with prolonged survival was R0 resection [hazard ratio (HR): 0.01, 95% confidence interval (CI): 0.001-0.20, $P = 0.002$]; presentation with acute cholecystitis was associated with poor survival (HR: 36.12, 95% CI: 2.82-463.27, $P = 0.006$) (Table 4).

DISCUSSION

The present retrospective analysis of 9 years' experience at a single tertiary hospital contributes to our understanding

Table 3 Univariate analysis of the factors associated with survival after a diagnosis of MGB

Factor	Median survival ¹	P value
Sex		0.794
Male (n = 14)	11.2	
Female (n = 6)	6.2	
Primary origin		0.224
Gastrointestinal organs (n = 15)	8.2	
Non-gastrointestinal organs (n = 5)	35.0	
Chronological relationship between primary malignancy and MGB		0.913
Synchronous (n = 8)	11.2	
Metachronous (n = 12)	6.9	
Asymptomatic upon diagnosis of MGB		0.023
Yes (n = 5)	40.0	
No (n = 15)	6.9	
Presentation with acute cholecystitis		< 0.001
Yes (n = 4)	2.8	
No (n = 16)	15.6	
Preoperative diagnosis of MGB		0.007
Yes (n = 9)	35.0	
No (n = 11)	4.7	
Age at diagnosis of the MGB (yr)		0.009
≥ 70 (n = 5)	6.2	
< 70 (n = 15)	21.2	
Secondary involvement of organs other than the GB		0.586
Yes (n = 11)	15.6	
No (n = 9)	8.7	
Recurrence at the remaining primary site		0.252
Yes (n = 1)	4.7	
No (n = 19)	11.2	
R0 resection		< 0.001
Yes (n = 9)	40.0	
No (n = 11)	6.2	

¹Value in months. Results of log-rank test. MGB: Metastasis to the gallbladder; GB: Gallbladder.

of the clinicopathologic features of MGBs. MGBs accounted for 4.8% of all GB malignancies. The most common primary origin was the stomach. Twelve cases (60%) of MGBs were diagnosed metachronously with a median interval of 24.9 mo. The median survival after the diagnosis of MGB was 8.7 mo. On multivariate analysis, R0 resection was the only factor associated with prolonged survival; presentation with acute cholecystitis was associated with poor survival.

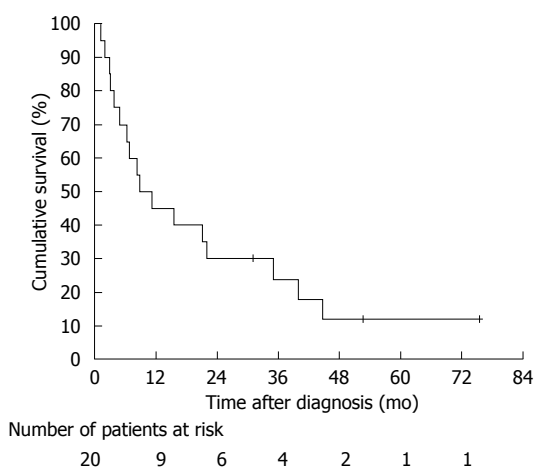
Malignant melanoma and renal cell carcinoma are reported to metastasize to the GB. In an autopsy series of 125 patients with malignant melanoma, nineteen (15%) had MGBs^[2]. In an analysis of 687 necropsies of patients with renal cell carcinoma, four cases had MGBs^[3]. However, the data available on MGBs are only from autopsy series of certain malignancies such as melanoma or renal cell carcinoma, or reviews of a certain metastatic carcinoma to the GB such as melanoma^[6,7], or in the form of single case reports of tumors such as melanoma^[8-11], breast cancer^[4], and hepatocellular carcinoma^[5]. Furthermore, there is limited data on the proportion of MGBs among GB malignancies.

Malignant melanoma is reported to be the most common origin of MGBs^[1]. In this study, malignant melanoma accounted for 10% of primary malignancies.

Table 4 Multivariate analysis of the factors associated with survival after a diagnosis of MGB

Factor	HR	95% CI	P value
Male sex	5.78	0.84-39.87	0.075
Asymptomatic upon diagnosis of the MGB	0.79	0.15-4.18	0.781
Presentation with acute cholecystitis	36.12	2.82-463.27	0.006
Preoperative diagnosis of MGB	0.58	0.13-2.62	0.477
Age at diagnosis of the MGB ≥ 70 yr	3.69	0.42-32.38	0.239
R0 resection	0.01	0.001-0.20	0.002

HR: Hazard ratio; CI: Confidence interval.

**Figure 1** Cumulative survival after the diagnosis of metastasis to the gallbladder.

Interestingly, the stomach was the most common primary origin of MGB in our study. This may result from bias arising from the small number of patients. However, the fact that stomach cancer is the most common cancer in Korea may have resulted in this finding^[12].

In the analysis of the factors associated with survival, R0 resection was the only factor associated with prolonged survival, which can be taken as a matter of course. The presentation of MGB with acute cholecystitis was associated with poor survival. A possible explanation for this finding is that a major infectious episode, such as acute cholecystitis, may have had a major negative impact on the patient's survival.

Our study has certain limitations. This study is based on information collected from patients with pathologically diagnosed GB malignancies only. The number of patients is small. In addition, the primary malignancies and treatments are varied. Nevertheless, this study provides some insight into the nature of MGBs.

In conclusion, MGBs accounted for 4.8% of all pathologically diagnosed GB malignancies over a period of 9 years. The stomach was the most common site of the primary malignancy. The overall median survival after diagnosis of the MGB was 8.7 mo. R0 resection was the only factor associated with a prolonged survival.

COMMENTS

Background

Metastases to the gallbladder (MGBs) are rare. There is limited data on the characteristics of patients with MGBs.

Innovations and breakthroughs

Although malignant melanoma and renal cell carcinoma are reported to metastasize to the GB, these data are based on autopsy series of these tumors. Despite the small number of cases, this report of 20 MGB cases is one of the largest among reports on MGBs.

Applications

The proportion of MGBs among pathologically confirmed GB malignancies is 4.8%. R0 resection is the only factor associated with a prolonged survival in MGB patients.

Peer review

This is a well-written paper presenting material on patients with metastasis of the gallbladder. Although the series of patients is rather small, which is also discussed in the paper, it gives some information which is new and of scientific and clinical interest as well.

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S- Editor Li LF L- Editor Webster JR E- Editor Zheng XM



BRIEF ARTICLES

Three-dimensional endoanal ultrasonographic assessment of an anal fistula with and without H₂O₂ enhancement

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Received: June 22, 2009 Revised: July 22, 2009

Accepted: July 29, 2009

Published online: October 14, 2009

helpful at times and selective use in difficult cases may be economical and reliable.

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Key words: Anal fistula; Endoanal ultrasound; H₂O₂ enhancement

Peer reviewer: Subbaramiah Sridhar, MB, BS, MPH, FRCP (Edin), FRCP (Glasg), FRCP (Lond), FRSS (Lond), FRCPC (Medicine & Gastroenterology), FACP, FACG, FASGE, AGAF, Section of Gastroenterology, BBR 2544, Medical College of Georgia, 15th Street, Augusta, GA 30912, United States

Abstract

AIM: To evaluate the effectiveness of three-dimensional endoanal ultrasound (3D-EAUS) in the assessment of anal fistulae with and without H₂O₂ enhancement.

METHODS: Sixty-one patients (37 males, aged 17-74 years) with anal fistulae, which were not simple low types, were evaluated by physical examination and 3D-EAUS with and without enhancement. Fistula classification was determined with each modality and compared to operative findings as the reference standard.

RESULTS: The accuracy of 3D-EAUS was significantly higher than that of physical examination in detecting the primary tract (84.4% vs 68.7%, $P = 0.037$) and secondary extension (81.8% vs 62.1%, $P = 0.01$) and localizing the internal opening (84.2% vs 59.7%, $P = 0.004$). A contrast study with H₂O₂ detected several more fistula components including two primary suprasphincteric fistula tracks and one supralelevator secondary extension, which were not detected on non-contrast study. However, there was no significant difference in accuracy between 3D-EAUS and H₂O₂-enhanced 3D-EAUS with respect to classification of the primary tract (84.4% vs 89.1%, $P = 0.435$) or secondary extension (81.8% vs 86.4%, $P = 0.435$) or localization of the internal opening (84.2% vs 89.5%, $P = 0.406$).

CONCLUSION: 3D-EAUS was highly reliable in the diagnosis of an anal fistula. H₂O₂ enhancement was

Kim Y, Park YJ. Three-dimensional endoanal ultrasonographic assessment of an anal fistula with and without H₂O₂ enhancement. *World J Gastroenterol* 2009; 15(38): 4810-4815 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4810.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4810>

INTRODUCTION

Despite the fact that anal fistulae are very common and have been studied extensively, some complex forms still continue to pose a difficult surgical problem. The aim of treatment for an anal fistula is to permanently eliminate abscess formation and achieve healing while preserving anal function and continence. Overly aggressive fistulotomy can lead to postoperative fecal incontinence, whereas inappropriate conservative treatment could lead to fistula recurrence. Therefore, accurate preoperative assessment of a fistula is necessary for optimal surgical results.

Inspection and digital examination with or without anesthesia are basic diagnostic methods. However, digital examination may fail to detect complex fistulae or to localize the internal opening. It is now well established that preoperative imaging modalities can alert the surgeon to fistula components that might otherwise be missed^[1,2].

Endoanal ultrasonography (EAUS) has been increasingly used in the preoperative evaluation of anal fistulae. Initial EAUS evaluation was not satisfactory^[3], but the diagnostic accuracy of EAUS has improved with technical advancements in ultrasonography, including the use of H₂O₂ as a contrast agent and 3D image reconstruction^[4,5]. The image is no longer limited to the axial plane in 3D-EAUS. Instead, it is possible to cut

across any part of the data set in the coronal, sagittal, or oblique plane. This property is expected to be helpful in tracing the tract and internal opening.

The H₂O₂-enhanced 3D-EAUS is the latest development in EAUS and is expected to diagnose anal fistulae with high accuracy. However, there is limited data on the accuracy and clinical usefulness of 3D-EAUS and H₂O₂-enhanced 3D-EAUS. The purpose of this study was to evaluate the accuracy of 3D-EAUS with or without H₂O₂ enhancement in identifying the primary tract, secondary extension, and internal opening in an anal fistula, compared to surgical findings as the standard reference.

MATERIALS AND METHODS

Patients and clinical evaluation

Between January 2007 and February 2009, 61 patients (37 men and 24 women; mean age 39 years, range 17-74 years) with anal fistulae were preoperatively evaluated with physical examination, 3D-EAUS with and without enhancement, and subsequently underwent surgery. EAUS was performed when the surgeon thought preoperative imaging was clinically necessary. EAUS was not performed in patients with obvious simple fistulae with low, straight configuration and external openings close to the anus. Patients without visible external openings were also excluded.

Three patients had known Crohn's disease, and two patients had a history of anal trauma leading to anal fistula formation. All other patients were believed to have fistulae of cryptoglandular origin. Twelve patients, including the three patients with Crohn's disease, had previously undergone 15 fistula operations.

Physical examination was performed during the first visit to the outpatient clinic. Patients were examined in the left lateral position without any anesthesia. By inspection and palpation of the perianal area and by digital rectoanal examination, the fistula anatomy was determined by a single surgeon. Insertion of a probe was attempted on each occasion, provided it did not cause significant discomfort. The relationship between the components of the anal fistula and the sphincter complex was categorized (whether diagnosed with full confidence or by suspicion only) and recorded.

Physical examination aimed to determine the following fistula characteristics: (1) the primary tract, categorized according to the criteria of Parks *et al.*^[6] as intersphincteric, transsphincteric, extrasphincteric, or suprasphincteric; (2) secondary extension, including horseshoe tract and abscess formation; and (3) the internal opening, localized with respect to a clock face and categorized as anterior, left lateral, posterior, or right lateral. The anatomic location of any secondary extension arising from the primary fistula track was recorded as intersphincteric, ischiorectal, or supralevator. A horseshoe extension was defined as any extension from the primary track that appeared to extend to both sides of the internal opening, and such an extension was classified as intersphincteric or ischiorectal.

Patients underwent surgical treatment after these ex-

aminations, and each component of the anal fistula was categorized and recorded using the same criteria.

EAUS

Images were acquired with a 10-MHz, 360°, rotating endoprobe (type 2050, BK Medical, Herlev, Denmark). The data from a series of closely spaced EAUS images (0.25 mm) were combined to create a 3D volume displayed as a cube. The endoprobe was introduced into the anal canal with the patient in the left lateral position. Serial radial images were made of the distal part of the rectum and the anal canal using the automatic probe withdrawal system. Three-dimensional ultrasound images were obtained with a software program used for 3D reconstruction (Life Imaging System 2000, L3D1 version 3.5.5; B-K Medical). Fistula tracks were visualized as tube-like, hypoechoic lesions. The internal fistula opening was identified as a hypoechoic area in the intersphincteric plane, as a defect in the internal anal sphincter, or as a subepithelial breach that connected to the fistulous tract through an internal sphincter defect^[7].

After the pre-enhanced data set was saved, H₂O₂-enhanced sonography was performed as previously described, with some modifications^[8]. Briefly, a flexible intravenous cannula (16-21 G Angiocath) was inserted into the fistula tract through the external opening and fixed with adhesive tape. This cannula was connected to a 10 mL syringe containing 3% H₂O₂. Ultrasonic scanning was started approximately 20 s after instillation of H₂O₂ to allow time for bubble release. The contrast study was usually performed with infusion of a small amount (0.5-3.0 mL) of H₂O₂. Further injection of H₂O₂ at a little higher pressure was performed when contrast was not sufficient. The low echogenic tract seen on the non-enhanced study became bright due to reflection from the gas, with acoustic shadowing deep to the track after H₂O₂ installation.

After the EAUS procedures, the characteristics of the fistula were classified according to the same criteria used in the clinical evaluation.

Statistical analysis

The accuracy of the preoperative diagnosis established through physical examination, 3D-EAUS, and H₂O₂-enhanced 3D-EAUS was compared against the surgical findings. Primary fistula tracts were considered correctly classified when placed in the correct anatomic group of the Parks classification^[6]. Secondary extensions (abscess and/or horseshoe extension) were considered to be correctly classified when placed in the correct anatomical compartment and quadrant, and internal openings were considered to be correctly classified when placed in the correct quadrant and level. The accuracy of 3D-EAUS and H₂O₂-enhanced 3D-EAUS in detecting and classifying components of a given fistula was compared with the surgical findings as a reference standard. Fisher's exact test was used to detect significant differences in diagnostic accuracy. Statistical significance was defined as $P < 0.05$.

RESULTS

Preoperative clinical assessment and 3D-EAUS were

Table 1 Results of physical examination and 3D-EAUS findings in the identification of the primary fistula tract

	Classification based on surgical findings			Overall accuracy
	Intersphincteric (<i>n</i> = 26)	Transsphincteric (<i>n</i> = 34)	Suprasphincteric (<i>n</i> = 4)	
Physical examination				
Intersphincteric	20 ¹	9	0	68.7% (44/64) ^a
Transsphincteric	7	23 ¹	2	
Suprasphincteric	0	2	1 ¹	
3D-EAUS				
Intersphincteric	24 ¹	4	0	84.4% (54/64) ^b
Transsphincteric	4	29 ¹	1	
Suprasphincteric	0	1	1 ¹	
H ₂ O ₂ 3D-EAUS				
Intersphincteric	24 ¹	3	0	89.1% (57/64) ^c
Transsphincteric	2	30 ¹	1	
Suprasphincteric	0	1	3 ¹	

Data represent number of primary tracts. ¹Absolute agreement; $P = 0.037$ between a and b, $P = 0.005$ between a and c, $P = 0.435$ between b and c.

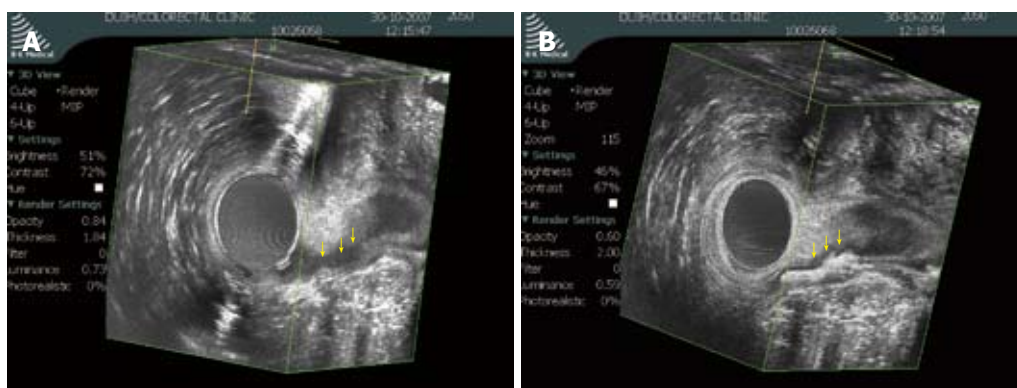


Figure 1 Pre-enhanced and H₂O₂ enhanced 3D-EAUS image of fistula tract in the same patient. A: Hypoechoic fistula tract is seen at pre-enhanced scan (arrows); B: The fistula tract became hyperechoic due to the gas generated from H₂O₂; the tract is more clearly discriminated from adjacent tissue than at the pre-enhanced scan (arrows).

conducted in all 61 patients. One patient complained of severe local pain, and another patient noted lower abdominal pain during the instillation of H₂O₂. The instillation was stopped instantly, and the patient with abdominal pain was closely monitored. The symptoms spontaneously subsided the next day without any problems. There were no adverse effects from the instillation of H₂O₂ except in these cases. The mean duration between ultrasound and surgery was 6 d (range, 0-18 d).

Primary tract

After the surgical procedures, we identified a total of 64 primary fistula tracts in 61 patients: 26 intersphincteric fistulae, 34 transsphincteric fistulae, and 4 suprasphincteric fistulae. The accuracy of physical examination in detecting the primary fistula tract was 68.7%, with surgical findings serving as the standard reference.

The concordance rates of 3D-EAUS and H₂O₂-enhanced 3D-EAUS with surgical findings with respect to identification of the primary tract were 84.4% and 89.1%, respectively (Table 1). These figures indicate that both 3D-EAUS and H₂O₂-enhanced 3D-EAUS

were superior to physical examination in detecting the primary tract ($P = 0.037$ and $P = 0.005$, respectively). The H₂O₂ enhancement enabled detection of one more transsphincteric fistula and two more suprasphincteric fistulae. Apart from the effect on accuracy, H₂O₂ enhancement made the tract more conspicuous in ambiguous cases (Figure 1). However, there was no statistically significant difference between 3D-EAUS and H₂O₂-enhanced 3D-EAUS with respect to accurate detection of the primary tract ($P = 0.435$).

Secondary extension

Twenty-nine secondary extensions, including abscesses and horseshoe tracts, were detected in 26 patients during surgical exploration: 7 intersphincteric, 19 ischiorectal, and 3 supralelevator. The overall accuracy of clinical evaluation in identifying secondary extension was 62.1%, as shown in Table 2. The overall accuracy of 3D-EAUS (81.8%) was significantly higher than that of physical examination ($P = 0.01$). The use of H₂O₂ did not further improve the diagnostic accuracy ($P = 0.482$), but one ischiorectal extension and one supralelevator type

Table 2 Results of physical examination and 3D-EAUS findings in classification of secondary extensions

	Classification based on surgical findings (No. of secondary extensions detected)				Overall accuracy
	Absent (37)	Intersphincteric (7)	Ischiorectal (19)	Supralelevator (3)	
Physical examination					
Absent	24 ¹	1	3		62.1% (41/66) ^a
Intersphincteric	5	4 ¹	3	2	
Ischiorectal	8	2	12 ¹		
Supralelevator	0	0	1	1 ¹	
3D-EAUS					
Absent	32 ¹	0	2	1	81.8% (54/66) ^b
Intersphincteric	2	5 ¹	1	2	
Ischiorectal	3	2	16 ¹	0	
Supralelevator	0	0	0	1 ¹	
H ₂ O ₂ 3D-EAUS					
Absent	33 ¹	0	1	1	86.4% (57/66) ^c
Intersphincteric	1	5 ¹	1	0	
Ischiorectal	3	2	17 ¹	0	
Supralelevator	0	0	0	2 ¹	

¹Absolute agreement; $P = 0.01$ between a and b, $P = 0.001$ between a and c, $P = 0.482$ between b and c.

Table 3 Results of physical examination and 3D-EAUS findings in localization of the internal opening

	Classification based on surgical findings (No. of internal openings detected)				Overall accuracy
	Anterior (12)	Left lateral (8)	Posterior (31)	Right lateral (6)	
Physical examination					
Anterior	6 ¹	0	0	0	59.7% (34/57) ^a
Left lateral	2	5 ¹	1	0	
Posterior	0	1	20 ¹	1	
Right lateral	0	0	2	3 ¹	
3D-EAUS					
Anterior	10 ¹	0	1	0	84.2% (48/57) ^b
Left lateral	0	6 ¹	0	0	
Posterior	0	1	26 ¹	0	
Right lateral	0	0	1	6 ¹	
H ₂ O ₂ 3D-EAUS					
Anterior	10 ¹	0	1	0	89.5% (51/57) ^c
Left lateral	0	7 ¹	0	0	
Posterior	0	0	28 ¹	0	
Right lateral	0	0	1	6 ¹	

¹Absolute agreement; $P = 0.004$ between a and b, $P = 0.001$ between a and c, $P = 0.406$ between b and c.

extension that were not clear on non-enhanced study were identified in enhanced study.

Internal opening

A total of 57 internal openings were detected in 54 patients at the time of surgery. No internal openings were found in seven patients, while two openings were found in three patients. The internal opening was most frequently found in the posterior position (36 patients), followed by anterior, right, and left positions. The concordance between physical examination and surgery with respect to the location of the internal opening was 59.7%. The concordance between surgical finding and 3D-EAUS was 84.2%, and that between surgical finding and H₂O₂-enhanced 3D-EAUS was 89.5%. Statistical analysis indicated that both 3D-EAUS and H₂O₂-enhanced 3D-EAUS were more accurate than physical examination (Table 3). No difference in diagnostic accuracy was noted

between 3D-EAUS and H₂O₂-enhanced 3D-EAUS, with respect to identifying the internal opening ($P = 0.406$).

DISCUSSION

There has been much controversy regarding the accuracy and usefulness of EAUS in anal fistula. According to our literature review, the accuracy of EAUS compared with surgery in the detection of the internal opening has varied between 28% and 94%, and the accuracy in the detection of the primary tract and secondary extension have been in the range of 36%-100% and 23%-92%, respectively^[9-12]. Such a wide range of accuracy rates could be related to differences in the criteria used to identify the internal opening, differences in operator experience, and differences in the complexities of fistulae recruited. In addition, the use of H₂O₂ and variable ultrasound equipment in individual studies may also

be responsible for such differences.

The most noticeable improvement in the accuracy of EAUS may derive from the use of H₂O₂ as a contrast material. Many studies have shown significantly superior results in H₂O₂-enhanced EAUS, compared to unenhanced study^[4,13,14]. Another technical development is the use of high frequency transducers and the introduction of 3D technology in EAUS^[15].

In the present study, the agreement of 3D-EAUS with surgery was excellent: 84% in the detection of primary tracts, 84% in internal openings, and 82% in secondary extensions. These outcomes are consistent with recent data concerning 3D-EAUS^[16-18].

Most reports using conventional 2D-EAUS have shown superior results with H₂O₂ enhancement. However, only a few reports have addressed the effect of H₂O₂ in 3D-EAUS. Ratto *et al*^[16] obtained improved diagnostic accuracy by using H₂O₂, and West *et al*^[5] reported high diagnostic accuracy with H₂O₂ enhanced 3D-EAUS, comparable to MRI. However, the improved accuracy was not apparent in the study of Buchanan *et al*^[17]. We identified a few more primary tracts and secondary extensions by using H₂O₂. However, there was no statistically significant difference between 3D-EAUS and H₂O₂ enhanced 3D-EAUS with respect to classifying primary tracks, internal openings, and secondary tracks.

Several explanations may be offered concerning the lack of favorable results with H₂O₂. First, 3D-EAUS was already highly accurate prior to the use of H₂O₂ in this study. The already elevated baseline may have left little room for improvement on the contrast study.

One of theoretical limitations of non-contrast EAUS is difficulty discriminating between an active tract and scar tissue since both tissues appear hypoechoic on noncontrast EAUS^[19]. The gas generated after H₂O₂ instillation makes the active tract hyperechoic. In this regard, contrasting with H₂O₂ could be more useful in patients with recurrent fistulae, which usually accompany previous operative scars. Several authors have suggested that H₂O₂ might be more helpful in recurrent cases^[8,17]. The number of recurrent or Crohn's disease cases in this study was relatively low. The paucity of recurrent and complicated cases might have limited the advantages of H₂O₂ enhancement.

Instillation of H₂O₂ is generally accepted to be very safe, but several complications have been reported, including air embolism^[20,21]. Such complications have been associated with large infusion volume or forceful instillation. Of note, one patient in this study complained of abdominal pain in the early period of this study, although symptoms subsided spontaneously. The cause for the abdominal pain is not clear, but it might be associated with the relatively large volume (10 mL) of H₂O₂ and the high injection pressure. We were very careful not to inject forcefully after that event, and no patients experienced complications thereafter. Such gentle instillation might negatively influence the usefulness of enhancement.

Another point to consider is that although H₂O₂ enhancement had little influence on diagnostic accuracy improvement, H₂O₂ enhancement often improved

the quality of images in cases where the unenhanced study barely delineated the tract as shown in Figure 1 of this study and in another report^[17]. The presence of gas in the internal opening or fistula track made the tract more obvious. It is notable that this beneficial effect was observed often in difficult cases including suprasphincteric fistula tract and recurrent fistulae, in which accurate anatomical assessment is far more important.

Considering these results, selective use of H₂O₂ in difficult cases rather than routine use may be economical and helpful.

EAUS was inferior to MRI in most earlier studies^[11,22], and it was inferior^[2,23] or equivalent^[24,25] in subsequent studies. A conventional 2D-EAUS was used in most of these comparative studies. In the only prospective comparative study evaluating 3D-EAUS and MRI, both modalities were shown to be equally accurate^[18]. The high accuracy of 3D-EAUS, which is comparable with MRI, indicates that 3D-EAUS may be the first choice in the assessment of anal fistulae, since it has advantages over MRI including easier use and lower cost.

In addition to the high accuracy and low cost, EAUS provides additional usefulness in clinical practice. Yee *et al*^[26] recognized a high incidence (92%) of coexisting occult sphincter defects in the evaluation of rectovaginal fistulae. Sphincter defects were noted more frequently based on EAUS than based on manometry or a history of fecal incontinence. In fact, the ability to accurately display the anal sphincter muscle anatomy is an inherent advantage of EAUS. This method clearly shows the overall volume of the sphincter muscle, as well as sphincter injury. Knowledge of sphincter status and associated injuries recognized on EAUS facilitates surgical decisions on whether to proceed with a sphincter-saving procedure or lay open. Surgical treatment in this study was chosen largely based on preoperative EAUS findings. Seton or mucosal advancement flap was favored over lay open when there was associated sphincter damage or a high type fistula.

Most previous reports have regarded surgical results as the reference standard. However, surgery as a gold standard has been questioned, as studies have shown that EAUS and MRI are able to detect fistula tracts that are not seen on surgical exploration^[13,18]. Buchanan *et al*^[2] suggested using clinical outcomes rather than surgical findings as a reference standard because missed occult infection is possible during surgical exploration. In view of the high accuracy of 3D-EAUS, the lack of data on clinical outcomes and the use of surgical findings as the gold standard in this study might in part have biased our results. This study was also limited by the retrospective study design and the low prevalence of high type fistulae. The latter made it difficult to draw clear conclusions as to how adequate 3D-EAUS was in detecting high type fistulae.

In conclusion, 3D-EAUS is highly reliable in the preoperative evaluation of anal fistulae. The use of H₂O₂ for enhancement offers some benefits, although it did not significantly improve the diagnostic accuracy in this

study. The selective use of H₂O₂ may be economical and reliable in difficult cases.

COMMENTS

Background

Accurate preoperative assessment of anal fistula is very important for optimal surgical results. Physical examination is a basic diagnostic method, but it often fails to accurately diagnose complex fistula. Endoanal ultrasonography (EAUS) is a useful tool in this situation, and recently introduced 3D-EAUS is expected to further increase diagnostic accuracy.

Research frontiers

In this study, the authors demonstrated that 3D-EAUS is highly accurate in the evaluation of anal fistula without or without H₂O₂ enhancement. They also showed that the selective application of H₂O₂ enhancement in difficult cases is an effective and economic strategy.

Innovations and breakthroughs

Recently, EAUS has been widely applied in the diagnosis of anal fistula. However, only a few reports have been published regarding the diagnostic accuracy of 3D-EAUS. This report would be helpful in accumulating data in this field.

Applications

This study would be a useful guideline to the surgeon dealing with anal fistula.

Peer review

The author evaluated the effectiveness of 3D-EAUS in the assessment of the anal fistula with and without H₂O₂ enhancement. This paper is written well and is an important paper for further studies.

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S- Editor Li LF L- Editor Webster JR E- Editor Ma WH

BRIEF ARTICLES

Hepatoprotective evaluation of *Anogeissus latifolia*: *In vitro* and *in vivo* studies

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Received: June 13, 2009 Revised: September 7, 2009

Accepted: September 14, 2009

Published online: October 14, 2009

Abstract

AIM: To evaluate the hepatoprotective activity of a hydroalcoholic extract of the bark of *Anogeissus latifolia*; *in vitro* in primary rat hepatocyte monolayer culture and *in vivo* in the liver of Wistar rats intoxicated by carbon tetrachloride (CCl₄).

METHODS: In the *in vitro* study, a primary hepatocyte monolayer culture was treated with CCl₄ and extract of *Anogeissus latifolia*. Hepatoprotective activity was demonstrated in the CCl₄ damaged primary monolayer culture. In the *in vivo* study, the hepatoprotective activity of a hydroalcoholic extract of *Anogeissus latifolia* was analyzed in liver injured CCl₄-treated rats. Biochemical parameters including serum transaminases [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] and alkaline phosphatase (ALP) in serum were

analyzed. The biochemical findings were supplemented with histopathological examination of rat liver sections.

RESULTS: *In vitro*: primary hepatocyte monolayer cultures were treated with CCl₄ and extract of *Anogeissus latifolia*. A protective activity could be demonstrated in the CCl₄ damaged primary monolayer culture. *In vivo*: Hydroalcoholic extract of *Anogeissus latifolia* (300 mg/kg) was found to have protective activity in rats with CCl₄-induced liver damage as judged from serum marker enzyme activity.

CONCLUSION: The above findings lead to the conclusion that the hydroalcoholic extract of *Anogeissus latifolia* is hepatoprotective. Hence, we suggest that the inclusion of this plant in the management of liver disorders is justified.

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Key words: *Anogeissus latifolia*; Hepatoprotective; Carbon tetrachloride

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Pradeep HA, Khan S, Ravikumar K, Ahmed MF, Rao MS, Kiranmai M, Reddy DS, Ahamed SR, Ibrahim M. Hepatoprotective evaluation of *Anogeissus latifolia*: *In vitro* and *in vivo* studies. *World J Gastroenterol* 2009; 15(38): 4816-4822 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4816.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4816>

INTRODUCTION

Oxidative stress has been implicated in the pathogenesis of acute and chronic liver injury in a variety of pathophysiological conditions such as hepatotoxin exposure, intrahepatic cholestasis, alcoholic liver injury, liver ischemia and viral hepatitis^[1-4]. Over-production of reactive oxygen species (ROS) and nitrogen species (RNS), along with significant decrease of antioxidant defense in these pathological conditions, impairs

various cellular functions through the processes of lipid peroxidation, protein oxidation and nucleic base oxidation. Lipid peroxidation causes changes in the physical and chemical properties of cellular membranes, thus altering their fluidity and permeability, leading to impairment in membrane signal transduction and ion exchange, resulting in swelling, cytolysis, and finally, cell death. The oxidation of proteins and DNA also relates directly to cellular dysfunction and death^[5]. Accordingly, effects of antioxidants or free radical scavengers have been widely tested for the prevention and treatment of acute and chronic liver injuries. In some of the studies, antioxidants have shown beneficial effects, specifically for prevention and treatment of chronic liver injury^[6-8].

Anogeissus latifolia Wall (Combretaceae) is a large or moderate-sized tree characteristic of dry deciduous forests and available throughout India. The plant is traditionally used for the treatment of dysentery, snakebite, leprosy, diabetes, wounds and ulcers and skin diseases, in addition to hepatopathy^[9]. The hydroalcoholic extract is reported to have antioxidant activity. It has been studied for total antioxidant activity, hydrogen-donating ability, nitric oxide, superoxide scavenging activity and hydrogen peroxide decomposition activity. Integral antioxidative capacity has been determined by chemiluminescence assay. It has also been studied in a lipid peroxidation assay with a thiobarbituric acid-reactive substances (TBARS) method using rat liver homogenate^[10]. A variety of substances which might contribute to hepatoprotective activity have been identified in extracts of *Anogeissus latifolia* including tannins, gallic acid, ellagic acid and flavonoids such as lutein and quercetin, which are potential antioxidants^[11-16]. The bark of the plant is also reported to possess several biological activities such as antiulcer, antimicrobial and wound healing activities. Gastroprotective potential of *Anogeissus latifolia* extract has been studied in aspirin-, cold-resistant stress (CRS)-, pylorus ligated- and ethanol-induced ulcers. The status of the antioxidant enzymes, superoxide dismutase and catalase, has also been studied in CRS-induced ulcers^[17,18]. The bark of the plant was standardized for the presence of chemical constituents such as gallic acid and ellagic acid (0.95% w/w and 0.25% w/w, respectively) using High Performance Thin Layer Chromatography (HPTLC) by Govindrajan *et al.*^[17]. Further, we identified and quantified the other constituents of the bark, quercetin and rutin (1.875% w/w, 0.1617% w/w, respectively) using HPTLC; these are reported as potent antioxidants^[15,16] and hepatoprotective agents^[19,20]. Antioxidant action has been reported to play a crucial role in hepatoprotection^[6-8]. The hydroalcoholic extract of *Anogeissus latifolia* is reported to have chemoprotective activity in paracetamol-induced toxicity in a rat model^[21]. Thus, the present study was therefore undertaken to investigate the hepatoprotective activity of hydroalcoholic extract of *Anogeissus latifolia* *in vitro* and *in vivo* against CCl₄ intoxicated rats.

MATERIALS AND METHODS

Materials

Plant material and extraction: Bark of *Anogeissus latifolia* was collected from Chikmagalur, Karnataka, South

India during the month of May. It was authenticated by Botanical survey of India, Coimbatore, Tamilnadu, India (No. BSI/SC/5/23/06-07/Tech.880).

The bark was shade-dried and powdered coarsely. The coarse powder (250 g) obtained was treated with n-hexane to remove the fatty substances; the bark was further submitted to exhaustive lipid extraction with 70% ethanol in Soxhlet apparatus and filtered. The extract was concentrated under reduced temperature and pressure to obtain dry residue (26.8 g)^[10].

Chemicals: All chemicals and solvents used were obtained from S.D. Fine Chemicals, Mumbai, Loba Chemie Indo Austranel Co., Mumbai, Ranbaxy laboratories Ltd., Punjab, Sigma Fine Chemicals, Mumbai and Hi media Laboratories, Mumbai, India. For various biochemical estimations, kits were procured from Ecoline, E. Merck Ltd., M.I.D.C., Taloja. Liv-52 syrup was procured from Market, manufactured by Himalaya Drug Company, Bangalore.

Animals: Healthy, adult female albino rats of Wistar strain, weighing 180-220 g were obtained from the animal house of J.S.S College of Pharmacy, Ooty, India. The animal house was well ventilated and the animals were exposed to 12 h day and night cycles at a temperature of 20 ± 2°C. The animals were housed in large spacious, hygienic polypropylene cages during the course of the experimental period. The animals were fed with water and standard rat pellet obtained from M/s Hindustan Lever Ltd., Bangalore, India (CPCSEA-JSSCP/IAEM/PHY.PHARM/2006-07).

Autoanalyser and UV spectrophotometer

Microlab 100, manufactured by M/s Vital Scientific N.V., The Netherlands, was used to estimate biochemical parameters for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP). UV-160 Spectrophotometer, manufactured by Shimadzu Corporation, Japan, was used to estimate total phenol content, total flavonol content and lipid peroxidation.

Methods

Estimation of total phenolic content: Phenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activity^[22]. Total phenol was determined using the Folin-Ciocalteu method. This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation a green blue complex is measured at 750 nm. The total phenol content of a tested material is related to its antioxidant activity^[23].

Estimation of total flavonol content: Total flavonol content was determined by the method of Woisky^[24]. This involved an aluminum chloride colorimetric method. The principle of this method is that aluminum chloride forms an acid stable complex with the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum

chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids^[25].

Preparation of drug solution: The 70% ethanolic extract of *Anogeissus latifolia* was suspended in 1% carboxy methyl cellulose (CMC), for oral administration. The concentrations of extract selected were 100 mg and 300 mg/kg body weight. Liv-52 syrup was administered orally at 2 mL/kg body weight.

Carbon tetrachloride-induced hepatotoxicity: It is emphasized that hepatotoxins which cause acute hepatitis should have close resemblance with viral hepatitis, clinically, biochemically and histologically. Certain drugs are responsible for chronic hepatic disease. Chemically-induced hepatic injury for experimental studies should be severe enough to cause cell death or to modify hepatic functions. The mechanism of acute hepatic injury depends upon the chemical compounds used to induce toxicity. Carbon tetrachloride (CCl₄) is one of the most powerful hepatotoxins in terms of severity of injury. It causes toxic necrosis leading to biochemical changes, having clinical features similar to those of acute viral hepatitis^[26,27]. CCl₄ at a dose of 0.5 mL/kg was dissolved in olive oil (1:1) and 0.1 mL was administered for each 100 g of rat body weight intraperitoneally.

Standard Liv-52 Syrup: Liv-52 is a poly herbal formulation introduced in 1954 as a specially formulated ayurvedic herbal remedy for the treatment of viral hepatitis and has been widely prescribed for infective hepatitis ever since^[28]. It is an ayurvedic formulation available as tablets and syrup containing the following herbs: *Caparis spinosa*, *Cichorium intybus*, *Solanum nigrum*, *Terminalia arjuna*, *Cassia occidentalis*, *Achillea millefolium*, *Tamarix galica* and *Phyllanthus amarus*.

In-vitro hepatoprotective activity

Hepatotoxin and test substances: For *in vitro* studies, CCl₄ (0.1 mol/L), was used to produce submaximal toxicity in isolated rat hepatocytes. The test solutions were administered at dose levels of 125, 250 and 500 µg/mL. Liv-52 was used as a positive control at a dose level of 250 µL/mL. All the substances were dissolved in DMSO^[29].

Isolation of rat hepatocytes: The rat hepatocytes were isolated according to the method of Seglen *et al*^[30]. The livers were isolated under aseptic conditions and placed in HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffer I containing HEPES (0.01 mol/L), NaCl (0.142 mol/L) and KCl (0.0067 mol/L), pH 7.4. The livers were cut into small pieces and then incubated with a second buffer containing HEPES (0.1 mol/L), NaCl (0.0667 mol/L), KCl (0.0067 mol/L) and 0.5% Collagenase type IV, pH 7.6, for about 45 min at 37°C in an incubator with constant shaking. Hepatocytes were obtained after filtration and cold centrifugation (4°C, 200 rpm for 2 min, three times) and suspended in HEPES buffer I. The viability of the hepatocytes was assessed by trypan blue (0.2%) exclusion method^[30].

Primary cultures of rat hepatocytes: The method of Tingstrom and Obrink^[31] with slight modifications was used for the culturing of rat hepatocytes. The freshly isolated viable hepatocytes were suspended in culture medium RPMI-1640 supplemented with calf serum (10%), HEPES and gentamycin (1 µg/mL). These cells (approximately $1-1.2 \times 10^6$ /mL) were then seeded into culture bottles and incubated at 37°C in an atmosphere of 5% CO₂ in a carbon dioxide incubator. Upon incubation for 24 h the hepatocytes formed a monolayer. The newly formed cells were round and most appeared as individual cells. These cells were 95%-96% viable as confirmed by trypan blue exclusion test.

Hepatic cytotoxicity testing: The hydroalcoholic extract of *Anogeissus latifolia* was tested for hepatic cytotoxicity at 250, 500 and 1000 µg/mL on isolated rat hepatocytes. After 24 h of incubation at 37°C in a CO₂ incubator, the percentage viability of hepatocytes was tested using trypan blue exclusion^[32].

Hepatoprotective activity: Twenty-four hours after the establishment of the monolayer of hepatocytes, the medium was decanted and the culture was washed with HEPES buffer-I and finally the hepatocytes were suspended in Buffer-I. The hepatic cytotoxicity was induced with CCl₄ (0.1 mol/L). Triplicate hepatocyte suspensions (0.1 mL) from different cultures were distributed into various culture tubes labeled as control, toxicant, standard (Liv-52 + toxicant) and test (test sample + toxicant). The control group received 0.1 mL of vehicle (30% DMSO) and toxicant groups received 0.1 mL of CCl₄, while the test groups received 0.1 mL of respective test solutions (250, 500 and 1000 µg/mL) followed by 0.1 mL (0.1 mol/L) of hepatotoxin. The standard groups received 0.1 mL of Liv-52 (250 µL/mL) followed by hepatotoxin. The contents of all culture tubes were made up to 1 mL with HEPES buffer I. The contents of all the tubes were mixed well and incubated in a CO₂ incubator for 24 h at 37°C. In test and standard groups the hepatocytes were preincubated with respective solutions for 30 min and then exposed to hepatotoxin. After incubation, hepatocyte suspensions were collected to assess cell damage. Cell viability was evaluated by trypan blue exclusion method. Hepatocyte suspensions were centrifuged at 200 rpm. The leakage of the enzymes ALT and AST secreted outside the cells was determined from the supernatant.

Assessment of hepatoprotective activity: The effect of different extracts on liver protection was determined by measuring an increase in the percentage of viable cells in that group of cells incubated with extracts, compared with the control and toxicant-alone groups. Reversal of toxin-induced elevations in the level of enzymes was also considered to assess hepatoprotective activity. Kits procured from Ecoline, E. Merck Ltd., using an auto-analyser, carried out the biochemical estimations (Table 1).

In vivo acute toxicity studies: Acute oral toxicity was induced according to the Organization for Economic Co-

Table 1 Effect of *Anogeissus latifolia* extract (ALE) on CCl₄-induced toxicity in rat hepatocytes (mean \pm SE)

Treatment	Viable cells (%)	ALT (IU/L)	AST (IU/L)
Cell control	93.88 \pm 0.81	18.70 \pm 0.46	21.53 \pm 0.63
Toxicant (1 mol/L CCl ₄)	33.06 \pm 114	50.20 \pm 0.61	57.50 \pm 0.94
Std Liv52 (250 μ L/mL)	85.84 \pm 1.19 (86.7) ^a	19.46 \pm 0.35 (97.59) ^a	24.10 \pm 0.37 (92.85) ^a
ALE (250 μ g/mL)	57.63 \pm 1.58 (40.39) ^a	41.33 \pm 0.62 (28.16) ^a	44.53 \pm 1.25 (36.05) ^a
ALE (500 μ g/mL)	77.60 \pm 0.57 (73.72) ^a	38.50 \pm 0.45 (37.14) ^a	32.80 \pm 0.65 (68.66) ^a
ALE (1000 μ g/mL)	81.24 \pm 1.67 (79.20) ^a	22.23 \pm 0.50 (88.48) ^a	27.33 \pm 0.69 (83.87) ^a

Values in brackets indicate percentage protection against toxicant. Significant reduction compared to hepatotoxin (^a $P < 0.05$). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Operation and Development (OECD) 423 guidelines procedure^[33]. Healthy, young adult Wistar albino rats of weight variation not exceeding $\pm 20\%$ of the mean weight were selected. The animals were fasted for 4 h with free access to water only. *Anogeissus latifolia* was administered orally at a dose of 5 mg/kg initially. Mortality, if any, was observed for 3 d. If mortality was observed in two out of three animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one animal out of three animals then the same dose was repeated again to confirm the toxic effect. If no mortality was observed, then higher doses (50, 300, 2000 mg/kg) of *Anogeissus latifolia* were employed for further toxicity studies. *Anogeissus latifolia* did not produce any behavioral changes and mortality up to the dose of 3000 mg/kg body weight. Hence, 1/10th of this dose, i.e. 300 mg/kg (high dose) was used for the study.

In-vivo hepatoprotective activity and estimation of biochemical parameters

Experimental design: Twenty four albino Wistar rats, weighing about 180–220 g were divided into 4 groups of six animals each. Group I served as solvent control (normal animals). Group II served as CCl₄ toxicant control and received 1% CMC, 2 mL/kg b.w. Group III served as positive control and received Liv-52, 2 mL/kg b.w., while Group IV received the freshly prepared *Anogeissus latifolia* suspended in 1% CMC at a dose level of 300 mg/kg b.w. The animals were treated for 7 d and on the 7th d after one hour of dosing, the toxicant CCl₄ (500 μ L/kg i.p.) was administered to all the groups except Group I. After 24 h, the animals were anesthetized and blood was collected by sino-orbital puncture for the assessment of various enzyme activities. The blood was centrifuged at 2000 rpm for 10 min. The serum was separated and was used for various biochemical estimations such as AST, ALT, and ALP. The animals were sacrificed later and the liver was perfused and excised. Part of the liver was stored in 10% formalin saline for histopathological studies. The remaining was frozen at -70°C and was used for the estimation of lipid peroxidation.

Estimation of lipid peroxidation by thiobarbituric acid reactive substances (TBARS): The level of lipid peroxidation in liver homogenate was determined by the method of Niehaus and Samuelson^[34]. Malondialdehyde

and other thiobarbituric acid reactive substances were quantified by their reactivity with thiobarbituric acid in acidic conditions. The reaction generates a pink colored chromophore, which can be read in a colorimeter at 535 nm.

Statistical analysis

The statistical analysis was carried out by one-way analysis of variance (ANOVA). The values are represented as mean \pm SE. Comparison of mean values of different groups treated with different dose levels of extracts and positive controls were estimated by Tukey's Multiple Comparison Test. $P < 0.05$ was considered significant.

RESULTS

Hepatic cytotoxicity

When normal hepatocytes were treated with the extracts under test conditions, there were no alterations in the values of percentage viable cells as compared to the control at the dose level up to 1000 μ g/mL, indicating that the extracts were not toxic to the cells.

Effects against CCl₄-induced toxicity

Incubation of hepatocytes with CCl₄ (0.1 mol/L) resulted in 65% depletion in viability of hepatocytes. Similarly an elevation of about 268.45% and 267.06% of ALT and AST levels were observed, respectively, upon intoxication with CCl₄. Hepatocytes treated with *Anogeissus latifolia* showed a concentration-dependant (100–1000 μ g/mL) protective effect by restoring the viability of hepatocytes (40.36%–79.20%), AST (28.16%–88.48%) and ALT (36.05%–83.87%) levels, while the positive control Liv-52 showed good protective effect by restoring viability (86.7%), AST (97.5%) and ALT (92.8%). The maximum protection was seen with 1000 μ g/mL of *Anogeissus latifolia*. Results are represented in Table 1.

In vivo hepatoprotective screening

Single administration of CCl₄ (500 μ L/kg, i.p) to vehicle control rats showed significant increases in ALT (222.8 \pm 10.14 IU/mL, $P < 0.001$), AST (254.9 \pm 19.3 IU/mL, $P < 0.001$) and ALP (328.5 \pm 25.36 IU/mL, $P < 0.01$) levels when compared to normal control rats (54 \pm 2.7 IU/mL, 88.17 \pm 5.47 IU/mL and 249.5 \pm 18.2 IU/mL, respectively) (Figure 1). *Anogeissus latifolia* administered at 300 mg/kg produced significant reductions in ALT 66.2 \pm 6.1 IU/mL

Table 2 Effect of *Anogeissus latifolia* on plasma biochemical parameters and lipid peroxidation in CCl₄-intoxicated rats liver (*n* = 6, mean \pm SE)

Treatment (<i>n</i> = 6)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Lipid peroxidation nmol of MDA/mg protein
Normal	88.17 \pm 5.47	54 \pm 2.7	249.5 \pm 18.2	4.1 \pm 0.5
CCl ₄ (500 μ L/kg)	254.9 \pm 19.3 ^c	222.8 \pm 10.14 ^c	328.5 \pm 25.36 ^a	14.8 \pm 1.3 ^c
Liv52 (2 mL/kg)	111.7 \pm 8.7 ^b	71.4 \pm 5.6 ^b	255 \pm 24.1 ^b	5.4 \pm 0.6 ^b
ALE (300 mg/kg)	117 \pm 6.7 ^b	66.2 \pm 6.1 ^b	258 \pm 15.54 ^b	6.1 \pm 0.5 ^b

^a*P* < 0.01, ^c*P* < 0.001 vs normal; ^b*P* < 0.01 vs carbon tetrachloride. ALP: Alkaline phosphatase.

(*P* < 0.01), AST 117 \pm 6.7 IU/mL (*P* < 0.01) and ALP 258 \pm 15.54 IU/mL (*P* < 0.01) levels when compared to CCl₄-administered rats. Liv-52 also showed significant reductions in ALT 71.4 \pm 5.6 IU/mL (*P* < 0.01), AST 111.7 \pm 8.7 IU/mL (*P* < 0.01) and ALP 258 \pm 15.54 IU/mL (*P* < 0.01) levels when compared to CCl₄-administered rats (Table 2).

Lipid peroxidation

Lipid peroxidation was significantly elevated following CCl₄ administration (14.8 \pm 1.3 nmol, *P* < 0.001) when compared to normal control (4.1 \pm 0.5 nmol) (Figure 1). *Anogeissus latifolia* at a dose of 300 mg/kg, b.w., resulted in significant (*P* < 0.01) reductions in lipid peroxidation (6.1 \pm 0.5 nmol) when compared to toxicant control. Liv-52 at 2 mL/kg b.w., showed significant (*P* < 0.01) reductions in lipid peroxidation (5.4 \pm 0.6 nmol) when compared to CCl₄-administered rats (Table 2).

Histopathology

The protective effect of the hydroalcoholic extract of *Anogeissus latifolia* was further confirmed by histopathological examination of the control (Figure 1A), CCl₄-treated and extract-treated groups. The liver of CCl₄-treated rats (Figure 1B) shows damaged liver cells and ballooning changes of the hepatocytes. The histopathological pattern of the livers treated with extracts at 100 mg and 300 mg/kg (Figure 1C) shows mild feathery changes, little ballooning degeneration of hepatocytes along with normal hepatocytes. Positive control liver treated with Liv 52 (Figure 1D) shows a normal lobular pattern with minimal pooling of blood in the sinusoidal spaces. The present study reveals the hepatoprotective activity of the hydroalcoholic extract of *Anogeissus latifolia* against well-known hepatotoxin CCl₄.

DISCUSSION

The bark of *Anogeissus latifolia* was selected to evaluate its antihepatotoxic effect in preclinical models on the basis of its utility profile in the traditional system of medicine. Subsequently, a survey of literature suggested that it has been used for different diseases including inflammation, diabetes, diarrhoea and skin diseases, as well as hepatopathy. The bark has been evaluated scientifically for its antioxidant and wound healing activity. There was, however, no evidence of any scientific studies on its hepatoprotective action. A qualitative chemical examination showed the presence of carbohydrates, glycosides, phenolic compounds, flavonoids and tannins. The presence of

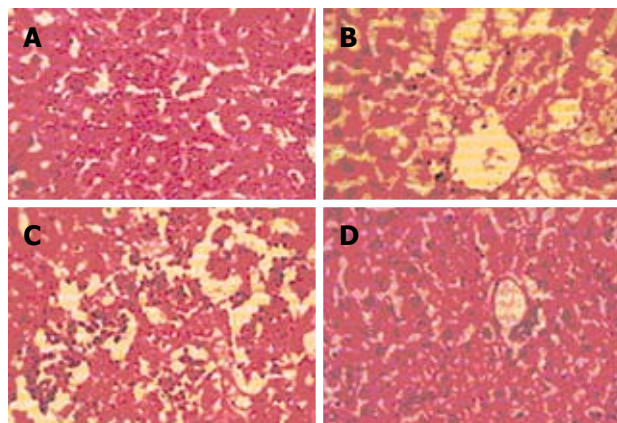


Figure 1 Photograph of rat liver (\times 100). A: Liver of a control rat showing normal hepatocytes and normal architecture; B: Liver section from a CCl₄-treated rat demonstrating damaged liver cells and ballooning changes in the hepatocytes; C: Liver section from a Liv-52-treated rat showing mild feathery change, little ballooning degeneration of hepatocytes with associated normal hepatocytes; D: Liver section from a latifolia-treated rat showing a normal lobular pattern with minimal pooling of blood in the sinusoidal spaces.

polyphenols and flavonoids supports its antioxidant potential. Total phenol content and total flavonol content was estimated in the extract, and found to be 64.43% and 43.9 mg/g of extract respectively. Since the bark has been reported to contain quercetin and rutin^[9], we estimated the quantity of these substances in the extract by HPTLC and this was found to be 1.875% w/w, and 0.1617% w/w respectively. The drug also contains gallic acid. The high percentage of quercetin, rutin and gallic acid in the extract justifies the potent antioxidant activity^[13,15,16] which results in the hepatoprotective potential of the extract. Quercetin and rutin are reported to be potential therapeutic agents as they reduce oxidative DNA damage, lipid peroxidation and quench free radicals^[35,36]. The drug, thus, is a rich source of various antioxidant chemicals which may exert a cumulative antioxidant effect producing favourable actions in various disease conditions such as hepatopathy, diabetes, inflammation and wound healing.

The hepatotoxicity induced by CCl₄ is due to its metabolite CCl₃^{*}, a free radical that binds to lipoprotein and leads to peroxidation of lipids of the endoplasmic reticulum^[37]. The ability of a hepatoprotective drug to reduce the injurious effects, or to preserve the normal hepatic physiological mechanisms which have been disturbed by a hepatotoxin, is an index of its protective effects. Although serum enzyme levels are not a direct measure of hepatic injury, they show the status of the liver. The lowering of enzyme levels is a definite indication of hepatoprotec-

tive action of the drug. The serum ALT, AST, and ALP levels are reliable markers of liver function^[38]. In our study, an increase in LPO level in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms resulting in excessive free radical. In CCl₄-induced hepatitis, administration of *Anogeissus latifolia* at 300 mg/kg b.w. produced significant reductions in ALT, AST, ALP levels and lipid peroxidation. Thus it could be suggested that *Anogeissus latifolia* has hepatoprotective activity in this model, a concept which was further supported by the histopathological results. The reactive species-mediated hepatotoxicity can be effectively managed upon administration of agents possessing antioxidant^[39], free radical scavenger^[40] and anti-lipid peroxidant^[41] activities. The inhibitors of cytochrome P450 isoenzymes (CYPs) are known to reduce the toxicity of CCl₄^[19]. Rutin and quercetin, which are constituents of *Anogeissus latifolia* extract, have been reported to inhibit CYPs^[42] and might have contributed favorably toward the observed hepatoprotection. *Anogeissus latifolia*, being a potent antioxidant, free radical scavenger, contributed favourably in this regard towards the observed hepatoprotection. The *in vitro* and histopathological studies are direct evidence of efficacy of this drug as a hepatoprotectant. Thus, the presence of rutin, quercetin and other antioxidants in *Anogeissus latifolia* may be the contributing factor towards its hepatoprotective activity and justifies the folkloric use of the plant in liver diseases.

ACKNOWLEDGMENTS

We are very grateful to Mr. Mohammed Jafer, Chairman, Nizam group of Institutions, Mr. Mohammed Fathe Azmath, Secretary, Nizam group of Institutions, and Mr P Chakrabarty, Director, Nizam group of Institutions, for patronage, encouragement, guidance, and financial support extended to us during the course of the study.

COMMENTS

Background

To evaluate the hepatoprotective activity of a hydroalcoholic extract of the bark of *Anogeissus latifolia*; *in vitro* in primary rat hepatocyte monolayer culture and *in vivo* in the liver of Wistar rats intoxicated by CCl₄.

Research frontiers

Great effort has been and is still being made to minimize costs and side effects of synthetic drugs, which are being used in the treatment of liver diseases.

Innovations and breakthroughs

This product is effective both in *in vitro* and *in vivo* studies with no side effects and is cost effective. The product was observed to have an excellent reparative effect on the CCl₄-damaged hepatocytes.

Applications

This article helps to understand and implement the process of treatment with herbal medicines in liver diseases, which is safe in every aspect.

Peer review

The author/authors of this manuscript have assessed the protective effect of *Anogeissus latifolia* in CCl₄-induced hepatotoxicity of rats. Their study has shown that *Anogeissus latifolia* contributes to increased viability of cultured hepatocytes *in vitro* and also causes reduced ALT and AST levels in intoxicated rats.

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S- Editor Tian L L- Editor Logan S E- Editor Ma WH

Treating delayed endoscopic sphincterotomy-induced bleeding: Epinephrine injection with or without thermotherapy

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Received: July 6, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: October 14, 2009

group and one patient (3%) in the combination therapy group required angiographic embolization or surgery ($P = 0.31$). The total number of blood transfusions was not significantly different between the two groups (3.5 ± 4.6 U vs 3.5 ± 4.5 U, $P = 0.94$). There was no bleeding-related death in either group.

CONCLUSION: Epinephrine injection alone is as effective as epinephrine injection combined with thermotherapy for the management of delayed post-ES bleeding.

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Key words: Bleeding; Endoscopic retrograde cholangiopancreatography; Endoscopic sphincterotomy; Epinephrine; Thermotherapy

Peer reviewer: Marko Duvnjak, MD, Department of Gastroenterology and Hepatology, Sestre milosrdnice University Hospital, Vinogradska cesta 29, 10 000 Zagreb, Croatia

Tsou YK, Lin CH, Liu NJ, Tang JH, Sung KF, Cheng CL, Lee CS. Treating delayed endoscopic sphincterotomy-induced bleeding: Epinephrine injection with or without thermotherapy. *World J Gastroenterol* 2009; 15(38): 4823-4828 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4823.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4823>

Abstract

AIM: To compare the hemostatic efficacy between epinephrine injection alone and epinephrine injection combined with thermotherapy for delayed post-endoscopic sphincterotomy (ES) bleeding.

METHODS: Cases with delayed post-ES bleeding undergoing epinephrine injection alone (epinephrine injection group, $n = 26$) or epinephrine combined with thermotherapy (combination therapy group, $n = 33$) in our institution between 1999 and 2007 were retrospectively investigated. The main outcome measurements were: initial endoscopic hemostasis, re-bleeding, complications, requirement of angiographic embolization or surgery, requirement for blood transfusion, and mortality.

RESULTS: The initial hemostatic efficacy was 96.2% for epinephrine injection alone and 100% for combination therapy ($P = 0.44$). There were four patients with re-bleeding in each group (16.0% vs 12.1%, $P = 0.72$). There was only one complication of pancreatitis from the combination therapy group. Three patients (11.5%) in the epinephrine injection

INTRODUCTION

Endoscopic sphincterotomy (ES) is the cornerstone of therapeutic endoscopic retrograde cholangiopancreatography (ERCP). The incidence of post-ES bleeding varies from 0.76%-2% to 10%-48%, depending on the definition applied^[1-6]. Post-ES bleeding is classified as immediate or delayed according to the timing of presentation^[7]. Although endoscopically observed bleeding at the time of ES occurs in approximately 10%-30% of cases, the majority of immediate post-ES bleeding episodes are self-limiting and can usually be managed conservatively^[5,8-10]. In contrast, delayed post-ES bleeding is often of clinical significance and requires more invasive intervention^[10-13].

The methods of endoscopic hemostasis for post-ES bleeding mirror those for peptic ulcer bleeding. For a bleeding peptic ulcer, epinephrine injection therapy is effective to stop bleeding and additional endoscopic treatment such as thermocoagulation after epinephrine injection can reduce the re-bleeding rate, need for surgery, and mortality^[14]. However, the bleeding mechanism for post-ES bleeding is different from that of peptic ulcer bleeding^[15]. It is unclear whether the hemostatic effect for post-ES bleeding would be similar to that of peptic ulcer bleeding if the same endoscopic methods are applied. There are a few series that specifically describe endoscopic hemostasis of post-ES bleeding, but none of them have a comparative study design^[4,5,9,13,15-17]. Therefore, this retrospective study was conducted to compare the hemostatic efficacy between epinephrine injection alone and epinephrine injection combined with thermotherapy in patients with delayed post-ES bleeding.

MATERIALS AND METHODS

Ethics

The study protocol was approved by the ethical committee at Chang Gung Memorial Hospital (IRB No.: 98-0734B).

Definitions in this study

Immediate post-ES bleeding: Any hemorrhage induced by ES and warranting endoscopic hemostasis within the procedure of ERCP.

Delayed post-ES bleeding: Any hemorrhage after completion of ERCP and manifested by melena, hematemesis or hematochezia associated with a decreased hemoglobin level from baseline.

Re-bleeding: Patients had clinical evidence of recurrent bleeding after initial successful endoscopic hemostasis, and underwent another endoscopy or procedure for the treatment of bleeding.

Coagulopathy: Patients with thrombocytopenia (defined as platelet count $< 80\,000/\mu\text{L}$), coagulopathy (defined as prolonged prothrombin time > 3 s of the control value) and renal failure requiring hemodialysis were considered for statistical analysis as a combined “coagulopathy” group^[18].

Patients

Between 1999 and 2007, 3542 patients underwent 3654 biliary ES procedures at Chang-Gung Memorial Hospital, Linkou Medical Center. ES was performed with a standard pull-type sphincterotome (KD-6G10Q-1; Olympus Co., Tokyo, Japan, or Ultratome and Tapertome; Boston Scientific Co., Spencer, IN, USA). A feedback-controlled generator was used (Erbe, ICC350; Erbe, Tübingen, Germany) and set for Endocut

mode (output limit 120 W). In difficult cases (279 cases; 7.64%) for deep cannulation, precut sphincterotomy was carried out using a needle-knife papillotome (267 cases, Huibregtse single lumen needle knife; Cook Medical Inc., Winston-Salem, NC, USA) or a standard pull-type sphincterotome (12 cases). Sixty-six patients (1.86% of patients or 1.81% of procedures) met the criteria of delayed post-ES bleeding during the study period. Seven of the 66 patients were excluded because they underwent thermal therapy alone (five cases), epinephrine injection plus hemoclippping (one case), or epinephrine injection combined with thermocoagulation and hemoclippping (one case). The remaining 59 patients were enrolled in the study. Of these 59 patients, 26 patients who underwent epinephrine injection therapy alone were classified as the “epinephrine injection group” and 33 patients who underwent epinephrine injection combined with thermotherapy were classified as the “combination therapy group”.

This study retrospectively analyzed the hemostatic efficacy for control of delayed post-ES bleeding between epinephrine injection therapy alone and epinephrine injection combined with thermotherapy. The outcome assessments compared between the groups were; initial endoscopic hemostasis, re-bleeding, complications, requirement of angiographic embolization or surgery, requirement for blood transfusion, and mortality.

Endoscopic therapy

Dilute epinephrine (1:10000) was injected in 0.5-2 mL aliquots into and around the bleeder at the sphincterotomy site until the bleeding was controlled. The group undergoing dual treatments received additional thermotherapy, including heat probe coagulation (18 cases), bipolar coagulation (gold probe, three cases), monopolar coagulation (hot biopsy forceps, 11 cases), and argon plasma coagulation (APC, one case). The heat probe device was not available in our institution after September 2007, and the gold probe was used as the substitute instrument. The settings and application were similar to those used for peptic ulcer bleeding^[19,20]. Endoscopic therapies were carried out within 24 h after initial symptoms of bleeding for inpatients (50 cases), or after arrival in the emergency room for outpatients (nine cases). Epinephrine monotherapy or dual therapy was carried out according to the endoscopist's preference. There was a trend towards performing combination therapy after 2004 (only five patients underwent combination therapy before 2004).

Statistical analysis

Data in the text and tables are expressed as mean \pm SD. The difference was compared using the two-sample *t*-test for continuous variables and the χ^2 -test or Fisher's exact test for categorical variables. The analyses were performed with statistical software of SPSS 15.0 version for Windows. A *P* value of < 0.05 was considered statistically significant.

Table 1 Clinical characteristics of patients in the two groups (mean \pm SD)

	Epinephrine injection group (<i>n</i> = 26)	Combination therapy group (<i>n</i> = 33)	<i>P</i> value
Age (yr)	60.6 \pm 18.1	56.1 \pm 14.2	0.30
Gender (Male/Female)	14/12	20/13	0.60
Indications			0.48
Choledocholithiasis	22	24	
Malignant obstruction	2	3	
Others	2	6	
Possible bleeding risk factors			
Coagulopathy ¹	6	7	0.86
Bile duct stones	22	24	0.27
Precut sphincterotomy	1	6	0.12
Periampullary diverticulum	4	4	0.72
Bleeding during ES ²	11	15	0.81
Cholangitis before procedure	11	16	0.64

¹Coagulopathy including: prolonged prothrombin time $>$ 3 s of the control value; Platelet count $<$ 80000/ μ L; end-stage renal disease requiring hemodialysis; ²All post-ES bleeding occurred during ERCP and required endoscopic hemostasis. ES: Endoscopic sphincterotomy.

Table 2 Bleeding severity, and bleeding stigmata at initial endoscopy

	Epinephrine injection group (<i>n</i> = 26)	Combination therapy group (<i>n</i> = 33)	<i>P</i> value
Bleeding severity			0.61
Mild	9	9	
Moderate	10	17	
Severe	7	7	
Bleeding stigmata			0.70
Active oozing	12	12	
Oozing under an adherent clot	6	7	
Non-bleeding visible vessel	0	1	
Non-bleeding clot	4	9	
Non-bleeding red spots	4	4	

Table 3 Clinical outcomes according to endoscopic therapy (mean \pm SD) *n* (%)

	Epinephrine injection group (<i>n</i> = 26)	Combination therapy group (<i>n</i> = 33)	<i>P</i> value
Initial hemostasis	25 (96.2)	33 (100)	0.44
Re-bleeding ¹	4 (16.0)	4 (12.1)	0.72
Embolization or surgery	3 (11.5)	1 (3.0)	0.31
Bleeding-related death	0	0	1
Transfusion requirement (U)	3.5 \pm 4.6	3.5 \pm 4.5	0.94

¹Re-bleeding after initial successful therapeutic endoscopy.

RESULTS

During ERCP, 854 (24.11%) patients experienced immediate post-ES bleeding. All patients underwent epinephrine injection, and 32 had additional endoscopic therapy. By definition, delayed bleeding occurred in 26 (3.04%) of the 854 patients. The time to onset of delayed bleeding was not significantly different between patients with and without immediate post-ES bleeding

(2.8 \pm 2.7 d *vs* 3.3 \pm 2.7 d, *P* = 0.5).

Clinical characteristics of the 59 patients with delayed post-ES bleeding are outlined in Table 1. There were no significant differences in the mean age, sex distribution, and indications for ERCP between the two groups. None of the participants used anticoagulants from 3 d before or till 3 d after ERCP, and all the ES procedures were performed by experienced endoscopists. Thus, the established risk factors of post-ES bleeding included coagulopathy, bile duct stones, precut sphincterotomy, periampullary diverticulum, immediate post-ES bleeding, and cholangitis before ERCP^[9,21]. There was no statistical difference between the two groups with regard to these parameters. The drop in hemoglobin from baseline was 31 \pm 22 g/L in the epinephrine injection group and 37 \pm 22 g/L in the combination therapy group (*P* = 0.26). The time period between ES and hemorrhage ranged from 9 h to 16 d, and was not statistically different between the two groups (3.5 \pm 3.6 d *vs* 2.8 \pm 1.6 d, *P* = 0.32).

Data regarding bleeding severity and bleeding stigmata at initial endoscopy are listed in Table 2. Bleeding severity was classified according to the established criteria^[10]. There were 9, 10 and 7 cases of mild, moderate, and severe bleeding, respectively, in the epinephrine injection group, and 9, 17 and 7 cases, respectively, in the combination therapy group (*P* = 0.6). At initial endoscopy for delayed post-ES bleeding, the bleeding stigmata were classified as active oozing, oozing under an adherent clot, non-bleeding visible vessel, non-bleeding adherent clot, and non-bleeding red spots. There was no statistically significant difference between the two groups when the bleeding stigmata were compared with respect to these parameters (*P* = 0.70).

Clinical outcome data are summarized in Table 3. The total injected volume of dilute epinephrine was 7.8 \pm 5.8 mL (range: 3-30 mL) in the epinephrine injection group and 9.1 \pm 6.2 mL (range: 3-30 mL) in the combination therapy group (*P* = 0.48). Initial hemostasis was successfully attained in 25 patients from the epinephrine injection group and 33 patients from the combination therapy group (96.2% *vs* 100%, *P* = 0.44). Initial hemostasis was not achieved in 1 patient treated with epinephrine injection alone and the patient went directly to surgery.

The re-bleeding rate was 16% (4 of 25) for the epinephrine injection group and 12.1% (4 of 33) for the combination therapy group. The difference, however, was not significant (*P* = 0.72). The treatment for the four patients with re-bleeding from the epinephrine injection group was as follows: 2 underwent 1 session of endoscopic treatment and the bleeding stopped; 1 went directly to angiographic embolization at re-bleeding; and 1 underwent 1 session of endoscopic treatment for the first re-bleeding - surgery rather than endoscopic treatment was performed to control bleeding at the second re-bleeding. The treatment for the four patients from the combination therapy group was as follows: 1 underwent 1 session of endoscopic treatment and the bleeding stopped; 2 underwent 3 sessions of endoscopic

treatment due to repeated re-bleeding and the bleeding was finally controlled, and 1 underwent 2 sessions of endoscopic combination therapy and surgery was required to finally control bleeding.

None of the patients from the epinephrine injection group experienced any complications of endoscopic hemostasis. One of the patients from the combination therapy group experienced mild pancreatitis after initial endoscopic hemostasis (epinephrine injection plus APC). Another patient from the combination therapy group developed mild pancreatitis after endoscopic treatment (epinephrine injection + heat probe + APC + hemoclip) for the third episode of re-bleeding. The total number of patients requiring angiographic embolization or surgery to control bleeding was 3 in the epinephrine injection group and 1 in the combination therapy group (11.5% *vs* 3.0%, $P = 0.31$). The total number of blood transfusions was not significantly different between the two groups (3.5 ± 4.6 U *vs* 3.5 ± 4.5 U, $P = 0.94$). There was no bleeding-related death in either group.

DISCUSSION

Although the endoscopic approach for post-ES bleeding is similar to that for peptic ulcer bleeding, there is no consensus with regard to the optimal endoscopic hemostasis for treating post-ES bleeding^[7]. For peptic ulcer bleeding, epinephrine injection is the most commonly used, and highly effective, method for control of bleeding; its hemostatic efficacy is comparable to that of epinephrine in combination with additional thermotherapy^[14,19]. From the literature review, it appears that epinephrine injection is the most widely used method for hemostasis of post-ES bleeding. Its success rate in the cases reported in two large series was 97.5% and 100%^[5,9]. The present study shows that epinephrine monotherapy is also highly effective for controlling delayed post-ES bleeding. In addition, the results demonstrate that epinephrine monotherapy is as effective as epinephrine injection combined with thermotherapy, similar to that for peptic ulcer bleeding.

Data regarding bleeding peptic ulcer management suggest that epinephrine injection alone does not achieve permanent hemostasis, and an additional endoscopic treatment such as thermotherapy can reduce the re-bleeding rate^[14]. There are only a few studies that discuss re-bleeding after endoscopic treatment for post-ES bleeding. Ferreira *et al*^[13] reported a re-bleeding rate of 28.4% for 74 patients undergoing various endoscopic treatments for delayed post-ES bleeding. The present study found that the re-bleeding rate between epinephrine monotherapy and combination therapy is not significantly different (16% *vs* 12.1%, $P = 0.72$), implying that additional thermotherapy does not seem to reduce the risk of re-bleeding. Interestingly, the additional thermotherapy used by the endoscopists in this study was most likely performed with the intention of reducing the re-bleeding risk rather than primary

hemostasis, since epinephrine injection alone was highly effective for initial hemostasis. This practice was probably due to the experience of the endoscopists; they applied lessons learned in treating bleeding peptic ulcers.

Thermotherapy alone using multipolar electrocoagulation or a heat probe device has been reported to be effective for controlling post-ES bleeding^[16,17]. Contact thermal therapy, however, has an accurate placement problem, as has been described in bleeding peptic ulcers^[19,22]. In our experience, using a duodenal scope to perform contact thermal therapy is technically difficult when massive bleeding obscures the visual field. In contrast, epinephrine injection does not require accurate targeting. Injection close to the bleeding point will suffice to control bleeding, resulting in a better endoscopic view for more accurate targeting of the additional contact thermal therapy. Under such circumstances, combination therapy is a reasonable alternative.

In this study, none of the patients developed any clinically significant complications after endoscopic epinephrine injection therapy alone. This result confirms those reported in the literature that epinephrine monotherapy is very safe for hemostasis of post-ES bleeding^[5,9,13]. Pancreatitis was the most common complication of endoscopic combination therapy for delayed post-ES bleeding^[13]. In the present study, the only two patients who experienced pancreatitis after endoscopic hemostasis also underwent combination therapy. It is reasonable to consider that an additional thermal procedure would increase risk of complication(s), as has been described in the management of bleeding peptic ulcers^[14,22].

Any bleeding that occurs during ES increases the risk for occurrence of delayed bleeding, and it is suggested that treating "endoscopically significant" immediate bleeding may reduce the risk of delayed bleeding^[11,5,9]. However, the results of the present study do not support this idea: 24.11% of the patients had undergone endoscopic hemostasis for immediate post-ES bleeding, but the rate of clinically significant delayed bleeding was still high (1.81%). The discrepancy between this result and others is possibly because there is no consensus on what is endoscopically significant bleeding and who should receive endoscopic treatment. Furthermore, endoscopically significant bleeding may not become clinically significant^[9,23].

There were more patients requiring angiographic embolization or surgery in the epinephrine injection group (3/26 *vs* 1/33). This result should be interpreted with caution. At re-bleeding, endoscopic treatment was not offered to two of the three patients from the epinephrine injection group: one patient went directly to angiographic embolization at re-bleeding and the other one patient received surgery at the second re-bleeding. In contrast, two patients from the combination therapy group underwent three sessions of endoscopic treatment rather than surgery at their repeated re-bleeding, and the bleeding episodes were finally controlled. Surgery was once the only treatment choice for post-ES bleeding

in early ES, but its usage has fallen from 3% to less than 0.1% because of the improvements in endoscopic techniques and equipment^[1,10,24]. Therefore, endoscopic treatment may be offered to patients with re-bleeding prior to more invasive therapy.

The current study has several limitations. Firstly, it is not a prospective, randomized study. We do not know if there were any of the patients undergoing dual therapy because of epinephrine monotherapy failure. However, as discussed above, an additional thermotherapy was performed possibly to reduce re-bleeding risk rather than epinephrine monotherapy failure. Secondly, four different thermal methods resulted in the heterogeneity of the combination therapy group. It is not known whether different thermal methods would have had similar hemostatic efficacy, although there are no published data indicating that one method is superior to the others.

In summary, the present results show that epinephrine injection is as effective as epinephrine in combination with thermotherapy for treating delayed post-ES bleeding. Considering that epinephrine injection is safe and easy to perform, and that an additional thermotherapy may increase the risk of complications, we would suggest epinephrine injection alone as the first-line therapy for patients with delayed post-ES bleeding.

COMMENTS

Background

With the improvements of endoscopic techniques and equipment, the management of post-endoscopic sphincterotomy (ES) bleeding has shifted from surgery to endoscopic therapy. Delayed post-ES bleeding is less prevalent than immediate post-ES bleeding but it is often of clinical significance and requires more invasive intervention. The endoscopic treatments for delayed post-ES bleeding mirror those for peptic ulcer bleeding. However, the optimal method for treating this type of bleeding has not been determined.

Research frontiers

This is the first study to compare the hemostatic efficacy between epinephrine injection alone and epinephrine injection combined with thermotherapy for delayed post-ES bleeding.

Innovations and breakthroughs

From the literature review, epinephrine injection is the most commonly used and highly effective method to control post-ES bleeding. The study results further demonstrate that epinephrine monotherapy is as effective as epinephrine injection combined with thermotherapy for controlling delayed post-ES bleeding. In addition, an additional thermotherapy does not seem to reduce the risk of re-bleeding.

Applications

The results of this study suggest that epinephrine injection alone can be the first-line therapy for patients with delayed post-ES bleeding.

Terminology

Immediate post-ES bleeding: Any hemorrhage induced by ES and warranting endoscopic hemostasis within the procedure of endoscopic retrograde cholangiopancreatography (ERCP). Delayed post-ES bleeding: Any hemorrhage occurring after completion of ERCP and manifested by melena, hematemesis or hematochezia associated with a decreased hemoglobin level from baseline.

Peer review

Title reflects the major topic and contents of the study. Abstract gives a clear description of the materials and methods, results and conclusions. Significant points have been convincing. Detailed description of methods is provided and statistical methods used are appropriate. Results provide sufficient data to draw firm conclusions. In discussion valuable conclusions are provided. References are appropriate, relevant, and updated. Tables are appropriately presented.

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S- Editor Li LF **L- Editor** Logan S **E- Editor** Zheng XM



Efficacy and safety of transnasal butorphanol for pain relief after anal surgery

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Received: July 12, 2009

Revised: August 31, 2009

Accepted: September 7, 2009

Published online: October 14, 2009

Abstract

AIM: To compare the analgesic properties and efficacy of transnasal butorphanol with intramuscular meperidine after anal surgery.

METHODS: Sixty patients who underwent fistulectomy were enrolled in the study from January 2006 to December 2007. They were randomly divided into transnasal butorphanol ($n = 30$) or intramuscular meperidine ($n = 30$) treatment groups. Assessment of postoperative pain was made using a visual analogue scale (VAS). The VAS score was recorded 6 h after the completion of surgery, before receiving the first dose of analgesic, 60 min after analgesia and the next morning. Any adverse clinical effects such as somnolence, dizziness, nausea or vomiting were recorded. Satisfaction with narcotic efficacy, desire to use the particular analgesic in the future and any complaints were recorded by patients using questionnaires before being discharged.

RESULTS: Forty-two men and eighteen women were included in the study. There were no significant differences in VAS scores between the groups within 24 h. Length of hospital stay and the incidence of adverse effects between the groups were similar. In addition, most

patients were satisfied with butorphanol nasal spray and wished to receive this analgesic in the future, if needed.

CONCLUSION: Butorphanol nasal spray is effective for the relief of pain after fistulectomy. However, it offered patients more convenient usage and would be suitable for outpatients.

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Key words: Butorphanol; Fistulectomy; Meperidine; Opioid

Peer reviewers: Dr. Marc D Basson, Department of Surgery, Wayne State University and John D Dingell VA Medical Center, Detroit, Michigan, 48201, United States; Walter Edwin Longo, Professor, Department of Surgery, Yale University, New Haven, Connecticut, 06510, United States

Mai CM, Wan LT, Chou YC, Yang HY, Wu CC, Jao SW, Hsiao CW. Efficacy and safety of transnasal butorphanol for pain relief after anal surgery. *World J Gastroenterol* 2009; 15(38): 4829-4832 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4829.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4829>

INTRODUCTION

Patients who undergo anal surgery always complain of intractable pain and appropriate analgesia is an important issue for these patients. Butorphanol is a synthetic opioid analgesic with agonist activity for the κ opioid receptor and antagonist activity for the μ opioid receptor^[1,2]. Butorphanol nasal spray is an alternative method which avoids hepatic metabolism and provides easier use^[1]. Several reports have demonstrated that butorphanol nasal spray is beneficial in treating pain after cesarean section, migraine headache, dental surgery, acute musculoskeletal pain and biliary colic^[3-10]. However, no studies have been reported for pain control after fistulectomy. The prospective objective of this study was to evaluate the analgesic properties and efficacy of butorphanol nasal spray after fistulectomy.

MATERIALS AND METHODS

After approval from the institutional review board of Tri-

Service General Hospital and on receipt of each patient's written informed consent, 60 patients (fistula in ano, intersphincteric type) scheduled for fistulectomy were enrolled from January 2006 to December 2007. Patients were randomly divided into the transnasal butorphanol treatment group ($n = 30$) and the intramuscular meperidine control group ($n = 30$) using a random number table. Exclusion criteria were an American Society of Anesthesiologists physical classification $> II$, any history of atrophy sinusitis or repeated epistaxis, previous anorectal surgery, inflammatory bowel diseases, hematologic disorders, significant cardiovascular disease, impaired renal function (serum creatinine > 1.5 mg/dL), hepatic disease (twice the upper normal limit of AST or ALT levels) or psychiatric disorders.

During surgery, a monitoring system was attached to each patient. This included pulse oximetry and noninvasive blood pressure measurement. A standardized heavy station intramuscular analgesia (meperidine 1 mg/kg, midazolam 0.08 mg/kg), local perianal anesthesia (2% lidocaine, 0.5% bupivacaine, 1:200 000 epinephrine) and surgical technique (fistulectomy) were prescribed for all patients. A gauze roll was placed in the anal canal for compression and this was removed 8 h later. At 6 h after completion of surgery, all patients were given oral analgesia (tolfenamic acid 10 mg). Subsequently, oral analgesia was prescribed regularly every 6 h. The patients in the butorphanol group received one spray (1 mg) at least every 4 h if they were in pain. In the control group, the patients received intramuscular meperidine (0.8 mg/kg) at least every 4 h if in pain.

Postoperative pain was assessed using a 10-point subjective visual analogue scale (VAS, 0 = "no pain" and 10 = "maximum pain"). The VAS score was recorded 6 h after the completion of surgery, before receiving the first dose of butorphanol or meperidine, 60 min after the analgesic prescribed and the next morning after removal of the gauze roll. Length of hospital stay and any adverse effects of the medicines such as somnolence, dizziness, nausea or vomiting were recorded. The VAS score and adverse effects were measured by an experienced nurse. Before discharge, the patients were asked three questions using questionnaires: (1) Are you satisfied with the narcotic efficacy? (2) How do you feel about the adverse effects? (3) Would you ask for the medicine again if necessary?

Statistical analysis was performed using SYSTAT (version 9.0, SPSS, Inc., Chicago, IL, USA). Each patient's demographic information and the VAS scores were compared between groups using Student's t or χ^2 tests. Side effects between groups were analyzed using the Fisher's exact test. The Wilcoxon rank-sum test was used to compare length of hospital stay between the groups and $P < 0.05$ was considered statistically significant.

RESULTS

Of the 60 patients, 42 (70%) were men and 18 (30%) were women. The mean age of the patients in the butorphanol group was 37.7 years and was 38.9 years in the meperidine

Table 1 Patient characteristics ($n = 30$, mean \pm SD)

	Butorphanol group	Meperidine group	<i>P</i> value
Age (yr)	37.70 \pm 10.76	38.90 \pm 10.89	0.669
Sex (%)			0.778
Male	20 (66.7)	22 (73.3)	
Female	10 (33.3)	8 (26.7)	
Weight (kg)	62.70 \pm 10.29	64.83 \pm 12.01	0.463
Height (cm)	164.57 \pm 7.03	165.73 \pm 7.18	0.528

Table 2 Results (mean \pm SD)

Variable	Butorphanol group	Meperidine group	<i>P</i> value
Operative time (min)	46.17 \pm 15.59	48.43 \pm 10.80	0.515
Frequent of analgesia (times)	3.03 \pm 1.67	1.23 \pm 0.504	< 0.001
VAS (6 h after surgery)	5.67 \pm 0.88	5.77 \pm 1.00	0.684
VAS (before analgesia)	8.17 \pm 1.66	8.31 \pm 1.36	0.719
VAS (after analgesia)	5.93 \pm 1.41	5.52 \pm 1.12	0.216
VAS (the next morning)	6.13 \pm 1.47	5.93 \pm 1.14	0.560
Hospital stay (d)	3.63 \pm 0.69	3.73 \pm 0.74	0.585

Table 3 Adverse effects n (%) ($n = 30$)

Adverse effect	Butorphanol group	Meperidine group	<i>P</i> value
Any side effect	16 (53.3)	10 (33.3)	0.192
Somnolence	10 (33.3)	5 (16.7)	0.23
Dizziness	6 (20.0)	4 (13.3)	0.73
Nausea	5 (16.7)	4 (13.3)	1.00
Vomiting	2 (6.7)	2 (6.7)	1.00

group. The mean surgical time was 46.17 min in the butorphanol group compared with 48.43 min in the meperidine group. The demographic details did not differ significantly between two groups (Table 1). The overall frequency of analgesic usage was 3.03 times in the butorphanol group compared with 1.23 times in the meperidine group ($P < 0.001$). The VAS scores were similar between the groups (Table 2). The mean VAS score 6 h after completion of surgery was 5.67 in the butorphanol group compared with 5.77 in the meperidine group. The mean VAS score before analgesic was 8.17 in the butorphanol group compared with 8.31 in the meperidine group. The mean VAS score after analgesia was 5.93 in the butorphanol group compared with 5.52 in the meperidine group. The mean VAS score the next morning was 6.13 in the butorphanol group compared with 5.93 in the meperidine group (Figure 1). The mean hospital stay was 3.63 d in the butorphanol group compared with 3.73 in the meperidine group.

Several adverse effects were recorded (Table 3). Sixteen patients complained of side effects in the butorphanol group and 10 patients complained of side effects in the meperidine group. The incidence of somnolence, dizziness and nausea was higher in the butorphanol group than in the meperidine group. However, no significant difference in adverse effects between the groups was observed. In the questionnaires, most patients reported good satisfaction with the analgesic they received; 22 patients in the butorphanol group (73%) would be happy

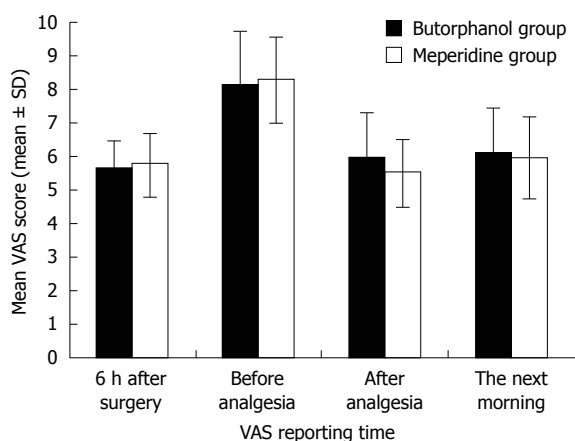


Figure 1 Pain level was scored from 0 to 10.

to receive butorphanol nasal spray for analgesia in the future if necessary. However, in the butorphanol group, two patients felt they had a poor analgesic response and four patients complained of multiple side effects. Three patients in the meperidine group complained of multiple side effects such as somnolence, dizziness and nausea.

DISCUSSION

Butorphanol nasal spray proved effective in relieving pain after fistulectomy. These data also demonstrate that butorphanol was equivalent to meperidine for analgesia. This is consistent with the literature on the treatment of moderate to severe pain^[3-10]. Butorphanol has been approved in an injectable formula in Taiwan since 1979. Initially, it was prescribed for intravenous or intramuscular administration to avoid the problem of hepatic first-pass metabolism^[1]. In 1992, a transnasal formulation was developed to avoid the reduced bioavailability of oral administration. Compared with the oral formulations, transnasal administration produces higher maximum concentration, rapid absorption and better pain relief^[1]. It is also 4-8 times more potent than morphine and 30-40 times more potent than meperidine^[11]. Peak plasma concentration is reached 30-60 min after 1 mg transnasal administration^[12,13]. Moreover, butorphanol nasal spray can be self-administered, is noninvasive and allows convenient usage for patients, especially outpatients.

In both treatment groups, the VAS scores were lower 6 h after completion of surgery. This could reflect the long anesthetic effect of bupivacaine. Subsequently, the VAS score increased and then decreased. The frequency of requests for butorphanol was higher than for meperidine ($P < 0.001$). However, the reduced VAS pain scores were similar in both groups. Moreover, transnasal butorphanol was noninvasive, convenient and more acceptable to patients. Thus, butorphanol nasal spray could be considered as an alternative device for patient-controlled analgesia.

From the questionnaires, we found that 22 patients (73%) would prefer to receive butorphanol nasal spray in the future, if necessary. However, two patients in the

butorphanol group felt that they had a poor analgesic response and these patients developed severe adverse effects. Therefore, to achieve the maximum benefit from butorphanol nasal spray, clinicians should inform patients about the possible adverse effects. Patients must also be alerted to the sedative properties of butorphanol. Most importantly, patients should be cautioned to avoid activities such as driving or operating equipment until the analgesia has worn off.

This study had several limitations, the main limitation being the small sample size. A larger sample size would have offered better information on efficacy and adverse effects of the medications. In addition, with a larger sample size statistically significant differences between the groups may have been observed. Secondly, the VAS pain scores were subjective, possibly reflecting inadequate instruction. Moreover, we did not design a placebo group as it would be unethical to withhold analgesia. Thus, we cannot draw any conclusion as to how butorphanol or meperidine might compare with placebo treatment.

In conclusion, butorphanol nasal spray was effective for relief of pain after fistulectomy. In addition, it offers patient-controlled and more convenient usage than intramuscular meperidine.

ACKNOWLEDGMENTS

The butorphanol nasal spray was donated by the Lotus Pharmaceutical Co., Ltd., Taipei, Taiwan, China.

COMMENTS

Background

Postoperative pain is the major patient complaint after anal surgery. Our purpose was to investigate the analgesic properties and efficacy of transnasal butorphanol after anal surgery.

Research frontiers

Postoperative pain was assessed using a visual analogue scale (VAS). However, VAS scales are subjective and the results possibly reflect inadequate instruction.

Innovations and breakthroughs

Several reports have demonstrated that butorphanol nasal spray is beneficial in treating postoperative pain. This is the first study to report that it is also effective for pain relief after anal surgery.

Applications

As butorphanol nasal spray is safe and effective for relief of pain after anal surgery, it might offer convenient usage for outpatients.

Peer review

It is a small and possibly worthwhile clinical trial with a novel analgesic regimen.

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S- Editor Cheng JX L- Editor Webster JR E- Editor Zheng XM



Clinicopathological significance of LRP16 protein in 336 gastric carcinoma patients

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Supported by Grant from the Ministry of Science and Technology of China, No. 2010CB912802

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Received: June 19, 2009 Revised: August 22, 2009

Accepted: August 29, 2009

Published online: October 14, 2009

Abstract

AIM: To investigate the expression of leukemia related protein 16 (LRP16), and the possible relationship between LRP16 expression and clinicopathological indices in 336 gastric carcinoma patients.

METHODS: Immunohistochemistry was used to detect LRP16 expression in 336 cases of paraffin-embedded gastric carcinoma tissues and 60 cases of distal normal mucosa. The relationships between LRP16 expression and patients' age, tumor size, histological grade, clinical stage, metastatic status and prognosis were analysed.

RESULTS: The expression of LRP16 was 58.6% (197/336) in gastric carcinoma and 31.7% (19/60) in distal normal gastric mucosa. The expression of LRP16 in carcinoma was significantly higher than that in normal mucosa tissues ($\chi^2 = 14.929$, $P = 0.001$). LRP16 protein expression was found in 44.1% (63/143) carcinomas at stage I and II, and 69.4% (134/193) carcinomas at stage III and IV ($\chi^2 = 21.804$, $P = 0.001$), and in 56.9% (182/320) of cancers without metastasis but 93.8% (15/16) of those with metastasis ($\chi^2 = 8.543$, $P = 0.003$). The expression of LRP16 was correlated with tumor size, infiltrative depth, clinical stage, lymphatic invasion and distant metastasis (all

$P < 0.05$). Follow-up data showed that there was a significant difference in median survival time between cancer patients with expression of LRP16 (27.0 mo) and those without (48.0 mo, Log rank = 31.644, $P = 0.001$).

CONCLUSION: The expression of LRP16 may be associated with invasion, metastasis and prognosis of gastric cancer.

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Key words: Gastric neoplasms; Immunohistochemistry; Leukemia related protein 16; Prognosis

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Li YZ, Zhao P, Han WD. Clinicopathological significance of LRP16 protein in 336 gastric carcinoma patients. *World J Gastroenterol* 2009; 15(38): 4833-4837 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4833.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4833>

INTRODUCTION

Leukemia related protein 16 gene (*LRP16*), localized on chromosome 11q12.1, is an important estrogen-responsive gene^[1-5]. It has been found expressed at a high level in testicles, ovaries and mucosa of colon, at a moderate level in prostate, small intestine, spleen and thymus, and at a low level in peripheral blood leucocytes^[2,3]. LRP16 is also an estrogen receptor alpha ($ER\alpha$), coactivator^[6], and it may play an important role in ER signaling pathways. Since gastric cancer is the second leading cause of cancer-related death worldwide^[7], it is important for us to assess its prognosis according to the expression of some markers. It has been reported that ER is expressed in gastric cancer^[8], but no reports have ever evaluated the expression of *LRP16* gene in gastric carcinoma so far. In this study we retrospectively analyzed the relationships between the expression of LRP16 and clinicopathological factors in 336 Chinese patients with gastric cancer.

MATERIALS AND METHODS

Biopsy specimens

Paraffin embedded sections of 336 gastric carcinomas and 60 distal normal gastric tissues were obtained from the Department of Pathology, Chinese People's Liberation Army (PLA) General Hospital (Beijing, China) from 1998 to 2001. Of these patients, 17 were grade I, 65 grade II and 254 grade III, according to histological grading; 66 were stage I, 77 stage II, 147 stage III and 46 stage IV, according to clinical TNM stage revised by UIAC in 2003; and 75 were tubular adenocarcinoma (well-moderately differentiated adenocarcinoma), 32 were mucinous adenocarcinoma, 183 were poorly differentiated adenocarcinoma, 35 were signet-ring cell carcinoma, 11 were other gastric carcinoma according to histological type, respectively. By March, 2008 (the time of data analysis), 251 patients were dead, and 85 patients were alive. The median survival time was 36 mo (range, 0.17-120 mo).

Immunohistochemistry

All samples were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut 4 μ m/L thick from wax blocks, mounted on to APES-coated glass slides. Slides were deparaffinized in xylene twice for 10 min, rehydrated through graded ethanols to distilled water before incubation for 10 min with 3% hydrogen peroxidase-methanol to inhibit endogenous peroxidase activity, and heated in 0.01 mol/L citrate buffer (pH 6.0) in a microwave oven for 5 min at 100°C after reaching boiling point for antigen retrieval. Then the slides were taken out of microwave oven to be cooled at room temperature for 15 min. After incubating for 20 min in a blocking solution containing 10% normal goat serum in PBS, sections were incubated at 4°C overnight in a humidified chamber with rabbit polyclonal antibody to human LRP16 (recognized and isolated in 1999 by Department of Molecular Biology of our hospital) diluted 1:400 in blocking solution. The sections were rinsed in PBS and incubated for 30 min with biotinylated secondary antibody (Poly peroxidase anti-mouse/rabbit IgG, Zymed). After washing in PBS, the sections were then incubated for 30 min at 37°C. 3,3'-Diaminobenzidine was used as the chromogen. Slides were counterstained for 3 min with hematoxylin solution. Normal ovarian tissue was used as a positive control for every lesion, whereas the primary antibody was replaced by PBS as a negative control.

Evaluation of score

In scoring LRP16 protein expression, both the extent and intensity of immunopositivity in the cell nucleus were considered. The intensity of staining was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong. The extent of staining was scored as follows: 0, < 5%; 1, > 5%-25%; 2, > 25%-50%; 3, > 50%-75%; 4, > 75% of the cells in the respective lesions. The final score was determined by multiplying the intensity of staining and the extent of staining scores, yielding a range from 0

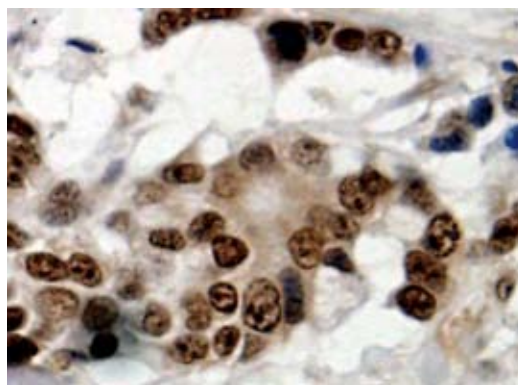


Figure 1 Positive LRP16 expression in gastric adenocarcinoma (IHC, $\times 400$). The case received 3 scores (strong) relating to intensity of staining, and 3 scores (> 50%-75%) relating to extent of staining. Thus the final score was 9, which was defined as strong staining pattern (++).

to 12. Scores 9-12 were defined as preserved or strong staining pattern (++), 5-8 were defined as weak staining pattern (+) and 0-4 were defined as markedly reduced or negative expression (-).

Statistical analysis

For the statistical analysis, SPSS 13.0 for Windows (SPSS Inc, Chicago, Illinois) was used. The clinical variables were analyzed with the χ^2 test. The survival rates were calculated by the Kaplan-Meier method, and the outcomes of treatment were evaluated with the log-rank test. Finally, multivariate analysis was performed to determine the independent prognostic factors by Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

RESULTS

LRP16 expression in gastric carcinoma and normal mucosas

LRP16 was assessed by IHC in 336 gastric cancer cases, with the following results: negative expression (-) in 41.4% (139/336) cases, weak staining (+) in 37.2% (125/336) cases, strong staining (++) in 21.4% (72/336) cases. In normal cases, LRP16 showed a negative expression in 68.3% (41/60) cases, a weak expression in 21.7% (13/60) cases, and a strong expression in 10.0% (6/60) cases. In total, LRP16 protein was expressed (+ or ++) in 58.6% (197/336) gastric carcinoma, but was expressed (+ or ++) only in 31.7% (19/60) of distal normal stomach mucosa (Figures 1 and 2). LRP16 protein was localized mainly in the nucleus of cancer cells or normal epithelial cells. A greatly significant difference was found in the expression of LRP16 protein between gastric carcinoma and normal gastric mucosa tissues ($\chi^2 = 14.929$, $P = 0.001$).

Relationships between LRP16 expression and histological grade, clinical stage and prognosis

The proportion of LRP16 expression showed an increasing trend from smaller tumor to bigger tumor. Significant positive correlations were found between

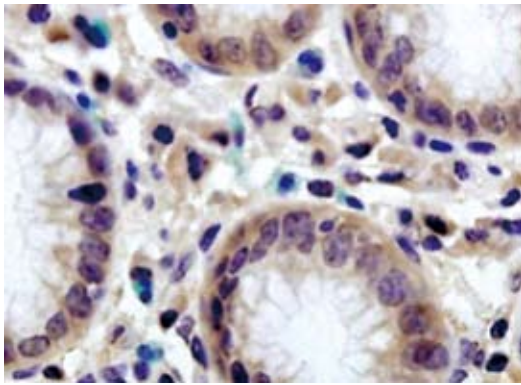


Figure 2 Negative LRP16 expression in normal gastric mucosa (IHC, $\times 400$). The case received 1 score (weak) relating to intensity of staining, and 0 scores ($< 5\%$) relating to extent of staining. Thus the final score was 0, which was defined as negative expression (-).

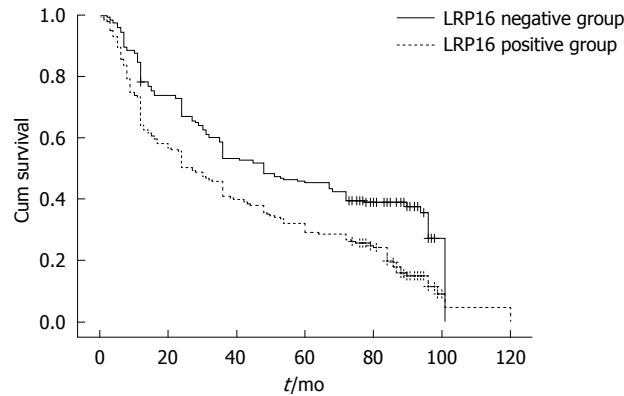


Figure 3 Survival status in LRP16 positive and negative groups of gastric cancer patients.

Table 1 Comparison of clinicopathological factors between LRP16 positive and LRP16 negative patients with gastric carcinoma *n* (%)

Variable	LRP16		Statistics value
	Positive group (<i>n</i> = 197)	Negative group (<i>n</i> = 139)	
Gender			
Male	165 (60.2)	109 (39.8)	$\chi^2 = 1.544$ $P = 0.214$
Female	32 (51.6)	30 (48.4)	
Age (yr)			
≤ 40	19 (55.9)	15 (44.1)	$\chi^2 = 1.544$ $P = 0.756$
41-65	114 (57.6)	84 (42.4)	
> 65	64 (61.5)	40 (38.5)	
Tumor size (cm)			
< 4	36 (46.8)	41 (53.2)	$\chi^2 = 11.593$ $P = 0.003^a$
4-7	117 (58.2)	84 (41.8)	
≥ 8	44 (75.9)	14 (24.1)	
Histologic differentiation			
Well differentiated	8 (47.1)	9 (52.9)	$\chi^2 = 1.165$ $P = 0.558$
Moderately differentiated	40 (61.5)	25 (38.5)	
Poorly differentiated	149 (58.7)	105 (41.3)	
Depth of invasion, T stage			
T1	10 (43.5)	13 (56.5)	$\chi^2 = 9.041$ $P = 0.029^a$
T2	55 (53.4)	48 (46.6)	
T3	113 (60.4)	74 (39.6)	
T4	19 (82.6)	4 (17.4)	
Lymph node metastasis			
0	49 (44.1)	62 (55.9)	$\chi^2 = 18.946$ $P = 0.000^a$
1-6	77 (60.2)	51 (39.8)	
7-15	49 (68.1)	23 (31.9)	
> 15	21 (84.0)	4 (16.0)	
Distant metastasis			
Negative	182 (56.9)	138 (43.1)	$\chi^2 = 8.543$ $P = 0.003^a$
Positive	15 (93.8)	1 (6.2)	
TNM stage			
I - II stage	63 (44.1)	80 (55.9)	$\chi^2 = 21.804$ $P = 0.000^a$
III-IV stage	134 (69.4)	59 (30.6)	

^a $P < 0.05$, statistically significant.

LRP16 expression and depth of invasion, lymph node metastasis, and distant metastasis (all $P < 0.05$). Meanwhile, an increasing trend in LRP16 expression was also observed in clinical stage, from 44.1% (63/143) at stage I and II to 69.4% (134/193) at stage III and IV carcinomas ($\chi^2 = 21.804$, $P = 0.001$) (Table 1). Moreover, follow-up data showed that there was a significant difference in

Table 2 Cox regression analysis of prognostic factors in gastric carcinoma

Prognostic variables	B	SE	Wald value	P value	RR
Gender	0.041	0.112	0.134	0.714	1.042
Age	0.523	0.078	45.169	0.000 ^a	1.687
Histologic differentiation	0.143	0.105	1.830	0.176	1.153
Histologic type	0.192	0.051	14.344	0.000 ^a	1.212
Depth of invasion	0.240	0.119	4.063	0.044 ^a	1.271
Lymph node metastasis	0.278	0.100	7.746	0.005 ^a	1.321
Distant metastasis	1.058	0.214	24.495	0.000 ^a	2.881
TNM stage	-0.016	0.131	0.015	0.901	0.984
Tumor size	0.686	0.082	70.279	0.000 ^a	1.986
LRP16 expression	0.174	0.094	3.466	0.063	1.190

^a $P < 0.05$, statistically significant. B: Partial regression coefficient; RR: Relative risk.

median survival time between the carcinomas with LRP16 expression (27 mo) and those without (48 mo), and the overall 5-year survival rate (45.8%) of the LRP16 positive group was better than that of the LRP16 negative group (39.1%) (Log rank = 31.64, $P = 0.001$) (Figure 3).

LRP16 expression and Cox proportional hazards model

By Cox proportional hazards model, age, tumor size, histological type, depth of invasion, lymph node metastasis, and distant metastasis were proved to be statistically significant, but LRP16 was not an independent prognostic indicator (Table 2).

DISCUSSION

LRP16 was originally isolated from lymphocytes in order to identify a leukemia relapse-related gene, but there was no difference between patients primarily diagnosed with acute myeloid leukemia (AML)^[9]. Later some studies demonstrated that *LRP16* gene plays an important role in the carcinogenesis and progression of hormone-dependent breast cancer^[5,6]. LRP16 overexpression markedly promoted the proliferation of MCF-7 human breast cancer cells by promoting G1/S transition through increasing the cyclin E and cyclin D1 protein level^[4,6]. On the contrary, suppression of *LRP16* gene

expression inhibited MCF-7 cell growth and sensitized tumor cells to radiation^[10]. It has been proposed that estrogen affects the expression of *LRP16* gene, and its expression was strongly dependent on the estrogen activities^[9,11]. However, ectopic expression of *LRP16* in ER α -negative cells has no effect on proliferation^[6]. Among breast cancer patients, *LRP16* expression was significantly correlated with tumor size, lymph node metastasis, and clinical stage^[12]. Clinical data has shown that *LRP16* is overexpressed in primary breast cancer samples compared with their matched normal tissues^[12]. In our study, *LRP16* expression is in relation to tumor size, depth of invasion, lymph node metastasis, distant metastasis and TNM stage. The expression of *LRP16* in carcinoma is significantly higher than that in normal mucosa. Two types of ERs, ER α and ER β , have both been identified in non-cancerous and cancerous gastric tissue^[13,14]. Therefore, we propose that *LRP16*, a coactivator of ER α , may have a similar function on gastric cancer as on breast cancer. Activation of the ER signaling pathway plays important roles in multi-tissue development^[15-18], which implies that *LRP16* may display an important function in ER α target tissue development.

In conclusion, *LRP16* protein may play an important role in the carcinogenesis, progress and prognosis of Chinese gastric carcinoma and *LRP16* expression detected by immunohistochemistry may be a simple and useful molecular marker to predict the prognosis in gastric carcinoma patients. The association between *LRP16* and ER in gastric cancer development needs to be further investigated, from which *LRP16* targeting with anti-estrogen therapy may be applied in gastric cancer patients.

ACKNOWLEDGMENTS

The authors thank all colleagues (Department of Molecular Biology, Chinese People's Liberation Army General Hospital) who have done much work on *LRP16*. We greatly appreciate the comments from the reviewers for improving the manuscript.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer death worldwide and it is important for us to assess its prognosis according to the expressions of some markers. The expression of *LRP16* may be associated with invasion, metastasis and prognosis of gastric cancer. Therefore, *LRP16* may play a significant role in the evolution of gastric carcinoma.

Research frontiers

LRP16 is an important estrogen-responsive gene. Some studies have demonstrated that *LRP16* gene plays an important role in the carcinogenesis and progression of hormone-dependent breast cancer. It has also been reported that ER is expressed in gastric cancer, but no reports have evaluated the expression of *LRP16* gene in gastric carcinoma so far. In this study the authors retrospectively analyzed the relationships between the expression of *LRP16* and the clinical and pathological factors for Chinese patients with gastric cancer.

Innovations and breakthroughs

Recent reports have demonstrated the important roles of *LRP16* in *in vitro* cell studies. Particularly in breast cancers, *LRP16* is over-expressed. This is the

first study to report that *LRP16* is also over-expressed in gastric carcinoma. Furthermore, our study shows that *LRP16* expression is in relation to tumor size, depth of invasion, lymph node metastasis, distant metastasis and TNM stage in gastric cancer.

Applications

The *LRP16* expression status detected by immunohistochemistry may be a simple and useful molecular marker to predict the prognosis in gastric carcinoma patients.

Terminology

LRP16, *Leukemia related protein 16* gene, localized on chromosome 11q12.1, is an important estrogen-responsive gene. It has been proposed that the expression of *LRP16* gene is strongly dependent on the estrogen activities. *LRP16* gene plays an important role in the carcinogenesis and progression of hormone-dependent breast cancer. Estrogen receptors (ERs), including ER α , ER β and the recently discovered ER β cx, have been identified in non-cancerous and cancerous gastric tissue. The biological mechanisms behind this are not yet clear.

Peer review

The authors examined the expression of *LRP16* protein, and the possible relationship between *LRP16* expression and clinicopathological indices in gastric carcinoma patients. It revealed that the expression of *LRP16* might be associated with invasion, metastasis and TNM stage of gastric cancer. The results are interesting and may provide us with a new molecular marker to assess the prognosis of gastric carcinoma.

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S- Editor Li LF **L- Editor** O'Neill M **E- Editor** Zheng XM



BRIEF ARTICLES

Research on focal nodular hyperplasia with MSCT and postprocessing

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Received: April 18, 2009 Revised: August 12, 2009

Accepted: August 19, 2009

Published online: October 14, 2009

were seen in 24 lesions, and were integrated into the parenchymatous lesions with a gradual and smooth course. On the contrary, there were no artery penetrated into the lesion found in any of comparative hepatic tumors.

CONCLUSION: Doctors could make a correct diagnosis and differentiation of FNH on evaluation of the characteristic appearance on MSCT with postprocessing.

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Key words: Angiography; Computer-assisted image processing; Focal nodular hyperplasia; Liver diseases; X-ray; Computed tomography

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Liu YJ, Fan WJ, Yuan ZD, Liu PC, Wang CR, Yan WQ, Wang SM, Chen JH, Liu Z. Research on focal nodular hyperplasia with MSCT and postprocessing. *World J Gastroenterol* 2009; 15(38): 4838-4843 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4838.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4838>

Abstract

AIM: To investigate and evaluate the pathological features and diagnostic value of focal nodular hyperplasia (FNH) with multi-section spiral computed tomography (MSCT) and postprocessing.

METHODS: A total of 25 patients with FNH who had undergone MSCT and postprocessing were included in the investigation. All patients had been pathologically or clinically confirmed with FNH. A number of 75 cases of hepatic carcinomas, hemangiomas and adenomas were randomly selected at a same period for a comparative study.

RESULTS: There was a single focus in 22 cases and multiple foci in 3 cases. On the plain scan, 17 lesions showed hypodensity, 7 isodensity and 4 hyperdensity (the case with fatty liver). With contrast, 28 lesions were enhanced evenly or in the nodules in the arterial phase; 13 lesions still showed hyperdensity, 11 lesions isodensity and 4 lesions hypodensity in the parenchymatous phase; in the delayed phase only 5 lesions showed hyperdensity but 9 lesions showed isodensity or slight hypodensity and 14 lesions showed hypodensity. Twelve lesions of 28 had central asteroid scars. Thickened feeding arteries in postprocessing

INTRODUCTION

Hepatic focal nodular hyperplasia (FNH) was first described and proposed by Edmondson^[1] in 1958, then verified a decade later by the World Health Organization in 1975. At that time cases were too few to be investigated, and were considered as "local sclerotization", "hepatic hamartoma", "hepatic adenoma" and so on^[2]. With the improvement in imaging techniques, the number of cases diagnosed with FNH have been gradually increasing. At present, the incidence of FNH is just less than that of hemangiomas and has become the second most common benign hepatic disease^[3]. Although there have been a number of papers on FNH with different modalities in recent years^[4-7], multi-section spiral computed tomography (MSCT) was considered as one of the common facilities and could demonstrate some radiological characteristics

to diagnose with FNH^[8]. This article discusses more critical imaging findings on relating to the histological features in FNH through analyzing a total of 25 patients with 28 lesions and 75 cases of other hepatic tumors by MSCT and postprocessing.

MATERIALS AND METHODS

Study source

Authors collected and analyzed data of 25 FNH patients with 28 lesions within 2 institutions, the Cancer Center of Sun Yat-sen University and Peking University Shenzhen Hospital, from January 2005 to December 2007. The patients underwent CT examination and were confirmed with FNH pathologically or clinically. None of the patients had hepatitis, and all alpha-fetoprotein tests were normal. There were 14 men and 11 women, age range 14-59 years, and mean age 35.5 years. Of the 25 patients, 11 patients were confirmed with FNH by pathology after surgery resection, 14 patients were ascertained to have FNH by relative examinations and clinical follow-up for at least 18 mo with a stable shape and no marked change or diminution of the lesion. Meanwhile 30 hepatic carcinomas, 30 hemangiomas and 15 adenomas were randomly selected at a same period with MSCT and postprocessing for a control group.

CT scan protocols

All 25 patients underwent 16-section spiral CT scanning (Aquilion 16, Toshiba Medical Corporation). CT projection radiographs were scanned firstly for range confirmation, from the apex of the diaphragm to the base of the liver. After the plain scan, a bolus of about 100 mL (average 1.5 mL/kg body weight) contrast media of iodixanol (Iohexol, 300 mgI/mL) was injected into the antecubital vein at a flow rate of 3-5 mL/s for the enhanced scan. Bolus tracking was performed with the region of interest on the descending aorta at the level of diaphragm apex, and the arterial phase scan was automatically started 6 s after attenuation in the region of interest reached a pre-defined threshold of 120 HU. Three-phase scans were performed after contrast media injection: arterial phase scan (about 20-25 s), parenchymatous or vein phase scan (about 65-70 s), delayed scan (about 150-180 s). Scan parameters: tube voltage 120 kVp, tube current 300 mA, gantry rotation time 0.5 s per rotation, matrix 512 × 512, beam pitch 0.9375, detector collimation thickness 16 × 1 mm, abdomen standard reconstruction algorithm, and effective thickness of reconstruction images 1 mm.

Imaging postprocessing and analysis

To study the multi-phase images and analyze the dynamic changes in the lesions, the images from the arterial phase and parenchymatous phase were transferred to workstation (Vitrea2, Vital, USA) for postprocessing, with the use of volume rendering technique (VRT) and maximum intensity projection (MIP) to display the arteries supplying the lesions. Three radiologists evaluated the images in a double-blind manner, recorded the main parameters and analyzed the results.

RESULTS

Size and shape of FNH

A total of 25 patients with 28 lesions were studied, among them 22 patients with single lesions, the other 3 with multiple lesions. The sizes of the lesions ranged from 1.0 to 8.3 cm. All lesions assumed a circular or circular-type shape with a clear boundary except 2, which showed irregular lobulated shapes.

Location of FNH

There were 6 lesions at the right anterior lobe, 13 at the right posterior lobe, 6 at the left lobe and 2 near the diaphragm in the right lobe, one projecting outside the hepatic boundary.

Pathology of FNH

Specimens showed some pale yellow tubercular structures with many separations, part of which were found with asteroid scars, but no real fibrillar involucrem. Under microscopy fibroplasia, permeated with acute or chronic inflammation, could be seen. The center or periphery of the scars were rich in fibrous tissue and small vessels. No malignant tumor cytology was observed.

MSCT and postprocessing findings

Of the 25 patients with 28 FNH lesions, on plain CT 17 lesions showed hypodensity, 7 isodensity and 4 hyperdensity (in the 4 patients with fatty liver, one of whom had multiple lesions). With contrast, 28 lesions showed even or nodular enhancement in the arterial phase; 13 showed hyperdensity, 11 isodensity and 4 hypodensity in the parenchymatous phase; 5 showed hyperdensity, 9 isodensity or slight hypodensity and 14 hypodensity in the delayed phase. Of the 28 lesions, 12 were found to have a central asteroid scar (Figure 1A-D), with radiating form, and among these, 4 scars were reduced in size in the parenchymatous and delayed phases (Figure 2), 5 scars vanished in the parenchymatous and delayed phases with the lesions showing isodensity, and the other 3 scars showed high density (Figure 1D) in the late delayed phase; also, blood vessels could be seen in some central asteroid scars. The authors also found one example of FNH simultaneously accompanied by hemangiomas (Figure 3). Exceptionally, 6 lesions assumed a ring-like enhancement (Figure 4) in the parenchymatous and delayed phases which were likely mixed with a false involucrem as seen in some hepatic tumors. Thickened blood-supplying arteries were demonstrated in 24 lesions with VRT or MIP in postprocessing (Figure 5A and B), and the arteries followed a gentle line with gradual and direct entry into the body of the lesion. On comparative study, there were no such an artery penetrated into the lesion found in any of other hepatic carcinomas, hemangiomas or adenomas.

DISCUSSION

FNH manifestation and characteristics in this group

FNH pathogenesis was once thought to occur most



Figure 1 A patient with FNH on the left hepatic lobe. A: Hypodensity of the lesion with lower density of the central asteroid scar; B: High enhancement with a thickened blood-supplying artery detected in the arterial phase; C: Normal density of the lesion and lower density of the central scar in the parenchymatous phase; D: Normal density of the lesion and slightly higher density of the central scar in the delayed phase.

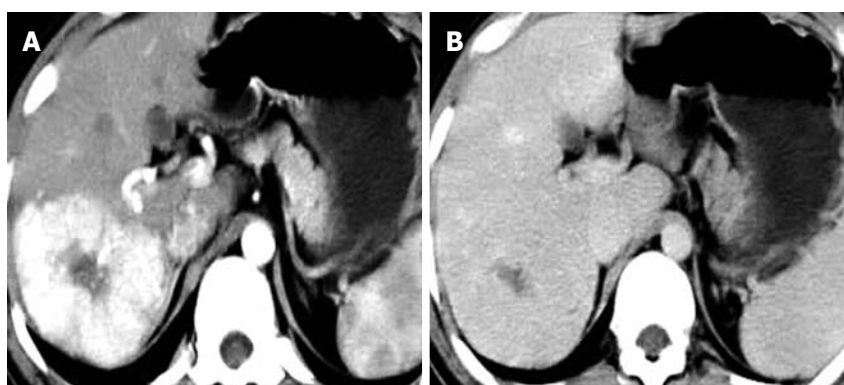


Figure 2 A FNH lesion located on the hepatic right posterior lobe was evenly highly enhanced except for the central asteroid scar in the arterial and parenchymatous phases (A), the scar being reduced in size in the delayed phase (B).

frequently in young women^[9], whereas in this study it occurred in more males than females, but the shape of the lesion was larger in females than in males. A relationship between FNH and oral contraceptives remains inconclusive. The use of contraceptives does not increase FNH incidence, but may possibly promote FNH growth^[10]. A recent report indicates that FNH is possibly linked with hereditary factors in an unusual manifestation of hepatic organization^[11].

This FNH group primarily appeared with a single lesion (22/25). Most of these cases had no marked clinical symptoms, and only a minority of this group presented with pain and feeling unwell, or a suspicious lump in the right upper abdomen, one of which was clinically thought to be in danger of rupture or hemorrhage. The majority of FNH cases were discovered on routine physical examination and then investigated further.

The FNH pathology has 3 major characteristics: (1) The pathological change is not a real tumor, but mainly normally functioning hepatic cells, with proliferation of the connectivum, the abnormal bile duct and

macrophages; (2) The existence of abnormal blood vessels and fiber structure, namely a scar in the center of the pathological change; (3) The blood supply pattern radiated from the inside to the periphery^[12-14]. Under the microscope may be seen fibrous divisions and an area of proliferating hepatic cells in FNH, which lacks a normal vein and portal vein, and sometimes has acute or chronic inflammatory cells^[15]. In addition there is a special type of FNH called multiple FNH syndrome^[16,17], which comprises more than 2 FNH lesions, simultaneously merged at least with one of the following pathological changes: hepatic angiocavernoma, artery structure damage, blood vessel abnormality in the central nervous system, meningioma or neurogliocytoma. This group included an example of multiple FNH combined with hepatic angiocavernoma (Figure 3).

It should be noted that on the border of the FNH lesions, there were expanded blood vessels or blood sinuses, which assumed an incomplete enhanced link round the tumor (Figure 4), and could easily be taken for a malignant tumor with involucrum enhancement.

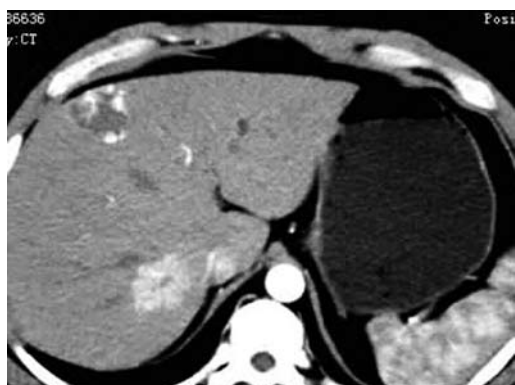


Figure 3 A patient with FNH (hepatic right posterior lobe) accompanied by hemangiomas (left medial lobe). In the arterial phase a border nodular enhancement can be seen in the hemangiomas but even enhancement was demonstrated in FNH except for the central scar.



Figure 4 An arrow shows the ring-like enhancement mimicking a false involucreum.

Appearance of FNH in MSCT scanning with postprocessing

The majority of FNH showed as normal or slightly low density tumors in CT scans, demonstrating a clear boundary, even density, and no calcification^[18,19]. In enhanced scanning, the FNH characteristics alternated as follows: in the arterial phase the lesion quickly and obviously increased evenly or in the nodules from the central area; in the parenchymatous and delayed phases the contrast agent flowed out evenly and the low density lesion appeared. These enhanced characteristics were the result of the rich arterial blood supply and major vein drainage and generous blood sinuses within FNH. The center asteroid low density scar in 12 of 28 (42.8%) lesions has a distinctive feature to differentiate FNH from other tumors with a rich blood supply. MIP or direct volume rendering (DVR) could demonstrate the supplying artery to the central scar (Figure 5). In the parenchymatous and delayed phase the scars were enhanced gradually to equilibrium or to slightly high density, and only 2 remained at low density. The authors believe that it might be possible that scar proliferation could lead to obliteration of the vessel lumen, which could easily be confused with necrosis or a collagen scar within fibrous lamina-like cancer, hepatocellular carcinoma or cholangiocellular carcinoma^[20].

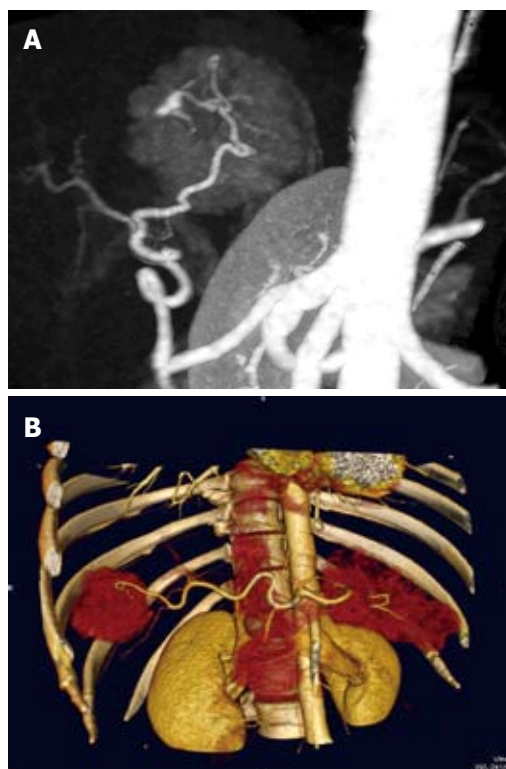


Figure 5 Imaging with use of MSCT postprocessing technology. A: A thickened blood-supplying artery was demonstrated in the FNH lesion on maximum intensity projection, and was centrifugal type with radiating blood supply within the lesion; B: Another case of FNH, the volume rendering technique showed the artery following a gentle line gradually entering into the body of the lesion.

The technology of DVR or MIP may directly demonstrate the pattern of blood supply in FNH with just the enhanced scanning raw data, the results of which provide new significant evidence for FNH diagnosis. The arteries supplying blood in FNH often appeared thickened, and had a smooth, gradual course, entering directly into the central area of the lesion. The pathology demonstrated that FNH has one or several blood-supplying arteries, which radiate from the center to the circumference^[21]. The major blood supply and drainage vessels have been demonstrated by color Doppler^[22,23]. In the scar center were vasa vasorum which proliferated and radiated throughout. All of these characteristics showed marked features different from hemangiomas or other cancers of the liver.

Differential diagnosis

The central scar may either appear in FNH or fibrous lamina-like hepatocellular carcinoma, and their imaging appears extremely similar. However, the FNH may occur as a single or multiple lesion, and in the arterial phase the lesions are markedly enhanced, their density approaching that of the aorta, whereas the fibrous lamina-like hepatocellular carcinoma usually appears as a single lesion with a diameter sometimes greater than 10 cm, with the central scar seldom or slightly enhanced. FNH may present false involucreum signs in certain case which could be confused with malignancy^[24], but, by

postprocessing, arterial vessels of hepatic carcinoma were revealed to be unevenly thickened, damaged, and discontinuous^[25].

Both FNH and hemangiomas of the liver could be markedly enhanced but in different ways. The typical enhancement of hemangiomas started from the periphery, with nodular or ring-like enhancement, advancing gradually to the center, with delayed phase backfill to equal density or high density. In contrast, enhancement of FNH started at the center of the lesion, rapidly and evenly spreading throughout, except for the central scar, which consistently strengthened then abated on the delayed phase. The process in hemangiomas enhanced slowly (representing slow bloodstream) and some atypical small hemangiomas may backfill completely in the arterial phase, but in the parenchymatous and delayed phases still assumed a high or equal density, which was a crucial difference from FNH^[26].

A patient with hepatic adenoma commonly has a history of taking contraceptives and/or has had celiacgia for a long time. Sometimes a ring-like bright band may be seen at the edge of an adenoma. As they bleed easily, some hematoma within the lesion result in a very different outcome of imaging compared with FNH^[27]. The peripheral enhancement in adenoma possibly indicated a wealth of blood-supplying vessels under the big involucre. The enhancement in adenoma was mostly centripetal, in contrast to the FNH centrifugal type^[28].

There are a variety of imaging techniques for visualizing FNH in different aspects^[29]: single photon emission CT (SPECT) can make a differentiation according to whether or not there are Kupffer cells in the lesion^[30]; color ultrasound may allow a rudimentary diagnosis based on finding radiating blood flow; magnetic resonance imaging emphasizes the special signal intensity in the FNH and its central scar and even its spectrum^[31]. However, MSCT with postprocessing technology provides a common mean of entirely recognizing the pathological characteristics of FNH, not only demonstrating the central asteroid scar and shape of the lesion, but also the tissue enhancing process, the blood-supplying model and hemodynamics^[32]. The following points should be used clinically to consider as FNH: (1) The MSCT plain scan presents a low or slightly low density lesion with an even, clear boundary and lower density central asteroid scar, which shows delayed enhancement; (2) In the arterial phase, the lesion is rapidly and evenly enhanced except for the central scar, in the parenchymatous phase, the lesion has normal or slightly high density, and in the delayed phase it has low density; (3) The lesion has a thickened but symmetrical blood-supplying artery^[33] entering gradually into the lesion. These are FNH pathological characteristics, on which clinicians could make a definite FNH diagnosis, no longer requiring further auxiliary investigations, biopsy or surgery.

diseases of the liver. As a benign disease, correct diagnosis of focal nodular hyperplasia (FNH) is vital for choosing the appropriate treatment protocol.

Research frontiers

Imaging techniques have been used to investigate the variants of FNH, e.g. single photon emission CT (SPECT) examined whether or not there were Kupffer cells in the lesion, color ultrasound investigated radiating blood flow, magnetic resonance imaging measured particular signal intensity in the central scar, whereas MSCT, by intravenous injection of contrast medium, can delineate the lesion shape, size, supplying arteries and hemodynamics, which could reflect the pathological manifestations of FNH.

Innovation and breakthroughs

On detailed FNH case observation, this paper has introduced different enhanced modes in the lesion and the central scar. By postprocessing in a workstation, the authors found a thickened but symmetrical artery entering into the lesion with a radiating blood supply, which is crucial in revealing the pathological characteristics of FNH and in making a conclusive diagnosis.

Applications

Based on the results of this study, a contrast method with postprocessing is recommended in diagnosis of hepatic lesions with MSCT, in order to reveal the features of enhancement, the central scar and supplying artery in FNH.

Terminology

FNH was one of the more recently recognized diseases, but its pathogenesis was still unknown. It is not a real tumor, but a mass of normal functional hepatic cells, with proliferation of the connectivum. FNH was often confused clinically with some other malignant and benign tumors such as hepatocellular carcinoma, hepatic hemangioma, etc.

Peer review

An important imaging technique has been addressed in this paper. Using a contrast method and postprocessing in a workstation, multi-section spiral computed tomography offers significant features which may reveal the characteristics of FNH pathology, and therefore the technique could make a reliable FNH diagnosis before biopsy or surgery.

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COMMENTS

Background

There are several kinds of lesions which provoke a conflict in diagnosis of

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S- Editor Tian L L- Editor Cant MR E- Editor Ma WH

BRIEF ARTICLES

Decreased expression of *Neurensin-2* correlates with poor prognosis in hepatocellular carcinoma

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Received: July 17, 2009 Revised: August 24, 2009

Accepted: August 31, 2009

Published online: October 14, 2009

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Key words: *Neurensin-2*; Hepatocellular carcinoma; Expression; Immunohistochemical analysis; Prognosis

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Ma HQ, Liang XT, Zhao JJ, Wang H, Sun JC, Chen YB, Pan K, Xia JC. Decreased expression of *Neurensin-2* correlates with poor prognosis in hepatocellular carcinoma. *World J Gastroenterol* 2009; 15(38): 4844-4848 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4844.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4844>

Abstract

AIM: To investigate the expression of *Neurensin-2* (*NRSN2*) in hepatocellular carcinoma (HCC) and its prognostic values in predicting survival.

METHODS: The expression and prognostic significance of *NRSN2* in HCC was examined by performing immunohistochemical analysis using a total of 110 HCC clinical tissue samples, and Western blotting analysis to further confirm the result.

RESULTS: Decreased *NRSN2* expression was shown in 70.9% cases. Loss of *NRSN2* expression in HCC was significantly related to tumor size ($P = 0.006$). Larger tumor size was related to negative expression of *NRSN2*. Patients showing negative *NRSN2* expression had a significantly shorter overall survival than those with positive expression ($P = 0.008$). Multivariate Cox regression analysis indicated that *NRSN2* expression level was an independent factor of survival ($P = 0.013$). Western blotting analysis further confirmed decreased expression of *NRSN2* in tumor tissues compared with non-tumorous tissues.

CONCLUSION: Our study indicated that *NRSN2* could be a tumor suppressor gene for HCC and a candidate biomarker for long-term survival in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related death in the world^[1,2]. In China, it is the second leading cause of cancer death among males^[3]. Factors associated with increased risk of HCC include HBV infection, HCV infection, chronic alcohol consumption, cirrhosis, hemochromatosis and aflatoxin *etc.* Among them, chronic HBV infection is the most common cause of HCC especially in China. However, there are currently very limited therapeutic options for advanced or metastatic HCC. It is therefore critical to understand the genetic background and molecular pathogenic processes involved in the carcinogenesis of HCC, to aid the development of rational, targeted therapies^[4,5].

In a recent publication, Zender *et al*^[6] combined an integrated cancer genomic analysis, RNA interference (RNAi) technology and cancer-susceptible mouse models to discover and validate tumor suppressor genes contributing to HCC. The approach resulted in the functional validation of 13 tumor suppressor genes, including *Exportin 4* (*XPO4*), *DEAD box polypeptide 20* (*DDX20*), *Gap junction protein, delta 4* (*GJD4*), *Follistatin-like 5* (*FSTL5*) and *Neurensin-2* (*NRSN2*) *etc.*

Interestingly, the vast majority of these identified genes had not previously been linked to cancer.

To validate and further study the potential value of these tumor suppressors, we randomly selected *NRSN2* from those genes showing a higher possibility of being tumor suppressors, and performed immunohistochemical analysis and Western blotting analysis to determine the expression of *NRSN2* in HCC, further identified its relationship to clinicopathological features and evaluated its prognostic value to post-resectional survival in HCC.

MATERIALS AND METHODS

Patients and tumor tissue sample

A total of 110 HCC surgical resection specimens were collected at the Sun Yat-sen University Cancer Center between January 2001 and December 2002. The 110 patients included 96 males and 14 females with a median age of 45 years (range, 22-74 years). None of the patients had received radiotherapy or chemotherapy prior to surgery. Both tumor and corresponding non-tumorous tissues not less than 2 cm away from the HCC were sampled, respectively, and proved by pathological examination. All tissue samples were fixed in 10% formalin and embedded in paraffin, and consecutive 4 μ m sections were cut. The histological types were assigned according to the criteria of the World Health Organization classification. The diagnosis of liver cirrhosis was based on the case records and pathological data of HCC patients from Sun Yat-sen University Cancer Center.

Immunohistochemical analysis

The sections were deparaffinized and rehydrated through graded ethanol, then endogenous peroxidase was inhibited with 0.3% hydrogen peroxide. For antigen retrieval, slides were boiled in EDTA (1 mmol/L, pH 8.0) for 15 min in a microwave oven. After rinsing with PBS, the sections were incubated with primary antibody (rabbit anti-NRSN2, Sigma-Aldrich, Inc. USA) in PBS (1:100) at 37°C for 3 h, and then incubated with horseradish peroxidase (ChemMate™ DAKO EnVision™ Detection Kit) at 37°C for 30 min. Finally, the visualization signal was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and then all of the slides were counterstained in hematoxylin. For negative controls, tissue sections were immunoreacted without anti-NRSN2 antibody under the same experimental conditions.

Semi-quantitative method

The total *NRSN2* immunostaining score was calculated as the sum of the percent positivity of stained tumor cells and the staining intensity. The percent positivity was scored as "0" (< 5%, negative), "1" (5%-25%, sporadic), "2" (25%-50%, focal), "3" (> 50%, diffuse). The staining intensity was scored as "0" (no staining), "1" (weakly stained), "2" (moderately stained), and "3" (strongly stained). Both percent positivity of cells and staining intensity were decided under double-blind conditions. The final *NRSN2* expression score was calculated using

the value of percent positivity score \times staining intensity score, which ranged from 0 to 9. We defined *NRSN2* expression level as follow: "-" (score 0-1), "+" (score 2-3), "++" (score 4-6) and "+++" (score > 6).

Western blotting analysis

The frozen HCC samples including tumor or non-tumorous tissue were homogenated in a RIPA lysis buffer, and lysates were cleared by centrifugation (14000 rpm) at 4°C for 30 min. About 40 μ g protein samples were run on a 15% SDS-PAGE gel and transferred to PVDF membrane. After blocking non-specific binding sites for 60 min with 5% non-fat milk, the membranes were incubated overnight at 4°C with primary polyclonal antibody against *NRSN2* (at 1:200 dilution). The membrane was then washed three times with TBST for 10 min and probed with HRP-conjugated secondary antibody (at 1:2000 dilution) for 30 min at room temperature. After being washed three times, the membrane was developed by an enhanced chemiluminescence system (ECL, Pierce).

Statistical analysis

Quantitative values were expressed as mean \pm SD or median (range). Categorical variables were enumeration data from counting the number of samples. The χ^2 test for proportion and Pearson's correlation were used to analyze the relationship between *NRSN2* expression and various clinicopathologic characteristics. For survival analysis, the main outcome was overall survival rates which were calculated from the date of surgery to the date of death. Follow-up time was censored if the patient was lost to follow-up. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. Cox proportional-hazard analysis was used for univariate and multivariate analysis to explore the effect of clinicopathological variables and *NRSN2* expression on survival. The SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses and a *P*-value < 0.05 was considered significant.

RESULTS

Expression of NRSN2 in HCC tissues

NRSN2 immunostaining was mostly in the cytoplasm. Overall, 32 of 110 (29.1%) cases had positive expression (*NRSN2* + or ++) in tumor cells (Figure 1A and B), 78 of 110 (70.9%) cases had negative expression (*NRSN2*-). In cases with adjacent hyperplastic tissue, we often observed a sharp contrast between infiltrative tumor areas of negative staining and the adjacent non-tumorous tissue of positive staining (Figure 1C). In addition, we further performed Western blotting analysis to detect the expression of *NRSN2* and the result was consistent with that of immunohistochemical analysis (Figure 2). Correlations between the expression of *NRSN2* and various clinicopathologic parameters are listed in Table 1. The *NRSN2* expression was significantly related to tumor size (*P* = 0.006). Negative expression of *NRSN2* was associated with larger tumor size. However, there was

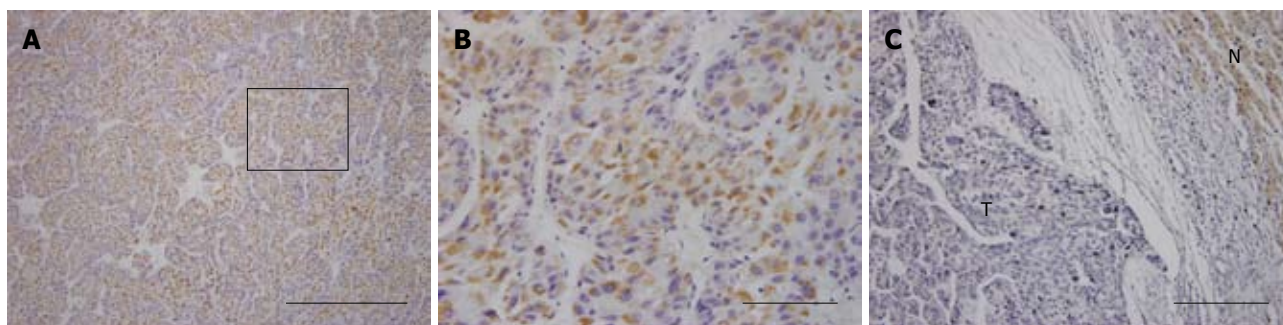


Figure 1 Immunohistochemical staining of *NRSN2* in HCC. A and B: Positive staining of *NRSN2* at two different magnifications ($\times 100$ and $\times 400$), respectively, scored as *NRSN2* (++); C: Immunostaining of HCC and adjacent surrounding non-tumorous tissue showing a sharp contrast between infiltrative tumor area of negative staining ("T") and the adjacent non-tumorous tissue of positive staining ("N") ($\times 200$). The length of the bar in A, B and C is 500, 100 and 200 μm , respectively.

Table 1 Correlations between *NRSN2* expression and clinicopathologic variables of 110 cases of HCC

Clinicopathologic variables	n	<i>NRSN2</i> expression		χ^2	P-value
		Negative	Positive		
All cases	110	32	78		
Age				0.037	0.848
< 50	60	43	17		
≥ 50	50	35	15		
Gender				0.341	0.559
Female	14	9	5		
Male	96	69	27		
Tumor size (cm)				7.678	0.006 ^a
< 5	37	20	17		
≥ 5	73	58	15		
Histological differentiation				0.879	0.258
Well	18	12	6		
Moderate	69	49	20		
Poor	23	17	5		
Liver cirrhosis				0.176	0.675
Yes	55	38	17		
No	55	40	15		
Metastasis				0.152	0.697
Yes	16	12	4		
No	94	66	28		
HBsAg status				3.400	0.065
Positive	96	71	25		
Negative	14	7	7		
Serum AFP ($\mu\text{g/L}$)				0.360	0.549
Positive (≥ 25)	80	58	22		
Negative (< 25)	30	20	10		

^a $P < 0.05$. *NRSN2*: Neurensin-2; HCC: Hepatocellular carcinoma.

no statistically significant difference between *NRSN2* expression and age, gender, histological differentiation, liver cirrhosis, metastasis, HBsAg status, or serum AFP.

Univariate and multivariate analyses of prognostic variables in HCC patients

The 5-year overall survival rates were 61.2% and 29.8%, respectively, in patients with positive and negative *NRSN2* expression. Patients showing negative *NRSN2* expression had a significantly shorter overall survival than those with positive expression ($P = 0.008$, log-rank test; Figure 3). Univariate Cox regression analysis also identified that clinical variables including tumor size, liver cirrhosis, serum AFP and *NRSN2* expression were significantly associated with overall survival (Table 2).

Table 2 Overall survival Cox regression analysis

Variables	Relative risk (95% CI)	P-value
Univariate		
Gender	0.674 (0.308-1.474)	0.320
Age	0.689 (0.423-1.123)	0.133
Tumor size	1.752 (1.028-2.986)	0.037 ^a
Histological differentiation	1.728 (0.855-3.495)	0.083
Liver cirrhosis	1.689 (1.047-2.725)	0.030 ^a
HBsAg	1.488 (0.680-3.252)	0.316
Serum AFP	1.990 (1.105-3.585)	0.019 ^a
Metastasis	1.368 (0.733-2.554)	0.323
<i>NRSN2</i>	0.470 (0.265-0.836)	0.008 ^a
Multivariate		
Liver cirrhosis	1.739 (1.076-2.808)	0.024 ^a
Serum AFP	1.957 (1.086-3.528)	0.025 ^a
<i>NRSN2</i>	0.481 (0.270-0.854)	0.013 ^a

^a $P < 0.05$.

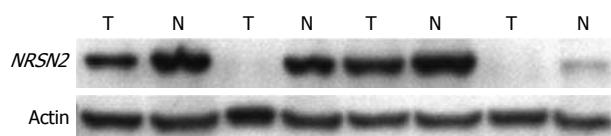


Figure 2 Western blotting analysis of *NRSN2* expression in HCC. Protein extracted from paired tumor tissues (T) and surrounding non-tumorous liver tissues (N) in four patients with HCC.

Furthermore, to evaluate the potential of *NRSN2* expression as an independent predictor for overall survival of HCC, multivariate Cox regression analyses (Forward: LR) were performed. While the others failed to demonstrate independence, liver cirrhosis, serum AFP level and *NRSN2* expression may play a role in predicting the overall survival in HCC ($P = 0.024$, 0.025 , and 0.013 , respectively, Table 2).

DISCUSSION

HCC is one of the most deadly human carcinomas. Even with improved diagnosis and compositive therapy, the prognosis of HCC remains dismal^[7]. Therefore, prognostic molecular biomarkers are invaluable for the clinician to evaluate patients and to aid in tumor control.

Recently, Zender *et al*^[6] introduced a new, effective and high-yield approach for identifying liver tumor

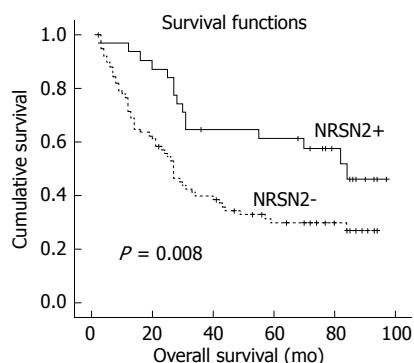


Figure 3 Kaplan-Meier survival analysis of primary hepatocellular carcinoma patients ($n = 110$) after surgical resection with negative *NRSN2* expression ($n = 78$) and positive *NRSN2* expression ($n = 32$). The survival rate for patients in the *NRSN2* negative group ("−") was significantly lower than that for patients in the *NRSN2* positive group ("+") (log rank, $P = 0.008$).

suppressors. They first used representational oligonucleotide microarray analysis (ROMA), a high-resolution array-based comparative genomic hybridization (CGH) platform, to narrow down the field of potential candidate genes. To further accelerate the study of cancer genes *in vivo*, they adapted stable RNAi technology, utilizing microRNA-based short hairpin RNAs (shRNAs), that are potent triggers of the RNAi machinery and can efficiently suppress gene expression when expressed from a single genomic copy^[8,9], to downregulate tumor suppressor genes in mice^[10]. In addition, to facilitate a more rapid and cost-effective analysis of cancer gene action *in vivo*, they developed a "mosaic" mouse model of HCC^[11]. In this mouse model, HCCs with different oncogenic lesions can be rapidly produced by genetic manipulation of cultured embryonic liver progenitor cells (hepatoblasts) followed by their retransplantation into the livers of recipient mice^[11,12].

According to Zender^[6], they selected 16 shRNAs targeting 14 different genes for validation. The result showed that many of the candidate shRNAs triggered tumor growth above background, with those targeting *Xpo4*, *Ddx20*, *Gjd4*, *Fstl5*, and *Nrsn2* showing the most prominent acceleration of tumor growth. Interestingly, most of these genes previously have not been linked to cancer.

To validate and further study the potential value of tumor suppressors in the results of Zender, we randomly selected *NRSN2* as a sample, and performed immunohistochemical analysis and Western blotting analysis to determine the expression of *NRSN2* in HCC and evaluate its potential clinical relevance.

To our knowledge, there are no studies of *NRSN2* in cancer, not to mention HCC. *NRSN2* encodes a 21 983 Da protein composed of 204 amino acids, belongs to the vesicular membrane protein (VMP) family, and shows a high sequence homology to Neuensin-1. So far, there is no definite function for *NRSN2*. After retrieval in UniProt Knowledgebase, it may play a role in maintenance and/or transport of vesicles, according to its sequence similarities with Neuensin-1, and it is uncertain whether Met-1 or Met-2 is the initiator (<http://www.uniprot.org/>

[uniprot/Q9GZP1#section_comments](http://www.uniprot.org/entry/UniProtKB:Q9GZP1#section_comments)).

In the present paper, using immunohistochemistry technology, we demonstrated that negative *NRSN2* protein expression was found in 70.9% of HCC samples (78/110). This was significantly associated with larger tumor size ($P = 0.006$) and was significantly correlated with poor patient outcome ($P = 0.008$). Regardless of whether *NRSN2* has prognostic significance, decreased expression of *NRSN2* was observed in larger tumors, which does support the hypothesis that *NRSN2* may play a role in inhibiting tumor progression. Metastasis is one of the characteristics of progression of HCC. However, in our studies, no significant correlation was observed between *NRSN2* expression and metastasis, indicating that some other mechanisms may be more important in moderating the metastasis of HCC, since hepatocarcinogenesis is a multi-step and complex process associated with accumulation of genetic and epigenetic changes^[13]. Further multivariate Cox regression analysis indicated that *NRSN2* expression level was an independent factor of survival and may constitute a prognostic factor for patients with HCC after surgery. Western blotting analysis further confirmed decreased expression of *NRSN2* in tumor tissues compared with paired non-tumorous tissues.

At the same time, we also performed some experiments with the gene *XPO4* to investigate its expression and prognostic values in HCC, and the result was similar to that of *NRSN2* (data not shown). All these data supported the result of Zender and further validated the utility of this new approach. Therefore, we have reason to believe other genes identified by Zender are tumor suppressors, although more studies are still needed to further confirm this.

What's more, in the study of Zender, *NRSN2* was also found to be frequently deleted in human breast cancer, indicating that *NRSN2* may be relevant to other epithelial malignancies as a tumor suppressor. However, to elucidate the molecular mechanism role of *NRSN2* in liver carcinogenesis and its relation with other tumor types, more studies still should be done.

ACKNOWLEDGMENTS

We gratefully acknowledge Professor Min-Shan Chen for his help in collecting tumor tissue samples.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most deadly human carcinomas. Even with improved diagnosis and compositive therapy, the prognosis of HCC remains dismal. Therefore, prognostic molecular biomarkers are invaluable for the clinician to evaluate patients and to aid in tumor control.

Research frontiers

A research article published on November 26, 2008 in "Cell" introduced a new, effective and high-yield approach for identifying liver tumor suppressors by combing an integrated cancer genomic analysis, RNA interference (RNAi) technology and cancer-susceptible mouse models. The approach resulted in identification of 13 tumor suppressor genes, including *Exportin 4* (*XPO4*), *DEAD box polypeptide 20* (*DDX20*), Gap junction protein, delta 4 (*GJD4*), *Follistatin*

like 5 (*FSTL5*) and *Neurensin-2* (*NRSN2*) etc. Interestingly, the vast majority of these identified genes had not previously been linked to cancer. Therefore, more work is needed to validate and further study the potential value of these tumor suppressors.

Innovations and breakthroughs

In the present study, for the first time, by using immunohistochemistry technology, *NRSN2* expression was found to be decreased in 70.9% of cases ($n = 110$). Loss of *NRSN2* expression in HCC was significantly related to tumor size ($P = 0.006$). Larger size tumors were related to negative expression of *NRSN2*. Patients showing negative *NRSN2* expression had a significantly shorter overall survival than those with positive expression ($P = 0.008$). Multivariate Cox regression analysis indicated that *NRSN2* expression level was an independent factor of survival. Western blotting analysis further confirmed decreased expression of *NRSN2* in tumor tissues compared with paired non-tumorous tissues.

Applications

Because *NRSN2* down-regulation frequently occurs in HCC, the authors propose that *NRSN2* may be a candidate tumor suppressor gene for HCC, and may be used as a candidate biomarker for long-term survival in HCC.

Terminology

NRSN2 encodes a 21983 Da protein composed of 204 amino acids, belongs to the vesicular membrane protein (VMP) family, and shows a high sequence homology to *Neurensin-1*. So far, there is no definitive function for *NRSN2*.

Peer review

Ma *et al* investigated the expression of *NRSN2* in 110 cases of HCC immunohistochemically and by Western blotting, and found a correlation between its negative expression and tumor progression. Although the function of *NRSN2* has not been clarified, their result was interesting and this molecule might be a new prognostic marker of HCC.

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S- Editor Cheng JX L- Editor O'Neill M E- Editor Lin YP



Acute chylous ascites mimicking acute appendicitis in a patient with pancreatitis

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Received: June 14, 2009 Revised: August 3, 2009

Accepted: August 10, 2009

Published online: October 14, 2009

Abstract

We report a case of acute chylous peritonitis mimicking acute appendicitis in a man with acute on chronic pancreatitis. Pancreatitis, both acute and chronic, causing the development of acute chylous ascites and peritonitis has rarely been reported in the English literature. This is the fourth published case of acute chylous ascites mimicking acute appendicitis in the literature.

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Key words: Chylous ascites; Pancreatitis

Peer reviewer: Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Smith EK, Ek E, Croagh D, Spain LA, Farrell S. Acute chylous ascites mimicking acute appendicitis in a patient with pancreatitis. *World J Gastroenterol* 2009; 15(38): 4849-4852 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4849.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4849>

INTRODUCTION

Chylous ascites is the accumulation of chyle in the peritoneal cavity. It develops following disruption of the lymphatic system caused by traumatic injury or by either benign or malignant processes^[1]. It is usually a chronic process. This phenomenon is rarely associated with the symptoms and signs of peritonitis. Acute abdominal pain with peritonism due to sudden extravasation of chyle into the peritoneal cavity is rare but has been described in the literature.

CASE REPORT

A 38-year-old indigenous Australian man presented to the emergency department of our institution with a 24-h history of generalised abdominal pain localizing to the right iliac fossa. Associated symptoms included anorexia and nausea but no vomiting or diarrhoea. On examination he was systemically unwell with a temperature of 38.6 and was tachycardiac with a pulse of 115 beats per minute. On examination the patient was exquisitely tender in the right iliac fossa with rebound tenderness and peritonism, consistent with a diagnosis of acute appendicitis. His medical history was significant for chronic alcohol dependence and chronic pancreatitis. The patient admitted to drinking in excess of 120 g of alcohol daily but denied alcohol consumption for the two days prior to presentation. He had no significant surgical history.

Haematology and biochemistry investigations on presentation demonstrated deranged liver function, a macrocytosis and thrombocytopenia. Gamma glutamyl transaminase was elevated at 1392 U/L, with lesser elevations of alkaline phosphatase and alanine transferase, consistent with his known history of alcohol (ETOH) abuse and probable underlying alcoholic liver disease. C reactive protein (CRP) and white cell count (WCC) were not elevated. Blood film was normal and blood cultures negative. Lipase was elevated at 153 U/L.

The patient was taken to theatre with a clinical diagnosis of acute appendicitis. Subsequently a grid-iron incision was made revealing "milky" peritoneal fluid which at the time was presumed to be pus secondary to a perforated viscus despite its atypical appearance.

The appendix was not inflamed. Specimens were taken for biochemistry and microbiology. The decision was then made to perform a laparoscopy with a view to identifying the source of the fluid. When no obvious cause could be found, a midline laparotomy incision was made. The duodenum and stomach were mobilized, the lesser sac entered to exclude a perforated peptic ulcer while the remainder of the small bowel and colon were examined with no evidence of perforation. The only significant finding was that of a malrotated pancreas with an indurated and erythematous head consistent with acute pancreatitis. At this stage a presumed diagnosis of chylous peritonitis was made. Drain tubes were inserted and the midline incision was closed. The patient was discharged on day 4 after an uneventful post-operative course. In the initial few days post-operatively, there was significant drainage of the small blood-stained milky fluid seen at laparotomy. Interestingly, after several hours of stasis there was sedimentation of the blood from chyle within the drain tube bags. Fluid was sent for lipoprotein electrophoresis to confirm the diagnosis of chylous ascites.

A CT scan was completed on day 1 post-operatively to define the anatomy of the pancreas and exclude other pathology. The CT report confirmed an annular pancreas with changes surrounding the pancreatic head representing focal pancreatitis. There were no pancreatic calcifications seen.

The peritoneal fluid was significant for cholesterol of 7.3 mmol/L and triglycerides of 26.0 mmol/L (2280.7 mg/dL), highly suggestive of chylous effusion. Fluid amylase and lipase levels were elevated at 115 U/L and 2165 U/L, respectively, suggesting pancreatitis as the aetiology in this case. Plasma triglyceride levels were not measured.

DISCUSSION

Pathophysiology and aetiology

Chylous ascites is the accumulation of a milk-like peritoneal fluid rich in triglycerides, due to the presence of thoracic or intestinal lymph in the abdominal cavity^[1]. Causes of chylous ascites (Table 1) relate to disruption of the lymphatic system due to traumatic injury or obstruction.

Chronic chylous ascites is usually asymptomatic, however, abdominal pain from chylous peritonitis, with sudden outpouring of chyle into the peritoneum, has been described in the literature. A review of 140 cases of all forms of chylous ascites showed that 21% of adults and 14% of children with chylous ascites have the acute form^[2].

Pancreatitis is a recognised but rare cause of chylous effusion. In most published cases pancreatitis, usually chronic, results in the development of chronic chylous ascites without acute abdominal pain. Until 1984 only two cases of pancreatitis, both chronic, had been reported as causes of chylous effusions^[3]. Goldfarb^[4] described the first case of acute pancreatitis associated with acute chylous ascites, abdominal pain and

Table 1 Aetiology of chylous ascites^[3]

Congenital (most common in the paediatric population)
Congenital idiopathic
Intestinal lymphangiectasia (mega lymphatics)
Primary lymphatic hypoplasia
Chyle cysts
Lymphangiomatosis
Acquired
Neoplastic (most common in adult population)
Malignant
Lymphoma
Kaposi's sarcoma
Lymphangiomyomatosis
Carcinoid tumours
Other cancers (breast, pancreatic, colon, renal, testicular, ovarian, prostate)
Benign
Postoperative
Resection of the abdominal aorta
Retroperitoneal lymphadenectomy
Pancreaticoduodenectomy
Vagotomy
Radical nephrectomy
Warren shunt
Nissen fundoplication
Placement of peritoneal dialysis catheter
IVC resection
Inflammatory
Radiation therapy
Tuberculosis
Pancreatitis
Filariasis/ascariasis
Peritoneal dialysis
Sarcoidosis
Constrictive pericarditis
Retroperitoneal fibrosis
Coeliac sprue
Whipple's disease
Retractile mesenteritis
Traumatic
Blunt (including Battered Child Syndrome)
Shear force to the root of the mesentery
Penetrating
Obstructive
Adhesions
Volvulus
Intussusception
Aortic aneurysm
Haemodynamic
Cirrhosis
Right heart failure
Dilated cardiomyopathy
Jugular, innominate, left subclavian, or portal vein thrombosis
Nephrotic syndrome

peritonism in 1984.

In his review of the literature, Goldfarb discussed three other cases of chylous effusion which were likely secondary to pancreatitis. Acute abdominal pain and peritonism were not features of these presentations. Since 1984 few cases of pancreatitis associated chylous ascites have been described. In 1999, Ben-Ami *et al*^[5] described acute chylous ascites secondary to acute pancreatitis. This was diagnosed during elective cholecystectomy and did not present with symptoms of peritonitis. In 2006, Chuang *et al*^[6] described a case of hypertriglyceridemia-associated acute pancreatitis with chylous ascites in pregnancy. In this

Table 2 Characteristics of ascitic fluid in chylous ascites^[1]

Colour	Milky and cloudy
Triglyceride level	Above 200 mg/dL (2.28 mmol/L)
Cell count	Above 500 (predominance of lymphocytes)
SAAG	Below 1.1 g/dL
Cholesterol	Low (ascites:serum < 1)
LDH	Between 110-200 IU/L
Culture	Positive in some cases of tuberculosis
Amylase	Elevated in cases of pancreatitis
Glucose	Under 100 mg/dL
Cytology	Positive in some cases of malignancy

review there appeared to be a lack of correlation between the formation of chylous ascites and the severity of the pancreatitis.

Aalami proposed two mechanisms believed to play a role in the development of acute chylous ascites in the setting of pancreatitis. These are: the compression of lymphatic channels by an inflamed pancreas and the direct damage of channels by pancreatic enzymes^[3].

Clinical features

Chronic chylous ascites frequently presents with progressive and painless abdominal distension. As with other types of ascites, respiratory embarrassment is a common feature secondary to diaphragmatic splinting. Constitutional symptoms such as anorexia, weakness and malaise are very common, but non-specific^[1]. Other features include abdominal pain, weight loss, diarrhoea and steatorrhoea, malnutrition, oedema, enlarged lymph nodes, early satiety, fevers and night sweats^[1].

In cases of acute chylous ascites symptoms of anorexia, nausea, vomiting and severe abdominal pain are reported. A high fat meal has been reported in the literature as a common precipitant in the development of symptoms. Examination findings of peritonism have been described in the literature. Interestingly, symptoms are often maximal in the right iliac fossa^[7] and most likely a result of pooling of chylous fluid in the right paracolic gutter, mimicking acute appendicitis. Three cases have been reported in which acute chylous peritonitis presented clinically with acute appendicitis. In two cases, the patient underwent open appendectomy, while, in the third patient a midline laparotomy was performed for what was suspected to be appendicitis complicated by appendiceal perforation. In all three cases a white milky fluid was found in the peritoneal cavity, biochemical assessment of this fluid confirmed chylous ascites. The appendix was normal and exploration of the abdomen could not find any cause for the acute chylous effusion in all of the three cases described^[7-9].

Diagnosis

Laboratory testing is rarely useful. The white cell count may be elevated, but other findings are non-specific^[10]. Radiological investigations are of limited benefit although CT of the abdomen has been reported as being useful in identifying pathological lymph nodes and masses and in determining the extent and localisation of the fluid.

Paracentesis is the most useful diagnostic test. Typically chyle has a cloudy and turbid appearance. Table 2 shows the characteristics of ascitic fluid in chylous ascites.

Blood tests including a complete blood count, electrolytes, liver function tests, total protein, albumin, lactate dehydrogenase (LDH), triglycerides, cholesterol, amylase and lipase should be preformed but are by no means diagnostic.

Management

The underlying cause should be addressed whenever feasible. In patients with an acute abdomen, immediate exploration should be performed^[9]. Laparotomy usually allows a definitive diagnosis and provides an opportunity to address the underlying cause.

In chronic chylous ascites which cannot be managed surgically, the goals of treatment are (1) the maintenance of adequate nutrition, (2) decreasing the rate of chyle formation and (3) correcting the underlying disorder^[3]. Considerable controversy exists regarding the effectiveness of a high-protein, low-fat diet with medium-chain triglycerides and diuretics, or total parenteral nutrition (TPN) alone as a means to reducing chyle formation. Guidelines for management published by Aalami in 2000 recommend the commencement of TPN only if no improvement is observed after three weeks on a low-fat, medium-chain triglyceride diet. Multiple case reports describe the use of octreotide in the management of chylous ascites. Somatostatin receptors have been described in the lymphatic vessels of the intestine and it may be that octreotide is effective in managing chylous ascites because it helps to decrease lymph flow through these vessels. The use of octreotide with TPN for the treatment of chylous ascites has been described with clinical improvement in the ascites and reduction in TPN requirements and paracentesis frequency^[11].

In patients with a large amount of ascites a large volume paracentesis to relieve discomfort and dyspnoea can be performed and repeated as needed, however, the risk of infection and fat emboli should be noted^[9]. Peritoneo-venous shunting may be an option, although this is controversial. Although there is some evidence of high initial success rates after insertion, as high as 75%^[3], these shunts carry a high rate of complications which include fever, sepsis and DIC. The high viscosity of chyle has rendered shunt patency disappointing with an eventual occlusion rate approaching 100%.

In conclusion, acute chylous ascites can present with symptoms and signs of peritonism and can mimic acute appendicitis. Chylous effusions, both acute and chronic, are a complication of pancreatitis and may confuse clinical assessment and diagnosis.

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S- Editor Tian L L- Editor Webster JR E- Editor Lin YP



Perforated duodenal ulcer presenting with massive hematochezia in a 30-month-old child

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Author contributions: All authors contributed equally to this work.

Supported by (in part) The Chung-Ang University Research Grant in 2009

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Received: June 11, 2009 Revised: September 8, 2009

Accepted: September 15, 2009

Published online: October 14, 2009

Peer reviewers: Frank I Tovey, OBE, ChM, FRCS, Honorary Research Fellow, Department of Surgery, University College London, London, United Kingdom; Dr. Philip Abraham, Professor, Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

Lee NM, Yun SW, Chae SA, Yoo BH, Cha SJ, Kwak BK. Perforated duodenal ulcer presenting with massive hematochezia in a 30-month-old child. *World J Gastroenterol* 2009; 15(38): 4853-4855 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4853.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4853>

INTRODUCTION

Upper gastrointestinal (GI) bleeding in children accounts for approximately 10% of all GI bleeding in children^[1,2]. Gastric and duodenal ulcers are infrequent causes of GI bleeding in children^[3-5]. The majority of children with active gastritis and ulcers of the stomach or duodenum have an associated systemic condition, such as overwhelming sepsis, severe head or body trauma, or burns^[6]. A history of taking medications such as corticosteroids or nonsteroidal anti-inflammatory drugs (NSAIDs) is also important. However, in the absence of such significant history, symptoms of childhood peptic ulcer disease can easily be overlooked in the initial stages, which can result in catastrophic consequences such as perforation or hemorrhage^[3,7].

We report here on a 30-month-old boy with duodenal perforation due to an ulcer. The patient presented to the emergency department (ED) with massive hematochezia (gastrointestinal bleeding) and ongoing active bleeding, and was subsequently found to have a perforated duodenal ulcer. This patient had an atypical presentation in that there were no antecedent signs or symptoms of peptic ulcer disease before the perforation.

CASE REPORT

A 30-month-old boy visited the ED with acute massive hematochezia. Two days before, he had watery diarrhea twice per day for 2 d, and was successfully managed with fluid therapy without any other medications at the ED. The patient was doing well before the incident except for a mild decrease in appetite. On arrival to the ED, he

Abstract

Peptic ulcer disease is uncommon in children and rarely suspected as a cause of abdominal complaints in this age group; the diagnosis is therefore made almost exclusively when complications develop. Peptic ulcer disease is usually not considered in the differential diagnosis of pediatric patients. We present the case of a 30-month-old boy with duodenal perforation due to a peptic ulcer without a known etiology. The patient was admitted through the emergency department due to severe hematochezia and ongoing anemia; he presented with neither abdominal pain nor abdominal distension. There were no medical problems, and no drugs, such as corticosteroids or nonsteroidal anti-inflammatory drugs, had been prescribed or administered recently. We tried to control the active bleeding by medical treatment including arterial embolization, but the active bleeding was not controlled. Finally, an exploratory laparotomy was performed. A discrete anterior perforation with active bleeding of the duodenal wall was found. After the operation, there were no complications and the patient recovered fully.

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Key words: Duodenal ulcer; Peptic ulcer perforation; Children; Hematochezia; Hemorrhage

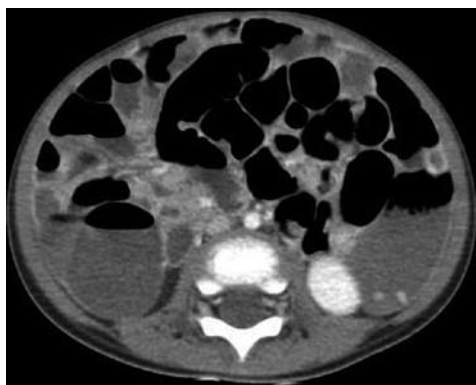


Figure 1 Air-fluid level with distension of the small bowel and colon and a decrease in the diameter of the abdominal aorta.



Figure 2 After coiling, extravasation at the duodenal branch of the gastroduodenal artery was still present (white arrows). A: Before coiling; B: After coiling.



Figure 3 The ulcer base of about 1.5 cm size and bleeding at the posterior wall of the perforation site is shown.

was alert, but he looked acutely ill. The patient presented with only hematochezia without abdominal pain or abdominal distension.

The medical history revealed that he was delivered vaginally at term, and otherwise had no significant prenatal or postnatal complications. There were no previous hospitalizations or medical problems, his immunization status was up-to-date, and no drugs had been prescribed or administered recently (especially NSAIDs).

On admission, the body weight was recorded to be 11.0 kg (25-50th percentile), height was 84 cm (3-10th percentile), and head circumference was 53.5 cm (25-50th

percentile). His vital signs were as follows: blood pressure 110/60 mmHg; pulse rate 80 beats/minute (bpm); respiration rate 20 breaths/min; temperature 36.6°C.

The patient looked acutely ill and had pale conjunctiva. Chest examination revealed regular heart beats without murmurs and clear breath sounds. Bowel sounds were hyperactive without localized tenderness or rebound tenderness. The abdomen was flat and soft without palpable mass.

The hemoglobin (Hb) was 11.9 g/dL two days ago, but dropped to 7.9 g/dL when checked on ED arrival. However, after 2 h, it dropped further to 7.3 g/dL. Therefore, an ongoing loss of blood was suspected and a transfusion started. Despite massive transfusions, Hb continued to decrease and fell to 4.8 g/dL, and platelets were also reduced to 75 000/mm³ after 2 h. Blood pressure could not be measured and heart rate was more than 160 bpm. A femoral central line catheter was inserted to transfuse packed red blood cells (P-RBC) and platelets. Even with ongoing transfusions, vital signs remained unstable and hematochezia was not improving.

Emergency abdominal CT was performed, which revealed an air-fluid level with distension of the small bowel and colon; in addition, the diameter of the abdominal aorta was decreased. Based on the CT scan, acute blood loss was strongly suspected (Figure 1). To determine the bleeding focus, angiography was performed, which showed extravasation at the duodenal branch of the gastroduodenal artery. Embolization was attempted with 3 coils; however, this could not stop the continuous arterial bleeding (Figure 2). The patient was transfused with 19 pints of P-RBC, 16 pints of platelets and 7 pints of fresh frozen plasma (FFP) because his vital signs were unstable before the operation started.

On hospital day #2, an exploratory laparotomy was performed 20 h after arrival at the ED. On entering the peritoneum, a large amount of blood-stained fluid was encountered. A discrete anterior perforation of the duodenal wall was found; there was a perforation injury that was half the size of the whole duodenal diameter at the anterior wall of the second portion of the duodenum. Serosal ulceration and active arterial bleeding were also seen at the gall bladder neck which was in contact with the perforation site. It showed an ulcer base of about 1.5 cm and bleeding in the posterior wall of the perforation site (Figure 3).

After the operation, the vital signs were stable. Hb was maintained in the normal range. We confirmed that the *Helicobacter pylori* (*H. pylori*) IgM was negative. The patient fully recovered and was eventually discharged without any sequelae.

DISCUSSION

Peptic ulcer disease is uncommon in children and rarely suspected as a cause of abdominal complaints in this age group^[2,4,8]. The diagnosis is therefore made almost exclusively when children develop complications; peptic ulcer disease is rarely included in the differential diagnosis of pediatric patients^[2,6,9,10]. Peptic ulcer disease is classified as either gastric or duodenal, based on the

location, and either primary (intrinsic) or secondary (extrinsic), depending on the etiology. Ulcer disease in children less than 10 years of age is usually secondary and is located predominantly in the duodenum. However, if a primary ulcer is present in this age group, it is usually gastric in origin. In children 10 years or older, primary ulcer disease is more common^[3,4,11,12].

The vast majority of primary duodenal ulcers are associated with *H pylori* infection of the gastric antral mucosa^[7,12-16]. Wong *et al*^[17] reported that patients with perforation underwent laparoscopic patch or open repair. All patients had a course of proton pump inhibitors postoperatively and in 90% of the patients *H pylori* was identified. In Hong Kong^[2], acute upper gastrointestinal bleeding in children was dominated by duodenal ulcers in 75% of the patients and *H pylori* infection was identified in 55% of the patients.

Secondary ulcer disease occurs as a result of some external predisposing cause, such as medications or stress. Associated medications include aspirin, NSAIDs, and steroids. In infants, stress-induced ulcers are often caused by traumatic delivery, respiratory or cardiac distress, sepsis, hypoglycemia, or dehydration. In older children, life-threatening illness and trauma are the main causes; ulcers associated with intracranial pathology (Cushing's ulcer) or burns (Curling's ulcer) have been well described. Since secondary ulcer disease predominantly occurs in the duodenum, it is more likely to present catastrophically, with hemorrhage or perforation as the initial features^[5,11,12]. In one study, 30% of patients had perforation on initial presentation^[8]. Often, exploratory laparotomy is the only way to diagnose patients with secondary ulcer disease because of the presentation of patients with an acute abdomen.

Moon *et al*^[3] reported the case of a 3-year-old boy presenting with shock who was diagnosed as having a perforated duodenal ulcer. Wilson *et al*^[4] reported the case of a 7-year-old boy who was admitted with gastroenteritis that was complicated by an acute perforated duodenal ulcer. In addition, Sisil Kumara *et al*^[6] reported on a 3-year-old boy who had taken prednisolone for a skin eruption and was diagnosed with a perforated duodenal ulcer. In all three of the above cases, chest radiographs showed air under the diaphragm. However, in our case, the gallbladder shielded the perforation site, and we did not see signs of a perforation. Barandica *et al*^[9] reported that CT was associated with a 26% false-negative rate in the detection of hollow viscus injuries in children.

Chan *et al*^[18] reported that 32 children with endoscopically proven ulcers of the duodenum were evaluated for their long-term outcome after H₂-receptor antagonist (H₂RA) treatment. In that study, 12.5% of the patients presented with a perforation. All four patients that had perforations were initially treated with a patch repair; two had persistent ulceration despite H₂RA treatment and required a proximal gastric vagotomy.

In summary, our patient did not have any of the known causes to explain the development of secondary

ulcer disease such as medications and stress, and *H pylori* infection was not detected in the duodenum. The patient did not exhibit symptoms of a bowel perforation such as abdominal tenderness or rebound tenderness; he presented with lower gastrointestinal bleeding symptoms including massive hematochezia rather than hematemesis or melena. The upright abdominal radiographs did not show free air under the diaphragm. Because the gallbladder wall covered the site of the duodenal perforation, the symptoms associated with bowel perforation were not apparent.

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CASE REPORT

Malignant mesothelioma of the greater omentum mimicking omental infarction: A case report

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Received: June 11, 2009 Revised: August 26, 2009

Accepted: September 2, 2009

Published online: October 14, 2009

Peer reviewer: Gianlorenzo Dionigi, MD, FACS, ESES, ETA, Associate Professor of Surgery, Department of Surgical Sciences, University of Insubria, Ospedale di Circolo, v. guicciardini, 21100 Varese, Italy

Shin MK, Lee OJ, Ha CY, Min HJ, Kim TH. Malignant mesothelioma of the greater omentum mimicking omental infarction: A case report. *World J Gastroenterol* 2009; 15(38): 4856-4859 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4856.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4856>

Abstract

Mesothelioma develops most commonly in the pleura, and less frequently in the peritoneum. Usually, it manifests as diffuse peritoneal thickening and multiple nodules, and rarely as a solitary mass. We report a rare case of primary malignant mesothelioma of the greater omentum, which mimicked omental infarct. A 54-year-old Korean man was admitted because of severe abdominal pain of sudden onset. A tender mass with indistinct margins was palpated in the upper abdomen. Abdominal ultrasound and computed tomography showed an ill-defined mass in the greater omentum and little ascites in the peri-hepatic space, and neutrophil-dominant exudates were documented on paracentesis. Intravenous antibiotics and analgesics were given for omental infarction with superimposed infection, which resulted in symptomatic improvement. The imaging studies after a week revealed a growing mass and ascites. Laparoscopic surgery was performed and an 8 cm × 3.3 cm greater omental mass was found, with multiple small nodules on the peritoneum, diaphragm, and pelvic cavity wall. Histological examination showed proliferating malignant epithelioid cells that stained strongly for calretinin, which was compatible with malignant mesothelioma. We recommend that primary omental mesothelioma should be included in the differential diagnosis of patients with omental infarction, despite its rarity.

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Key words: Infarction; Mesothelioma; Omentum

INTRODUCTION

Malignant mesothelioma originates from the mesothelial lining cells of serous cavities. The disease develops most commonly in the pleura, but the peritoneum is involved in 20%-40% of cases^[1-4]. Besides its rarity, the disease has no specific clinical or radiological manifestations, therefore, diagnosis is very difficult in the absence of a history of exposure to asbestos^[4]. Usually, it manifests as multiple peritoneal nodules or plaques, which may later coalesce to produce diffuse neoplastic thickening of the peritoneum, with encasement of the abdominal viscera. However, a solid mass-like presentation is unusual, and furthermore, primary greater omental tumor is rare. Only a few cases of malignant mesothelioma of the greater omentum have been reported in the English-language literature^[5,6]. The omentum is composed mainly of adipose tissue; however, tumors that arise from adipose tissue are less common than smooth muscle tumors, and those that originate from the mesothelial lining are very rare^[7,8].

We report here a case of primary malignant mesothelioma that originated from the greater omentum, which manifested as omental infarction.

CASE REPORT

A 54-year-old Korean man was admitted to Gyeongsang National University Hospital because of severe abdominal pain of sudden onset. He had been suffering from vague abdominal pain of unknown cause during the previous 3 mo. He was given thorough medical examinations including abdominal ultrasonography (US) and computed tomography (CT) at several hospitals and clinics, but no cause for abdominal pain had been found. On the day prior to admission, the pain exacerbated suddenly and did

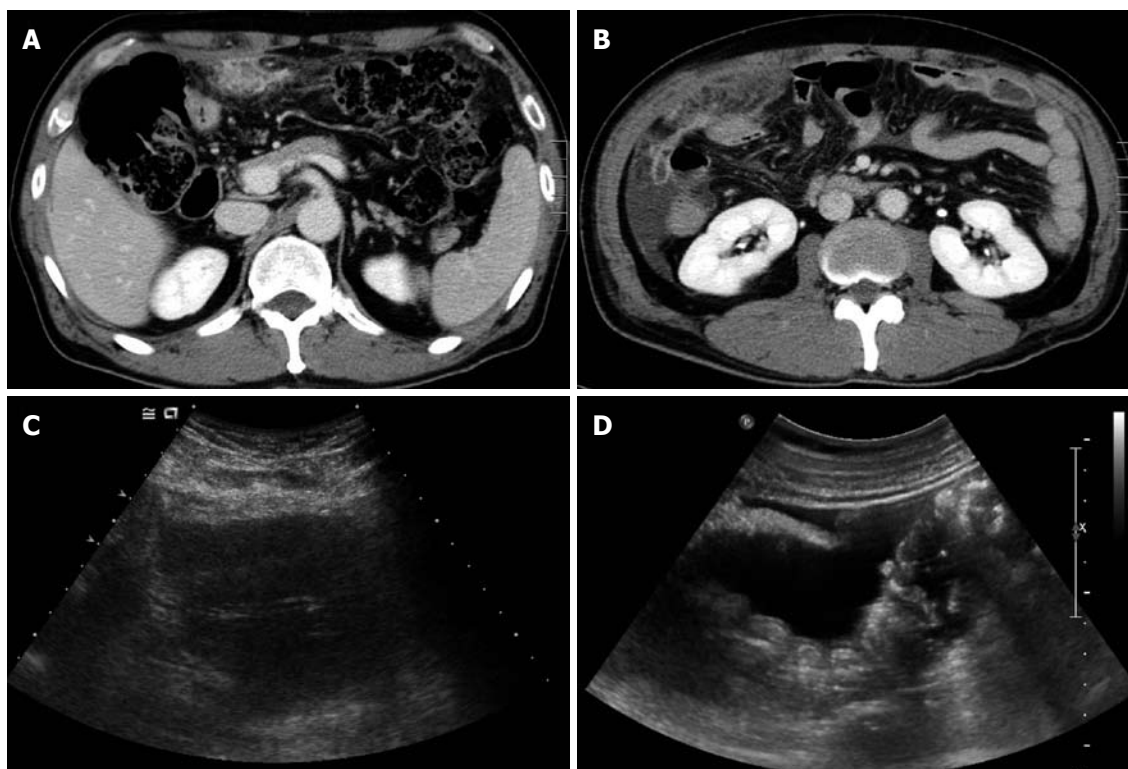


Figure 1 Abdominal computed tomography (CT) and ultrasonography (US). A: Initial abdominal CT revealed a mass-like lesion of about 7 cm × 3 cm at the greater omentum; B: Abdominal CT follow-up after 1 wk antibiotic therapy showed an increased omental mass lesion, with a shift to the right lower abdomen, and ascites; C: Abdominal US showed a heterogeneous echoic mass at the greater omentum, and ascites; D: Multiple round nodules at the peritoneum and ascites.

not subside with analgesics. The patient was working at a construction company as a chief executive officer but had no apparent history of exposure to asbestos. He had diabetes mellitus controlled with an oral hypoglycemic agent but had no specific family history. He was a nonsmoker and denied drinking alcohol. He looked very nervous and agitated. An indistinct mass-like lesion was palpated, which showed severe tenderness in the upper abdomen. The blood count was normal. Serum glucose was 252 mg/dL (normal range: 70-110 mg/dL), total protein 5.8 g/dL (6.4-8.3 g/dL), albumin 3.1 g/dL (3.4-4.8 g/dL), and C-reactive protein 252 mg/L (0-5 mg/L), but there were no remarkable abnormalities in the other biochemical tests including renal and hepatic function tests. Cancer antigen 125 (CA 125) was 186.7 U/mL (0-5 U/mL). A chest X-ray film was normal, while abdominal sonography and CT revealed an ill-defined mass with heterogeneous echogenicity/attenuation, about 3 cm × 7 cm in size, located in the greater omentum, and a little ascites in the peri-hepatic space (Figure 1). Diagnostic paracentesis of ascites yielded polymorphonuclear neutrophil (PMN)-dominant exudates (white blood cells 2525/mm³, PMN 79%, protein 4.4 g/dL, glucose 234 mg/dL, lactate dehydrogenase 136 U/L). Broad-spectrum antibiotics and analgesics were given for omental infarction with superimposed infection, which resulted in symptomatic improvement. However, the imaging studies after administration of antibiotics for 1 wk showed increased mass size and ascites. Laparoscopic surgery was performed and an 8 cm × 3.3 cm greater-omental mass was found (Figure 2A), with a moderate amount

of turbid ascites. Multiple, variable-sized, nodular lesions were scattered diffusely on the surface of the parietal peritoneum, mesentery, diaphragm, and pelvic cavity wall (Figure 2B). No visceral neoplastic lesion was detected. Partial omentectomy and excisional biopsy of nodules were performed. The main omental mass and nodules were composed of proliferating epithelioid cells that showed tubular, cystic or papillary structures. The tumor cells were strongly positive for calretinin (Figure 3), cytokeratin and vimentin, and negative for S-100 and carcinoembryonic antigen (CEA) upon immunohistochemistry. Diagnosis of malignant mesothelioma was made based on histopathological findings.

The patient recovered uneventfully and was referred to an oncologist for adjuvant therapy. He underwent eight cycles of treatment with pemetrexed and cisplatin. Serum CA 125 after three cycles of chemotherapy decreased to 14.9 U/mL, and positron emission tomography performed after six cycles showed no demonstrable abnormal fluorodeoxyglucose uptake. He has been followed every 3 mo on an outpatient basis after completion of eight cycles of palliative therapy. He is alive and well almost 14 mo after operation.

DISCUSSION

The occurrence of mesothelioma is known to be associated with environmental factors and carcinogens. The association between mesothelioma and asbestos exposure was first reported by Wagner *et al*^[1] in 1960. However, the relationship between peritoneal mesothelioma and asbestos

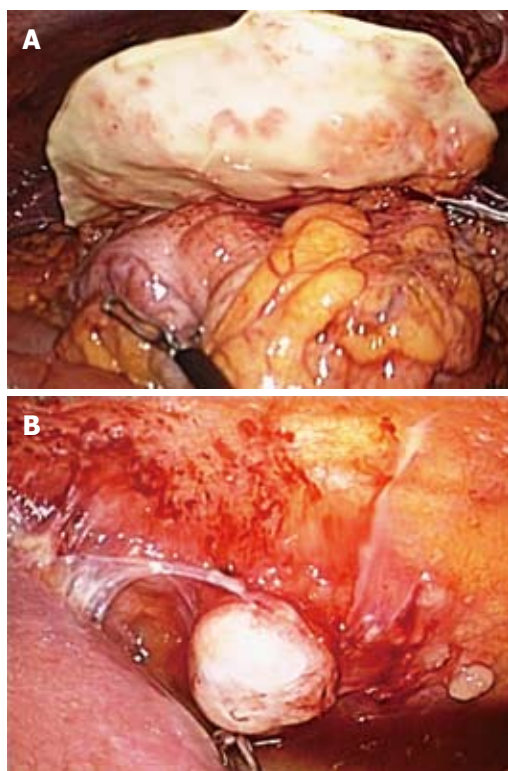


Figure 2 Laparoscopic findings. A: Greater omental mass of 8 cm × 3.3 cm with coarsely nodular surface; B: Variable-sized metastatic nodules adhered to the parietal peritoneum.

is less obvious than that of pleural mesothelioma^[10]. Liu *et al*^[6] also have reported a non-asbestos-related primary mesothelioma of the greater omentum. Another causative etiology is the simian virus 40 (SV40) TAG sequences^[11]. A multi-institutional international study has revealed that mesothelioma frequently expresses SV40 TAG sequences^[12]. Although our patient had no history of direct exposure to asbestos, he worked in a construction company, so that asbestos could not be excluded as a possible etiological factor. Unfortunately, SV40 studies were not performed in our case.

Primary greater omental tumors are rare, while metastatic tumors in the greater omentum are not uncommon. Several types of primary greater omental tumor have been reported: leiomyosarcoma, hemangiopericytoma, myosarcoma, fibrosarcoma, rhabdomyosarcoma, leiomyoma, leiomyoblastoma, lipoma, fibroma, mesothelioma, and endothelioma^[13-16]. In particular, greater omental malignant mesothelioma is very uncommon.

Although patients with primary greater omental tumors may be asymptomatic, most present with abdominal discomfort, nausea, early satiety, weight loss, and a palpable abdominal mass^[5,13]. Our patient presented with a greater omental mass with severe pain and PMN-dominant exudative ascites. Furthermore, he showed symptomatic improvement with antibiotic therapy. These findings made us suspect initially complicated omental infarction, making preoperative diagnosis difficult. US and CT of the abdomen can provide important information during the diagnostic process, as in the present case. Short-term follow-up imaging showed no improvement with regard to

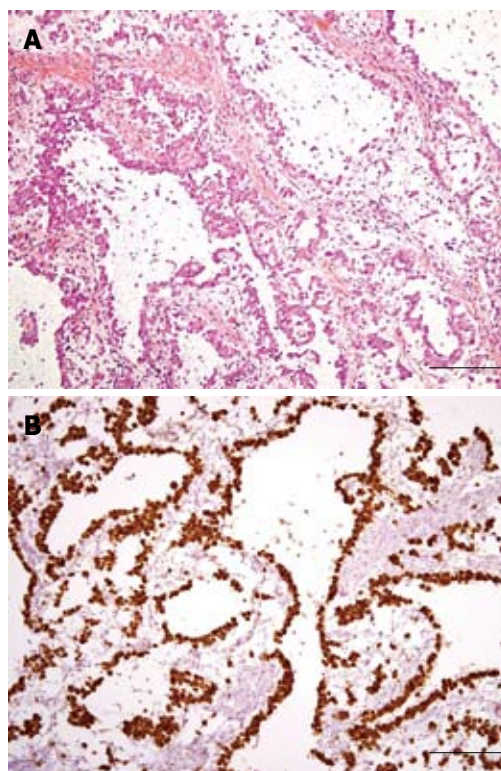


Figure 3 Photomicrographs of laparoscopically resected omental mass. A: Proliferating epithelioid tumor cells formed tubular, cystic or papillary structures (HE, × 100, bar 100 μm); B: Tumor cells were strongly positive for calretinin upon immunohistochemical staining (× 100, bar 100 μm).

omental mass and ascites despite symptomatic amelioration, and we therefore decided to perform a laparoscopy to make a definitive diagnosis.

A definitive diagnosis can be established only by laparoscopy or open surgery with biopsy. Histological examination may reveal an epithelial, sarcomatoid, or biphasic pattern^[17]. Epithelial-type neoplasms account for 75% of cases and vary from a relatively well-differentiated tumor with a tubulopapillary pattern to solid sheets of rounded or polygonal cells. This type may mimic carcinoma, such that the distinction between epithelioid mesothelioma and metastatic carcinoma, particularly adenocarcinoma, is perhaps the most frequently encountered diagnostic dilemma^[18]. The sarcomatoid-type neoplasms also may be indistinguishable from fibrosarcoma upon histology alone. Immunohistochemistry can be helpful in differential diagnosis of sarcoma and adenocarcinoma. Positive immunoreactivity for calretinin markedly increases the accuracy of diagnosis^[18-21]. Mesothelioma cells are diffusely positive for calretinin, cytokeratin and epithelial membrane antigen, and negative for S-100 protein, Leu-M1, CEA, thrombomodulin and placental alkaline phosphatase^[22]. The tumor cells in our case also showed positive immunoreactivity for calretinin, cytokeratin and vimentin, but were negative for S-100 and CEA.

The prognosis of this malignant tumor is extremely poor because of the lack of effective treatment, with most patients dying within 1 year of diagnosis^[3]. Retrospective studies have shown that median survival after palliative

surgery and systemic and/or intraperitoneal chemotherapy is about 1 year, ranging from 9 to 15 mo^[23-25]. Recently, however, several independent phase I / II prospective trials have reported improved survival with an intensive loco-regional treatment strategy, including cytoreductive surgery along with perioperative intraperitoneal chemotherapy in the form of hyperthermic intraperitoneal chemotherapy, with or without early postoperative intraperitoneal chemotherapy^[23]. The median survival after aggressive surgery combined with hyperthermic intraperitoneal chemotherapy has approached 5 years and seems to improve with subsequent reports^[22,23,26-28]. Meanwhile, Vogelzang *et al.*^[29] have demonstrated in a multicenter, controlled, randomized phase III trial that pemetrexed-cisplatin is the gold standard for the non-operable malignant pleural mesothelioma. The combination of pemetrexed and cisplatin chemotherapy yielded an objective response in the present case.

Yan *et al.*^[28] have found by multivariate analysis that a small nuclear size is the only good independent prognostic determinant. The 3-year survival rate with a nuclear size of 10-20, 21-30, 31-40 and > 40 μm were 100%, 87%, 27% and 0%, respectively. The present patient survived for 14 mo without clinical and radiological evidence of recurrence, but his prognosis was poor because he was only able to undergo palliative surgery, despite his cells having a small nuclear size. We recommend that primary omental mesothelioma should be included in differential diagnosis of cases of omental infarction, despite its rarity.

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ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Meetings

Events Calendar 2009

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Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
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Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

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British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
<http://www.easl.ch/>

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
<http://www.ddw.org>

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27, 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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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 PMID: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 1274 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]

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- 10 **Sherlock S,** Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S,** Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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ISI, Thomson Reuters, 2008 Impact Factor: 2.081 (32/55 Gastroenterology and Hepatology).

Volume 15 Number 39
October 21, 2009

World J Gastroenterol
2009 October 21; 15(39): 4865-4992

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World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 39
October 21, 2009



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PUBLISHING

The WJG Press and Beijing Baishideng
BioMed Scientific Co., Ltd., Room 903,
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Telephone: +86-10-59080039
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PRINTING

Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR

Beijing Bureau for Distribution of
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(Code No. 82-261)
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Corporation PO Box 399, Beijing,
China (Code No. M4481)

PUBLICATION DATE

October 21, 2009

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SUBSCRIPTION

RMB 50 Yuan for each issue, RMB
2400 Yuan for one year

CSSN

ISSN 1007-9327
CN 14-1219/R

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Biochemical mechanisms in drug-induced liver injury: Certainties and doubts

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Received: July 28, 2009 Revised: September 4, 2009

Accepted: September 11, 2009

Published online: October 21, 2009

in hepatic necrosis or cholestasis, in which different HLA genotypes might play a major role. This review focuses on current knowledge of the mechanisms of drug-induced liver injury and recent advances on newly discovered mechanisms of liver damage. Future perspectives including new frontiers for research are discussed.

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Key words: Adverse effects; Apoptosis; Drug toxicity; Liver diseases; Microsomes; Mitochondria; Necrosis

Peer reviewer: Yukihiro Shimizu, MD, PhD, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyō, Kyoto 615-8256, Japan

Grattagliano I, Bonfrate L, Diogo CV, Wang HH, Wang DQH, Portincasa P. Biochemical mechanisms in drug-induced liver injury: Certainties and doubts. *World J Gastroenterol* 2009; 15(39): 4865-4876 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4865.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4865>

Abstract

Drug-induced liver injury is a significant and still unresolved clinical problem. Limitations to knowledge about the mechanisms of toxicity render incomplete the detection of hepatotoxic potential during preclinical development. Several xenobiotics are lipophilic substances and their transformation into hydrophilic compounds by the cytochrome P-450 system results in production of toxic metabolites. Aging, preexisting liver disease, enzyme induction or inhibition, genetic variances, local O₂ supply and, above all, the intrinsic molecular properties of the drug may affect this process. Necrotic death follows antioxidant consumption and oxidation of intracellular proteins, which determine increased permeability of mitochondrial membranes, loss of potential, decreased ATP synthesis, inhibition of Ca²⁺-dependent ATPase, reduced capability to sequester Ca²⁺ within mitochondria, and membrane bleb formation. Conversely, activation of nucleases and energetic participation of mitochondria are the main intracellular mechanisms that lead to apoptosis. Non-parenchymal hepatic cells are inducers of hepatocellular injury and targets for damage. Activation of the immune system promotes idiosyncratic reactions that result

INTRODUCTION

Drug-induced liver injury is the leading cause of acute liver failure and transplantation in western countries. The detection of subtle mechanisms that lead to potential drug hepatotoxicity is of key importance and remains a major challenge in clinical practice.

The frequent involvement of the liver in drug-induced toxicity depends on its anatomical location (the liver is the primary port of entry for ingested drugs) and its physiological and biochemical functions because of the abundance of metabolizing enzymes.

The spectrum of injury secondary to drug reaction ranges from mild damage to massive hepatic destruction. However, if one considers the large consumption of drugs, the latter possibility is rather infrequent^[1]. While direct toxic damage is dose-dependent, predictable and experimentally reproducible, idiosyncratic damage is rather supported by the innate and the adaptive immune system. With few exceptions of intrinsic hepatotoxicity, most cases of drug-induced liver injury are idiosyncratic. Toxicity can be experimentally tested by administering the compound at increasing doses, in the presence of

metabolic inducers or inhibitors or toxicity enhancers, with depletion of protective systems, or by co-administering the drug with a known toxic compound. In general, *in vitro* tests precede *in vivo* experiments. Intracellular organelles and their functions are often the primary targets of hepatotoxicity^[2]. Not only hepatocytes, but also cholangiocytes, Kupffer cells, Ito cells and sinusoidal endothelial cells can be involved in the process of drug-induced hepatotoxicity. Some drugs can induce cholestasis by impairing bile secretion or by causing obstruction of extrahepatic bile ducts^[3].

This review deals with the main mechanisms associated with drug-induced hepatic injury, by discussing current views on intra- and extracellular mechanisms of damage and cell death with respect to different drugs. Future perspectives on emerging problems, namely liver steatosis and genetic polymorphisms, are also discussed.

RISK FACTORS

As toxicity is exerted mostly through metabolites rather than the parent drugs, factors affecting metabolite formation are of key importance. Accordingly, genetic polymorphisms and environmental influences on metabolizing enzymes play an important role. Of note, drug-induced hepatotoxicity occurs mainly in women^[4], and this points to the existence of hormonal conditioning factors. Additional genetic, metabolic and immunological factors also may have a role in idiosyncratic hepatotoxicity. All such mechanisms can occur if specific metabolic pathways are activated and previous exposure has sensitized the organ with the formation of specific antibodies (e.g. halothane). In addition, the intrinsic toxicity of some molecules can depend on the expression of genetic variants, as occurs for paracetamol^[5]. Although preexisting liver disease generally is believed to play a minor role as a risk factor for hepatotoxicity, there are some well-documented exceptions. Hepatotoxicity caused by isoniazid, for example, is more common among patients with viral hepatitis and/or human immunodeficiency virus (HIV) infection^[6]. Patients undergoing antiretroviral treatment for HIV infection are at higher risk for severe hepatotoxicity when co-infected with hepatitis B or C viruses, particularly if therapy includes protease inhibitors^[7]. Fatty liver is another condition that is particularly prone to stress damage^[8]. Further studies are needed urgently in this respect, linking toxic injury to liver steatosis, which is becoming an emerging health problem, because of the increasing epidemic of obesity and diabetes as part of the metabolic syndrome^[9].

GENERAL MECHANISMS OF DAMAGE

Although major pathways leading to drug-induced liver injury include necrosis and/or apoptosis, a net distinction between these two processes is sometimes difficult and both events often coexist in the same microscopic field^[10]. Several factors may influence the hepatocyte response to a toxic insult and the extent

of damage results from the intervention of intrinsic and extrinsic cell factors. A combination of age, sex, genetics, hormones, cell energetic status, underlying liver disease, environmental factors, and local O₂ supply, strongly contributes to the expression of cell death mediators^[11]. Less frequently, hepatocyte injury follows on from vascular damage as a consequence of the occlusion of the centrilobular vein (i.e. azathioprine, estrogens, progesterone, pyrrolidine alkaloids). Generally, hepatocytes react to toxic aggression by activating defense mechanisms that include hypertrophy of the endoplasmic reticulum, induction of protective systems (glutathione, GSH), and synthesis of heat shock and acute phase proteins.

Apoptosis and necrosis initially may follow common metabolic pathways. When the injury affects the maintenance of functional cell programs, hepatocytes preferentially die *via* apoptosis, thus limiting the extent of the injury. Necrotic damage generally begins at the cytoplasmic level and thus involves mitochondria and the nucleus in determining swelling and loss of plasma membrane integrity. It becomes irreversible when cytosolic Ca²⁺ concentration increases^[12,13] for increased release by mitochondria and endoplasmic reticulum, or increased extracellular influx. Apoptosis determines cytoplasmic and nuclear condensation and fragmentation without loss of membrane integrity. Drug-induced apoptosis is generally spotty, whereas necrosis is zonal.

The mechanisms of damage include interference with hepatic transport proteins (i.e. organic anion transporting polypeptides), bile salt export pump, or with the nuclear receptor-mediated regulation of drug metabolism and transport^[14,15].

MECHANISMS OF CELL DEATH

Hepatocyte death typically follows an apoptotic or necrotic pathway^[16], mainly depending on predisposing factors^[10]. General mechanisms of hepatotoxicity include reactive metabolite formation, antioxidant depletion, and protein alkylation. Intracellularly generated signaling can activate B-cell CLL/lymphoma 2 (Bcl-2) family members (Bax and Bid) which form pores in the outer mitochondrial membrane. This condition favors the release of intramembrane proteins and promotes chromatin condensation and DNA fragmentation. Alternatively, mitochondrial dysfunction, through reactive oxygen species (ROS) delivery and peroxynitrite formation, triggers membrane permeability transition and leads to membrane potential collapse with decrease of energy production and release of nucleases^[17].

Apoptosis

Apoptosis results from an ATP-dependent death program that is characterized by activation of specific pathways involving death ligands and death receptors (e.g. Fas ligand with Fas) with activation of the caspase cascade (Figure 1). There are two different activating pathways of drug-induced hepatocyte apoptosis. The “intrinsic way” is triggered by intracellular signals

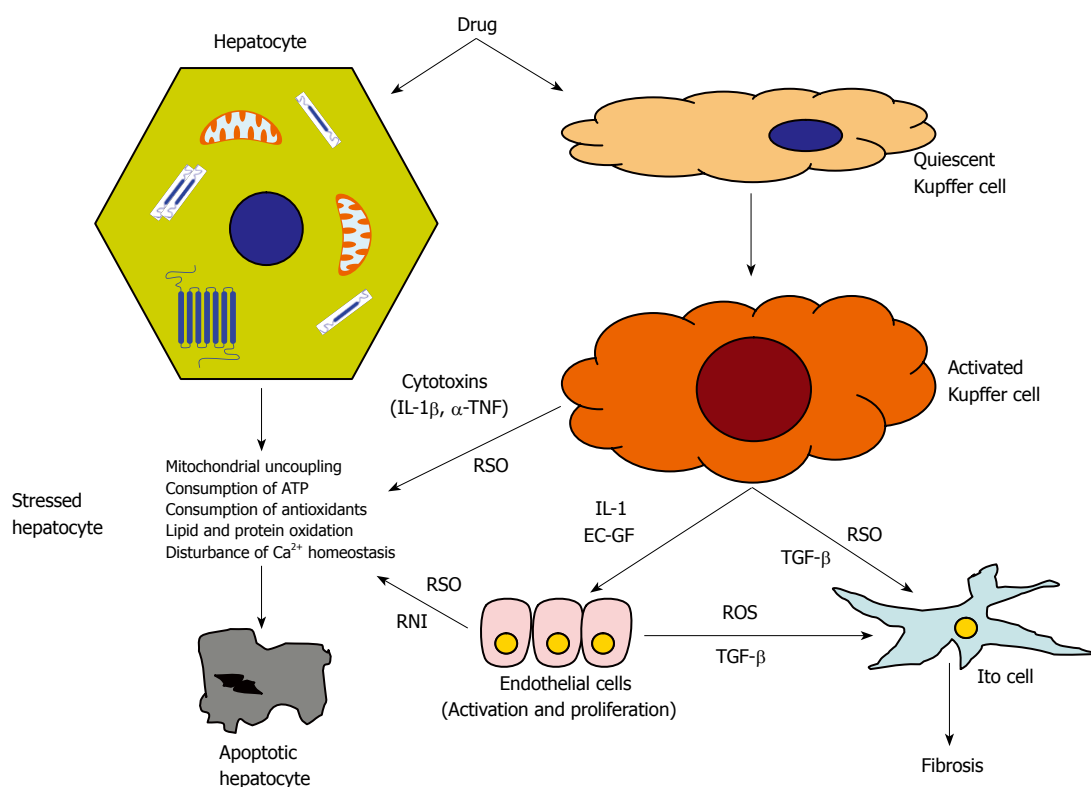


Figure 1 Schematic representation of subtoxic damage of hepatocyte in response to moderate dose of drug. Drug molecule activates Kupffer cells is metabolically processed by hepatocytes. These events may result in hepatocyte stress which is worsened by the intervention of reactive oxygen species (ROS) and nitrogen species from activated endothelial cells. Final result is apoptotic death and Ito cells activation with promotion of fibrosis. EC-GF: Endothelial cell growth factor; IL1: Interleukin 1; $\text{IL1}\beta$: Interleukin 1 β ; RNI: Reactive nitrogen intermediates; ROS: Reactive oxygen species; TGF- β : Transforming growth factor β ; TNF: Tumor necrosis factor α .

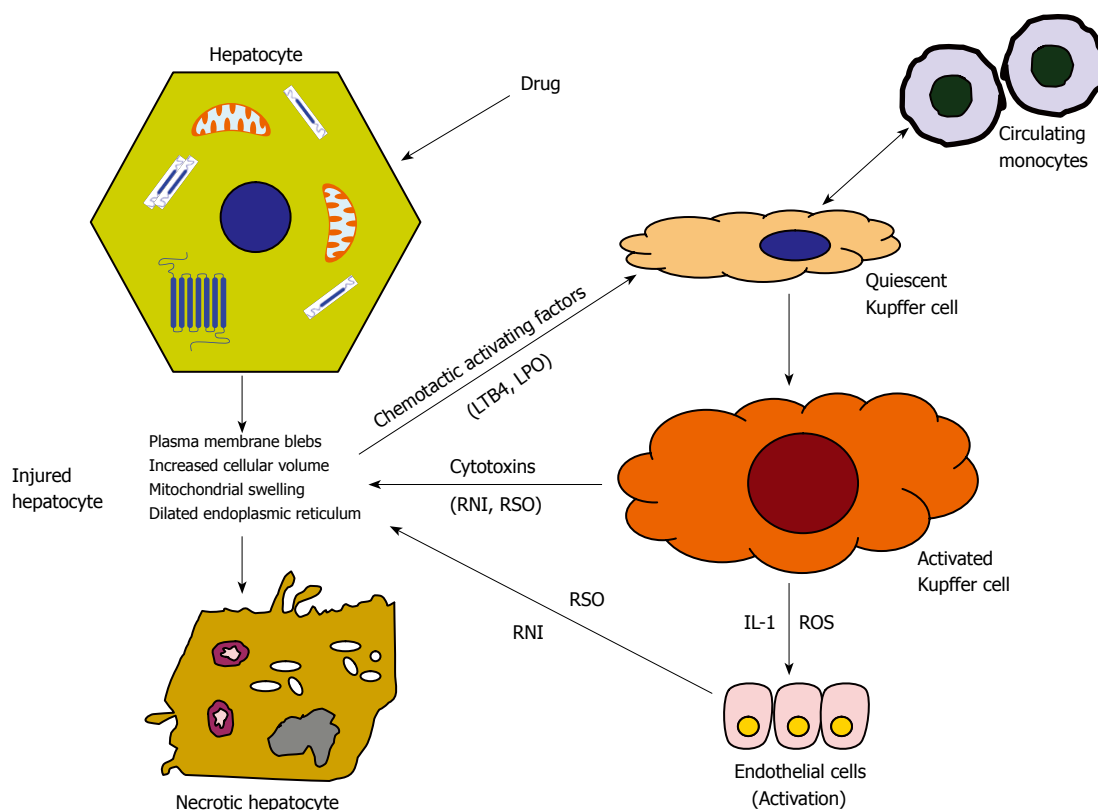


Figure 2 Schematic representation of toxic damage of hepatocyte in response to high dose of drug. High drug amount is processed by hepatocytes with production of reactive metabolites which induce cell injury. Toxic products and chemotactic factors released by damaged hepatocytes stimulate the activation of Kupffer and endothelial cells with a subsequent delivery of reactive oxygen (ROS) and nitrogen species. The intracellular damages result in necrotic death. LPO: Lipid peroxidation; LTB_4 : Leukotriene B $_4$.

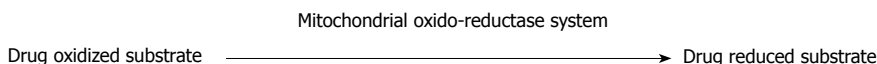


Figure 3 Schematic representation of mitochondrial oxido-reductase system. Several drug molecules directly or after metabolic release of toxic intermediates can cause mitochondrial alterations at different levels. The following impairment of the energetic and redox balance finally triggers apoptotic or necrotic processes according to a poor or sufficient ATP level. Important regulatory mechanisms rely on the glutathione dependent redox status of proteins. GSH: Reduced glutathione; GSSG: Oxidized glutathione; GSH-Px: Glutathione peroxidase; GSSG-RX: Glutathione reductase; iNOS: Inducible nitric oxide synthetase; NO: Nitric oxide; PSH: Protein sulphhydryls; PS-SG: Protein mixed disulfides; PS-SP: Protein-protein disulfides; TRx: Thioredoxin; TRx-R: Thioredoxin reductase; TR-S₂: Oxidized thioredoxin.

scattered directly by the drug or its metabolites, with activation of a cascade of reactions that damage nuclear and/or mitochondrial DNA directly. Single-stranded DNA subsequently will stimulate intracellular sensor systems and induce the expression of the effector p53. In the “extrinsic way”, new surface antigens on hepatocyte membranes work as receptors. The interaction with ligands, such as tumor necrosis factor alpha (TNF- α) or Fas, activates cytotoxic T cells and liver non-parenchymal cells, with release of cytokines^[18] that engage death receptors on the cell surface^[19]. After binding, the receptor trimerizes and leads to a clustering of death domains. Intrinsic and extrinsic ways finally promote the activation of interleukin (IL)-1 β converting enzyme, which activates caspases and nucleases.

Generally, hepatocytes are resistant to TNF- α -induced cytotoxicity^[20]. In fact, under normal conditions, the activation of membrane receptors stimulates the synthesis of anti-apoptotic molecules and enzymes (e.g. Bcl-2, NO synthase), mediated by the intervention of the nuclear transduction factor nuclear factor- κ B (NF- κ B). Therefore, increased cell sensitivity to TNF- α or to other specific ligands is required to trigger subsequent events^[21]: a strong signaling response with activation of the executioner caspases^[22], and the involvement of mitochondria to amplify death mechanisms in the presence of a poor caspase activation^[23].

Necrosis

Drug-induced cell necrosis results from an intense and massive perturbation of cell homeostasis, with ATP depletion (Figure 2) associated with cytoskeletal alterations, cellular swelling and bleb formation^[16]. The next steps include lysosomal breakdown, bleb rupture,

and irreversible collapse of electrical and ion gradients.

When a high amount of toxicant reaches the liver, necrosis occurs because of dramatic intracellular alterations, or as a consequence of oxygen and nitrogen radical attack from activated Kupffer and endothelial cells^[24]. On this occasion, drugs are oxidized by the cytochrome P-450 (CYP-450) enzymes, with release of a large amount of reactive metabolites, with promotion of lipid and protein oxidation and depletion of GSH. Oxidized proteins and protein adducts may have immunogenic properties and activate Kupffer and polymorphonuclear cells, with subsequent release of ROS. The formation of protein disulfides results in increased permeability of the inner mitochondrial membrane, with loss of membrane potential, decrease of ATP synthesis, inhibition of Ca^{2+} -dependent ATPase, decreased capability to sequester Ca^{2+} , oxidation of actin, microfilament breakage, and membrane bleb formation^[25].

Abnormal control of cell volume is a major factor that promotes hepatocyte necrosis. Oxidative stress, very fast consumption of cellular energy, and mitochondrial dysfunction activate anaerobic glycolysis, which results in decreased intracellular pH. The incoming acidosis is contrasted partially by H^+/Na^+ and Na^+/HCO_3^- exchanges with influx of Na^+ . As a result of low ATP availability, Na^+ cannot be further exchanged and accumulates within the cell. The consequent osmotic load results in cell swelling and blocks the apoptotic process, which requires a reduction of the cell volume. This osmotic stress is worsened by the increase of cytosolic Ca^{2+} and results in plasma membrane rupture^[26].

Additional mechanisms include nucleotide alterations

and protein synthesis disruption. In most cases, these actions follow drug-induced mitochondrial injury. However, discriminatory nucleotide alterations and oxidation of protein sulfhydryls (γ -glutamyl synthetase and glucose 6-phosphate dehydrogenase) are promoted selectively by some drugs; one example is the damage to the ATPase complex observed after cisplatin intoxication^[27].

CELLULAR AND INTRACELLULAR TARGETS OF DRUG HEPATOCELLULAR INJURY

Aspects to discuss include non-parenchymal hepatic cells, microsomes, mitochondria, and nuclear receptors. Much evidence suggests the participation of non-parenchymal hepatic cells in drug-induced hepatocellular injury^[28], which depends on factors such as the intrinsic characteristics of the drug, its dose, its metabolites, and the local O₂ supply^[29]. Activation of Kupffer cells results in the release of inflammatory mediators and ROS, and modulates hepatocyte injury^[30]. It has been shown that inhibition of macrophage activation or administration of TNF- α antagonists protects hepatocytes against paracetamol toxicity^[31], and that depletion of Kupffer cells attenuates thioacetamide hepatotoxicity^[32]. Indeed, both Kupffer and endothelial cells can be activated secondarily by chemotactic factors (i.e. leukotriene B₄) released by injured hepatocytes^[24,33], which in turn, can be damaged by TNF- α and IL-1 β released from activated non-parenchymal cells. Examples of drug hepatotoxicity that involves non-parenchymal cells are that seen with methotrexate (activation of hepatic stellate cells to myofibroblasts, and liver fibrosis may develop even in the absence of liver enzyme elevation); bosentan (inhibition of transport proteins including the bile salt export pump^[34]); sulindac (competitive inhibition of canalicular bile salt transport, a contributing factor to cholestatic liver injury^[35]); cyclophosphamide and azathioprine (sinusoidal obstruction syndrome, veno-occlusive disease, follows a severe depletion of GSH in sinusoidal endothelial cells. This damage results in fibrosis of the hepatic sinusoids).

Microsomes are another target of hepatocellular damage induced by drugs. Biotransformation of lipophilic drugs *via* CYP-450 metabolic pass and the subsequent excretion of their metabolites are essential to avoid intracellular accumulation of toxic compounds. Less than 10 CYP-450 enzymes accounts for > 90% of all drug oxidation. Most adverse drug reactions depend on the release of reactive metabolites and ROS, which may overwhelm lethal insult, sensitize the innate immune system, or haptenize, thus eliciting immunoallergic reactions^[36]. If metabolites have a particularly high reactivity, they can even bind and inactivate the metabolic enzymes^[37]. This occurs with drugs that show a narrow therapeutic index (e.g. terfenadine and astemizole). Several factors may affect the efficiency of the microsomal metabolism: namely

aging, liver disease, enzyme induction and inhibition, genetics (existence of slow and fast acetylators), and O₂ supply. Changes in the level of CYPs may have a dramatic impact on drug metabolism. P-450 enzymes are subjected to multiple levels of regulation and expression; the latter being dominant in zone 3 just surrounding the centrilobular vein. Expression of P-450 isoforms varies with age; therefore, the capacity for drug metabolism is a function of age^[38,39]. Polymorphisms in P-450s or induction/inhibition account for the appearance of adverse reactions. In this regard, it has been noted that the constitutive androstane receptor (CAR) binds drugs and regulates the expression of the genes that code for CYP3A and CYP2B^[40]. Also, induction or inhibition of CYPs by herbal remedies accounts for the increasing number of case reports of hepatotoxicity^[41]. In fact, some herbal components are converted to toxic metabolites by P-450 enzymes; this is the case of aristolochis acid, which generates the highly reactive cyclic nitrenium ions^[42]. Upregulation of specific P-450 enzymes has been described during rifampicin treatment^[43] in experimental models of obesity and fatty liver^[44] and in humans with nonalcoholic fatty liver disease (NAFLD)^[45].

Mitochondria are often a major target of drug toxicity, and therefore mitochondrial dysfunction represents a major determinant of hepatotoxicity^[46,47] (Figure 3). Indeed, mitochondria are the gateway at which signals that initiate cell death converge^[3,48]. By integrating signaling networks, mitochondria have an active role in several metabolic pathways^[49]. Signals may damage mitochondria directly or act indirectly by activating death receptors. In particular, reactive metabolite formation, GSH depletion and protein alkylation are associated with mitochondrial dysfunction, and represent critical initiating events for drug-induced toxicity. Opening of pores in the outer mitochondrial membrane, release of proteins and cytochrome c, imbalance in intracellular Ca²⁺ homeostasis, and intracellular accumulation of Na⁺ are essential steps in hepatocyte death^[17,50]. In this context, the maintenance of the mitochondrial GSH pool^[21,51] is important to detoxify ROS and maintain the reduced status of membrane protein sulfhydryls, including the ATP synthase complex and the Ca²⁺-dependent ATPase. A fall of total cellular GSH below 15% (< 1 μ mol/g) inevitably is associated with lethal cell damage by involving the mitochondrial stores^[52,53]. Common events that lead to apoptosis and necrosis act through mitochondrial permeabilization and dysfunction. In particular, it seems that the number of mitochondria that undergo pore opening is associated with apoptosis or necrosis, according to ATP availability or deficiency^[47]. Some drugs exert toxic effects on mitochondria only after their metabolic activation at the microsomal level (isoniazid/rifampicin), after inducing endoplasmic reticulum stress (paracetamol) or even lysosomal dysfunction. The study of these mechanisms has revealed intriguing relationships between mitochondria and other intracellular organelles^[54-56].

Recent advances in molecular biology have revealed

that nuclear receptors such as the pregnane X receptor (PXR) and CAR act as intracellular sensors for lipophilic compounds by encoding proteins and regulating the expression of enzymes^[57,58] that are involved in drug oxidative metabolism, disposition and transport^[15]. Their incorrect activation may result in drug metabolism disturbance. PXR can be activated or inhibited by a variety of structurally different drugs. Its activation is associated with downregulation of several genes^[59] that influence mitochondrial ketogenesis^[60] and favor mitochondrial imbalance and hepatic steatosis^[61]. These receptors also represent important drug targets. In fatty livers, peroxisome proliferator-activated receptor (PPAR) activation/deactivation is particularly important, not only for the switch from simple steatosis to steatohepatitis, but also for maintaining the efficiency of specific metabolic drug pathways^[62]. PPARs and other oxidative stressors can be activated also by macrophage-released molecules (i.e. Stat-3 and NF- κ B)^[63]. The existence of single-nucleotide polymorphisms is associated in humans with drug transport alterations as a predisposing factor for drug-induced cholestasis^[14].

COMMON PATHWAYS OF DRUG-INDUCED LIVER DAMAGE

Immune system

The liver is a site of intense immunological activity and represents a tolerogenic immune organ for lymphocytes. Activation of Kupffer cells, and recruitment of macrophages and immune cells result in inflammation and injury caused by cytokines release^[64]. These events are major factors in initiating and maintaining drug-induced liver injury^[65].

The drug itself and its metabolites can activate an immune response in the liver: the molecule is processed by antigen-presenting cells in the central lymphoid tissue directly, or after the appearance of haptens or new antigens on the hepatocyte membrane. The latter case follows a covalent binding of the drug molecule with membrane constituents or intracellular proteins^[66]. This hypothesis is supported by the observation that neutrophil depletion protects against paracetamol toxicity^[67]. Also, idiosyncratic reactions are more likely to occur in the presence of an inflammatory state^[68]. Effectors are dendritic cells, which act by sensing pathogens and triggering adaptive immune responses. These responses are characterized by activation of B lymphocytes, which release immunoglobulins and kinins and activate the complement cascade, and of T lymphocytes, which produce lymphokines (CD4) or determine direct cytotoxicity (CD8) *via* surface-molecule expression and the release of mediators (e.g. perforin and granzyme)^[69]. As a consequence, inhibition of lymphocyte activation reduces the extent of drug-induced hepatocyte injury^[70].

The local O₂ supply has an important role in the progression of immune-mediated toxic liver injury. For example, metabolism of halothane under the anaerobic

conditions of the reductive pathway may result in mild hepatitis, whereas, in the presence of a high O₂ supply, the oxidative pathway may induce massive liver necrosis^[29]. These different effects may be explained by the higher immunogenicity of oxidized metabolites that form adducts with proteins. This example suggests the potential capacity of some drugs to trigger autoimmune hepatitis in some patients. In fact, statins, hydralazine and procainamide may trigger autoimmune reactions in predisposed patients^[71]. Most of these patients are positive for HLA-DR3, 4 or 7, which are known to be associated with increased risk of autoimmunity. Halothane toxicity rarely occurs after first exposure; but antibodies against CYP 2E1-mediated trifluoroacetylated metabolite-protein adducts can be detected after frequent exposures to halothane.

Direct toxicity

Paracetamol hepatotoxicity is the classical example of direct liver injury. Given at recommended doses, paracetamol is generally safe, but its intrinsic toxicity at higher doses represents the most important cause of acute liver failure and transplantation. Predominantly metabolized by conjugation with sulfate and glucuronide (metabolites are excreted into bile by Mrp2 and extruded into blood through Mrp3), only a small amount is degraded by CYP 2E1 to the highly reactive metabolite N-acetyl-benzoquinoneimine (NAPQI). NAPQI is, in turn, detoxified by binding with GSH. If the amount of paracetamol that reaches the liver exceeds 12-15 g, the conjugating capacity is overwhelmed and the remaining unbound NAPQI covalently binds to cellular and mitochondrial proteins, which leads to cell necrotic death. In the presence of CYP 2E1 hypertrophy and/or decreased GSH availability (e.g. chronic alcoholism, malnutrition, and prolonged intake of barbiturates), NAPQI formation is increased even at therapeutic doses, and after overwhelming the GSH stores, it may cause severe liver injury^[72,73].

Events start with disturbances of intracellular Ca²⁺ homeostasis, with an increase in cytosolic Ca²⁺ levels, Bax and Bid translocation into mitochondria, and mitochondrial oxidative changes with accumulation of oxidized GSH and peroxynitrite^[74,75]. The latter induces membrane permeability transition, with collapse of mitochondrial membrane potential, inability to synthesize ATP, release of mitochondrial proteins with calpain activation, and release of cytochrome C and endonucleases. ATP deficiency prevents caspase activation but induces nuclear DNA damage, and activates intracellular proteases that lead to cell membrane rupture and hepatocyte necrosis^[76,77]. These intracellular events explain the massive cell death and liver failure observed after paracetamol poisoning^[17]. The recent observation that paracetamol toxicity is modulated by CAR gives rise to new concepts that are important for the general understanding of drug-induced liver injury^[78]. Accordingly, the presence of gene polymorphisms may explain inter-individual differences

in susceptibility to paracetamol toxicity. Finally, a role for hepatic non-parenchymal cells in paracetamol-induced hepatocellular injury also has been suggested. In fact, the chemical elimination of Kupffer cells by gadolinium chloride has been observed to reduce the extent of paracetamol-induced liver injury^[31].

Direct toxicity of the liver is also induced by another drug, valproate, a branched medium-chain fatty acid with eight carbon atoms. Its chronic intake is associated with weight gain and it causes insulin resistance and NAFLD in 61% of treated patients^[79]. Mechanisms of toxicity rely on mitochondrial β oxidation inhibition followed by the appearance of microvesicular steatosis^[80]. Mitochondrial dysfunction follows the microsomal production of toxic metabolites (4-ene-valproate, 2,4-diene-valproate)^[81], decreased activity of complex IV of the respiratory chain, and depletion of coenzyme A (CoA) and carnitine^[80]. Preexisting mitochondrial impairment or deficiency of cofactors involved with valproate metabolism (e.g. carnitine) may represent risk factors for hepatotoxicity^[82].

Idiosyncratic reactions

Unpredictable idiosyncratic reactions can follow the administration of virtually any drug. As a consequence, an enormous number of hepatic reactions have been registered for practically all drug classes. Several mechanisms have been elucidated, including TNF- α -induced apoptosis, inhibition of mitochondrial function, and neoantigen formation. Here, we report some of the most representative cases. Hepatotoxicity associated with the non-steroid anti-inflammatory drug (NSAID) nimesulide has led recently to its commercial withdrawal in some countries^[83]. The mechanism is unknown, although liver histology has shown centrilobular and bridging necrosis^[84]. Diclofenac potentially leads to zone 3 necrosis, autoimmune hepatitis, or even cholestasis^[85] in predisposed individuals. The major pathway of diclofenac metabolism is through 40-hydroxylation by CYP 2C9^[86]. Diclofenac also undergoes oxidative metabolism by CYP 2C8 to form reactive diclofenac acyl glucuronide and 5-hydroxydiclofenac^[87]. Nucleophilic displacement can then replace the glucuronic acid moiety to form adducts with free cysteine thiols^[88], and act as a potential hapten that triggers autoimmunity. Studies with diclofenac-protein conjugates have shown that diclofenac-treated hepatocytes carry antigens that are recognized by T-cell- and non-T-cell-enriched splenocytes^[89]. As a consequence, changes in the activity of CYP 2C8, its haplotype distribution, or impairment in the clearance of acyl glucuronide may potentially increase the risk of hepatotoxicity. Polymorphisms, such as the presence of UGT2B7*2 allele, favor the development of diclofenac hepatotoxicity^[90].

EXAMPLES OF LIVER DAMAGE INDUCED BY COMMONLY USED DRUGS

Aspirin induces hepatotoxicity that is different

from that of other NSAIDs. Aspirin is hydrolyzed into salicylic acid, which is transformed actively by mitochondria into its salicyl-coenzyme A derivative. This compound indirectly inhibits the β oxidation of long-chain fatty acids and increases NADH availability, thus resulting in increased capacity of mitochondria to decarboxylate branched chain amino acids^[91,92]. The negative effect on mitochondrial β oxidation probably is augmented by concomitant viral infection that affects mitochondrial function. This combination may determine microvesicular steatosis known as Reye's syndrome^[93].

Nefazodone, a triazolopyridine trazodone, an antidepressant drug, recently has been withdrawn from the market because of hepatotoxicity. Mechanisms include inhibition of mitochondrial respiratory complex I and IV, associated with accelerated glycolysis. This effect leads to mitochondrial membrane potential collapse, GSH depletion and oxidative stress^[94].

Hepatotoxicity exerted by isoniazid, an anti-tuberculosis drug is related to its metabolite monoacetyl hydrazine, which is activated at the CYP-450 level and detoxified by N-acetyltransferase 2. These enzymes undergo genetic variability and environmental alterations; slow acetylator status and CYP 2E1 genetic polymorphism are risk factors for isoniazid hepatotoxicity^[95,96]. Concomitant therapy with rifampicin, a CYP-450 inducer, significantly increases the risk of liver injury^[56].

Amiodarone is a commonly used antiarrhythmic drug that consists of a benzofuran ring coupled with two iodine and diethyl-ethanolamine side chains substituted with a p-OH-benzene structure. Amiodarone accumulates within mitochondria and causes toxicity by inhibiting state 3 glutamate and palmitoyl-CoA oxidation and by decreasing mitochondrial respiration^[55]. Electron transport chain complexes and β oxidation are also inhibited by amiodarone^[96]. The chemical structure of benzarone resembles that of amiodarone. Benzarone, a non-halogenated benzobromarone derivative, is used for the treatment of vascular disorders. It decreases mitochondrial membrane potential, as well as state 3 oxidation and respiratory control ratio, uncouples oxidative phosphorylation, and inhibits β oxidation. Benzarone increases the production of ROS, as well as the leakage of cytochrome C, with final induction of mitochondrial permeability transition^[97].

Troglitazone, a PPAR agonist, causes hepatocyte injury by dissipating the mitochondrial transmembrane potential, which favors superoxide generation, thioredoxin oxidation and activation of the kinase-1-dependent apoptosis signaling pathway^[98].

HIV-1 protease inhibitors are essential components of antiretroviral therapy. However, mitochondrial toxicity represents a serious problem for patients taking antiretroviral drugs. It occurs most often with administration of a full dose of ritonavir and saquinavir^[7]. Genetic HLA variants of the immune system seem to participate in the hepatotoxicity induced

Table 1 Mechanisms that favor high sensitivity of fatty liver to drug toxicity and necrotic cell death

Initial change	Intermediate effects	Consequences
Increased bioactivation (microsomal CYP 450s)	Higher amount of toxic metabolites	Consumption of antioxidants
Mitochondrial dysfunction	Increased release of ROS	Lipid peroxidation
	Decreased energy production (ATP) and cytochrome c content	Over-expression of uncoupling protein 2
	Increased release of ROS and NO derivatives	Increased Ca ²⁺ efflux
	Pores opening and increased membrane permeability	Protein oxidation and nitration
Impaired intracellular signaling and trafficking	Altered expression of nuclear receptors and sensors	Expression of FAS ligands
	Increased DNA fragmentation rate	Calpain activation and protein cleavage
Activation of non-parenchymal cells (Kupffer cells) and enzymes	Increased release of transforming growth factor- β 1, p53, TNF- α	Defective transcription of repair mechanisms
	Increased NADPH oxidase activity	Inflammation and pro-oxidant attack

ROS: Reactive oxygen species; TNF: Tumor necrosis factor.

by abacavir, another antiretroviral drug. Co-infection with hepatitis viruses is known to increase the risk of mitochondrial toxicity induced by these nucleoside compounds^[6].

CHOLESTASIS

Hepatic clearance of drugs depends on the activity of transport proteins that are located on the hepatocyte canalicular membrane. Alterations of these transporters by drugs or genetic polymorphisms increase the susceptibility to cholestatic injury^[14]. As a consequence, cholestasis is one of the most important features of drug-induced hepatotoxicity^[99]. Substrates for hepatic transport proteins include indomethacin, statins, digoxin, enalapril, midazolam, tamoxifen, diclofenac, methotrexate, and troglitazone. Selective inhibition of ATP-dependent bile salt transport proteins represents an additional mechanism of damage; therefore, co-administration of drugs at this level may enhance the risk of cholestasis. Examples are troglitazone plus lisinopril, itraconazole and verapamil, bosentan and glyburide^[100,101]. Changes in the expression of drug transporters in conditions of chronic liver disease can also result in marked alterations in drug disposition^[102]. Examples are increased bioavailability of drugs with high hepatic extraction, and decreased hepatic clearance of drugs with a low hepatic extraction and of those with biliary excretion^[103]. Finally, cholangiocytes can also be damaged directly by drugs. Flucloxacillin, an isoxazolyl-penicillin, can cause cholestasis by injuring bile duct epithelial cells^[104].

FUTURE PERSPECTIVES

Drug-induced liver injury occurs when the organ defense systems are overwhelmed. Preexisting conditions may contribute to the extent of damage. Two examples in this respect are the existence of fatty liver disease (liver steatosis), and genetic polymorphisms.

The mechanisms that favor high sensitivity of fatty liver to drug toxicity and necrotic cell death are depicted in Table 1. It is known that fatty liver has a reduced

tolerance towards stress conditions, i.e. ischemia-reperfusion, prolonged fasting, and exposure to *t*-butyl-hydroperoxide^[105,106]. Potential mechanisms that favor increased susceptibility of steatotic liver to drug-induced toxicity include mitochondrial imbalance^[107], increased mitochondrial ROS production^[108], and deficient repair capacity^[109]. Indeed, a high incidence of hepatotoxicity has been observed in patients with type 2 diabetes^[110], a condition that is associated inevitably with fatty liver^[111]. Therefore, it is conceivable that hepatotoxic drugs might produce injury even at non-toxic doses in patients with fatty liver, although in a recent study^[112], steatosis appeared to protect against paracetamol toxicity through preserving microcirculatory alterations. Defective hepatobiliary transport as well as the downregulation of Mrp2, as observed in rats with fatty liver, may represent additional predisposing factors for damage in these organs^[113].

Genetic polymorphisms are another important issue. Polymorphisms of CYP-450s account, at least in part, for the variability of efficacy and for the occurrence of adverse drug reactions, and may explain the variety of effects exerted by the same drug in different subjects. Genetic variations in the glutathione S-transferases (GSTT1 and GSTM1) have been associated with drug-induced hepatotoxicity^[114]. Subjects who display mutations in some alleles that code for manganese superoxide dismutase have a higher risk of developing drug-induced liver injury^[115]. Genetic mitochondrial abnormalities are a major determinant of the high susceptibility towards idiosyncratic liver injury caused by drugs that target mitochondria, especially in aged and female subjects^[116]. Genetic polymorphisms associated with alteration of hepatobiliary transporters have implications in drug-induced cholestasis^[14].

CONCLUSION

The search for the underlying mechanisms of damage is expected to lead to new intriguing perspectives for diagnosing and treating toxic liver injury. Today, certain microsomal and mitochondrial metabolic pathways can be assessed easily *in vivo* by performing breath tests with

substrates that release CO₂ during their metabolism. Methionine and α -ketoisocaproate breath tests assess mitochondrial functions and are altered after exposure to alcohol or drugs, thus reflecting specific metabolic alterations induced by exogenous compounds^[92,117]. Such noninvasive diagnostic tools may guide evaluation of the effect of therapeutic strategies.

Future issues might include the use of cytokine and death receptor antagonists, strategies directed at factors that cause mitochondrial damage, and approaches that promote survival gene expression that may overcome drug-induced cell death. In this regard, toxicogenomics, a combination of toxicology and genomics, attempts to identify the effects of drugs on gene expression, and the role of genetic polymorphisms in drug-induced liver injury. However, although recent developments in genetics and toxicology have provided some new insights into drug hepatotoxicity, the complex interactions of hepatotoxins with genetic and environmental risk factors responsible for the onset of toxic injury have yet to be elucidated. Severe drug-induced liver diseases therefore remain unpredictable for most drugs. The identification of new risk factors and a better understanding of pathogenetic mechanisms will certainly have implications for health care and pharmaceutical developments in the near future.

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S- Editor Li LF L- Editor Kerr C E- Editor Yin DH

Surgical outcome of carcinosarcoma of the gall bladder: A review

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Received: July 23, 2009 Revised: August 26, 2009

Accepted: September 3, 2009

Published online: October 21, 2009

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Key words: Gall bladder; Carcinosarcoma; Spindle cell carcinoma; Surgery; Outcome

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Okabayashi T, Sun ZL, Montgomery RA, Hanazaki K. Surgical outcome of carcinosarcoma of the gall bladder: A review. *World J Gastroenterol* 2009; 15(39): 4877-4882 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4877.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4877>

Abstract

Carcinosarcoma, which comprises less than one percent of all gall bladder neoplasms, is characterized by the presence of variable proportions of carcinomatous and sarcomatous elements. Recently, several reports have described patients suffering from carcinosarcoma of the gall bladder. However, there are no large studies regarding the clinicopathologic features, therapeutic management, and surgical outcome of this disease because the number of patients who undergo resection of gall bladder carcinosarcoma at a single institution is limited. A Medline search was performed using the keywords 'gall bladder' and 'carcinosarcoma'. Additional articles were obtained from references within the papers identified by the Medline search. Optimal adjuvant chemotherapy and/or radiotherapy protocols for carcinosarcoma of the gall bladder have not been established. Curative surgical resection offers the only chance for long-term survival from this disease. The outcome of 36 patients who underwent surgical resection for carcinosarcoma of the gall bladder was poor; the 3-year overall survival rate was only 31.0% and the median survival time was 7.0 mo. Since the postoperative prognosis of carcinosarcoma of the gall bladder is worse than that of adenocarcinoma, new adjuvant chemotherapies and/or radiation techniques are essential for improvement of surgical outcome.

INTRODUCTION

Adenocarcinoma is the most common type of malignant tumor generated in the gall bladder, whereas carcinosarcoma is rare with an incidence of less than one percent of all malignant gall bladder neoplasms^[1]. Carcinosarcomas are composed of variable proportions of both carcinomatous and sarcomatous elements. Recently, several reports have described patients suffering from carcinosarcoma of the gall bladder. However, there are no large studies that report the clinicopathologic features, therapeutic management, and surgical outcome for this disease because the number of patients who undergo resection at a single institution is limited.

In the literature, there are well-presented data from 36 reported patients who underwent surgical resection for carcinosarcoma of the gall bladder with intent to cure^[2-28]. The purpose of the present study was to analyze these 36 cases to clarify the factors that might influence surgical outcome, including survival rates after surgery, and to determine the prognostic factors of carcinosarcoma of the gall bladder.

PATIENTS

We analyzed data from 36 patients reported in the literature from 1971 to 2009^[2-28] who underwent surgical management for carcinosarcoma of the gall bladder (Table 1). These patients consisted of 10 male and 26 female with a mean age of 67.7 years (range 45 to 90

Table 1 Thirty six reported cases of surgical resection for carcinosarcoma of the gall bladder

Author	Year	Age (yr)	Sex	Palpation	Position	Stone	Size (cm)	Depth	SCC	Stage	Survival (mo)
Mehrotra <i>et al</i> ^[2]	1971	45	F	+	Neck	+	10	si	+	III	4
Higgs <i>et al</i> ^[3]	1973	77	M	-	Neck	-	ND	bd	+	IVa	1
Mansori <i>et al</i> ^[4]	1980	81	M	+	Body	-	15	liver	ND	IVa	1.5
Aldovini <i>et al</i> ^[5]	1982	75	F	+	Body	+	9	mp	-	III	8 ¹
Von Kuster <i>et al</i> ^[6]	1982	77	F	-	Fundus	-	3	mp	ND	II	31 ¹
Born <i>et al</i> ^[7]	1984	90	F	+	Body	+	15	du	ND	IVa	3
Inoshita <i>et al</i> ^[8]	1986	53	M	+	Neck	-	11	bd	+	IVa	17
Suster <i>et al</i> ^[9]	1987	54	F	+	Body	+	8	si	+	III	ND
Lumsden <i>et al</i> ^[10]	1988	81	F	+	Neck	-	5	mp	ND	II	12 ¹
Guo <i>et al</i> ^[11]	1988	69	M	ND	ND	ND	ND	ND	-	ND	3
		61	M	ND	ND	ND	ND	ND	-	ND	3
		66	M	ND	ND	ND	ND	ND	+	ND	19 ¹
Ishihara <i>et al</i> ^[12]	1990	58	F	-	Fundus	-	8	mp	-	II	7 ¹
Nishihara <i>et al</i> ^[13]	1993	63	F	ND	ND	-	9.5	ND	-	ND	39 ¹
		66	F	ND	ND	+	5	ND	-	ND	1
		70	F	ND	ND	-	4.2	ND	-	ND	7
		75	F	ND	ND	-	16	ND	+	ND	6
		80	F	ND	ND	-	5	ND	-	ND	1.5
Fagot <i>et al</i> ^[14]	1994	83	F	-	Fundus	+	4.5	mp	ND	II	12 ¹
Nakagawa <i>et al</i> ^[15]	1996	66	F	-	Fundus	-	7	liver	ND	IVa	ND
Rys <i>et al</i> ^[16]	1998	67	F	+	Fundus	-	15	liver, cln	ND	IVa	2
Eriguchi <i>et al</i> ^[17]	1999	65	F	+	ND	+	10	mp	ND	II	16 ¹
Ajiki <i>et al</i> ^[18]	2002	69	F	-	Body	+	6	liver	-	IVa	7
Hotta <i>et al</i> ^[19]	2002	53	M	+	Body	+	11	mp	-	II	7
Kim <i>et al</i> ^[20]	2003	61	F	+	Neck	-	4.5	si	-	IVa	2 ¹
Takahashi <i>et al</i> ^[21]	2004	84	F	-	Body	-	8	si	+	IVa	2
Huguet <i>et al</i> ^[22]	2005	64	F	-	Body	-	12	panc	+	IVa	4
Sodergren <i>et al</i> ^[23]	2005	68	F	-	Neck	-	9	bd	-	IVa	5
Kubota <i>et al</i> ^[24]	2006	72	M	+	Body	-	7	liver, col	-	IVa	8
Liu <i>et al</i> ^[25]	2009	51	M	ND	ND	ND	ND	si	-	IVa	3
		65	F	ND	ND	ND	ND	si	-	IVa	0.7
		56	F	ND	ND	ND	ND	si	-	IVa	5
Agarwal <i>et al</i> ^[26]	2009	60	F	+	Body	-	7	mp	-	II	3 ¹
Uzun <i>et al</i> ^[27]	2009	70	M	+	Fundus	-	10	mp	-	II	54 ¹
Shimada <i>et al</i> ^[28]	2009	69	M	-	Body	+	9	si	-	II	6 ¹
Present case	2009	72	F	-	Body	-	2.5	mp	-	II	60 ¹

¹Alive patients. SCC: Squamous cell carcinoma component; si: Serosal invasion; bd: Bile duct; mp: Muscularis propria; cln: Colon; panc: Pancreas; ND: Not described; Stage: Classification according to UICC (International Union Against Cancer).

years). The outcome of each case was obtained from the published data. We evaluated the clinicopathological findings including clinical symptoms, tumor location, tumor size, the number and size of gall bladder stones, depth of the tumor invasion, tumor stage according to International Union Against Cancer (UICC) criteria, pathological features, and survival rates. All patients had undergone attempted curative resection for carcinosarcoma of the gall bladder. Survival rates were estimated by using the Kaplan-Meier method and were compared by using the log-rank test^[29]. Values were expressed as mean \pm SD. Differences in proportions were evaluated by the Pearson χ^2 test. $P < 0.05$ was considered to be statistically significant.

DIAGNOSIS OF CARCINOSARCOMA OF THE GALL BLADDER

Table 1 lists the 36 patients who underwent curative surgical resection for carcinosarcoma of the gall bladder and summarizes the clinical features and outcome. In these patients, carcinosarcoma was not associated with

any specific clinical syndromes. All patients presented clinical symptoms, such as abdominal pain, fever, anorexia, nausea, vomiting, painless jaundice, anorexia, and/or body weight loss (data not shown). In 56% of cases in which carcinosarcoma of the gall bladder was diagnosed, it was recognized as a palpable mass. The size of gall bladder carcinosarcomas appears to be larger than that of gall bladder carcinomas. The mean size of carcinosarcomas of the gall bladder was 8.4 ± 3.7 cm (range, 2.5-16 cm) in 29 patients with available data (Table 1).

Accurate preoperative diagnosis of carcinosarcoma of the gall bladder is very difficult because imaging studies cannot differentiate it from carcinoma of the gall bladder. Abdominal angiography often shows neovascularity and staining of carcinosarcomas of the gall bladder, whereas computed tomography (CT) shows an enhanced solid mass lesion. Differential diagnosis includes gall bladder carcinoma when there is calcification, calcified gall stones, or porcelain gall bladder, and carcinosarcoma of the gall bladder is suspected when calcification is observed within the tumor on CT examination^[15]. However, more detailed imaging

Table 2 Clinical characteristics after surgical resection for carcinosarcoma of the gall bladder

Characteristics	n	Survival rate (%)			Median survival in months (range)	P value
		1 yr	2 yr	3 yr		
Overall	36	37.2	31.0	31.0	7.0 (4.4-9.6)	
Age (yr)						
< 65	14	37.7	18.9	18.9	5.0 (0.3-9.7)	0.887
> 65	22	36.7	36.7	36.7	7.0 (4.2-9.8)	
Gender						
Male	10	36.0	24.0	24.0	6.0 (0.3-13.7)	0.877
Female	26	37.5	37.5	37.5	6.0 (3.3-8.7)	
Palpable mass						
Present	14	48.0	48.0	48.0	7.0	0.853
Absent	11	47.6	23.8	23.8	8.0 (0.0-16.9)	
Stone						
Present	10	40.0	-	-	7.0 (3.2-10.8)	0.937
Absent	20	42.1	33.7	33.7	7.0 (3.4-10.6)	
Size of the tumor (cm)						
< 5	8	60.0	60.0	60.0	-	0.361
> 5	21	36.6	24.4	24.4	7.0 (5.1-8.9)	
Si or organ invasion						
Present	18	9.2	0.0	0.0	4.0 (2.2-5.8)	0.001
Absent	10	88.9	88.9	88.9	-	
Scc component						
Present	8	28.6	14.3	-	4.0 (1.4-6.6)	0.291
Absent	16	31.2	31.2	31.2	7.0 (4.8-9.2)	
Stage						
II	10	87.5	87.5	87.5	-	0.001
III or IVa	18	13.8	0.0	0.0	4.0 (2.2-5.8)	
Changing trends						
1970-1989	12	45.5	30.3	-	4.0 (0.0-12.9)	0.920
1990-1999	10	44.4	44.4	-	7.0 (4.1-9.9)	
2000-2009	14	20.8	20.8	20.8	7.0 (4.8-9.2)	

Scc: Squamous cell carcinoma; Si: Serosal invasion; Stage: Classification according to International Union Against Cancer (UICC).

data is needed to improve diagnosis of carcinosarcoma of the gall bladder. Carcinosarcoma of the gall bladder is not associated with specific radiological findings or serum data, including tumor markers (carcinoembryonic antigen, carbohydrate antigen 19-9, or squamous cell carcinoma antigen). Carcinosarcoma of the gall bladder should be considered as a differential diagnosis of neoplasms of the gall bladder, especially when patients present with severe abdominal symptoms and/or a large tumor size.

Table 2 summarizes the trends of the incidence of carcinosarcoma of the gall bladder. These data reveal that despite advancements in diagnostic techniques and equipment in recent years, the frequency of resections in patients with gall bladder carcinosarcomas has not increased.

PATHOLOGICAL FEATURES OF GALL BLADDER CARCINOSARCOMA

Carcinosarcoma is characterized by malignancy of both the epithelial and mesenchymal components of the same tissue. Its diagnosis requires the presence and intermingling of both histological components. In most reported cases of gall bladder carcinosarcoma the epithelial component is adenocarcinoma, although a squamous cell carcinoma component is also often present. The mesenchymal component varies from

homogeneous sarcoma to more heterotopic elements such as malignant bone, cartilage, and other mesenchymal tissues^[22,27]. Sarcomatous change or squamous change from adenocarcinoma leads to aggressive spread and metastasis. Squamous cell carcinomas grow at twice the speed of adenocarcinomas^[30-32]. Therefore, once an adenocarcinoma transforms to an adenocarcinoma with a squamous component, the carcinoma exhibits a high degree of malignancy. For our series of carcinosarcoma of the gall bladder patients, the presence of either cartilage, rhabdoid tumor or a squamous component was not a significant prognostic factor.

The homogeneous sarcoma was usually a spindle cell type in these cases. The pathogenesis of carcinosarcoma is poorly understood. Several theories have been proposed to explain the admixture of epithelial and mesenchymal tissues in these neoplasms: (1) mesenchymal reaction, (2) true sarcoma (including the collision neoplasm hypothesis), (3) malignant proliferation of epithelial origin (including the stromal induction/metaplasia model), (4) an embryonic cell rest origin, and (5) the totipotent stem cell hypothesis^[24,33]. The third theory is supported by reports based on immunohistochemical findings^[19,24]. Sarcomatous change of carcinoma can be induced by radiotherapy, alterations to the *p53* gene, and the production of bone morphogenetic protein by cancer cells^[34-36]. A recent report suggests that genetic and gene expression alterations may underlie the

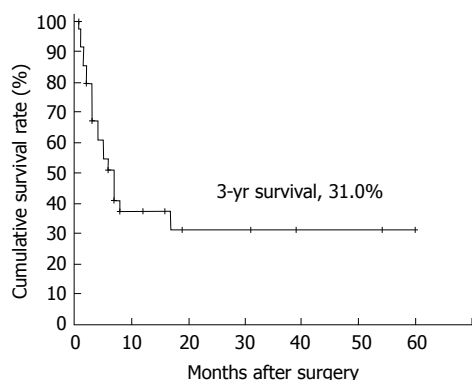


Figure 1 Survival after surgical resection for carcinosarcoma of the gall bladder ($n = 36$).

sarcomatous change or epithelial mesenchymal transition in cholangiocarcinoma^[37]. On the other hand, it has been speculated that these neoplasms arise from totipotent stem cells, rest cells of myoblasts that retain the capability of transformation, primitive undifferentiated müllerian stroma, or paramesonephric tissue^[5,22,38].

MANAGEMENT OF RESECTABLE CARCINOSARCOMA OF THE GALL BLADDER

Since carcinosarcomas are uncommon tumors with a poor prognosis, the outcomes related to various therapeutic interventions are not well defined and no optimal postoperative adjuvant therapy has been established.

Table 1 shows tumor location and the clinicopathological features of the 36 reported cases of carcinosarcoma of the gall bladder. Six cases (25.0%) arose in the neck of the gall bladder, 12 in the body of the gall bladder (50.0%), and the other 6 cases arose in the fundus of the gall bladder (25.0%). Among these cases, the incidence of tumor invasion of the muscularis propria was 35.7%, and in the remaining 63.3% of cases the tumor had perforated the visceral peritoneum or had invaded other organs. Although carcinosarcoma of the gall bladder has different clinicopathological features from adenocarcinoma, the treatment strategies are similar. It is considered that surgical treatment remains the only curative management option for carcinosarcoma of the gall bladder. Simple cholecystectomy and extended cholecystectomy, including cholecystectomy with the adjacent liver bed, with the extrahepatic bile duct, with partial resection of the small intestine and/or colon, and with pancreaticoduodenectomy, were performed in 9 (36.0%) and 16 (64.0%) of cases, respectively. Extended cholecystectomy was performed for carcinosarcoma of the gall bladder because most of these cases presented with a large mass invading adjacent organs.

PROGNOSIS AFTER SURGICAL RESECTION

The overall 1-, 2-, and 3-year survival rates after surgery

were 37.2%, 31.0%, and 31.0%, respectively (Figure 1). By comparing the survival rate among the subgroups identified by each predictive factor with the univariate analysis of the prognostic factors, two factors, namely (1) the presence of serosal invasion and/or involvement into other organs, and (2) advanced stage according to the classification of UICC in resected specimens, were found to be significantly associated with a poor outcome after surgery (Table 2).

The cases examined in the current study were patients recruited for surgical treatment of carcinosarcoma of the gall bladder with intent to cure; however, the current overall 5-year survival rate of 31.0%, which included an in-hospital mortality rate of 8.3%, was comparable to or worse than the reported rates for adenocarcinoma of the gall bladder. It is likely that the overall and median survival is poor because two-thirds of the patients with carcinosarcoma of the gall bladder in this study had serosal invasion and/or involvement of other organs. This finding suggests that carcinosarcoma has greater malignant potential than adenocarcinoma of the gall bladder. The 5-year survival rate after curative resection for carcinosarcoma of the gall bladder was 88.9% when tumor invasion was restricted to the muscularis propria.

Due to limited experience, the staging system could not be defined, nor has any consensus been established on the management of carcinosarcoma of the gall bladder. Here, we used statistical analysis to support a correlation between the staging system for carcinoma of the gall bladder according to UICC and the classification of carcinosarcoma of the gall bladder. In addition, the 5-year survival rate was 87.5% even when resection with intent to cure was performed for stage II tumors. The three cases surviving more than 3 years included a patient where tumor involvement was limited to the muscularis propria^[13,27]. Although curative resection provides the best hope for long-term survival with early stage tumors, only 35.7% of gall bladder carcinosarcoma cases are discovered at the early stage (stage II). It is likely that the overall and median survivals are poor in this study because 64.3% of patients with carcinosarcoma of the gall bladder had tumors at stage III or IV. As shown in Table 2, curative resection and stage II tumors are significant factors for a favorable prognosis for patients with carcinosarcoma of the gall bladder, thus an early diagnosis is required for a better outcome after treatment.

Recurrence was evaluated for twelve patients in this study. The major sites of recurrence were the liver (10 patients), lymph nodes (5 patients), and peritoneal cavity (4 patients). The median time to recurrence was less than one year. From the time of surgery, recurrence occurred within half a year in 8 patients (80.0%). The median time to recurrence for patients who died was only 1.5 mo. The invasive nature and aggressive malignant biology of carcinosarcoma explains the limited number of resectable cases. The results of this study suggest that adjuvant strategies would be beneficial for pre- and post-operatively diagnosed carcinosarcoma. Previous studies reported the use of chemoradiotherapy after

surgical resection of carcinosarcoma of the gall bladder, but this treatment did not significantly improve patient prognosis^[10,18,19,22,25].

CONCLUSION

Prognosis is poor following curative resection for carcinosarcoma of the gall bladder because of recurrence as systemic metastasis of the liver and peritoneal dissemination. In addition, a large proportion of these patients have recurrence during the postoperative early period. Consensus of opinion as to surgical indication for this tumor has not yet been achieved. Surgical treatment strategies based on the appropriate surgical indication are essential for improvement of surgical outcome because curative resection is usually not possible for advanced disease. For these reasons, once a diagnosis of carcinosarcoma of the gall bladder is made it is important to inform patients and their family regarding the biological behavior of this uncommon disease and the proposed prognosis following curative surgical treatment.

Exploration of new radiation techniques and of chemotherapeutic regimens with new drugs is required for the treatment of carcinosarcoma of the gall bladder because conventional chemotherapy and radiotherapy do not increase patient survival. Novel 'molecularly targeted' agents may improve surgical outcome. The prognosis of carcinosarcoma of the gall bladder remains poor despite curative resection, thus efforts to improve surgical outcome should continue for this rare, worldwide disease. Furthermore, the collection of epidemiologic data and pathologic findings will be required to determine the appropriate surgical indication for this tumor.

ACKNOWLEDGMENTS

We thank Toru Ando, Senko Fujiwara, Kengo Ichikawa, and Shinzo Ozaki (Kochi Prefectural Hata-Kenmin Hospital) for advice. This study was made possible with the support and suggestions of Junichi Miyazaki, and Norihito Kamioka (Kochi Prefectural Hata-Kenmin Hospital).

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Multifocal stenosing ulceration of the small intestine

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Received: May 27, 2009 Revised: September 14, 2009

Accepted: September 21, 2009

Published online: October 21, 2009

Abstract

Several reports have described an apparently uncommon clinicopathological disorder that is characterized by multifocal stenosing small-intestinal ulceration. Compared to Crohn's disease, the ulcers are not transmural and typically remain shallow, and involve only the mucosa and submucosa. The disorder seems to be localized in the jejunum and proximal ileum only, and not the distal ileum or colon. Only nonspecific inflammatory changes are present without giant cells or other typical features of granulomatous inflammation. Most patients present clinically with recurrent obstructive events that usually respond to steroids, surgical resection, or both. With the development of newer imaging modalities to visualize the small-intestinal mucosa, such as double-balloon enteroscopy, improved understanding of the long-term natural history of this apparently distinctive disorder should emerge.

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Key words: Ulcer; Stenosis; Intestinal diseases; Small intestine; Crohn's disease

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Freeman HJ. Multifocal stenosing ulceration of the small intestine. *World J Gastroenterol* 2009; 15(39): 4883-4885 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4883.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4883>

INTRODUCTION

A syndrome that is characterized by intermittent episodes of small-intestinal obstruction caused by benign multifocal ulcerated stenosis has been described, largely, but not entirely, in the French literature^[1-10]. This disorder has also been termed "cryptogenic multifocal ulcerous stenosing enteritis" (CMUSE) and has been reported to localize largely within the jejunum or proximal ileum. CMUSE typically is associated with shallow, rather than deep transmural ulceration, and has also been noted to be usually responsive to steroids, although occasionally, surgical resection is required. The location of ulceration and strictures in the more proximal small intestine, along with the absence of any associated granulomatous inflammatory changes in resected material are believed to differentiate this entity from Crohn's disease, which usually is localized in the distal ileum and the colon^[11]. CMUSE appears to be an entirely distinct disorder from other small-bowel disorders, which is characterized by multifocal ulceration with stricture formation. With new imaging modalities increasingly becoming available, particularly double-balloon enteroscopy, further appreciation of this entity and its natural history should result.

DIFFERENTIAL DIAGNOSIS

A number of other entities may cause multifocal small-bowel ulcers (Table 1). Idiopathic ulcerative jejunoileitis, described elsewhere in detail^[12], is a small-intestinal ulcerative disorder, often associated with celiac disease, or at least, with concomitant flattening of the villous architecture of the small bowel. Some believe that the disorder may represent a specific complication of celiac disease, but ulceration of the small intestine in this setting may also be caused by a difficult-to-diagnose focal lymphoma with lymphomatous cells localized at the ulcer edges^[13].

A variety of infectious agents, some common, may also cause small bowel ulceration^[7], but most of these (e.g. *Campylobacter*, *Shigella*, *Yersinia* and *Salmonella*) seem to resolve completely without stricture formation. Of course, the natural history of many infections in the small intestine are not particularly well defined (e.g. tuberculosis and cytomegalovirus infection), especially if immune

Table 1 Other causes of multifocal small-bowel ulceration and stenosis

Ulcerative jejunoileitis with celiac disease or sprue-like intestinal disease
Lymphoma (especially mucosa only), including T-cell enteropathy and α -chain disease (Mediterranean type)
Crohn's disease involving the small intestine
Infections (e.g. <i>Campylobacter</i> and <i>Shigella</i>)
Drug-induced type (especially with NSAIDs)
Zollinger-Ellison syndrome (gastrinoma) or other hypersecretory disorders
Traumatic injury (e.g. endoscopic or surgical treatment, seat-belt injury)
Ischemia related to vasculopathy (e.g. collagen vascular disease, coagulopathy, or inflammatory vasculitis)

NSAIDs: Nonsteroidal anti-inflammatory drugs.

suppression from a concomitant disease or drug treatment is present. Other infectious agents appear to cause a completely different pathological reaction, although ulceration may conceivably occur (e.g. *Tropheryma whipplei* or *Mycobacterium avium intracellulare*).

Drug-induced causes of small-bowel ulceration are numerous and include potassium, gold and chemotherapeutic agents. Currently, the most common causes of drug-induced mucosal injury are nonsteroidal anti-inflammatory drugs (NSAIDs) that may cause frank ulcers, erosions, broad strictures and so-called diaphragm disease^[8]. Alternatively, NSAIDs also have been associated with the small-bowel-mucosal lesion that is characteristic of untreated celiac disease in the absence of ulcer formation^[14].

Peptic ulceration may occur but this is unusual in the jejunum and proximal ileum^[7]. In this location, a peptic ulcer could hypothetically be a clue to an occult Zollinger-Ellison syndrome from a gastrinoma, or heterotopic functioning gastric mucosa. Meckel's diverticulum may be associated with small-bowel ulceration caused by heterotopic functioning gastric mucosa, but usually, the ulceration occurs in the more distal ileum.

Crohn's disease may occur in the jejunum without disease elsewhere, but this seems to be uncommon^[15], and as noted earlier, usually is associated with other clinical and pathological features of Crohn's disease (Table 2).

Traumatic injury may also occur. Surgical injury (including peri-anastomotic ulceration) and external trauma from seat belts in motor vehicle accidents are being increasingly recognized. Endoscopic biopsies for diagnostic purposes, therapeutic interventions (e.g. cautery for polypectomy) or foreign bodies (e.g. ingested suture materials) may induce or be associated with small-intestinal ulcerations.

Finally, ischemic pathogenesis that results from a variety of causes may occur. For example, some systemic diseases have been associated with multifocal ulcers and stricture formation including: thrombotic diseases, Dego's disease, pseudoxanthoma elasticum, myeloproliferative disorders, anti-thrombin III deficiency and vasculitis associated with an occult collagen vascular disease (e.g. systemic lupus erythematosus or polyarteritis nodosa).

CLINICAL FEATURES

A previous retrospective evaluation^[7] of reported

Table 2 Differentiation of CMUSE from Crohn's disease

Absence of clinical or laboratory features of an inflammatory syndrome
Absence of small-intestinal transmural inflammatory process or ulceration
Absence of small-intestinal giant-cell granulomatous inflammatory process
Absence of small-intestinal fistula formation despite recurrent chronic disease
Absence of disease in other parts of gastrointestinal tract (i.e. stomach or colon)
Absence of most extraintestinal features of Crohn's disease (e.g. skin manifestations)

CMUSE: Cryptogenic multifocal ulcerous stenosing enteritis.

cases of CMUSE has revealed that virtually all patients complained of abdominal symptoms and about 70% had extraintestinal symptoms that included weight loss, fever, malaise and joint symptoms. The lesions in the small intestine were considered characteristic of stenosis (1-25, mean 8) found in the jejunum or the proximal ileum. The remainder of the small intestine appeared to be completely normal. Ulceration was superficial, involved the mucosa, sometimes the submucosa, but did not extend deeper into underlying tissues. All of these stenosis were associated with a nonspecific inflammatory infiltrate only. About 40% had persistent pain. Abdominal pain appeared to resolve with steroids but ongoing treatment was reported to be required in 50% of patients. Surgical resection resulted in complete recovery in about 40% but a second resection for recurrent stenosis was needed in 25% of patients. Occasionally, some required multiple resection. Vascular changes and complement component deficiency also have been associated with CMUSE^[7].

FUTURE DIRECTIONS

CMUSE needs to be defined carefully and more precisely. Its etiology and pathogenesis are unknown. Diagnosis of CMUSE should be considered only after exclusion of each entity listed in the differential diagnosis above, especially Crohn's disease and drug-induced ulceration with stenosis. Nonspecific small-intestinal ulceration without stricture formation should be considered a separate entity. With the development of better imaging methods for the small intestine, especially double-balloon enteroscopy, the opportunity now may be present to further explore this intriguing entity.

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S- Editor Tian L L- Editor Kerr C E- Editor Lin YP



REVIEW

Systematic review of Chinese herbal medicine for functional constipation

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Received: June 19, 2009 Revised: August 13, 2009
Accepted: August 20, 2009
Published online: October 21, 2009

Abstract

Constipation is a common gastrointestinal complaint in clinical practice, affecting an estimated 27% of the population. Many patients are disappointed by current conventional treatments and, therefore, seek help from complementary and alternative medicine (CAM). Traditional Chinese medicine, is the most important part of CAM and has been practiced for treating diseases and promoting the health of humans for thousands of years, and has become a popular alternative choice. Although there are many Chinese herbal medicine (CHM) interventions available, and some have been verified by clinical trials, their efficacy and safety are still questioned by both patients and health care providers worldwide. The purposes of this review are, first, to appraise the qualities of individual study designs in the new Cochrane approach. Second, the benefits of individual CHM interventions or individual types of CHM intervention for the treatment of functional constipation are analyzed. Finally, valid and comprehensive conclusions are drawn, if applicable, in order to make clinical recommendations.

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INTRODUCTION

Constipation is a common gastrointestinal complaint in clinical practice. It affects an estimated 12%-19% of Americans^[1], 14% of Asian^[2], and up to 27% of the general population depending on demographic factors, sampling, and definition^[3]. A variety of over-the-counter medications are available. It is estimated that in the US alone, more than \$800 million is spent annually on laxatives^[4], with each constipated patient spending approximately \$7900, accounting for 6.5% of the total medical expenditure on lower gastrointestinal diseases^[5]. However, many patients are disappointed by current conventional treatments^[6,7] and, therefore, seek help from complementary and alternative medicine (CAM)^[8].

Many traditional Chinese medicine (TCM) interventions have been used for the treatment of constipation. A recent review listed the current clinical research findings of TCM interventions on treating functional constipation (FC)^[9]. However, an analysis on the benefits of individual interventions or individual types of interventions, and the qualities of individual study designs has not been undertaken. To draw valid and comprehensive conclusions and make clinical recommendations, a systematic review of Chinese herbal medicine (CHM) for FC is necessary.

This review aimed to determine the efficacy and safety of CHM for the treatment of FC by summarizing current available randomized controlled trials (RCTs) according to the Cochrane approach, newly revised in 2008.

CRITERIA AND METHODS FOR LITERATURE SEARCH

Criteria for considering studies for this review

The criteria for considering studies for this review are as follows. (1) Types of studies. Only RCTs without

restriction on language and publication types were included; pseudo-RCTs were not considered; (2) Types of participants. Patients of both sexes and of any age or any ethnic group with diagnosed FC according to the Rome criteria (Rome I, II or III) were included while those with secondary constipation due to medication and/or other diseases were excluded; (3) Types of interventions. Any form of CHM in any dose or as add-on combination treatment was considered, including oral and external preparations. Comparisons could include placebo, no intervention, acupuncture, massage, Western conventional medication (WCM) or any other interventions. Studies comparing one kind of CHM to another CHM were also included; (4) Types of outcomes. The responder rate of patients with a mean increase of ≥ 1 complete spontaneous bowel movement (CSBM) per week was considered a primary outcome. This outcome by combining a subjective measure of the completeness of defecation with an objective measure of stool frequency was considered to be clinically meaningful^[10]. If this outcome measure was not used in the study, the overall effectiveness assessment according to the references of Criteria of Diagnosis and Therapeutic Effect of Diseases and Syndromes in Traditional Chinese Medicine^[11], Guidelines for Clinical Research on New Chinese Herbal Medication^[12], Guidelines for Clinical Research on New Chinese Herbal Medication (Draft)^[13] or criteria made by the authors with details and comparable definitions were also considered. Based on the above criteria, interventions which resulted in improvement in general constipated symptoms and/or objective examination indices, for general improvement $\geq 30\%$ compared to their baselines, were counted as effective. Secondary outcomes including (a) Changes in individual symptoms, such as stool frequency, straining, completeness of defecation; (b) Changes in examination indices, such as blood nitric oxide (NO) and substance P (SP) levels, total colon transit test (TCTT) and anorectal pressure; (c) Changes in quality of life assessment as assessed with the Health Related Quality of Life (HRQOL) or other validated scales; (d) Adverse events (AEs), such as functional injury of liver or kidney, nausea, vomiting, diarrhea and allergic reaction.

Search methods for identification of studies

All relevant studies regardless of language or publication status were identified by searching the following databases from 1994, the year of the establishment of Rome criteria, up to the May 18 of 2009. (1) Ovid SP, which included the databases of Cochrane DSR (Cochrane Database of Systematic Reviews), ACP Journal Club, DARE (Database of Abstracts of Reviews of Effects), CCTR (Cochrane Central Register of Controlled Trials), CMR (Cochrane Methodology Register), HTA (Health Technology Assessment), and NHSEED (NHS Economic Evaluation Database), AMED, BIOSIS Previews (2001-2006), Biological Abstracts (1994-2000), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations (1950 to Present), Ovid MEDLINE(R) (1950 to Present), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations Present, and Ovid MEDLINE(R) Daily Update

Table 1 Search strategy for Ovid SP (advanced Ovid search)

No.	Searches
1	case*.m_titl
2	clinical observation.mp
3	clinical trial.mp
4	clinical study.mp
5	efficacy.mp
6	effectiveness.mp
7	1 OR 2 OR 3 OR 4 OR 5 OR 6
8	random.mp
9	randomi*ed.mp
10	randomi*zation.mp
11	8 OR 9 OR 10
12	functional constipation.mp
13	irritable bowel syndrome.m_titl
14	ibs.m_titl
15	13 OR 14
16	12 NOT 15
17	rome.mp
18	16 AND 17
19	Chinese medicine*.mp
20	herbal medicine*.mp
21	herb*.mp.
22	Chinese adj10 oriental medicine*.mp
23	19 OR 20 OR 21 OR 22
24	7 AND 11 AND 18 AND 23

""was used for truncation.

Table 2 Common search strategy for VIP, TCM Database System and CJN

Title contains "case (Li)" OR Title/Abstract/Keyword contains ["efficacy observation (LiaoXiao GuanCha)" OR "efficacy comparison (LiaoXiao BiJiao)" OR "efficacy analysis (LiaoXiao FenXi)" OR "clinical observation (LinChuang GuanCha)" OR "clinical research (LinChuang YanJiu)" OR "clinical trial (LinChuang ShiYan)"]
AND
Text word contains "random (SuiJi)"
AND
Title/Abstract/Keyword contains "functional constipation (GongNengXing BianMi)" NOT Title contains ["irritable bowel syndrome (ChangYiJi ZongHeZheng)" OR "ibs"]
AND
Text word contains ["rome (LuoMa)" OR "rome"]
AND
Text word contains ["Chinese medicine (ZhongYao/ZhongYiYao)" OR "herbs (CaoYao)" OR Chinese herbal medicine (ZhongCaoYao) OR "Chinese proprietary medicine (ZhongChengYao)"]

TCM: Traditional Chinese medicine; CJN: China Journal Net.

Present. Detailed search strategy presented in Table 1. (2) VIP Citation Databases (VIP); (3) Traditional Chinese Medical Database System (TCM Database System); (4) China Journal Net (CJN). The common search strategy for VIP, TCM Database System and CJN is listed in Table 2. The Chinese wordings were presented in Pinyin.

Data collection and analysis

The title and abstract of the search results were scanned and full articles for all potentially relevant trials were retrieved. A data extraction form was used to extract data on study characteristics including methods, participants, interventions and outcomes. The reasons for the exclusion of studies were recorded accordingly.

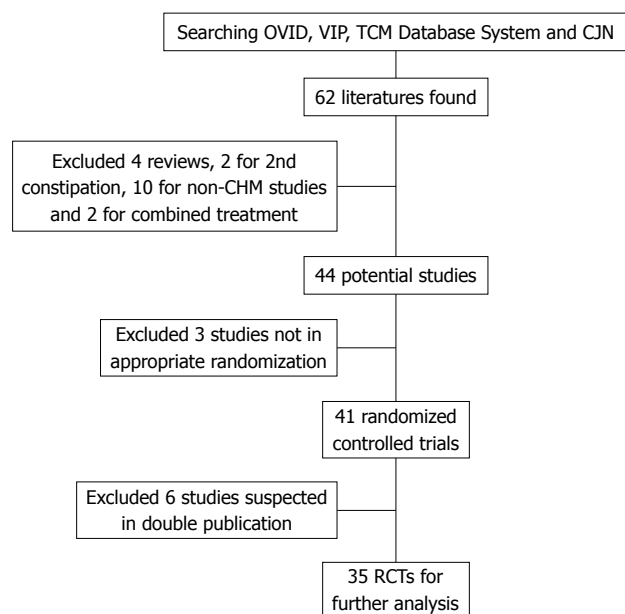


Figure 1 Flow diagram for literature search. TCM: Traditional Chinese medicine; CJN: China Journal Net; CHM: Chinese herbal medicine; RCTs: Randomized controlled trials.

The treatment effects of all CHM interventions were analyzed using Review Manager (Version 5.0). Mean difference with 95% confidence interval was used for continuous data while relative risks with 95% confidence interval was used for binary data. The risk of bias on sequence generation, allocation concealment, blinding, incomplete outcome data, selective outcome reporting and other potential threats to validity were assessed as “YES” (low risk of bias), “NO” (high risk of bias) and “UNCLEAR” (uncertain risk of bias) according to the criteria described in the Cochrane Handbook for Systematic Reviews of Interventions 5.0.1^[14].

SUMMARY OF LITERATURE

Description of studies

A total of 62 articles were identified. Of these, four articles were excluded because they were reviews^[9,15-17], two articles were excluded because they dealt with secondary constipation^[18,19], ten articles were excluded because they did not include CHM^[20-29], and two articles were excluded because they evaluated combination treatment of WCM and CHM by comparing with massage or WCM^[30,31]. This left 44 studies which claimed to be “randomized controlled” trials for FC.

Of these studies, three were not real RCTs because they used the admission sequence for treatment allocation, and thus were excluded^[32-34]. Six studies were suspected of being published more than once by the authors or publishers, and were excluded^[35-40]. This further screening left 35 studies for review. The screening process is summarized in a flow diagram (Figure 1).

Characteristics of included studies

A total of 3571 participants (ranging in age from 1 mo to 93 years) were included in these 35 studies. With the exception of two^[41,42] in 3 parallel groups, all studies used

Table 3 Details of CHM interventions in the included studies

Intervention	Preparation form	<i>n</i>
LiuWei Auxiliary	Capsule	4
LiuWeiAnXiao Capsule/ LiuWeiNengXiao Capsule		
MaRen Auxiliary		7
MaRen Pill/MaZiRen Pill	Pill	2
MaRenRunChang Wan	Pill	1
Modified MaRenRunChang Wan	Pill	1
MaRen Capsule	Capsule	1
MaRen Soft Capsule	Capsule	2
RunChangTongBianNongSuo Pill	Pill	2
Others (in single investigation)	Decoction	16
	(w/o modification)	
	Decoction	4
	(w/modification)	
	Capsule	4
	Pill	3
	Solution	1
	Granule	1

CHM: Chinese herbal medicine.

a 2 parallel group design. Thirty six CHM interventions, including add-on with WCM treatment, were investigated by comparing with another CHM and/or WCM. The details of CHM interventions are listed in Table 3.

Risk of bias: The methodological quality of each study’s randomization sequence, allocation concealment, blinding, incomplete outcome data, selective outcome reporting and potential threats are summarized in Figures 2 and 3.

Randomization & allocation concealment: Only two studies clearly stated a random component in the sequence generation process, Liu *et al*^[43] used randomization software while Xie *et al*^[44] used an open random allocation schedule in sequence generated with a random number table. For the others, the words “random allocation” were cited in abstracts and/or main texts but without description.

Blinding: None of the participants, personnel or outcome assessors were blinded in any of these studies. Although minority outcome measures were based on the objective examination results, such as blood NO and SP levels, total colon transit time and anorectal pressure, the risk of both performance bias and detection bias with regard to general symptom improvement and safety issues were deemed very high.

Flow of participants and intention-to-treat: None of the trials reported the withdrawal, drop-out and/or loss to follow up rates. The method of handling missing data regarding intention-to-treat or per protocol analysis was not addressed.

Selective outcome reporting: Five studies had a high risk of bias with regard to selective outcome reporting^[45-49] because the data on individual symptoms, overall improvement and colon transit test pre-specified were reported incompletely in the results. Thus further meta-analysis could not be implemented.

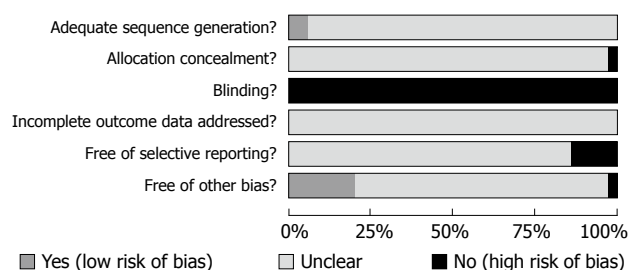


Figure 2 Methodological quality graph: judgments about each methodological quality item presented as percentages across all included studies.

Other potential threats to validity: MaRen Capsule, which is derived from the ancient formula MaZiRenWan, was used as the control for treating FC in the Syndrome of Qi and Yin Deficiency in the study by Fan *et al.*^[50]. As MaZiRenWan is the representative formula for treating heat constipation (excessive constipation), it is therefore, not suitable for patients suffering from FC with Qi and Yin deficiency, and such a study design was a potential source of bias in assessing the efficacy and safety of MaRen Capsule.

Effects of interventions

None of the trials reported the responder rate on complete spontaneous bowel movement; instead overall effectiveness in which patients with improvement in general constipation symptoms and/or objective examination indices, was commonly used as a primary outcome measure.

CHINESE HERBAL MEDICINE VS PLACEBO/NO TREATMENT (COMPARISON 01)

None of the included trials used a placebo control, but one used no treatment as a control^[51]. General treatment was allowed for all participants in both groups, such as increased fibre and liquid intake, physical exercise and defecation habit training. The total effectiveness rates of Modified MaRenRunChang Pill and the control were 91.4% and 59.6%, respectively ($P < 0.01$). Reported AEs included two cases of diarrhea and one case of nausea and loss of appetite.

CHINESE HERBAL MEDICINE VS WESTERN CONVENTIONAL MEDICINE (COMPARISON 02)

Twenty-six studies, including two with three parallel groups^[41,42], tested 24 different CHM interventions compared with cisapride, polyethylene glycol 4000 (PEG), mosapride, phenolphthalein, itopride and bifidobacterium.

CHM vs cisapride

Nine studies compared nine different CHM interventions with cisapride or add-on with cisapride and/or

	a	b	c	d	e	f
Cal ^[53] , 2004	?	?	-	?	?	?
Chen ^[74] , 2009	?	?	-	?	?	?
Chen <i>et al.</i> ^[54] , 2004	?	?	-	?	?	?
Fan <i>et al.</i> ^[50] , 2009	?	?	-	?	?	-
Gan <i>et al.</i> ^[48] , 2008	?	?	-	?	-	+
Guo ^[70] , 2003	?	?	-	?	?	+
Guo <i>et al.</i> ^[71] , 2006	?	?	-	?	?	?
Huang ^[75] , 2008	?	?	-	?	?	?
Jiang <i>et al.</i> ^[72] , 2007	?	?	-	?	?	?
Kang <i>et al.</i> ^[65] , 2004	?	?	-	?	?	?
Li <i>et al.</i> ^[51] , 2004	?	?	-	?	?	?
Li <i>et al.</i> ^[68] , 2008	?	?	-	?	?	?
Li <i>et al.</i> ^[55] , 2005	?	?	-	?	?	+
Li <i>et al.</i> ^[73] , 2008	?	?	-	?	?	+
Liang <i>et al.</i> ^[63] , 2008	?	?	-	?	?	?
Liu <i>et al.</i> ^[43] , 2004	+	?	-	?	?	?
Liu ^[41] , 2005	?	?	-	?	?	?
LWAX Collaboration ^[60] , 2004	?	?	-	?	?	+
Meng ^[69] , 2009	?	?	-	?	?	?
Qu <i>et al.</i> ^[42] , 2008	?	?	-	?	?	+
Shi <i>et al.</i> ^[46] , 2008	?	?	-	?	-	?
Sun <i>et al.</i> ^[58] , 2008	?	?	-	?	?	+
Wang ^[66] , 2004	?	?	-	?	?	?
Wang <i>et al.</i> ^[64] , 2007	?	?	-	?	?	?
Wang <i>et al.</i> ^[62] , 2006	?	?	-	?	?	?
Wu <i>et al.</i> ^[52] , 2003	?	?	-	?	?	?
Wu <i>et al.</i> ^[47] , 2008	?	?	-	?	-	?
Xie <i>et al.</i> ^[44] , 2008	+	-	-	?	?	?
Xin <i>et al.</i> ^[67] , 2008	?	?	-	?	?	?
Xiong ^[59] , 2008	?	?	-	?	?	?
Yan <i>et al.</i> ^[56] , 2007	?	?	-	?	?	?
Yan <i>et al.</i> ^[61] , 2006	?	?	-	?	?	?
Yang ^[49] , 2008	?	?	-	?	-	?
Zhan <i>et al.</i> ^[45] , 2005	?	?	-	?	-	?
Zhu ^[57] , 2008	?	?	-	?	?	?

Figure 3 Methodological quality summary: judgments about each methodological quality item for each included study. a: Adequate sequence generation? b: Allocation concealment? c: Blinding? d: Incomplete outcome data addressed? e: Free of selective reporting? f: Free of other bias?

lactulose^[45,52-59]. Eight studies reported total effectiveness rates in the group using CHM which varied from 83.3%-96.7%, while these rates varied from 39.6%-80.5% in the cisapride group (RR 0.24, 95% CI 0.17 to 0.34). The difference suggested that CHM was more effective than cisapride (Figure 4).

The study by Li *et al.*^[55] showed that 92.6% of the CHM group and 68.3% of the cisapride group had normal stool consistency on the fifteenth day of treatment (half of treatment course) ($P < 0.01$). Sustainable improvement was

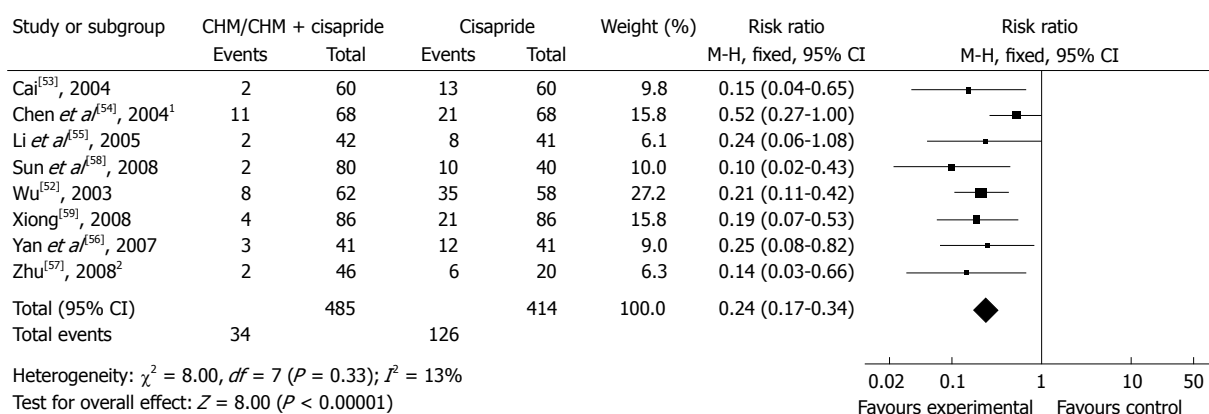


Figure 4 Comparison of CHM vs cisapride, failure to respond at endpoint. ¹Add-on treatment: CHM + cisapride vs cisapride; ²Add-on combined treatment: CHM + (cisapride + lactulose) vs cisapride + lactulose. CHM: Chinese herbal medicine.

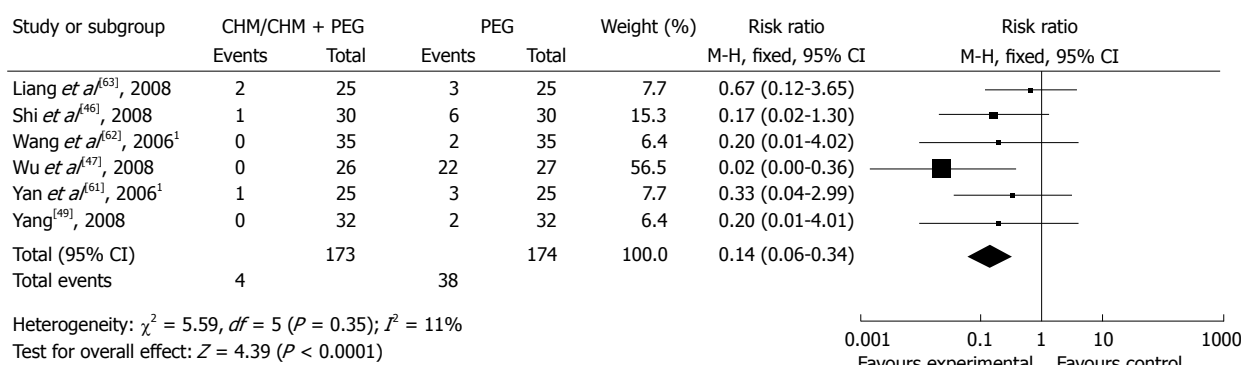


Figure 5 Comparison of CHM/CHM + PEG vs PEG, failure to respond at endpoint. ¹Add-on combined treatment: CHM + PEG vs PEG. PEG: Polyethylene glycol.

noted on the seventh day of the follow-up period when 95.2% and 80.3% of participants reported normal stool consistency, respectively ($P < 0.05$). The stool interval was shortened significantly from 4.4 ± 1.4 d to 2.2 ± 1.3 d during treatment and 2.1 ± 1.1 d during the post-treatment follow-up period for the CHM treatment group, but not for the cisapride control group. Both groups had shown a significant increase in barium strips excretion in the total colon transit test from $30.13\% \pm 9.2\%$ (before treatment) to $69.45\% \pm 11.32\%$ (during treatment) and $73.2\% \pm 12.16\%$ (after treatment) in the treatment group and from $29.86\% \pm 11.34\%$ to $41.43\% \pm 12.05\%$ and $48.01\% \pm 12.76\%$, respectively, for the controlled group ($P < 0.05$). In the study by Zhan *et al*^[45], patients showed a significant improvement in stool type, ease of defecation, stool frequency and total colon transit test in both groups ($P < 0.05$). Statistically significant differences between the groups were found only for stool type and difficulty of defecation ($P < 0.01$).

Six out of nine studies did mention safety measures. All adverse events were reported among patients receiving cisapride in two studies. 43 (43/68) cases in the study by Chen *et al*^[54] reported diarrhea, gas or abdominal pain and four needed to reduce the dose by half due to adverse reactions. In the study by Cai^[53], one (1/60) case reported headache and two (2/60) cases reported lassitude after taking cisapride. No cases were withdrawn due to adverse events.

CHM vs PEG

Eight studies compared seven different CHM interventions with PEG or add-on with PEG treatment^[43,46,47,49,60-63]. Six studies reported that the total effectiveness rates in the group using CHM or add-on with PEG varied from 92%-100%, while these rates were 18.5%-94% in the PEG group (RR 0.14; 95% CI 0.06-0.34). This finding suggested that CHM or add-on with PEG was more effective than PEG alone (Figure 5).

The study by Liang *et al*^[63] comparing CHM with PEG showed a statistically significant improvement with regard to abdominal bloating and TCM symptoms ($P < 0.05$), but not stool frequency, hardness of stool, straining and abdominal pain ($P > 0.05$). With the exception of time to defecation, the study by Wu *et al*^[47], showed that CHM, when compared with PEG significantly improved all symptoms, including stool frequency, sensation of urge to defecate, straining, dry stool, use of rescue drug and total symptom score ($P < 0.01$). The study by Yang^[49] comparing CHM with PEG control, reported that CHM resulted in significant benefit with regard to stool frequency, stool type, difficulty and time of defecation during treatment ($P < 0.05$). Liu *et al*^[43] showed that the effectiveness of the CHM intervention was equivalent to PEG with regard to stool frequency, stool type, straining, abdominal bloating, abdominal pain and loss of appetite and excretion rate of the total colon transit

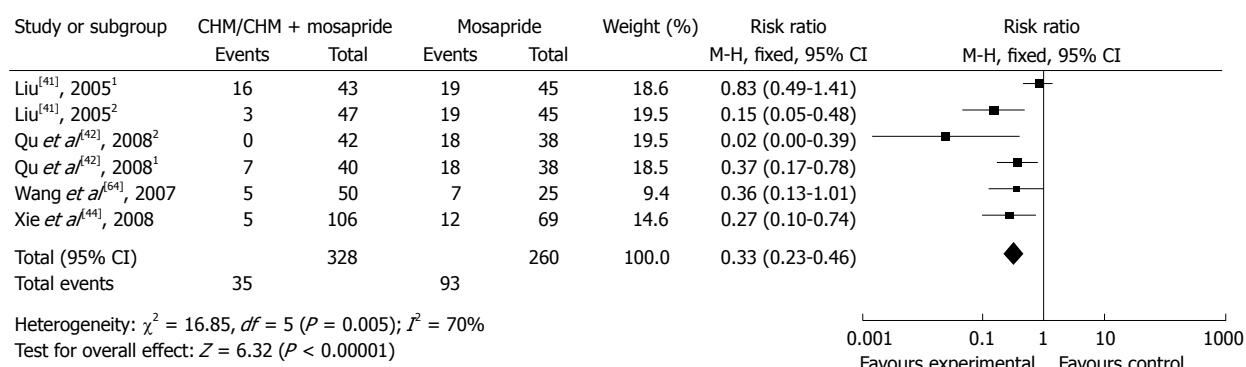


Figure 6 Comparison of CHM/CHM + mosapride vs mosapride, failure to respond at endpoint. ¹3 arms study: CHM vs mosapride; ²3 arms study: CHM + mosapride vs mosapride.

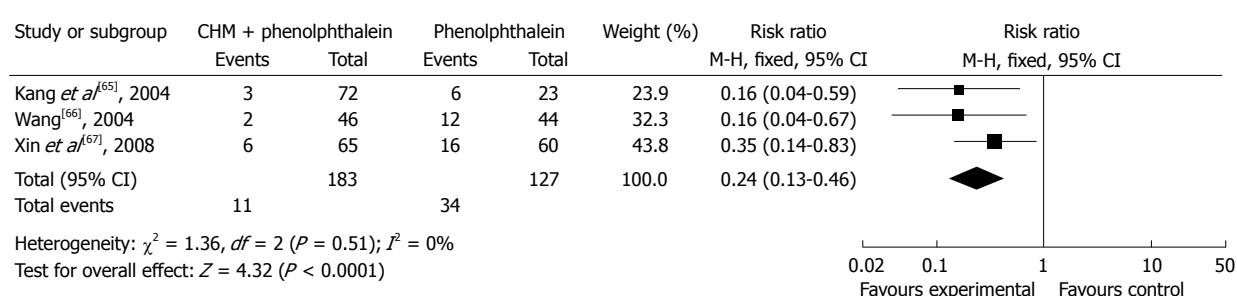


Figure 7 Comparison of CHM vs phenolphthalein, failure to respond at endpoint.

test. The LiuWeiAnXiao Capsule Collaboration Group^[60] reported that those who took CHM showed statistically significant improvement in QoL for components on general feeling, vitality, and daily activities ($P < 0.05$) and difficulty of defecation during follow-up ($P = 0.026$), but not on stool frequency, stool type, and excretion rate of the total colon transit test ($P > 0.05$). Since the outcome measures among these five studies were on different scales, further meta-analysis was not implemented.

Three studies mentioned the issue of safety^[43,60,61]. Only one AE (i.e. abnormal facial muscle tone) was reported by a patient receiving PEG from the study of LiuWeiAnXiao Capsule.

CHM vs mosapride

Four studies compared four different CHM interventions with mosapride^[41,42,44,64]. Two of them in three parallel groups included a CHM arm, a mosapride arm and a CHM plus mosapride treatment arm^[41,42]. All studies reported total effectiveness rates in the group using CHM or add-on with mosapride which varied from 65.2%-100%, while the effectiveness rate was 54.4%-82.6% in the mosapride group (RR 0.33; 95% CI 0.23 to 0.46). This suggested that CHM or add-on with mosapride was more effective than mosapride alone (Figure 6).

The study by Xie *et al*^[44] comparing CHM with mosapride showed a statistically significant improvement in the bothersome of constipation, straining and TCM Qi deficient symptoms ($P < 0.01$). The combined treatment group in Liu's study^[41] showed symptom relief with regard to abdominal pain, abdominal bloating and loss of appetite which was significantly better than both CHM

and mosapride alone ($P < 0.01$).

Two studies evaluated the safety of CHM interventions. Two patients (2/38) with abdominal pain from the CHM arm, two (2/40) with diarrhea from the mosapride arm and one (1/42) with diarrhea from the combined treatment arm were reported in the study by Qu *et al*^[42]. Liu^[41] reported 17 AEs, nine patients with abdominal pain (two from the CHM arm, two from the mosapride arm and three from the combined treatment arm), five with diarrhea (two from the CHM arm and three from the combined treatment arm), two with active bowel sounds and one with dry mouth (both of the latter from the mosapride arm).

CHM vs phenolphthalein

Three studies compared three different CHM interventions with phenolphthalein^[65-67]. The total effectiveness rates in the group treated with CHM were 90.8%-95.8%, while the comparable rates for phenolphthalein were 72.7%-73.9% (RR 0.24; 95% CI 0.13-0.46). Thus the results suggested that CHM was more effective than phenolphthalein (Figure 7). Only Kang *et al*^[65] mentioned that no AEs were observed.

Other

The study by Li *et al*^[68] showed that the total effectiveness of the combined treatment (a TCM intervention add-on with itopride) and itopride alone were 92% and 76%, respectively ($P < 0.05$). In total three cases of mild abdominal pain and two cases of loose stool were reported in the combined treatment arm while two cases of mild abdominal pain were reported in the itopride arm.

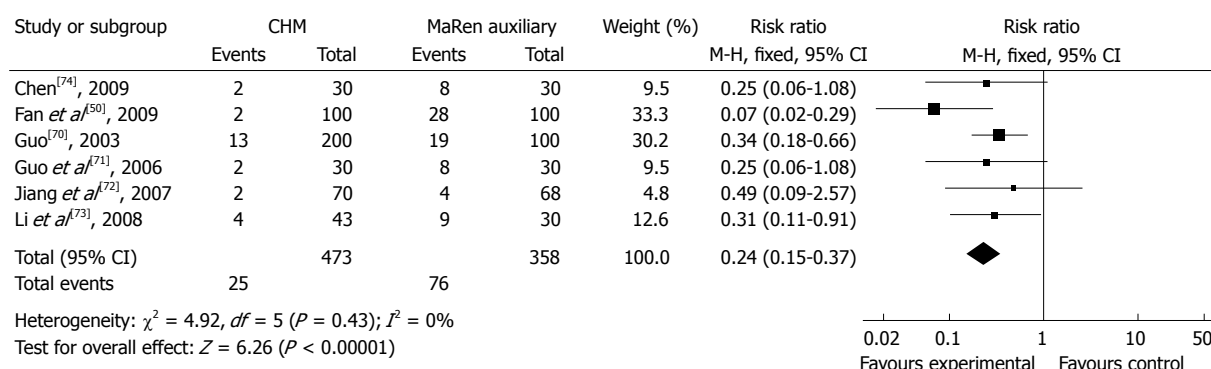


Figure 8 Comparison of CHM vs MaRen auxiliary, failure to respond at endpoint.

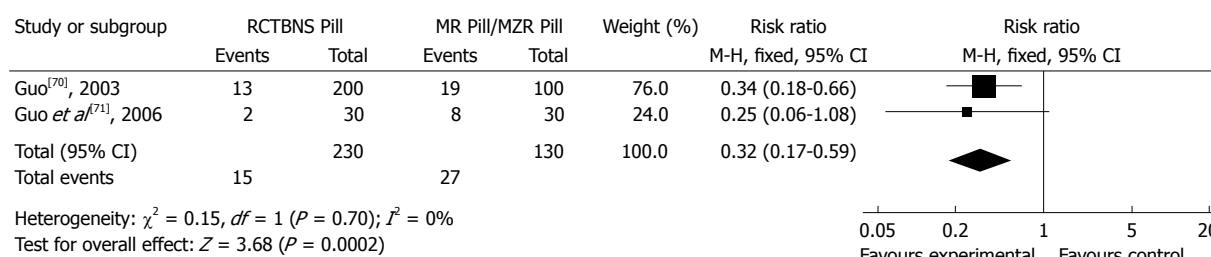


Figure 9 Comparison of RunChangTongBianNongSuo Pill vs MaRen Pill/MaZiRen Pill, failure to respond at endpoint.

The study by Meng^[69] showed that the total effectiveness of the combined treatment (a TCM intervention add-on with live bifidobacterium) and live bifidobacterium alone were 94% and 64%, respectively ($P < 0.01$). No studies reported safety issues.

CHINESE HERBAL MEDICINE VS CHINESE HERBAL MEDICINE (COMPARISON 03)

Ten different CHM interventions were tested in seven trials^[48,50,70-74]. Six of them used MaZiRenWan/MaRenWan or its modifications as control (MaRen auxiliary) while one used LuiWeiNengXiao capsules as control (LuiWei auxiliary).

CHM vs MaRen auxiliary

The total effectiveness rates in the group treated with CHM varied from 90.7%-98%, and was 70%-94.1% in the MaRen auxiliary (RR 0.24, 95% CI 0.15 to 0.37) (Figure 8). RunChangTongBian NongSuo Pill was the only intervention compared with the same control in two studies^[70,71] (RR 0.32, 95% CI 0.17 to 0.59) (Figure 9). These results suggested that the CHM interventions developed by the study authors were more effective than the MaRen auxiliary.

Li *et al*^[73] found that CHM resulted in a statistically significant improvement in time of defecation, abdominal bloating and pain, incompleteness of defecation, and total symptoms score by comparing QiLang Mixture with MaRenRuan Capsule ($P < 0.05$), but not on stool frequency, straining and stool type ($P > 0.05$). From the studies by Guo *et al*^[70,71] published in 2003 and 2006, RunChangTongBian NongSuoWan resulted in significant

improvement in the main constipation related symptoms, such as incompleteness and difficulty of defecation, when compared with MaRen Pill/MaZiRen Pill ($P < 0.05$), but it did not improve the minor symptoms of dry mouth, dizziness and palpitation, and blood NO and SP levels ($P > 0.05$).

Only two studies reported adverse effects^[50,70]. Three cases treated with YiQiRunChang Capsule reported diarrhea or abdominal pain while no AEs were observed in Guo's study.

CHM vs LiuWei auxiliary

The study by Gan *et al*^[48] compared TiaoChang Decoction with LuiWeiNengXiao capsules. The total effectiveness rates were 90% and 83.3%, respectively. Patients taking TiaoChang Decoction showed a significant improvement in constipation-related symptoms compared with LuiWeiNengXiao Capsule, and both were safe for consumption without any prominent AEs reported.

CHINESE HERBAL MEDICINE VS NON-PHARMACEUTICAL INTERVENTIONS (COMPARISON 04)

The study by Huang^[75] compared massage with FuFangLuHui capsules. The total effectiveness rates were 97.8% and 53.3%, respectively ($P < 0.05$). Massage was more effective in improving stool frequency, straining, lumpy or hard stool, time to defecation, sensation of anorectal blockage, manual maneuvers to facilitate the process, sensation of incomplete evacuation and stool weight for each defecation. No AEs were reported.

SUMMARY

This review analyzed 35 randomized trials that were conducted in China and published in Chinese medical journals. The results favored the tested CHM interventions in comparison with controls, WCM and some CHM interventions, but not when compared with massage; however, there was not enough replicable evidence to conclude that any specific CHM intervention is effective for FC.

Furthermore, the results of these trials should be interpreted with caution due to the generally low methodological quality of the included studies. First, all studies provided insufficient information on how the random allocation was generated and/or concealed, which is necessary to avoid selection bias. It has been shown that trials with inadequate concealment of allocation or unclear reporting of the technique used were on average 18% more “beneficial” than effect estimates from trials with adequate concealment (95% CI 5% to 29%)^[14]. Second, none of the studies used any blinding method. Lack of blinding to participants, healthcare providers and assessors can introduce performance bias and detection bias. Lack of blinding can also be associated with exaggerated estimated intervention effects-by 9% on average, measured as odds ratio^[14]. Third, none of the included studies addressed incomplete outcome data, such as missing data due to attrition or exclusions. Inadequate handling of missing data can compromise statistical analysis. Fourth, none of studies had been registered, despite a statement issued in 2004 by the International Committee of Medical Journal Editors (ICMJE) requiring that all clinical trials must be registered in order to be considered for publication^[76]. Therefore, protocols were not available to confirm free of selective reporting, especially for those trials which tended to address statistical conclusions instead of listing the details of individual outcomes^[46-48]. Fifth, the majority of experimental CHM interventions were prepared by the investigators without detailed information describing underlying rationales on formulation, dosage, manufacturing process, *etc.* The quality control processes of their tested interventions are unknown. For all these reasons, independent validation of the findings of these trials is necessary.

With regard to selection of an active control in the trials, it is necessary to consider whether there is evidence to support the efficacy of an active control. If no evidence supports the selection of a control, it may bias the trial results. Among all the active controls selected, only PEG had good supporting evidence for the treatment of constipation^[6]. Cisapride, mosapride and itopride have been used as gastroprokinetic agents for the symptomatic treatment of functional dyspepsia. They were thought to be useful for the treatment of constipation due to their stimulating effect on gastrointestinal motility as reported in recent research findings. However, cisapride was suspended by the US Food and Drug Administration in 2000 because of its side effects of heart rhythm disturbances, including QT prolongation^[77,78]. More

clinical data, especially evidence from systematic reviews, are urged to confirm the efficacy and safety of mosapride, itopride, phenolphthalein and bifidobacterium for the treatment of FC. Therefore, the effectiveness of tested CHM interventions is not conclusive, despite beneficial findings from meta-analyses.

The herbal medicines evaluated in this review generally appeared to be safe and well tolerated by patients. However, the safety of their use for FC could not be confirmed because only 48.6% studies (17/35) mentioned the safety of interventions or investigated AEs as one of the secondary outcome measures. It is recommended that more attention should be given to both recording and reporting the harmful effects of these interventions.

This systematic review has several methodological limitations. First, all the data were collected from the reports without directly contacting the trial authors. Therefore, many items of the “Risk of bias” assessment tool could only be classified as “unclear”. Second, most of the included studies were small and without formal sample size calculation. The results were likely to be underpowered. Third, in some cases, different CHM interventions were grouped together for analysis. The results might have been compromised by the heterogeneity within each CHM intervention and by the study design. Fourth, in general, the concept of TCM syndrome was not considered when analyzing the data, as some studies only targeted a WCM disease in a particular type of TCM symptom. Therefore, the actual therapeutic effect might not have been fully captured.

CONCLUSION

CHM interventions or CHM combined treatments showed benefit in the treatment of FC when compared with cisapride, PEG, mosapride, phenolphthalein, itopride and bifidobacterium alone, but not when compared with massage. However, the evidence and reliability of these conclusions are compromised by methodological flaws and lack of replicable validation. Further well-designed, randomized, double-blind, placebo-controlled trials need to be carried out and reported in detail according to the Consolidated Standards of Reporting Trials (CONSORT) and/or CONSORT for TCM Statements.

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S- Editor Tian L L- Editor Webster JR E- Editor Lin YP



ORIGINAL ARTICLE

Characterization of CD133⁺ parenchymal cells in the liver: Histology and culture

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Received: June 11, 2009 Revised: August 4, 2009

Accepted: August 11, 2009

Published online: October 21, 2009

Abstract

AIM: To reveal the characteristics of CD133⁺ cells in the liver.

METHODS: This study examined the histological characteristics of CD133⁺ cells in non-neoplastic and neoplastic liver tissues by immunostaining, and also analyzed the biological characteristics of CD133⁺ cells derived from human hepatocellular carcinoma (HCC) or cholangiocarcinoma cell lines.

RESULTS: Immunostaining revealed constant expression of CD133 in non-neoplastic and neoplastic biliary epithelium, and these cells had the immunophenotype CD133⁺/CK19⁺/HepPar-1⁻. A small number of CD133⁺/CK19⁺/HepPar-1⁺ cells were also identified in HCC and combined hepatocellular and cholangiocarcinoma. In addition, small ductal structures, resembling the canal of Hering, partly surrounded by hepatocytes were positive for CD133. CD133 expression was observed in three HCC (HuH7, PLC5 and HepG2) and two cholangiocarcinoma cell

lines (HuCCT1 and CCKS1). Fluorescence-activated cell sorting (FACS) revealed that CD133⁺ and CD133⁻ cells derived from HuH7 and HuCCT1 cells similarly produced CD133⁺ and CD133⁻ cells during subculture. To examine the relationship between CD133⁺ cells and the side population (SP) phenotype, FACS was performed using Hoechst 33342 and a monoclonal antibody against CD133. The ratios of CD133⁺/CD133⁻ cells were almost identical in the SP and non-SP in HuH7. In addition, four different cellular populations (SP/CD133⁺, SP/CD133⁻, non-SP/CD133⁺, and non-SP/CD133⁻) could similarly produce CD133⁺ and CD133⁻ cells during subculture.

CONCLUSION: This study revealed that CD133 could be a biliary and progenitor cell marker *in vivo*. However, CD133 alone is not sufficient to detect tumor-initiating cells in cell lines.

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Key words: Cholangiocarcinoma; Hepatocellular carcinoma; Keratins; Stem cells

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Yoshikawa S, Zen Y, Fujii T, Sato Y, Ohta T, Aoyagi Y, Nakanuma Y. Characterization of CD133⁺ parenchymal cells in the liver: Histology and culture. *World J Gastroenterol* 2009; 15(39): 4896-4906 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4896.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4896>

INTRODUCTION

CD133 (also known as prominin-1 or AC133) was the first identified member of the prominin family of pentaspan membrane proteins^[1-3]. In 1997, CD133 was reported as a marker of hematopoietic progenitor cells, using a novel monoclonal antibody that recognized the CD133 antigen^[1-3]. Subsequently, it was reported that CD133 was also expressed in epithelial and non-epithelial progenitor cells in murine or human tissues including brain, kidney, prostate, pancreas, and skin^[4-8].

The specific functions and ligands of CD133 have not been elucidated completely, although CD133 currently is recognized as a stem cell marker for normal and cancerous tissues in various organs^[9-13].

Until now, there have been several reports regarding CD133 expression in hepatocellular carcinoma (HCC)^[14-17]. Suetsugu *et al.*^[14] have examined CD133 expression in three cell lines of human HCC (HuH7, HepG2 and Hc). CD133 is expressed only on the surface of HuH7 cells. The CD133⁺ population of HuH7 cells is characterized by high proliferation activity and lower expression of mature hepatocellular markers. CD133⁺ cells can form tumors in SCID mice, whereas CD133⁻ cells induce a very small number of tumors or none at all. It has been concluded that CD133 could be useable as a marker of cancer stem cells in human HCC^[14]. Yin *et al.*^[15] and Ma *et al.*^[16] also have characterized CD133⁺ cells in HCC, and they have reached a conclusion similar to that of Suetsugu *et al.*^[14]. However, because these previous studies were mainly *in vitro*, the histological characteristics of hepatic CD133⁺ cells have not been fully examined so far. In particular, there are few data about CD133⁺ cells in non-neoplastic liver tissues and non-hepatocellular liver cancers.

In this study, CD133 expression in non-neoplastic and neoplastic liver tissues was examined. *In vitro* studies were also performed to examine the biological characteristics of CD133⁺ cells of HCC and cholangiocarcinoma cell lines. The goal of this study was to elucidate the histological and biological characteristics of CD133⁺ cells in non-neoplastic and neoplastic human livers.

MATERIALS AND METHODS

Histological studies

Case selection: A total of 52 samples of liver tissues were obtained from the hepatobiliary disease file of the Division of Pathology, Kanazawa University Hospital in Japan between 2005 and 2009. This study consisted of three cases of normal liver, five cases of chronic viral hepatitis or liver cirrhosis, 33 cases of HCC, six cases of intrahepatic cholangiocarcinoma, and five cases of combined hepatocellular and cholangiocarcinoma (combined carcinoma). All cases used in this study were surgically resected cases. Normal liver tissues used in this study were background liver tissues of metastatic colon cancers. Age, sex and clinicopathological characteristics are shown in Table 1.

Expression of CD133 (mRNA level): Total RNA was extracted from the frozen section of all 47 cases using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was dissolved in 50 μ L of distilled water that contained 0.1% diethylpyrocarbonate, and quantitated using a spectrophotometer at OD₂₆₀. Isolated RNA was used for the subsequent reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of CD133 mRNA was examined by nested RT-PCR using two sets of primers. The oligonucleotide sequences, numbers

of cycles, and annealing temperatures of these primers are shown in Table 2. After PCR, 5- μ L aliquots of the products were subjected to 1.5% or 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Immunostaining of CD133, cytokeratin 19 (CK19) and hepatocyte paraffin-1 (HepPar-1): Frozen sections of 52 samples of non-neoplastic and neoplastic liver tissues were used for immunostaining. Immunostaining for CD133, CK19 and HepPar-1 was performed using a mouse monoclonal antibody against human CD133 (clone AC133; Miltenyi Biotec, Auburn, CA, USA), a mouse monoclonal antibody against human CK19 (Dako Cytomation, Glostrup, Denmark), and a mouse monoclonal antibody against human HepPar-1 (Dako Cytomation).

Serial sections were used in each case to examine the co-localization of CD133, CK19 and HepPar-1 expression. Sliced frozen sections were fixed with acetone for 20 min. After blocking endogenous peroxidases, the sections were incubated in protein block solution (Dako Cytomation) for 20 min and incubated at 4°C with each primary antibody. These sections were incubated for 1 h at room temperature with goat anti-mouse immunoglobulins, which were conjugated to peroxidase-labeled polymer (Envision+; Dako Cytomation). 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen, followed by light counterstaining with hematoxylin. Negative controls were evaluated by substituting the primary antibody with similarly diluted non-immunized mouse serum.

Culture studies

Cell culture: Three human HCC cell lines (HuH7, PLC5 and HepG2) and two human cholangiocarcinoma cell lines (CCKS1 and HuCCT1) were used in this study. HuH7, PLC5 and HepG2 were obtained from the Health Science Research Bank (Osaka, Japan). HuCCT-1 was obtained from the Cell Resource Center for Biochemical Research, Tohoku University, Sendai, Japan. CCKS1 was established in our laboratory^[18]. HuH7 and PLC5 were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen Corp., Carlsbad, CA, USA), and HepG2 was maintained in minimum essential medium (Invitrogen Corp.) with 1% nonessential amino acids (Specialty Media, Phillipsburg, NJ, USA). CCKS1 and HuCCT1 were cultured in RPMI-1640 medium (Invitrogen Corp.) Each medium was supplemented with 10% fetal bovine serum (Invitrogen Corp.) and 1% antibiotic-antimycotic (Invitrogen Corp.).

Dual fluorescent immunostaining of CD133/CK19 and CD133/ α -fetoprotein (AFP): Cell lines were cultured on Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL, USA) for fluorescent immunostaining. After culturing for 2 d, the specimens were fixed in 4% paraformaldehyde for 10 min at 4°C. After incubation in protein block solution (Dako Cytomation) for 10 min, the specimens were incubated

Table 1 Age, sex, and etiology of liver diseases in our study

	<i>n</i>	Age (yr)	Male/Female	Etiology
Normal liver	3	50	2/1	
Chronic hepatitis/cirrhosis	5	58	3/2	HBV (3), HCV (2)
HCC				
Well-differentiated	3	62	2/1	HBV (1), HCV (2)
Moderately differentiated	24	62	20/4	HBV (11), HCV (7), alcohol (3), NASH (1), cryptogenic (1) ¹
Poorly differentiated	6	54	4/2	HBV (3)
Cholangiocarcinoma	6	60	3/3	HCV (4)
Combined carcinoma	5	59	4/1	HBV (1), HCV (4)

¹The remaining one case had no etiology of liver diseases and showed histologically normal liver. NASH: Nonalcoholic steatohepatitis; HCC: Human hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Sequences, annealing temperatures, cycle times, and product sizes of PCR primers

	F/R	Sequence	Temperature (°C)	Cycles	Size (bp)
CD133 ¹	1st F	GCCAGAACTGTAATCTTAG	48	35	275
	1st R	TTACCTGGTGATTGGCCACA			
	2nd F	CCTGGGGCTGCTGTTTATTA	55	35	153
	2nd R	ATCACCAACAGGGAGATTGC			
CK19	F	TCCCGCGACTACAGCCACTACTACACGACC	55	35	745
	R	CGCGACTTGATGTCCATGAGCCGCTGGTA			
CK7	F	GGATGCTGCCTACATGAGC	52	30	164
	R	CCAGGAGCGACTGTTGT			
AFP	F	GGGAGCGGCTGACATTATTA	50	30	231
	R	TCTTGCTTCATCGTTTGACG			
Albumin	F	TGCTTGAATGTGCTGATGACAGGG	50	30	161
	R	AAGGCAAGTCAGCAGGCATCTCATC			
β-actin	F	CAAGAGATGGCCACGGCTGCT	55	30	334
	R	TCCCTCTGCATCCCTGTCGGCA			

¹Examined by nested PCR using first and second sets of primers. F: Forward; R: Reverse.

with antibodies against CD133 and CK19, or antibodies against CD133 and AFP for 1 h at room temperature. The antibodies used were as follows: CD133, mouse monoclonal, clone AC133, Miltenyi Biotec; CK19, goat polyclonal, clone G-14, Santa Cruz Biotechnology (Santa Cruz, CA, USA); and AFP, a rabbit polyclonal, Dako Cytomation. The reaction product was visualized with fluorescent goat anti-mouse and anti-rabbit IgG antibodies (1:500, Molecular Probes Inc., Eugene, OR, USA). Specimens were counterstained with DAPI (Molecular Probes Inc.), and fluorescent signals were observed using a fluorescence microscope (Olympus, Tokyo, Japan).

Fluorescence-activated cell sorting (FACS) with reference to CD133 expression: HuH7 and HuCCT1 cells were used for FACS. Cultured cells were harvested after treatment with 0.25% of trypsin-EDTA solution (Sigma Chemical Co., St Louis, MO, USA) for 20 min, and washed three times in Hanks' Balanced Salt Solution (Invitrogen Corp.). Cultured cells were stained live in a staining solution containing bovine serum albumin, insulin, and phycoerythrin (PE)-conjugated monoclonal antibody to CD133 (clone AC133; Miltenyi Biotec) for 30 min at 4°C. As negative controls, cultured cells were incubated similarly with non-immunized mouse

immunoglobulin. Samples were analyzed and sorted by JSAN (Bay Bioscience, Kobe, Japan). Cell debris and cell aggregates were gated out electronically. For the positive population, only the top 5%-10% of the most brightly stained cells were selected. For the negative population, only the bottom 5%-10% of the most dimly stained cells were selected. Then, 1.0×10^5 cells were sorted from the positive or negative population at the most specific mode. Sorted cells were plated on culture dishes for subculture. After sorting, CD133⁺ and CD133⁻ cells were cultured separately. After 4-wk culture, cultured cells were sorted again into CD133⁺ and CD133⁻ cells using flow cytometry to evaluate how the CD133⁺ cell ratios were altered in each subpopulation. After subculturing for 3 or 4 wk, cultured cells were sorted again into CD133⁺ and CD133⁻ cells to evaluate how the CD133⁺ or CD133⁻ populations changed during subculture. The percentages of CD133⁺ cells were calculated in a total of 1000-5000 cells in each group.

RNA expression in culture cells: Total RNA was extracted from five types of cultured cells using an RNeasy Mini Kit (Qiagen). Total RNA was similarly extracted from CD133⁺ and CD133⁻ cells. RT-PCR was performed for CD133, hepatocyte makers (AFP and albumin), biliary markers (CK19 and CK7 and β-actin.

The oligonucleotide sequences, numbers of cycles and annealing temperatures of these primers are shown in Table 2. After PCR, 5- μ L aliquots of the products were subjected to 1.5% or 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Real-time RT-PCR: The alterations of CD133 expression levels were examined in non-sorted or sorted (CD133⁺ or CD133⁻) cultured cells time-dependently (days 0, 7, 14, 21 and 28) after the passage or sorting. Real-time analysis was performed using premade CD133 and β -actin-specific primers and probes with the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Warrington, UK). RT-PCR was done with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 2 μ L cDNA in a 25- μ L final reaction mixture. Cycling conditions were as follows: incubation at 50°C for 2 min, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. CD133 was normalized (Δ Ct) to β -actin from the Ct value of CD133. Each experiment was performed in triplicate, and the mean adopted.

Cell proliferation assay of CD133⁺ and CD133⁻ cells: CD133⁺ and CD133⁻ cells were plated on a Lab-Tek II chamber slide (Nalge Nunc International), and cultured for 7 d before the cell proliferation assay. Cell proliferation was assayed using BrdU. Cultured cells were incubated on slides with BrdU solution (10 mmol/L) at 37°C for 30 min. After fixing with 70% ethanol (50 mmol/L glycine buffer solution, pH 2.0) for more than 20 min, the slides were incubated with anti-BrdU solution at 37°C for 30 min. After additional incubation with IgG fluorochrome solution for 30 min, positive signals were detected by a fluorescence microscope (Olympus).

Relationship between side population (SP) and CD133⁺ cells: SP is currently estimated as one of the most reliable stem cell phenotypes^[19,20]. The relationship between SP and CD133⁺ cells was examined by FACS. After detaching and washing, the cultured cells were then incubated at 37°C for 90 min with 20 μ g/mL Hoechst 33342 (Sigma Chemical Co.), PE-conjugated monoclonal antibody to CD133 (clone AC133; Miltenyi Biotec), bovine serum albumin, in the presence or absence of 100 μ mol/L verapamil (Sigma Chemical Co.). After incubation, 1 μ g/mL propidium iodide (Sigma Chemical Co.) was added and the cells were filtered through a 40- μ m cell strainer (BD Biosciences, San Diego, CA, USA) to obtain single-cell suspensions. The relationship between SP and CD133 expression was analyzed by JSAN (Bay Bioscience). Hoechst 33342 was excited with a UV laser at 350 nm and fluorescence emission was measured with 405/BP30 (Hoechst blue) and 570/BP20 (Hoechst red) optical filters. Propidium iodide labeling was measured through a 630/BP30 filter for the discrimination of dead cells. Next, HuH7 cells were sorted into SP/CD133⁺, SP/CD133⁻, non-SP/CD133⁺,

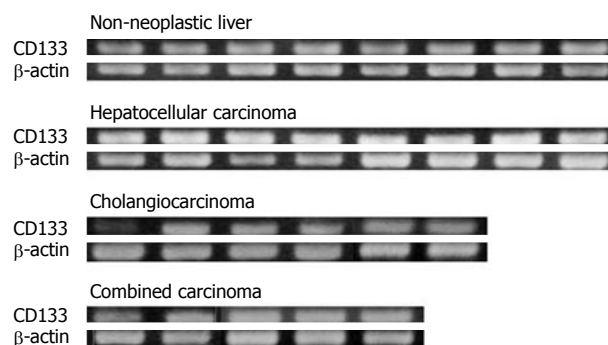


Figure 1 Expression of CD133 mRNA. Nested RT-PCR revealed CD133 mRNA expression in all cases of non-neoplastic liver tissue, HCC, intrahepatic cholangiocarcinoma, and combined hepatocellular and cholangiocarcinoma. Only eight cases of HCC are shown, although the remaining cases also expressed CD133 mRNA.

and non-SP/CD133⁻. After 4 wk subculturing, each population was analyzed again with regard to CD133 expression by FACS.

Statistical analysis

Differences between two groups were analyzed using the Mann-Whitney *U* test or χ^2 test. Statistical analysis was performed using Statcel 2 software (OMS publishing, Tokorozawa, Japan). *P* < 0.05 was considered to be significant.

RESULTS

CD133 expression in non-neoplastic and neoplastic liver tissues

The expression of CD133 mRNA was identified in all non-neoplastic and neoplastic liver tissues examined in this study by nested RT-PCR (Figure 1). The results of immunostaining of CD133 are shown in Figures 2 and 3. In normal livers, CD133 was expressed constantly in biliary epithelium of intrahepatic large and small bile ducts. Mature hepatocytes were negative for CD133. In the livers of chronic hepatitis and liver cirrhosis patients, CD133 was expressed in bile ducts and proliferating bile ductules. In addition, small ductal structures, resembling the canal of Hering, partly surrounded by hepatocytes were also positive for CD133 (Figure 2). CD133 was expressed on cellular membrane with accentuation on the luminal side. Immunostaining of CK19 and HepPar-1 on serial sections revealed that CD133 and CK19 expressions were closely co-localized (Figure 2). In contrast, mature hepatocytes that expressed HepPar-1 were negative for CD133. CD133 expression was not evident in mesenchymal or inflammatory cells upon immunostaining.

In HCC, eight of 33 cases (24%) had CD133⁺ cells. CD133⁺ cells were small in number and randomly distributed in these tumors. There were no morphological differences between CD133⁺ and CD133⁻ cells. Serial sections stained with CK19 and HepPar-1 revealed that CD133⁺ cells in HCC were HepPar-1⁺ and CK19⁺ (Figure 3). CD133⁺ cells were observed more often in

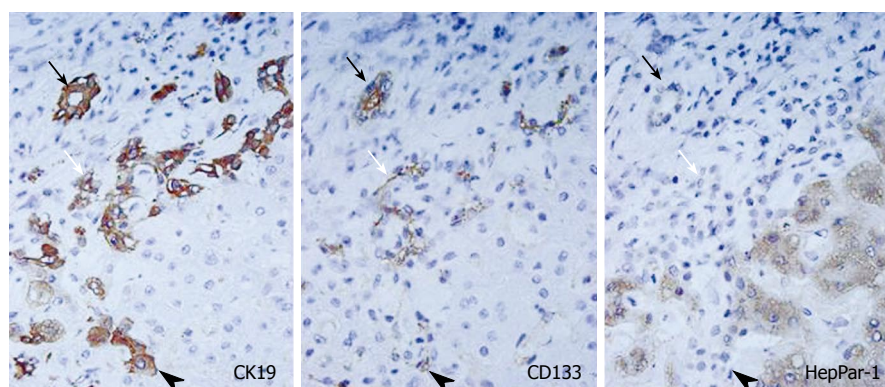


Figure 2 CD133 expression in liver cirrhosis (immunostaining). CD133 was expressed in bile duct (black arrows), bile ductules (white arrows), and small parenchymal cells surrounded by hepatocytes. CD133 was expressed on the cellular membrane with an accentuation on the luminal side. CD133⁺ cells were also positive for CK19 but not HepPar-1. All images, $\times 400$.

Hepatocellular carcinoma

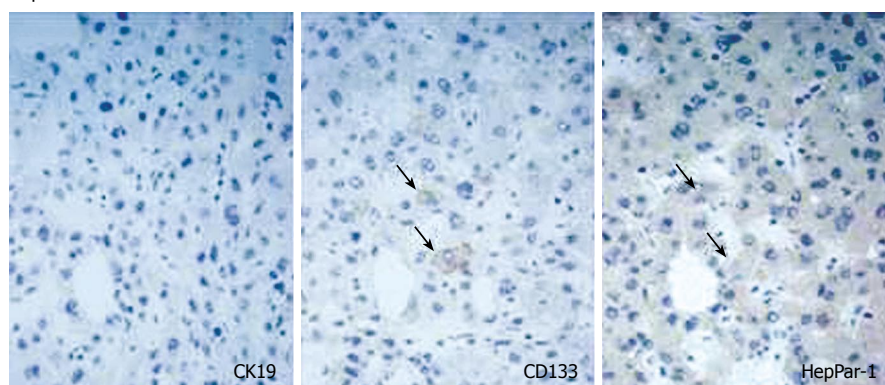
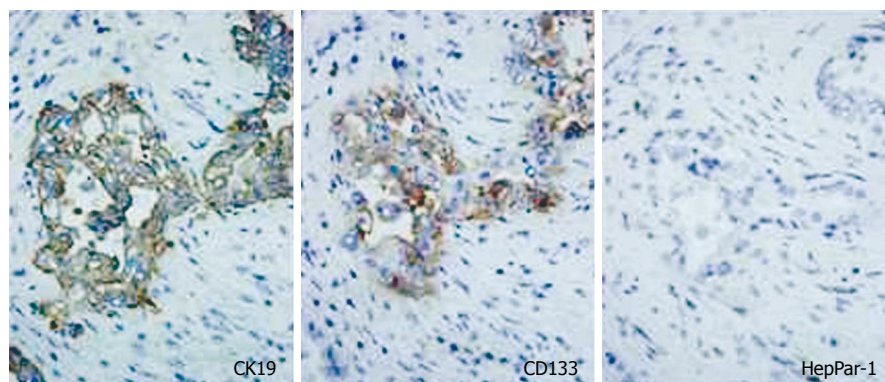
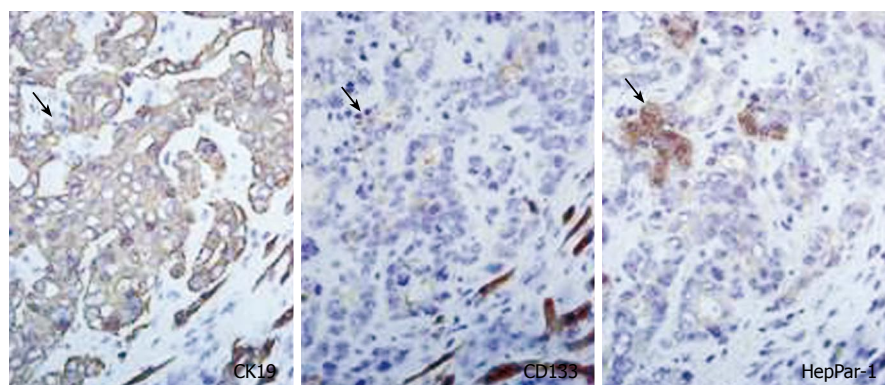


Figure 3 CD133 expression in HCC, intrahepatic cholangiocarcinoma, and combined hepatocellular and cholangiocarcinoma (immunostaining). In HCC, a few carcinoma cells expressed CD133, and those cells were CK19⁻ and HepPar-1⁺ (arrows). In cholangiocarcinoma, CD133 was expressed diffusely in carcinoma cells, and CK19 was also positive. In combined carcinoma, CD133 was expressed mainly in carcinoma cells positive for CK19, whereas some carcinoma cells were CD133⁺/CK19⁺/HepPar-1⁺ (arrows). All images, $\times 400$.

Intrahepatic cholangiocarcinoma



Combined hepatocellular and cholangiocarcinoma



less differentiated HCCs: 0/3 (0%) in well-differentiated, 4/24 (17%) in moderately differentiated, and 4/6 (67%) in poorly differentiated HCC cases. The expression of CD133 mRNA was detected in all HCC cases by nested RT-PCR, although CD133⁺ cells were identified in only

24% of cases by immunostaining. This discrepancy might have resulted from the small numbers of CD133⁺ cells in HCC.

In cholangiocarcinoma, CD133 was expressed diffusely in carcinoma cells in all cases examined (Figure 3).

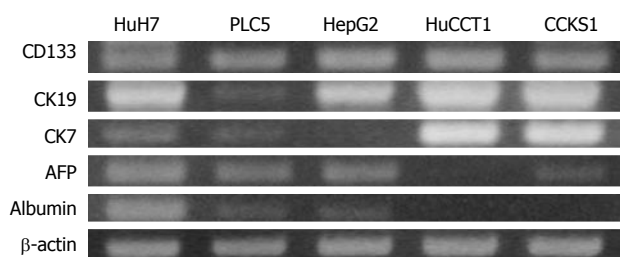


Figure 4 Expression levels of mRNA in cell lines. CD133 mRNA was expressed in all cell lines examined. Biliary markers (CK19 and CK7) were expressed more often in cholangiocarcinoma cell lines (HuCCT1 and CCKS1), whereas hepatocellular markers (AFP and albumin) were expressed constantly in HCC cell lines (HuH7, PLC5, and HepG2).

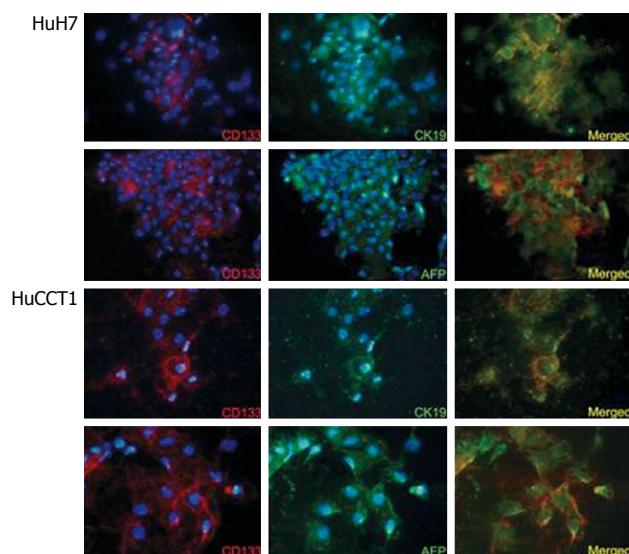


Figure 5 Dual immunofluorescence of CD133/CK19 and CD133/AFP in HuH7 and HuCCT1 cells. CD133⁺ carcinoma cells were positive for CK19 or AFP in both HuH7 and HuCCT1 cell lines. All images, $\times 400$.

CD133 expression was mainly on cellular membranes. HepPar-1 expression was not observed in any cases of cholangiocarcinoma, and CD133⁺ cholangiocarcinoma cells were CK19⁺ and HepPar-1⁻. In combined carcinoma, all cases had CD133⁺ carcinoma cells. CD133 expression was observed mainly in adenocarcinoma components. Most CD133⁺ cells were CK19⁺ and HepPar-1⁻, although some CD133⁺ cells were CK19⁻ and HepPar-1⁺ (Figure 3).

CD133 expression in cultured cells

The expression of CD133 mRNA was identified in all cell lines by RT-PCR (Figure 4). Biliary markers (CK19 and CK7) were expressed strongly in CCKS1 and HuCCT1 cells, whereas hepatocellular markers (AFP and albumin) were expressed constantly in HuH7, PLC5 and HepG2 cells. In addition, a cholangiocarcinoma cell line, HuCCT1, also expressed AFP. Similarly, HCC cell lines also expressed CK19 or CK7. Next, the relationships between CD133 and CK19 or AFP expression levels were examined using HCC (HuH7) and cholangiocarcinoma (HuCCT1) cell lines, both of which expressed hepatocellular and biliary markers. Dual immunostaining of CD133/CK19 or CD133/AFP revealed that CD133⁺/

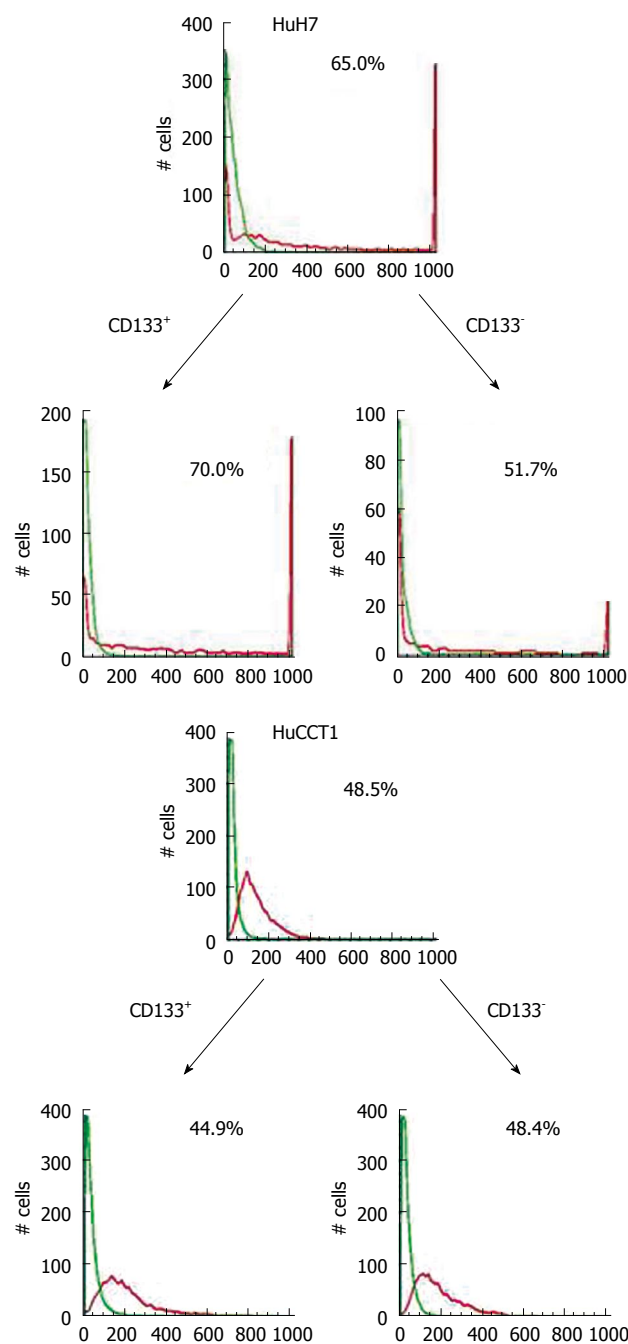


Figure 6 Flow cytometry analysis of CD133 in HuH7 and HuCCT1 cells. CD133⁺ cells comprised 65.0% of HuH7 and 48.5% of HuCCT1 cells. CD133⁺ and CD133⁻ cells could be generated from CD133⁺ and CD133⁻ subpopulations of HuH7 and HuCCT1 cells after 4 wk subculture.

CK19⁺ or CD133⁺/AFP⁺ cells were present in both HuH7 and HuCCT1 cells (Figure 5).

Cell sorting of cultured cells with regard to CD133 expression

FACS was performed using two cell lines (HuH7 and HuCCT1). The flow cytometry analysis with regard to CD133 expression is shown in Figure 6. The percentages of CD133⁺ cells from flow cytometry were 65.0% in HuH7 and 48.5% in HuCCT1 cells. After cell sorting, CD133⁺ and CD133⁻ cells derived from HuH7 or HuCCT1 cells were cultured separately for 4 wk. After

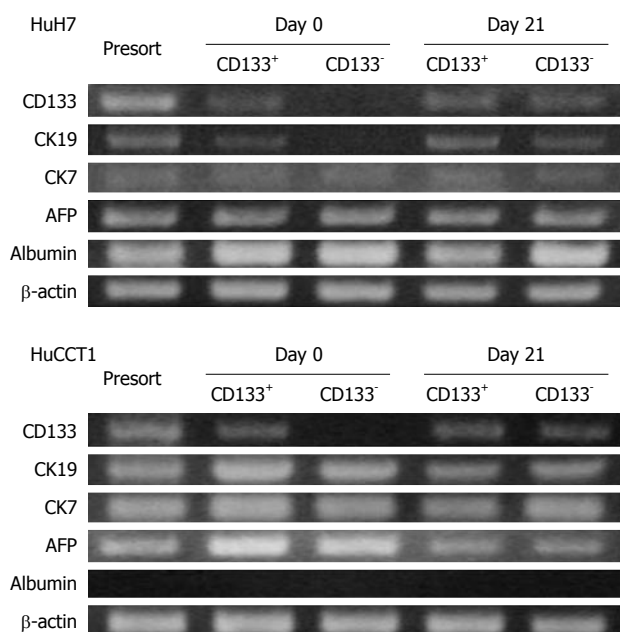


Figure 7 Expression CD133, CK19, CK7, AFP and albumin in HuH7 and HuCCT1 cells before sorting, just after sorting (day 0), and after 3 wk subculture (day 21). At day 0, expression of CD133 mRNA was observed in only the CD133⁺ population in HuH7 and HuCCT1 cells. However, CD133⁺ and CD133⁻ populations expressed similar levels of CD133 mRNA at day 21. At day 21, CD133⁺ and CD133⁻ HuH7 and HuCCT1 cells showed almost similar mRNA expression patterns.

4 wk subculturing, both CD133⁺ and CD133⁻ subpopulations returned to almost the pre-sorting cellular population that comprised both CD133⁺ and CD133⁻ cells (Figure 6). These results suggested that CD133⁻ HuH7 and HuCCT1 cells generated CD133⁺ and CD133⁻ progenies during subculture.

Expression patterns of mRNA in CD133⁺ and CD133⁻ cells

The expression patterns of CD133, CK19, CK7, AFP and albumin were examined in HuH7 and HuCCT1 cells before sorting, just after sorting (day 0), and after 3 wk subculture (day 21). At day 0, the expression of CD133 mRNA was observed in only the CD133⁺ population in both HuH7 and HuCCT1 cells. However, CD133⁺ and CD133⁻ populations expressed the CD133 mRNA at similar levels at day 21 (Figure 7). These results suggested that CD133⁻ cells began to express CD133 or produce CD133⁺ progeny during subculture. Acquisition of CD133 expression in CD133⁻ cells was consistent with the results of FACS.

On day 0, CK19 was expressed only in CD133⁺ HuH7 cells. However, CK19 expression was also identified in CD133⁻ cells at day 21. At day 21, CD133⁺ and CD133⁻ HuH7 or HuCCT1 cells showed similar expression patterns for mRNA, except for the slightly more intense expression of CK19 and albumin in CD133⁺ and CD133⁻ HuH7 cells, respectively (Figure 7).

Alteration of CD133 expression levels in CD133⁺ and CD133⁻ cells

Alterations of CD133 expression levels in HuH7 and

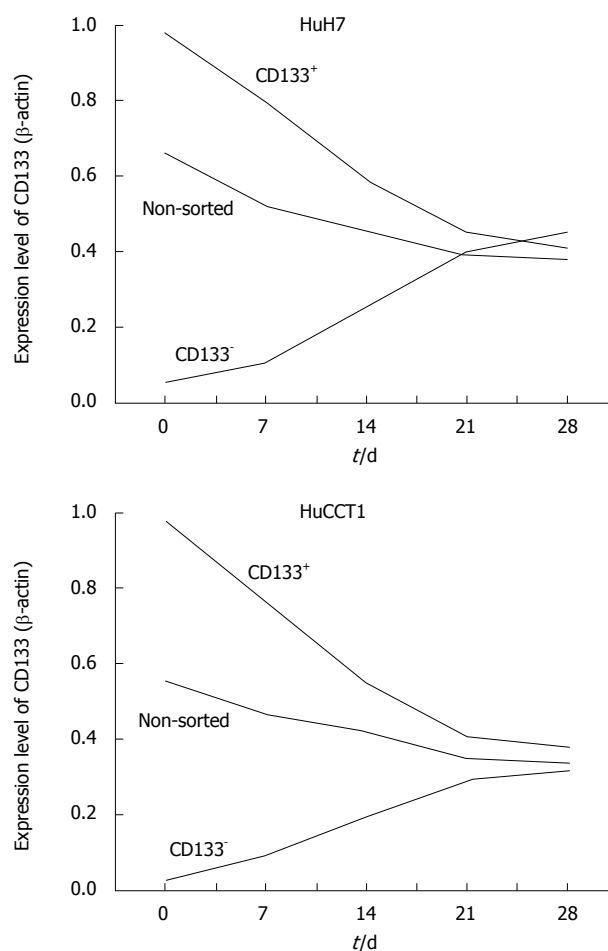


Figure 8 Time-dependent alteration of CD133 expression in HuH7 and HuCCT1 cells. Real-time quantitative RT-PCR revealed CD133 expression levels gradually decreased in non-sorted cells after passage. CD133⁺ HuH7 and HuCCT1 cells showed decreased expression of CD133. In contrast, CD133 expression increased in CD133⁻ cells in both cell lines. Around day 21-28, CD133 expression in three types of cells became similar to the level in both cell lines.

HuCCT1 cells were examined by real-time quantitative RT-PCR. HuH7 and HuCCT1 cells showed similar alteration patterns. As shown in Figure 8, CD133 expression levels in non-sorted HuH7 and HuCCT1 cells gradually decreased after passage. CD133 expression levels in CD133⁺ populations were high just after the sorting (day 0) in both cell lines. These expression levels decreased time-dependently. In contrast, CD133 expression levels in CD133⁻ cells were very low at day 0, and time-dependently increased. CD133 expression in CD133⁺ and CD133⁻ cells reached a similar level around day 21 or 28. In addition, their expression level was also similar to the level of CD133 expression in non-sorted cells at day 21 (Figure 8).

Proliferation assay of CD133⁺ and CD133⁻ cells

The proliferation of CD133⁺ and CD133⁻ cells were examined using BrdU after 7 d subculture. The percentages of BrdU-labeled cells were as follows: CD133⁺ HuH7 cells, 22%; CD133⁻ HuH7 cells, 24%; CD133⁺ HuCCT1 cells, 39%; and CD133⁻ HuCCT1 cells, 42%. No significant differences were observed in proliferation of CD133⁺ and CD133⁻ HuH7 and HuCCT1 cells.

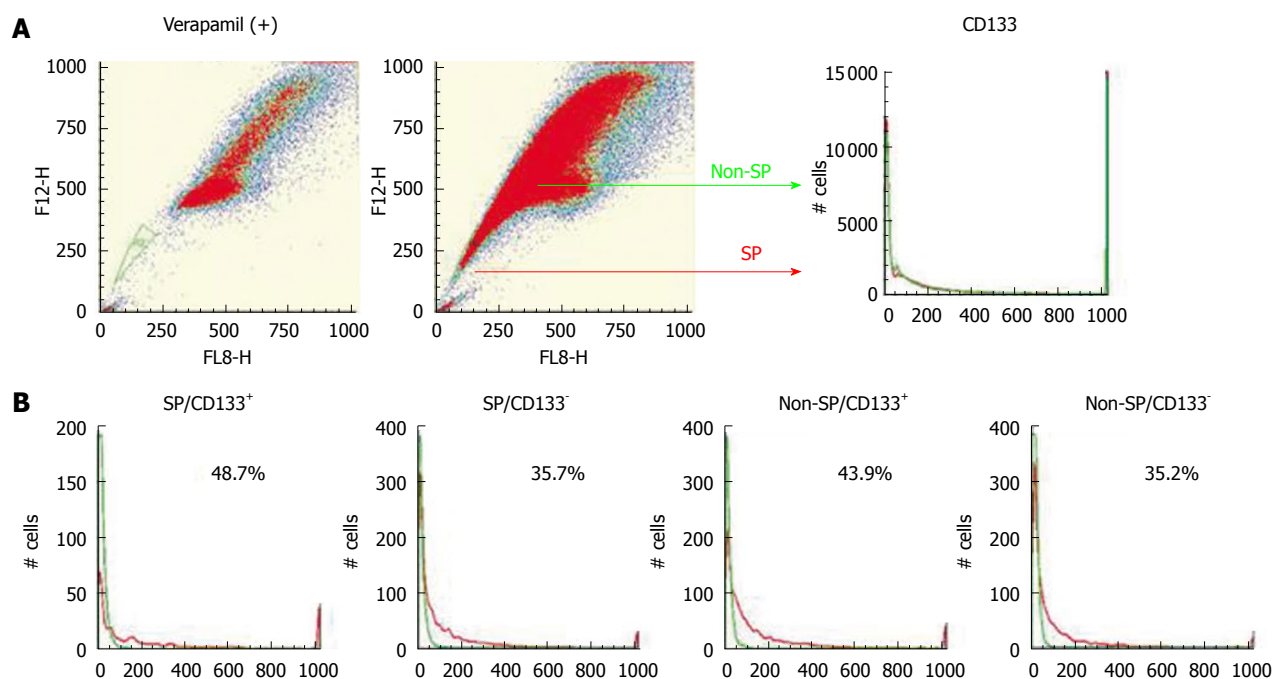


Figure 9 Relationship between CD133⁺ cells and SP phenotype. A: The percentages of CD133⁺ cells in SP and non-SP fractions of HuH7 cells were examined using Hoechst 33342 and a PE-conjugated antibody to CD133. The ratio of CD133⁺ cells was almost the same in the SP and non-SP fractions; B: HuH7 cells were sorted into four populations: SP/CD133⁺, SP/CD133⁻, non-SP/CD133⁺, and non-SP/CD133⁻. After 4 wk subculture, CD133⁺ and CD133⁻ cells were generated at similar levels from all populations.

Relationship between SP and CD133⁺ cells

Previous studies have reported that CD133⁺ HCC cells have a greater colony-forming efficiency, higher proliferative activity, and greater ability to form tumors *in vivo*^[14-16]. It has been suggested that CD133⁻ cells are not capable of producing CD133⁺ cells. However, CD133⁺ and CD133⁻ HuH7 cells returned to almost identical cell populations after 4 wk subculture in this study. To resolve this discrepancy, the relationship between SP and CD133⁺ cells in HuH7 cells was examined because SP is one of the most reliable stem cell markers currently available.

As in the previous study^[21], an SP fraction was identified in HuH7 cells. The percentages of CD133⁺ cells in SP and non-SP fractions were examined using Hoechst 33342 and a PE-conjugated antibody against CD133. The ratios of CD133⁺ cells were almost the same in both the SP and non-SP fractions (Figure 9). HuH7 cells were sorted into four populations: SP/CD133⁺, SP/CD133⁻, non-SP/CD133⁺, and non-SP/CD133⁻. Each population was cultured separately for 4 wk and was analyzed again with regard to CD133 expression by FACS. CD133⁺ and CD133⁻ cells were produced at similar levels in the four populations (Figure 9). These results suggested no relationship between SP phenotype and CD133 expression.

DISCUSSION

This study involved the histological characterization of CD133⁺ cells in the liver and the biological characteristics of CD133⁺ cells derived from human HCC and cholangiocarcinoma cell lines. The results

obtained can be summarized as follows. (1) CD133 was expressed constantly in the biliary epithelium in non-neoplastic liver tissues. Most of the CD133⁺ cells were CK19⁺ and HepPar-1⁻ in non-neoplastic liver tissues. (2) In HCC, the expression of CD133 mRNA was observed in all cases by nested RT-PCR, whereas CD133⁺ cells were identified in only 24% of cases by immunostaining. CD133⁺ cells were small in number in all the cases of HCC examined. (3) In cholangiocarcinoma, CD133 was expressed diffusely in most carcinoma cells. (4) In combined carcinoma, most of the CD133⁺ cells were CK19⁺ and HepPar-1⁻, although some CD133⁺ cells were CK19⁻ and HepPar-1⁺. (5) In human HCC and cholangiocarcinoma cell lines, CD133⁺ cells co-expressed CK19 and AFP. (6) CD133⁺ or CD133⁻ cells derived from HuH7 and HuCCT1 cell lines similarly produced CD133⁺ and CD133⁻ progeny during subculturing. (7) There was no relationship between CD133⁺ cells and SP phenotype.

In the histological examination, CD133 expression was related closely to CK19 expression. CK19 has been used as not only a biliary marker, but also as a progenitor cell marker. CK19 is expressed usually in the bile ducts, bile ductules, and the canal of Hering^[22-24]. Small ductal structures partly surrounded by hepatocytes (the canal of Hering) are currently estimated as hepatic stem/progenitor cells, and these structures are also positive for CD133^[25,26]. Before starting this study, it was speculated that CD133 was expressed only in hepatic progenitor cells. However, this study revealed that CD133 is not only a progenitor cell marker, but can also be used as a novel biliary marker.

Some might argue about the discrepancy between the

results with nested RT-PCR and immunohistochemistry for HCC. Nested RT-PCR could detect CD133 expression in all HCC cases; whereas, its expression was observed in only 24% of cases by immunostaining. We speculate that this difference might have been caused by the low expression level of CD133 in HCC. Indeed, non-nested conventional PCR showed CD133 expression in less than half of HCC cases in the preliminary study. That is, it might be difficult to detect CD133 expression in HCC by immunostaining because of the low expression level or the lower number of positive cells.

Other investigators have examined CD133 expression in liver tissues. Yin *et al*^[15] have reported that CD133 expression is observed in a small subset of hepatocytes, biliary epithelium, and epithelial clusters in the portal tracts in cirrhotic livers, but CD133 expression is not seen in normal liver^[15]. In addition, Ma *et al*^[16] have also reported that CD133⁺ cells are almost absent in non-neoplastic liver tissues. The discrepancy between the previous and current studies might have been caused by the method of immunostaining. Both previous studies used a goat polyclonal antibody and paraffin-embedded specimens, but preliminary trials in the current study could not detect any positive signals for CD133 in paraffin-embedded specimens using any antibodies for CD133; therefore, frozen sections were used instead. In addition, CD133, which is usually expressed on the cellular membrane, was detected in the cytoplasm in previous studies. More recently, Shmelkov *et al*^[27] have examined CD133 in various organs using a unique transgenic mouse model, in which endogenous promoters for CD133 drove the expression of the reporter gene *lacZ*. CD133 was expressed widely in differentiated ductal structures in various organs including bile ducts in the liver^[27]. These previous results are consistent with the results of the current study.

CD133 expression was also related closely to CK19 expression in neoplastic liver tissues. In cholangiocarcinoma and combined carcinoma, CK19⁺ cells constantly co-expressed CD133. CD133⁺ cells comprised 48.5% of cells in the HuCCT1 cell line, and there were no differences between CD133⁺ and CD133⁻ cells in terms of proliferation or mRNA expression. We speculate that CD133 expression in cholangiocarcinoma reflects the biliary phenotype and not the progenitor phenotype. In contrast, some CD133⁺ cells in HCC and combined carcinoma were CK19⁻ and HepPar-1⁺. CD133⁺/CK19⁻/HepPar-1⁺ cells could not be identified in non-neoplastic livers, although this suggests that CD133⁺ cells are pluripotent and can differentiate into CK19⁺/HepPar-1⁻ and CK19⁻/HepPar-1⁺ cells. In particular, it is interesting that CD133⁺/CK19⁻/HepPar-1⁺ cells are observed in combined carcinoma because the involvement of hepatic progenitor cells is suggested in tumorigenesis of combined carcinoma^[28-30].

Until now, some investigators have examined the characterization of CD133⁺ cells in HCC, and have suggested that CD133⁺ HCC cells are characterized by higher proliferative activity, expression of "stemness"

genes, the ability to self-renew, and greater ability to form tumors *in vivo*^[14-16]. They have concluded that CD133⁺ cells are tumorigenic cancer cells, and located at a higher rank in the cancer-cell hierarchy. However, in the current study, CD133⁺ HuH7 cells were not different from CD133⁻ cells in terms of proliferation. In addition, CD133⁻ cells could generate both CD133⁺ and CD133⁻ cells in subcultures, which did not support the existence of a cancer-cell hierarchy with respect to CD133 expression. As noted in previous studies, CD133⁺ cells comprised about half of the carcinoma cells in the HuH7 cell line (65.0% in the current study, 46.7% or 65.0% in previous studies^[14,16]). The percentage of CD133⁺ cells seems too high to suggest that CD133 cells are tumor-initiating cells. Indeed, CD133⁻ cells were able to give rise to CD133⁺ cells in the subculture system in the previous study^[16]. Moreover, a more recent study has revealed that CD133 expression is not restricted to stem cells, and CD133⁺ and CD133⁻ cells derived from colon cancer are capable of initiating tumors in immunodeficient mice^[27].

To resolve the uncertainties regarding CD133 expression and tumor-initiating cells, the relationship between CD133⁺ cells and SP phenotype was examined in our study. SP is a minor population with extreme tumorigenic potential, and it is supposed that tumor-initiating cells exist in SP cells. If CD133⁺ cells are tumor-initiating cells, CD133⁺ cells should be related closely to the SP phenotype, and CD133⁻ cells should not exist in the SP fraction. However, there was no difference in the CD133⁺/CD133⁻ cellular population between SP and non-SP fractions. In addition, four cell populations (SP/CD133⁺, SP/CD133⁻, non-SP/CD133⁺, and non-SP/CD133⁻) could similarly produce CD133⁺ and CD133⁻ cells during subculture. It is speculated that CD133 expression might reflect the progenitor phenotype in HCC; however, CD133 alone is not sufficient to detect tumor-initiating cells.

It seems important to know that CD133 is one of the progenitor cell markers in the liver, but this is not specific. We have to determine the conditions to identify a pure hepatic progenitor or stem cell population, using multiple surface markers including CD133. From the biliary aspect, CD133 could become a useful marker, because this is a surface antigen. We can use this molecule for sorting or purification of biliary epithelium.

In conclusion, this study revealed that CD133 can be a biliary and progenitor cell marker in liver tissues. However, CD133 alone is not sufficient to detect tumor-initiating cells in cultured cells.

COMMENTS

Background

CD133 is recognized as a stem cell marker for normal and cancerous tissues in various organs. The histological characteristics of hepatic CD133⁺ cells have not been examined fully, especially in non-neoplastic liver tissues and non-hepatocellular liver cancers.

Research frontiers

Previous studies have shown that CD133 can be used as a marker of cancer stem cells in human hepatocellular carcinoma (HCC). The current study

elucidated the histological and biological characteristics of CD133⁺ cells in non-neoplastic and neoplastic human livers.

Innovations and breakthroughs

Immunohistochemical analysis showed that CD133 was expressed constantly in the non-neoplastic biliary epithelium. In cholangiocarcinoma, CD133 was expressed diffusely in most carcinoma cells, whereas CD133⁺ cells were identified in only a small number of cases of HCC. In combined carcinoma, most of the CD133⁺ cells were CK19⁺. In human HCC and cholangiocarcinoma cell lines, CD133⁺ cells co-expressed CK19 or alpha-fetoprotein. CD133⁺ or CD133⁻ cells derived from human HCC and cholangiocarcinoma cell lines similarly could produce CD133⁺ and CD133⁻ progeny during subculturing, and there was no relationship between CD133⁺ cells and the side population (SP) phenotype.

Applications

This study demonstrated that CD133 could be a biliary and progenitor cell marker *in vivo*. However, CD133 alone is not sufficient to detect tumor-initiating cells in cell lines. These results may provide insights into understanding the pathogenesis of various hepatobiliary diseases.

Terminology

CD133 (also known as prominin-1 or AC133) is a marker of hematopoietic progenitor cells. It has also been reported that CD133 is expressed in epithelial and non-epithelial progenitors in various tissues, in which the specific functions and ligands of CD133 have not been fully elucidated. SP is a minor population with extreme tumorigenic potential, and it is supposed that tumor-initiating cells exist in SP cells.

Peer review

The article presents interesting and novel data about CD133 expression in non-neoplastic and neoplastic liver tissues, and examines some biological characteristics of CD133⁺ cells in HCC and cholangiocarcinoma cell lines. Although the authors presented some controversial data about the presence of CD133 expression in HCC, the experiments were designed appropriately, the methodology was precise, and the discussion supports the results.

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S- Editor Li LF L- Editor Kerr C E- Editor Ma WH

Short-term Celecoxib intervention is a safe and effective chemopreventive for gastric carcinogenesis based on a Mongolian gerbil model

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Supported by A grant from Kaohsiung Medical University (Q096014), and Kaohsiung Municipal Hsiao-Kang Hospital (kmhk-96-005, kmhk-95-005). This study was approved by the Institutional Animal Care and Use Committee, Kaohsiung Medical University

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Received: July 28, 2009

Revised: August 31, 2009

Accepted: September 6, 2009

Published online: October 21, 2009

METHODS: One hundred and twelve MGs were divided into six groups (A-F). One hundred gerbils were inoculated with *H pylori* (groups A-E). Twelve gerbils were inoculated with vehicle broth only (group F). After 4 wk, they were given N'-methyl-N'-nitro-N-nitroso-guanidine (MNNG) (50 µg/mL) in the drinking water for 20 wk. In groups B-E, the animals were given the stock Celecoxib (10 mg/kg per day) diet from the 21st, 31st, 21st and 41st week respectively. The periods of administering Celecoxib were 30, 20, 20, and 15 wk respectively. On the 51st week, the animals were sacrificed for histological examination. Local PCNA expression was examined by the immunohistochemistry method. The expression of COX-2 protein was assessed by Western Blot. Analysis used the χ^2 test. The difference was regarded as significant when *P* value was less than 0.05.

RESULTS: Seventeen percent (17/100) of *H pylori*-infected MGs developed gastric cancer. All of these lesions were well-differentiated adenocarcinoma. The incidence rates of adenocarcinoma in groups A-F were 40%, 0%, 0%, 20%, 25%, and 0% respectively. The inflammatory scores were higher in group B than in other groups. There was no inflammatory response noted in group F. Celecoxib treatment resulted in a significant reduction in the proliferation of *H pylori*-infected mucosal cells (groups B, C and D) (*P* < 0.01). The expression of COX-2 protein was significantly attenuated in the groups which were Celecoxib-treated for more than 20 wk (groups B, C, D). The groups treated with Celecoxib had a significantly lower rate of advanced gastric cancer (34% vs 75%, *P* < 0.001). There were no sudden deaths in any of the groups.

CONCLUSION: Short-term treatment with Celecoxib has an anti-carcinogenic effect, and resulted in less severe inflammation and inhibited the invasive degree of gastric cancer.

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Key words: Cyclooxygenase-2; Chemoprevention; *Helicobacter pylori*; Mongolian gerbil

Peer reviewer: Yuan Yuan, Professor, Cancer Institute of

Abstract

AIM: To evaluate the optimal intervention point of a selective cyclooxygenase-2 (COX-2) inhibitor, Celecoxib, for inhibiting *Helicobacter pylori* (*H pylori*)-associated gastric carcinogenesis in Mongolian gerbils (MGs).

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Kuo CH, Hu HM, Tsai PY, Wu IC, Yang SF, Chang LL, Wang JY, Jan CM, Wang WM, Wu DC. Short-term Celecoxib intervention is a safe and effective chemopreventive for gastric carcinogenesis based on a Mongolian gerbil model. *World J Gastroenterol* 2009; 15(39): 4907-4914 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4907.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4907>

INTRODUCTION

Since the isolation and culture of *Helicobacter pylori* (*H. pylori*) in 1983, this bacterium has become accepted as an important human pathogen for the development of gastritis, peptic ulcer, and gastric cancer^[1]. The worldwide prevalence rate of *H. pylori* infection is approximately 50%, with the highest being in developing countries. In Taiwan, the overall prevalence rate is 54% and this rises with age^[2]. Seven-day triple therapy [proton pump inhibitor (PPI), amoxicillin and clarithromycin] has been the main first-line therapy for *H. pylori* infection in Taiwan, Europe and many other countries as guided by the Maastricht-22 000 consensus^[3,4]. Despite this, although the eradication of *H. pylori* is still the most cost-effective method for prevention of gastric carcinogenesis, the antibiotic treatment of *H. pylori* infection is confronting a significant challenge associated with resistance to antibiotics. The first-line regimen continues to have a 10%-23% failure rate^[5-10]. Unfortunately, several studies have shown that rescue regimens have failed in 5%-63% of patients whose *H. pylori* cannot be eradicated by standard PPI-based triple therapies^[11,12].

If refractory *H. pylori* infection persists, *H. pylori*-dependent induction of cyclooxygenase-2 (COX-2) is associated with enhanced production of multidrug resistance-1 (MDR-1) and Bcl-xL proteins that may contribute to gastric tumorigenesis and resistance to therapy^[13]. Therefore, it is important to find an alternative chemoprevention for these patients.

COX-2 is a prostaglandin-synthesizing enzyme. Elevated expression of COX-2 is observed in a wide variety of human malignancies, including gastric cancer. Various *in vitro* and *in vivo* studies strongly suggest that COX-2 is involved in a major early oncogenic event in these human malignancies^[14-18]. *H. pylori*-induced chronic gastritis also shows elevated levels of COX-2 expression in the stomach mucosa^[17-22]. Enhanced expression of COX-2 is also observed in intestinal metaplasia, dysplasia, and gastric adenoma, which are regarded as precancerous lesions^[14,23]. Moreover, previous studies showed that regular intake of either nonselective or selective COX-2 inhibitors reduces the risk of several human cancers^[24-26].

The selective COX-2 inhibitors have been reported to prevent chemical carcinogen-induced carcinogenesis in C57/BL6 mice^[22] and Mongolian gerbils (MGs)^[27-29].

However, the adverse effects of COX-2 inhibitors on the cardiovascular system inhibit the application of this chemoprevention^[30-33]. There is no animal study focusing on the optimal therapeutic period of COX-2 inhibitors to prevent these possible severe adverse events.

In the present study, our aim is to evaluate the optimal intervention point of Celecoxib in order to inhibit *H. pylori*-associated gastric carcinogenesis in MGs. In addition, we also investigated the effects on tumor invasion.

MATERIALS AND METHODS

The experimental design was approved by the Animal Research Committee of Kaohsiung Medical University. One hundred and twelve gerbils were divided into six groups (A-F), and were inoculated with *H. pylori* [CagA(+)/VacA(+)] (groups A-E; *n* = 20 in every group) or vehicle (Brucella broth) alone (group F; *n* = 12). After 1 wk, groups A-E were given N'-methyl-N'-nitro-N-nitroso-guanidine (MNNG) at a concentration of 50 µg/mL in the drinking water for 20 wk (as shown in Figure 1). Then, all groups were switched to autoclaved distilled water as drinking water. In groups B-E, the animals started to be given the stock Celecoxib diet from the 21st, 31st, 21st and 36th week. The periods during which Celecoxib was given were 30, 20, 20, and 15 wk respectively. However, the animals in groups A and F received the control diet. The daily-administered dosage of Celecoxib was 10 mg/kg per day in groups B-E. On the 51st experimental week, the animals were fasted for 24 h before being sacrificed.

Histological evaluation of the gastric mucosa in *H. pylori*-infected gerbils

Samples of the gastric mucosa were excised from each gerbil stomach for the assessment of the presence of *H. pylori* and gastric inflammation using Giemsa and hematoxylin-eosin (HE) staining for histological examination, respectively. The samples were fixed in 10% buffered formalin and embedded in paraffin^[34]. The paraffin sections were cut at a thickness of 5 µm and stained. Two experienced pathologists, unaware of the treatment given, performed histological examinations blindly. Histological features of mucosal inflammation and intestinal metaplasia were evaluated for each specimen under a light microscope according to the classification of the Sydney system. The degree of inflammatory cell infiltration and the area of intestinal metaplasia were scored as follows: 0, normal; 1, mild; 2, moderate; 3, marked. For the evaluation of mucosal cell proliferation, the proportion of PCNA-positive cells per 1000 mucosal cells was assessed in the antrum and corpus as in a previous study by Suzuki *et al.*^[35]. We also recorded the size, depth and location of tumor.

Analysis of anti-proliferating cell nuclear antigen (PCNA) in gastric mucosa

Half of the excised stomachs were fixed in 10% neutral-

Table 1 Effect of Celecoxib treatment period on incidence of gastric cancer in Mongolian gerbils

Group	Regimen	Period of Celecoxib treatment (wk)	n	Rate of gastric cancer (%)	Type of cancer		
					Well	Poor	Sig
A	HP+MNNG	0	20	40 (8/20)	8	0	0
B	HP+MNNG+Celecoxib	30	20	0 (0/20) ^a	0	0	0
C	HP+MNNG+Celecoxib	20	20	0 (0/20) ^a	0	0	0
D	HP+MNNG+Celecoxib	20	20	20 (4/20)	4	0	0
E	HP+MNNG+Celecoxib	15	20	25 (5/20)	5	0	0
F	Vehicle	0	12	0	0	0	0

HP: *H. pylori* (intra-gastric); MNNG: N'-methyl-N'-nitro-N-nitroso-guanidine; Well: Well-differentiated adenocarcinoma; Poor: Poorly differentiated adenocarcinoma; Sig: Signet-ring cell carcinoma. ^a*P* < 0.05 vs group A.

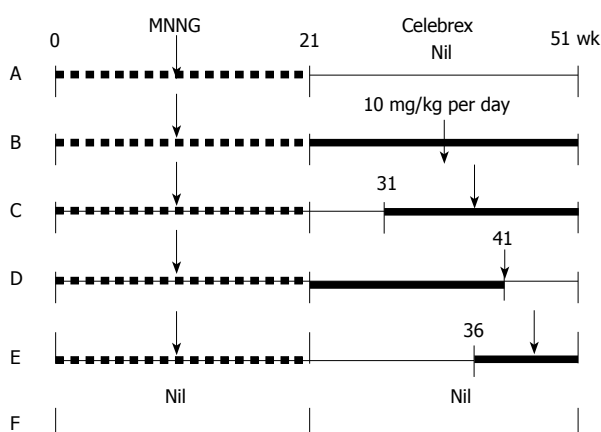


Figure 1 Design of the study. At the beginning of the experiment, gerbils were inoculated (i.g.) with *H. pylori* (Grps A-E) or vehicle (Brucella broth; Grp F). Until the 21st week, the animals were given drinking water containing no Grp F (open bars) or 50 µg/mL MNNG (Grp A-E). All groups were then switched to distilled water and given a diet containing no drug (Grps A and F) or Celecoxib 10 mg/kg per day for 30 (Grp B), 20 (Grp C), 20 (Grp D) or 15 (Grp E) weeks. The gerbils were sacrificed at week 51.

buffered formalin and embedded in paraffin. Tissues sections were stained with HE and were analyzed by immunohistochemistry with anti-proliferating cell nuclear antigen (PCNA) serum (Dako).

Protein extraction and analysis of COX-2 expression in the gastric mucosa by Western blotting

Frozen gastric tissue was homogenized in lysis buffer (100 mmol Tris-HCl, pH 7.4, 15% glycerol, 2 mmol EDTA, 2% SDS, 100 mmol DDT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol PMSF as described in previous studies^[32]. Approximately 100 µg of cellular protein extract was loaded into a well, separated electrophoretically on 13.5% SDS-polyacrylamide gel and transferred onto Sequi-Blot TMPVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting. Western blotting was performed with specific primary rabbit polyclonal antibody against COX-2 (dilution 1:500, Santa Cruz, USA) or anti-β-actin rabbit polyclonal antibody as primary antibody, and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (dilution 1:2000, Santa Cruz, USA). Visualization of immune complexes was achieved by chemiluminescence using BM Chemiluminescence Blotting Substrate (Boehringer, Mannheim, Germany).

and the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany). We did not perform Western blotting for group F.

Statistical analyses

We analyzed the collected data using the statistical software package STATA. An unpaired *t*-test or a Mann-Whitney *U* test was applied to determine the significance of differences between two groups. The incidence of cancer was assessed using χ^2 test. *P* < 0.05 was considered to be statistically significant.

RESULTS

In our study, all gerbils were alive till the end of this experiment; there was no significant difference in the survival rates among the various groups. Seventeen percent of *H. pylori*-infected gerbils developed gastric cancer. All of these lesions were well-differentiated adenocarcinoma (Figure 2). The incidence of cancer in every experimental group is shown in Table 1. As a result of long-lasting infection with *H. pylori*, 40% of the animals in group A developed cancer. There was no cancer found in groups B and C. However, the group treated with a shorter period of Celecoxib (group E) and the early-treatment group (group D) did not show obvious inhibitory effects on gastric carcinogenesis. Therefore, this meant that Celecoxib might have a chemopreventive effect on gastric carcinogenesis in some situations. Our data disclosed that the protective effect of Celecoxib might exist during long-term use or late-use of Celecoxib at a dose of 10 mg/kg per day (groups B and C).

We also evaluated the effect of Celecoxib on invasion of gastric cancer. Eight gerbils in the groups with no Celecoxib treatment and nine gerbils in groups with Celecoxib treatment developed gastric cancers. The incidence rate of advanced gastric cancer was higher in group A than groups D and E (75% vs 34%, *P* < 0.001).

In all of the *H. pylori*-infected animals (groups A-E), different degrees of infiltration of inflammatory cells were observed in the lamina propria and submucosa. The infiltration was predominantly lymphocytes, although some macrophages and neutrophils were also observed. The histological examination also revealed hyperplasia of the epithelia accompanied by erosions, lymphoid follicle formation, and intestinal metaplasia. These findings are mimicked in *H. pylori*-infected humans. The inflammatory

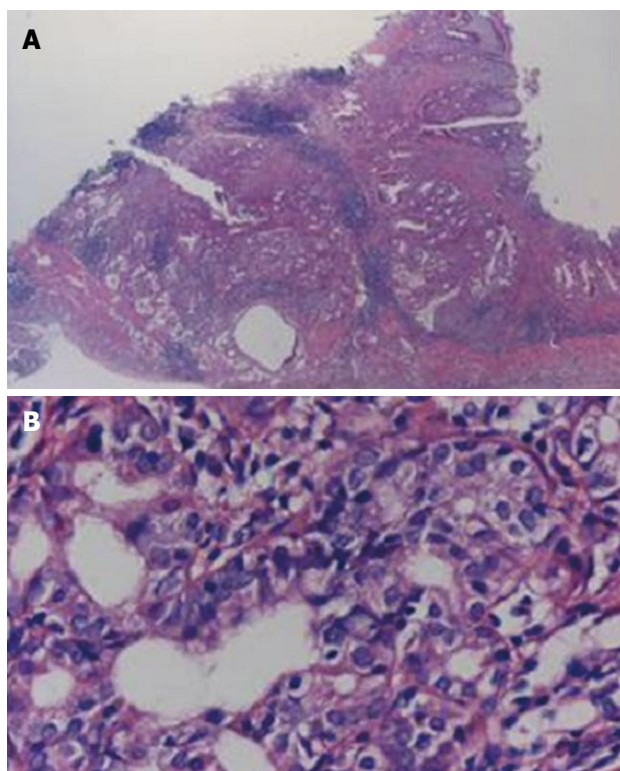


Figure 2 Typical adenocarcinoma in the pyloric mucosa of *H. pylori*-infected MGs. Shown is a typical well-differentiated adenocarcinoma (A, B) stained with HE. Images were obtained at $\times 100$ (A) and $\times 400$ (B).

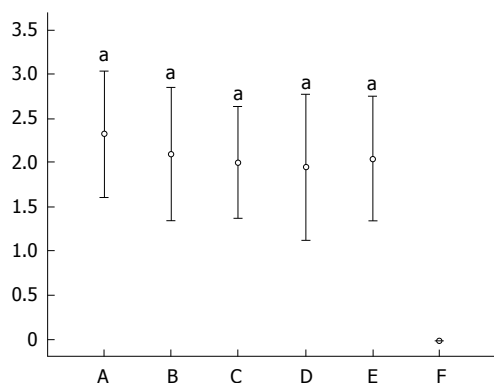


Figure 3 Effect of Celecoxib on inflammation of the stomach mucosa. No obvious inflammatory change was found in group F. Significantly obvious inflammation was shown in *H. pylori*-infected groups (A-E) vs group F ($^aP < 0.05$), but there was no significant difference among *H. pylori*-infected groups. Group B showed higher inflammatory response than group A, C, D, E.

score was higher in group B than other groups. However, there was no significant difference between groups A, B, C, D and E. No definite evidence of inflammatory response was found in group F (Figure 3).

Our data revealed that Celecoxib could repress the development of intestinal metaplasia. Intestinal metaplastic changes were observed after *H. pylori* infection in our study (groups A-E) (Figure 4). These changes were significantly reduced in groups B and C. We did not find similar reductions in groups D and E. No intestinal metaplasia was found in group F.

In this study, we found that infection with

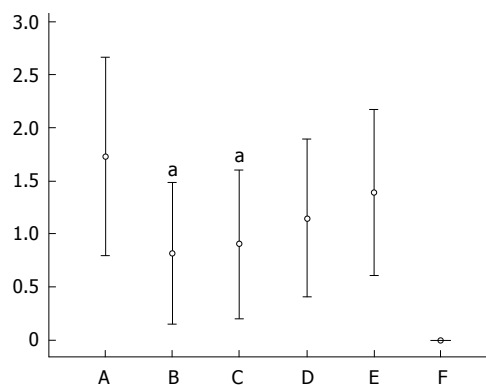


Figure 4 Effect of Celecoxib on the development of intestinal metaplasia (IM). Severe IM was found in group A. There was significantly lower rates of IM in groups B and C. A relatively lower rate of IM was found in groups D and E ($P > 0.05$ vs group A). There was no definite IM found in group F. $^aP < 0.05$ vs group A.

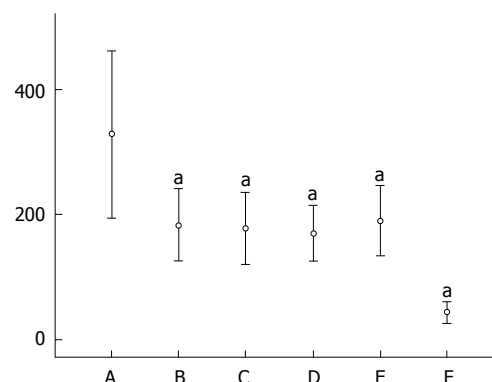


Figure 5 Effect of Celecoxib on the proliferation of gastric mucosal cells in gerbils. High PCNA positive ratio was found in group A, and the value decreased significantly after treatment with Celecoxib. The value was also lower in group F without *H. pylori* infection. $^aP < 0.05$ vs groups A.

H. pylori greatly enhanced the density of anti-PCNA immunohistochemistry in mucosal cells (groups A-E). This showed that proliferation of mucosal cells was promoted after *H. pylori* infection (Figures 5 and 6). Celecoxib treatment resulted in a significant reduction in the proliferation of *H. pylori*-infected mucosal cells (groups B, C, D and E), ($P < 0.01$). The group F showed lowest proliferation indices.

Results showed strong expression of COX-2 protein in those gerbils inoculated with *H. pylori*, but we did not detect any indication of this in gerbils treated with vehicle (Figure 7). Ratio of COX-2/ β -actin protein was significantly increased in group A, and the signal for COX-2 protein was significantly attenuated in the Celecoxib-treated groups (B, C, D). The value of COX-2 protein/ β -actin protein ratio was significantly lower in gerbils treated with long-term Celecoxib (groups B, C, D) compared to that treated with *H. pylori* alone (group A) or short-term Celecoxib (group E).

DISCUSSION

In this study, we attempted to evaluate the effect of short-term treatment of Celecoxib on prevention of

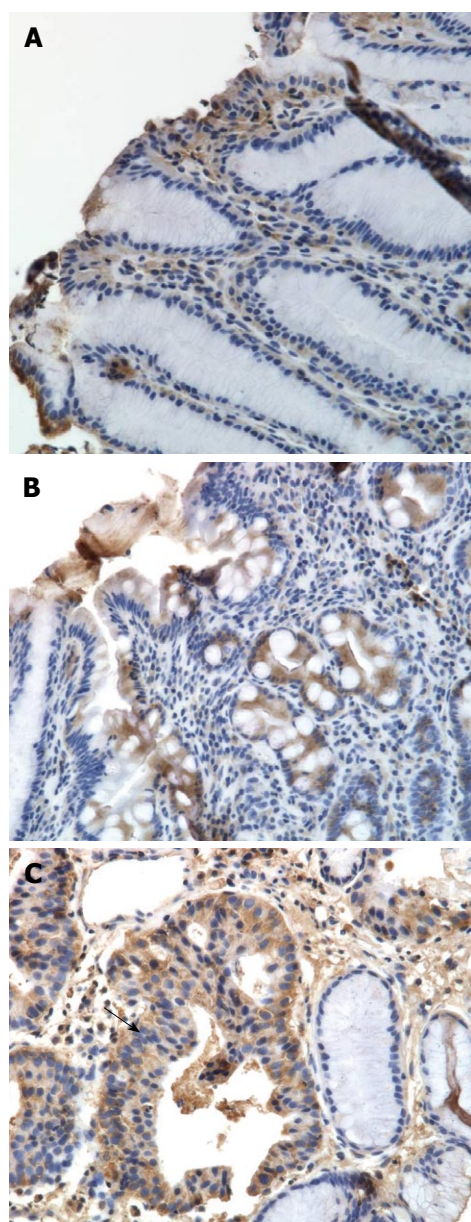


Figure 6 Expression of PCNA by immunohistochemistry method. A: Normal gastric mucosa; B: Intestinal metaplasia; C: Adenocarcinoma. Adenocarcinoma is pointed out by arrow. Images were obtained at $\times 400$.

gastric carcinogenesis in a Mongolian gerbil model. Our data support the concept that Celecoxib has a chemopreventive effect on *H. pylori*-associated stomach carcinogenesis. Besides this, we also showed that a short-term treatment period (20 wk) in the late infection phase could provide a similar chemoprevention effect as a longer treatment period (30 wk) reported in a previous study^[26]. According to this finding, we postulate that a COX-2 inhibitor could be used as chemoprevention for people older than about forty years old. However, this suggestion is not strongly definite due to the small scale of our study. The exact time-point of Celecoxib intervention needs further investigation.

This chemoprevention may play an important role for some people. For example, subjects with extensive metaplastic gastritis have the highest risk for the development of gastric cancer (annual cancer incidence,

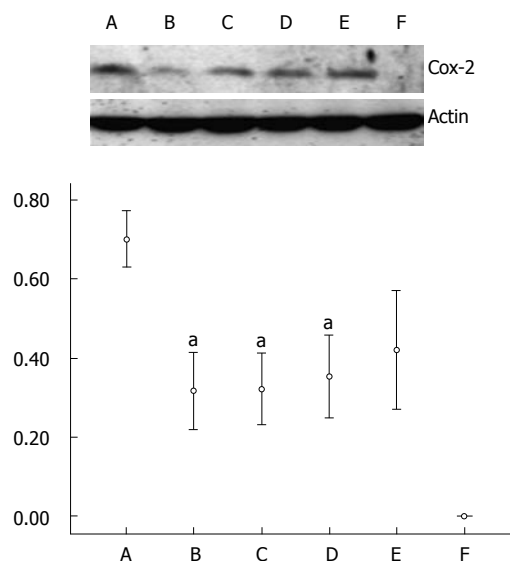


Figure 7 Analysis of COX-2 protein expression in gastric mucosa of gerbils. The expression of COX-2 protein in group A was high. There was no detectable expression in group F. The expression of COX-2 was inhibited significantly by Celecoxib. There was a significant decrease in groups B, C and D. ^a $P < 0.05$ vs group A.

0.87%), but they cannot be treated with therapy to eradicate *H. pylori* because the infection is no longer present^[36]. In this small population, inhibition of COX-2 expression may be useful for the prevention of gastric cancer. Our study supports the fact that a COX-2 inhibitor has a chemopreventive effect by inhibiting expression of COX-2.

Eradication of *H. pylori* is a well-known effective prevention of gastric cancer^[37]. COX-2 inhibitors are expensive and less cost-effective than therapy aimed at eradicating *H. pylori*. However, there are still people with refractory infection despite rescue eradicating regimens. So this chemoprevention is very important for those patients with refractory *H. pylori* infections at high risk of gastric cancer.

Various mechanisms have been proposed to explain the anti-tumor action of NSAIDs, including cell growth suppression, inhibition of angiogenesis and metastasis, and NSAID-induced apoptosis in cancer cells^[38,39]. Persistent activation of COX-2 is associated with oncogenesis as well as with increased invasive potential of tumor cells^[40-42]. In our study, we found that Celecoxib could inhibit the tumor invasion even with short-term use. According to our results, Celecoxib may have effects including anti-oncogenic effect, inhibition of angiogenesis and metastasis, and it was shown that these effects were obtained by both late and short-term use of Celecoxib. In our study, the treatment began at 30-40 wk of age of the gerbils which is equivalent to 35 to 45-years old in humans.

With regard to PCNA results in our data; these disclosed that the inhibitory effect of Celecoxib was related to the inhibition of cell proliferation. This was comparable with previous studies' findings^[43,44]. It is well-known that carcinogenesis in the *H. pylori*-infected stomach also results from an inflammation-mediated

sequence. Chronic inflammation is thought to play a cardinal role in the accumulation of genetic damages leading to transformation and cancer by inducing the proliferation of target cells^[45-47]. Consequently, it is important to find the suitable time-point at which COX-2 inhibitors prevent the carcinogenesis.

In our study, group D had a higher incidence rate of gastric cancer than group C (20% *vs* 0%). This finding supports the theory that the protective effect of Celecoxib is involved in an early oncogenic phase not in an early inflammation phase. This was comparable with findings of previous studies^[14-17]. According to our findings, Celecoxib could be used latterly and short term for refractory *H pylori* infection in a clinical situation; a point which has been seldom discussed in previous reports. This short term use is very important for decreasing the possible side effects of COX-2 inhibitors.

In our study, different periods of treatment with Celecoxib produce different results in inflammatory score. The longer treatment period results in more obvious gastric mucosal damages. Previous studies which used long-term treatment indicated that COX-2 inhibitors were not a placebo and had toxic effects. Accordingly, COX-2 inhibitors should not be used too long for chemoprevention of gastric cancer. Although we did not find any sudden death resulting from cardiovascular events in the gerbils, this may be due to the small sampling size. However, we found that the average ventricular size was large in group B (data not shown). Therefore, subclinical cardiovascular events may occur. The above findings show that the treatment period of Celecoxib should be as short as possible.

It should be noted that there could be other COX-2-independent mechanisms involved in stomach carcinogenesis because a relatively high dose of Celecoxib is needed for the anti-carcinogenic effect. Further studies are required to survey these mechanisms.

In conclusion, our study supports the hypothesis that Celecoxib has a potent anti-carcinogenic effect, and that short-term use could result in an almost equal effect to longer term use with less side effects. The protective effect of Celecoxib could be involved in the early oncogenic phase not in the early inflammation phase. This chemoprevention may be suitable for subjects with high risk for the development of gastric cancer: such as people with extensive metaplastic gastritis or refractory *H pylori* infection. Thus we suggest that Celecoxib could be used short-term for high-risk patients.

COMMENTS

Background

Long-term high dose cyclooxygenase-2 (COX-2) inhibitors can inhibit gastric carcinogenesis in animal models, but the possible life-threatening cardiovascular events limit its popular application. Therefore, in the present study, we wished to evaluate the optimal intervention point of a selective COX-2 inhibitor, Celecoxib, for inhibiting *Helicobacter pylori* (*H pylori*)-associated gastric carcinogenesis in Mongolian gerbils (MGs).

Research frontiers

COX-2 is a prostaglandin-synthesizing enzyme. Elevated expression of COX-2 is observed in a wide variety of human malignancies, including gastric cancer.

H pylori-induced chronic gastritis also shows elevated levels of COX-2 expression in the stomach mucosa. The selective COX-2 inhibitors have been reported as preventing chemical carcinogen-induced carcinogenesis in C57/BL6 mice and Mongolian gerbils. It is important to evaluate the optimal therapeutic period of COX-2 inhibitors to prevent possible severe adverse events.

Innovations and breakthroughs

Chronic inflammation is thought to play a cardinal role in the accumulation of genetic damages leading to transformation and cancer by inducing the proliferation of target cells. Therefore, it is important to find the suitable time-point at which COX-2 inhibitors prevent the carcinogenesis. Previous studies used relatively long-term periods of chemopreventive treatment. However, COX-2 inhibitors were not a placebo and had a toxic effect, so COX-2 inhibitors should not be used too long for chemoprevention of gastric cancer. According to our results, Celecoxib may have effects including anti-oncogenic effect, inhibition of angiogenesis and metastasis, and it was shown that these effects were obtained by both late and short-term use of Celecoxib. In our study, the treatment began at 30-40 wk of age of the gerbils which is equivalent to 35 to 45-years old in humans. In our study, we found that the protective effect of Celecoxib could be involved in an early oncogenic phase not in an early inflammation phase. The short-term use also resulted in less severe inflammation and inhibited the invasion degree of gastric cancer. According to our findings, Celecoxib could be used latterly and short-term for refractory *H pylori* infection in a clinical situation; a point which has been seldom discussed in previous reports. This is very important for decreasing the possible side effects of COX-2 inhibitors.

Applications

Our study supports the theory that short-term treatment with Celecoxib has an anti-carcinogenic effect. Consequently, Celecoxib could be used in the later stages of *H pylori* infection to achieve safe and effective chemoprevention of gastric adenocarcinoma. In addition to this finding, The authors would like to suggest that COX-2 inhibitor should be used as chemoprevention for people older than about forty years old. This chemoprevention may play an important role for people who have extensive metaplastic gastritis with the highest risk for the development of gastric cancer, and it is also very important for those patients with refractory *H pylori* infections at high risk of gastric cancer.

Peer review

In the present study, the author investigated the optimal intervention point of Celecoxib for inhibiting *H pylori*-associated gastric carcinogenesis in MGs. They found the animals with extensive metaplastic gastritis or refractory *H pylori* infection may be suitable for celecoxib chemoprevention. This result provided us with some new information about personalized therapy for gastric cancer prevention and will prove beneficial for clinical application in the future.

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S- Editor Li LF **L- Editor** Logan S **E- Editor** Yin DH



Milk protein IgG and IgA: The association with milk-induced gastrointestinal symptoms in adults

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Supported by Helsinki University Research Funds; Helsinki University Central Hospital Grant and The Research Foundation for Allergy, Finland; The Foundation for Promoting Occupational Medicine in Finland, Helsinki, Finland

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Received: February 26, 2009 Revised: September 16, 2009

Accepted: September 23, 2009

Published online: October 21, 2009

Abstract

AIM: To study the association between serum levels of milk protein IgG and IgA antibodies and milk-related gastrointestinal symptoms in adults.

METHODS: Milk protein IgG and IgA antibodies were determined in serum samples of 400 subjects from five outpatient clinics in Southern Finland. Subjects were randomly selected from a total of 1900 adults undergoing laboratory investigations in primary care. All 400 participants had completed a questionnaire on abdominal symptoms and dairy consumption while waiting for the laboratory visit. The questionnaire covered the nature and frequency of gastrointestinal problems, the provoking food items, family history and allergies. Twelve serum samples were disqualified due to insufficient amount of sera. The levels of specific milk protein IgG and IgA were measured by using the ELISA technique. The association of the milk protein-specific antibody level was studied in relation to the milk-related gastrointestinal symptoms and dairy consumption.

RESULTS: Subjects drinking milk ($n = 265$) had higher levels of milk protein IgG in their sera than non-milk drinkers ($n = 123$, $P < 0.001$). Subjects with

gastrointestinal problems related to milk drinking ($n = 119$) consumed less milk but had higher milk protein IgG levels than those with no milk-related gastrointestinal symptoms ($n = 198$, $P = 0.02$). Among the symptomatic subjects, those reporting dyspeptic symptoms had lower milk protein IgG levels than non-dyspeptics ($P < 0.05$). However, dyspepsia was not associated with milk drinking ($P = 0.5$). The association of high milk protein IgG levels with constipation was close to the level of statistical significance. Diarrhea had no association with milk protein IgG level ($P = 0.5$). With regard to minor symptoms, flatulence and bloating ($P = 0.8$), were not associated with milk protein IgG level. Milk protein IgA levels did not show any association with milk drinking or abdominal symptoms. The levels of milk protein IgA and IgG declined as the age of the subjects increased ($P < 0.004$).

CONCLUSION: Milk protein IgG but not milk IgA seems to be associated with self-reported milk-induced gastrointestinal symptoms.

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Key words: Abdominal symptoms; Cow's milk; Food hypersensitivity

Peer reviewers: Robert JL Fraser, Associate Professor, Investigations and Procedures Unit, Repatriation General Hospital, Daw Park 5041, Australia; Hitoshi Asakura, Director, Emeritus Professor, International Medical Information Center, Shinanomachi Renga Bldg.35, Shinanomachi, Shinjuku, Tokyo 160-0016, Japan

Anthoni S, Savilahti E, Rautelin H, Kolho KL. Milk protein IgG and IgA: The association with milk-induced gastrointestinal symptoms in adults. *World J Gastroenterol* 2009; 15(39): 4915-4918 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4915.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4915>

INTRODUCTION

More than 40% of adults in primary care suspect that milk ingestion is causative of their gastrointestinal symptoms^[1]. Furthermore, patients suffering from irritable bowel syndrome (IBS) often relate their symptoms to milk^[2]. To assess the impact of milk in

abdominal complaints is challenging. It is often difficult to distinguish the symptoms of milk hypersensitivity from other types of milk- or food-related gastrointestinal symptoms. Milk hypersensitivity in early childhood is mostly milk protein IgE-mediated^[3,4] causing immediate-type hypersensitivity reactions. The frequency of IgE-mediated cow's milk allergy decreases with increasing age and a high level of cow's milk-specific IgE is rare in adults^[5-8]. The impact of other types of immune reactions to cow's milk and, more specifically, the association of antibodies of IgG and IgA isotypes with cow's milk-induced adverse gastrointestinal symptoms in adults, is presently controversial^[9-13]. Ou-Yang *et al*^[14] have reported that an elimination diet based on the elevated level of food-specific IgG improved chronic diarrhea in children. Another recent study from China showed no association between symptom severity and food antigen-specific IgG in patients suffering either from IBS or functional dyspepsia (FD) although the levels of food-specific IgG titres were higher both in IBS and FD patients compared to healthy controls^[15].

The purpose of our study was to evaluate the association of serum level of milk protein IgG and IgA antibodies with gastrointestinal symptoms experienced by cow's milk ingestion in working age adults and to evaluate the milk IgA and IgG levels in relation to dairy consumption.

MATERIALS AND METHODS

We screened adults during spring 2004 in five different primary care centres for food-related symptoms, focusing on milk-related problems^[11,16]. Ethical approval was received from the Ethics Committee for outpatient clinics in Helsinki and surrounding areas (567/E1/03). Subjects who were referred to the laboratory for blood tests were invited to give a blood sample for the study purposes and to complete a questionnaire on gastrointestinal symptoms and dairy consumption as described recently^[1]. Of the 1900 adults who agreed to a blood sample within the three month study period, an exceptionally high proportion, 99%, returned the questionnaire. Randomly, serum samples from 400 of these subjects (198 women and 202 men) were selected for the measurement of milk protein IgG and IgA levels. Twelve samples were excluded due to an insufficient amount of sera. Thus, the study group comprised 388 adults (aged 18-64 years, mean age 40 years) of whom 119 informed us that they experienced gastrointestinal symptoms from consuming milk and 198 reported having no milk-related symptoms. The non-response rate was, in general, low per question. However, 71 (18%) did not answer the question on the presence of subjective milk-related symptoms, although they responded to questions on dairy consumption and gastrointestinal symptoms.

The reasons for laboratory testing were; gastrointestinal symptoms in 69/388 (18%), health check-up in 209/388 (54%), and follow up of an earlier diagnosed disease in 90/388 (23%). In 23/388 (6%) the indication for blood

test was not reported. All subjects had been genotyped for adult-type hypolactasia^[11] and screened for celiac disease^[16]. IgG antibodies to *Helicobacter pylori* (*H. pylori*) were determined with an in-house enzyme immunoassay as previously described^[17]. The lower limit for raised titres was 700 with a sensitivity of 99% and specificity of 93% as compared to histology^[17].

The milk protein IgG and IgA antibodies were measured by the ELISA technique using an adapted infant formula to coat the microtitre plates. Values are expressed as % of the standard with a very high titre of cow's milk antibodies^[18]. The major antigen in the formula was casein.

Statistical analyses

Kruskal-Wallis test, Spearman Rank Correlation, Mann-Whitney, Fisher's exact test, and ANOVA were used for analyzing the results. Significance was set at $P < 0.05$.

RESULTS

The levels of milk protein IgA and IgG antibodies declined as the age of the subjects increased, being lowest in the oldest age group, and the age-related decline was statistically significant with regard to milk protein IgG (ANOVA, $P < 0.004$; Table 1). Age and personally estimated milk-related gastrointestinal problems showed no correlation ($P = \text{NS}$, Spearman Rank). Men had higher milk protein IgA but not milk IgG levels in their sera than women (Mann-Whitney, $P = 0.04$; Table 1). Subjects drinking milk daily had higher levels of milk protein IgG in their sera than non-milk drinkers (Mann-Whitney, $P < 0.001$; Table 1). The daily consumption of milk was less frequent among subjects reporting gastrointestinal problems after drinking milk, but they had higher milk protein IgG levels than those who experienced no gastrointestinal symptoms (Table 1). Milk protein IgA levels did not show any association with milk drinking or abdominal symptoms (Table 1).

The association of high milk protein IgG levels with constipation was close to the level of statistical significance (Table 1). Diarrhea had no association with milk protein IgG level ($P = 0.5$). Regarding minor symptoms, flatulence and bloating ($P = 0.8$, Mann-Whitney), were not associated with milk protein IgG level. Subjects reporting dyspeptic symptoms had lower milk protein IgG levels than non-dyspeptics ($P < 0.05$). Furthermore, dyspepsia was not associated with milk drinking ($P = 0.5$, Fisher's exact test) or age ($P = 0.19$, Spearman Rank).

Milk protein IgG level was lower in subjects positive for antibodies to *H. pylori* ($n = 76/386$, $P < 0.05$ Mann-Whitney) although they drank milk more often than *H. pylori*-negative subjects ($n = 62/76$, $P < 0.006$ Mann-Whitney). However, the *H. pylori*-positive group was somewhat older (mean age 46 years) than the *H. pylori*-negative group (mean age 40 years, $P = 0.004$, ANOVA), which may explain the result. Accordingly, the presence of *H. pylori* antibodies in serum was associated

Table 1 Data of the study group, the experienced symptoms and correlation of milk protein IgG and IgA levels with different parameters

Subjects	<i>n</i> (% of the study group)	Mean IgG% (arbitrary units) 95% CI (lower-upper)	<i>P</i>	Mean IgA% (arbitrary units) 95% CI (lower-upper)	<i>P</i>
Study group (yr)	388	13.5 (11.5-15.5)		7.3 (5.5-9.2)	
18-34	139 (35)	16.6 (13.1-20.2)	< 0.004 ¹	9.4 (5.5-13.2)	0.16
35-49	123 (32)	14.7 (10.7-18.8)		7.4 (4.1-10.6)	
50-64	126 (33)	8.8 (6.5-11.1)		5.1 (3.1-6.9)	
Male	195 (50)	14.0 (11.2-16.7)	0.5	7.7 (5.1-10.3)	0.04 ²
Female	193 (50)	13.0 (10.2-15.9)		7.0 (4.4-9.6)	
Drinking milk daily	265 (68)	15.4 (12.8-18.0)	< 0.001 ³	7.8 (5.6-10.0)	
Not drinking milk	123 (32)	9.3 (6.5-12.1)		6.4 (3.2-9.7)	
Subjective symptoms from milk	119 (31)	10.5 (7.6-13.5)	0.02 ⁴	6.4 (2.5-10.3)	0.5
No subjective symptoms from milk	198 (51)	16.0 (12.8-19.1)		8.0 (5.7-10.4)	
Constipation	47 (12)	19.2 (11.7-26.8)	0.05	11.3 (3.7-18.8)	0.06
No constipation	282 (73)	13.3 (11.0-15.6)		7.5 (5.4-9.7)	
Diarrhea	133 (34)	14.4 (11.0-17.8)	< 0.05 ⁵	8.0 (4.4-11.6)	0.7
No diarrhea	196 (51)	14.0 (11.0-17.0)		8.1 (5.5-10.8)	
Dyspepsia	127 (33)	11.9 (8.5-15.3)		4.8 (3.00-6.68)	
No dyspepsia	202 (52)	15.6 (12.6-18.5)		10.1 (6.8-13.3)	

¹The level of IgG decreased according to the age statistically significantly. ²The IgA level was statistically significantly higher in men than in women. ³The milk drinking was associated with IgG level statistically significantly. ⁴The IgG level was statistically significantly higher in subjects with symptoms from milk. ⁵The level of IgG was statistically significantly lower in subjects with dyspepsia.

in a statistically significantly manner with a lower level of milk protein IgA antibodies ($P = 0.03$, Mann-Whitney).

There was no correlation between milk protein IgG or IgA antibodies and C/T-13910 genotype associated with adult type hypolactasia. Unexpectedly, none of these randomly picked subjects was screen-positive for celiac disease^[16]. There was no association of milk-specific IgG or IgA with a reported history of a diagnosed gastrointestinal disorder [irritable bowel syndrome $n = 12/388$ (3.0%) or inflammatory bowel disease $n = 4/388$ (1.0%)], since none of these patients had high levels of cow's milk-specific IgG or IgA. Irritable bowel syndrome was reported less in the study group than in an average western population (5%-10%) and inflammatory bowel disease more often than in an average western population (0.1%)^[19,20].

DISCUSSION

Milk protein IgG but not milk IgA seems to be associated with self-reported milk-induced gastrointestinal symptoms. The nature of these symptoms, however, is unclear. There was a clear association of milk protein IgG with milk drinking in our study, supporting the view that the presence of milk protein IgG-specific antibodies may, to a certain level, be a normal physiologic reaction to ingested milk protein. Regarding the gastrointestinal symptoms, dyspepsia but not diarrhea or constipation showed a statistically significant association with milk protein IgG level. However, the association with milk protein IgG and dyspepsia was confounding as it was negative and not attributed to milk drinking or age.

The serum samples and questionnaires of the 388 patients included in this study were randomly picked from a group of 1900 volunteers attending a larger study of milk-induced gastrointestinal symptoms^[1]. The blood

samples were initially obtained during a short period of three months. Thus, seasonal variation had minimal effect on the results. An exceptionally high proportion, 99% of the 1900 participants, returned the questionnaire on abdominal symptoms and dairy consumption. The questionnaires were well completed and no samples needed to be excluded because of missing questionnaire data. However, as the number of samples included was limited there may be bias in the results. The information about milk-related gastrointestinal problems was derived from the questionnaires, not from milk challenge and subsequent observation of the participants. However, questionnaires were well-formulated and the participants were all working age people capable of understanding the questions. Furthermore, the most common causes for milk-related symptoms such as adult-type hypolactasia or celiac disease had been screened out by blood tests in the study group^[1,16].

There are only a few studies which have been conducted in adults regarding milk protein antibodies, especially involving IgG and IgA. Although there are a number of studies in children, extrapolation of results from pediatric populations requires caution. There are some recent pediatric studies which imply that determination of milk-specific IgG4 might have a role in this field^[13,21,22]. In this study we did not have the opportunity to measure milk-specific IgG4. However, the results of the earlier studies conducted in adults provided controversial results^[9-13].

In our previous study, we showed that IgE antibodies to milk did not correlate with the self-reported, milk-related symptoms^[5]. Consistent with this, Peltó *et al*^[11] have shown that hypersensitivity to cow's milk does also occur in adults but the mechanism is most likely not milk-specific antibody-mediated but rather due to an increase in serum reactivity to milk protein. Moreover, the positive association between milk protein IgG level

and reported gastrointestinal symptoms from milk are in accordance with earlier findings showing that bowel irritation had a correlation with high mucosal IgG levels to food antigens^[10,18].

The importance of milk protein IgG and IgA antibodies in the etiology of gastrointestinal symptoms following cow's milk ingestion remains uncertain. At present however, it would appear that measurement of these antibodies in routine clinical practice is of limited value and cannot be recommended.

ACKNOWLEDGMENTS

The skilful technical assistance of Ms Sirkku Kristiansen is gratefully acknowledged. Also, the patients who voluntarily took part in the study are thanked for their support.

COMMENTS

Background

Cow's milk-related gastrointestinal symptoms are often complained about by primary care patients. In adult populations "lactose intolerance" or, more precisely, adult-type hypolactasia, is the most common cause of cow's milk induced gastrointestinal symptoms. Celiac disease, the most common cause of secondary hypolactasia, is encountered in 1%-2% of western populations and thus should be considered when milk-related symptoms are being diagnosed. Allergy to cow's milk protein is relatively rare in adults and the mechanisms by which it is mediated are not yet known. Milk protein-specific immunoglobulin (Ig)E, the most common mediator of milk hypersensitivity in children, is hardly ever the mediator in adults. There are a number of studies regarding milk protein hypersensitivity in children, but only a few conducted among adults and the results achieved from children cannot be extrapolated to adults without caution. This study was conducted in order to evaluate the roles of milk protein specific IgG and IgA in cow's milk hypersensitivity in adults.

Applications

Milk protein IgG is associated with self-reported milk-related gastrointestinal symptoms, whereas milk protein IgA has no such association. Milk protein IgG antibody levels also correlate with drinking milk. These findings imply that milk protein-specific IgG has a role in the humoral reaction to ingested milk. However, the measurement of milk protein IgG provides no accurate information on milk hypersensitivity.

Peer review

This is an interesting work that is concerned with a controversial area of gastroenterology.

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S- Editor Tian L L- Editor Logan S E- Editor Yin DH

Effects of fasting and preoperative feeding in children

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Received: August 5, 2009 Revised: August 21, 2009

Accepted: August 28, 2009

Published online: October 21, 2009

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Key words: Cortisol; Diet; Fasting; Food; Glucose; Liquids; Prealbumin

Peer reviewer: Luigi Bonavina, Professor, Department of Surgery, Policlinico San Donato, University of Milano, via Morandi 30, Milano 20097, Italy

Yurtcu M, Gunel E, Sahin TK, Sivrikaya A. Effects of fasting and preoperative feeding in children. *World J Gastroenterol* 2009; 15(39): 4919-4922 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4919.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4919>

Abstract

AIM: To investigate whether children should undergo surgery without a long period of fasting after feeding.

METHODS: Eighty children with inguinoscrotal disorders (aged 1-10 years) were studied prospectively. They were divided into eight groups that each contained 10 children who were fed normal liquid food (NLF) and a high-calorie diet (HCD) 2, 3, 4 and 5 h before surgery, in two doses at 6-h intervals. NLF was given to four groups and HCD to the other four. In all groups, glucose, prealbumin and cortisol levels in the blood were measured twice: just after oral feeding and just before the operation. After the establishment of adequate anesthesia, gastric residue liquid was measured with a syringe.

RESULTS: Blood glucose levels in all patients fed NLF and HCD were high, except in patients in the HCD-4 group. There was no significant difference in the blood prealbumin levels. There was a significant increase in the blood cortisol levels in the NLF-2 (14.4 ± 5.7), HCD-2 (13.2 ± 6.0), NLF-3 (10.9 ± 6.4), and HCD-5 (6.8 ± 5.7) groups ($P < 0.05$).

CONCLUSION: The stress of surgery may be tolerated by children when they are fed up to 2 h before elective surgery.

INTRODUCTION

For many years, overnight fasting has been recommended before elective surgery. This fasting period is applied to reduce the risk of aspiration of stomach contents during anesthesia^[1]. The routine use of perioperative oral dietary supplements in patients about to undergo gastrointestinal surgery confers no clinical or functional benefit^[2].

However, this routine is now being questioned, because fasting causes discomfort and unnecessary problems with routine oral medication^[3,4]. The free intake of water is allowed up to 3 h before surgery in children^[5,6] and adults^[7]. Thirst and anxiety are reduced in comparison with overnight fasting. Feeding with clear fluids does not increase gastric contents^[7,8].

Although they do not diminish the risk of aspiration, clear liquids given enterally 2 h before surgery appear to pose no additional risk for aspiration of gastric contents in normal healthy children, and may provide some psychological benefit, as demonstrated by a decrease in irritability before induction of anesthesia^[9]. It has been reported that a high-calorie diet (HCD) given enterally 2 h before surgery makes the surgery more comfortable^[10,11].

Furthermore, Powell-Tuck *et al*^[12] and Beier-Holgersen *et al*^[13] have reported that early enteral feeding decreases postoperative complications, regulates wound improvement rapidly, decreases the cost of hospitalization, and increases quality of life and postoperative surgical success.

Recent studies have shown that perioperative insulin and glucose infusion maintains normal insulin sensitivity after surgery^[14]. Prealbumin (transthyretin) level is used often as an indicator of protein status because of its relatively short half-life, high tryptophan content, high

proportion of essential to nonessential amino acids, and small pool size. A sensitive acute phase reactant such as C-reactive protein always should be assayed along with prealbumin if levels are to be used to estimate nutritional status^[15].

Although some studies have investigated the duration of fasting, to the best of our knowledge, there has been no research published about the metabolic changes in children fed normal liquid food (NLF) and an HCD, depending on the duration of fasting before an operation.

The aim of our study, therefore, was to compare NLF and HCD depending on the duration of fasting before surgery, and to decide whether children should undergo surgery without a long period of fasting after feeding them with an HCD.

MATERIALS AND METHODS

Ethics

This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study was approved ethically by Selcuk University Meram Medical School (2005/058). All families provided informed written consent for their children.

Patients

The study population consisted of 80 patients (58 male; mean \pm SD, 6.18 ± 1.25 years; range, 1-10 years). Blood samples were obtained from the patients by the same surgeon. The study was conducted as a randomized, single-blind clinical trial. The children included in the study were outpatients and they had inguinoscrotal disorders without any additional abnormality. Sixty-two (77.5%) patients who had inguinal hernias underwent high ligation (Ferguson procedure) and 18 (22.5%) who had undescended testes underwent orchidopexy. All patients were admitted on the day of surgery or underwent surgery as outpatients. Tracheal intubation was planned in all cases. General anesthesia was used. Patients taking medication or who had a disease known to delay gastric emptying or increase acid production were excluded.

Clinical design

The patients were divided into eight groups, each containing 10 patients who were fed NLF and an HCD 2, 3, 4 and 5 h before surgery. NLF was given to four groups and HCD to the other four (Figure 1). The food and liquid requirements of the children, which consisted of 10 mL/kg, were given orally in two doses at 6-h intervals, after calculating their carbohydrate, protein, lipid, and electrolyte needs according to body weight. Then, they were fasted for 2, 3, 4 and 5 h preoperatively. After the establishment of an adequate and stable level of anesthesia, a 14 or 16 Fr Levin multi-orificed gastric tube was passed orally into the stomach by the same investigator, who was unaware of the patient's fasting status, to determine whether there was any residue in the stomach before surgery. After confirmation of the gastric tube's position by auscultation, gastric fluid contents were

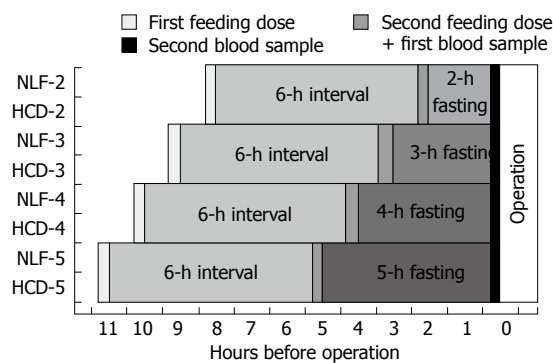


Figure 1 Groups, feeding procedures, and durations in the study design.

NLF: Normal liquid food; HCD: High-calorie diet; NLF-2: Fed with NLF 2 h before surgery; HCD-2: Fed with an HCD 2 h before surgery; NLF-3: Fed with NLF 3 h before surgery; HCD-3: Fed with an HCD 3 h before surgery; NLF-4: Fed with NLF 4 h before surgery; HCD-4: Fed with an HCD 4 h before surgery; NLF-5: Fed with NLF 5 h before surgery; HCD-5: Fed with an HCD 5 h before surgery.

obtained through the tube by gentle aspiration with a 60-mL syringe in several positions, with the patient tilted head-up, head-down, to the right, and to the left. Gastric residue liquid was measured using this syringe.

Biochemical analysis

Blood samples were obtained twice from the patients in all groups (Figure 1), when oral feeding was stopped and just before the operation (before the induction of anesthesia), to measure the values of blood glucose, prealbumin and cortisol. After all the blood samples were taken, blood glucose levels were determined in a Beckman Coulter Unicel DXC 800 Synchron Clinical System autoanalyzer (Beckman Coulter, Inc., Fullerton, CA, USA) using a Beckman Coulter test kit (catalog no: T709005). Prealbumin levels were determined using a Beckman Coulter test kit (catalog no: M605268) in a Beckman Array Protein System autoanalyzer. Cortisol levels were determined using a Beckman Coulter test kit (catalog no: 230) in Unicel DXI-800 Access Immunoassay System autoanalyzer (Beckman Coulter Inc. Immunodiagnostic Development Center, Chaska, USA).

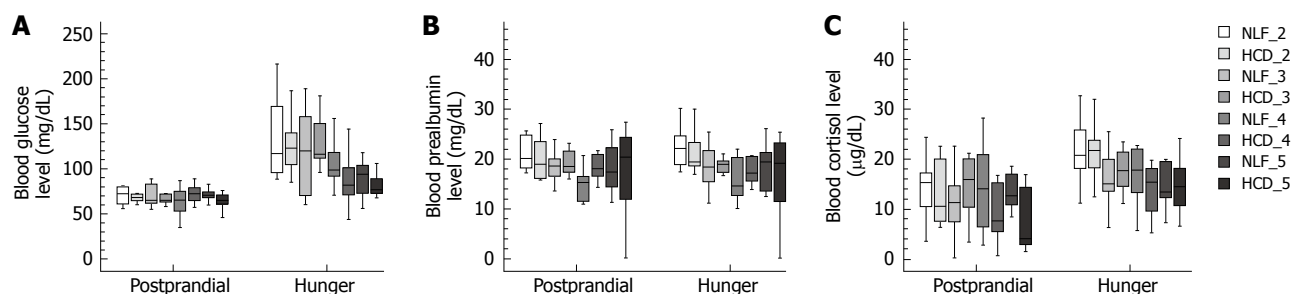
Statistical analysis

Power was calculated as 100% with a sample size of 10 patients in each group. All data were presented as mean \pm SD for biochemical analysis. Two-way repeated measures of ANOVA were used to compare postprandial (PP) and starvation values for each parameter in the eight groups. Post-hoc tests were used to identify the origin of the difference when a significant difference was identified. For this purpose, Bonferroni-corrected one-way ANOVA and Tukey-HSD test were used to identify whether there was a difference among the groups during the PP and fasting periods. Moreover, Student's paired *t* test was used to compare the PP and starvation values in each dependent group. $P < 0.05$ was considered statistically significant. However, $P < 0.025$ was considered statistically significant when Bonferroni correction was made. For all calculations, SPSS version 13.0 was used, and for calculation of power PASS version 08.07 was used.

Table 1 Blood glucose, prealbumin and cortisol changes investigated in children fed NLF and an HCD

Groups	Blood glucose (mg/dL)			Prealbumin (mg/dL)		Cortisol (μ g/dL)		
	PP (mean \pm SD)	Fasting (mean \pm SD)	<i>P</i> value (PP vs Fasting)	PP (mean \pm SD)	Fasting (mean \pm SD)	PP (mean \pm SD)	Fasting (mean \pm SD)	<i>P</i> value (PP vs Fasting)
NLF-2	70.8 \pm 9.6	133.2 \pm 44.5 ^a	0.003	21.2 \pm 3.4	27.1 \pm 16.4	14.4 \pm 5.7	22.6 \pm 7.8	0.014
HCD-2	69.0 \pm 11.8	126.7 \pm 29.5	0.000	19.8 \pm 3.8	21.4 \pm 4.8	13.2 \pm 6.0	21.9 \pm 5.9	0.008
NLF-3	70.7 \pm 12.2	119.7 \pm 48.0	0.005	18.5 \pm 2.8	18.4 \pm 4.3	10.9 \pm 6.4	18.1 \pm 10.2	0.022
HCD-3	64.5 \pm 9.6	126.2 \pm 25.9	0.000	19.2 \pm 2.3	18.7 \pm 1.4	14.9 \pm 5.9	17.5 \pm 4.1	0.219 ^{NS}
NLF-4	64.4 \pm 16.0	105.5 \pm 24.2	0.002	14.9 \pm 3.3	15.2 \pm 3.9	14.4 \pm 9.0	18.4 \pm 7.9	0.316 ^{NS}
HCD-4	71.7 \pm 9.7	94.9 \pm 41.4	0.120 ^{NS}	18.2 \pm 2.4	17.4 \pm 2.7	9.3 \pm 5.7	13.9 \pm 5.0	0.081 ^{NS}
NLF-5	70.1 \pm 11.0	89.7 \pm 19.7	0.019	18.3 \pm 4.9	18.7 \pm 4.9	12.8 \pm 5.2	14.4 \pm 4.2	0.531 ^{NS}
HCD-5	64.1 \pm 8.8	83.4 \pm 16.7	0.034	17.3 \pm 10.0	16.2 \pm 9.3	6.8 \pm 5.7	15.0 \pm 5.6	0.001
<i>P</i> value (groups)	0.570 ^{NS}	0.004		0.187 ^{NS}	0.158 ^{NS}	0.064 ^{NS}	0.027 ^{NS}	

^a*P* < 0.004, NLF-2 vs HCD-5 group fasting values according to Tukey-HSD test; No significant difference according to PP vs fasting, *P* = 0.162. PP denotes values of blood glucose, prealbumin, and cortisol 2, 3, 4 and 5 h before surgery (when oral feeding was stopped). Fasting denotes values of blood glucose, prealbumin, and cortisol before just surgery (before the induction of anesthesia). NS: not significant; NLF: Normal liquid food; HCD: High-calorie diet; PP: Postprandial.

Figure 2 Boxplot graph of blood glucose (A), prealbumin (B), cortisol (C) levels in the eight study groups (*n* = 10 each) during PP and fasting periods.

RESULTS

PP blood glucose levels within and between groups were significantly lower compared to the fasting blood glucose levels, except for HCD-4. There was no significant difference regarding PP blood glucose (*P* = 0.570), but there was a significant increase in the fasting values of blood glucose in the NLF-2 group when compared with the HCD-5 group (*P* = 0.004) (Table 1 and Figure 2A).

There was no significant difference between or within the groups (*P* = 0.162) regarding blood prealbumin (Table 1 and Figure 2B).

PP blood cortisol levels in the NLF-2, HCD-2, NLF-3, NLF-5 and HCD-5 groups were significantly lower compared to fasting blood glucose levels (Table 1 and Figure 2C). The stomach residue liquids of all patients were at tolerable levels (1-2 mL). All anesthesia induction was uneventful, and no patient suffered from coughing, laryngospasm, or vomiting. There was no problem regarding the outcomes of surgery or wound healing.

DISCUSSION

Preoperative fasting has been applied before elective surgery to prevent the risk of aspiration of stomach contents during anesthesia^[1], but no study has examined whether there were negative effects of an HCD on the effects of duration of fasting and preoperative feeding on metabolic changes and anesthesia in children^[3,4].

The ingestion of unlimited clear fluids by healthy 2-12-year-old children up to 2 h before elective surgery does not affect gastric contents^[6]. In addition, there is no correlation between the fasting interval and gastric fluid pH or volume, and 2 mL/kg of water may be administered to healthy, non-premedicated children 2 h before surgery, without decreasing gastric fluid pH or increasing gastric fluid volume compared to those fasting for up to 6 h^[5]. Drinking clear liquids up to 2 h before anesthesia induction is unlikely to affect substantially the volume of gastric fluid contents or the percentage of patients with gastric fluid pH \leq 2.5. Clear liquids appear to add no additional risk for aspiration of gastric contents in normal healthy children, and may provide some psychological benefit, as demonstrated by a decrease in irritability before induction of anesthesia^[9]. Partial feeding in the immediate (up to 2 h before premedication) preoperative period may become routine in the future^[11].

The values of blood glucose and insulin are known to be increased significantly 40 min after ingestion of a carbohydrate-rich drink^[11]. Perioperative glucose and insulin infusions minimize the endocrine stress response and normalize postoperative insulin sensitivity and substrate utilization^[14]. To achieve standardization in our study, the food and liquid requirements of the patients were given in two doses at 6-h intervals, and blood samples were obtained from all groups twice before surgery. It is novel to evaluate metabolic changes in children fed NLF and an HCD, depending on the

duration of fasting before surgery. In the present study, blood glucose levels increased and stomach residue liquids were at a tolerable level in all patients fed NLF and an HCD. These results indicate that children can tolerate the stress of surgery when they are fed until 2 h before surgery, because there was no difference regarding stomach residue and metabolic changes among patients that underwent surgery after fasting for short and long periods.

In conclusion, there is no need for more than 2 h of fasting before inguinoscrotal region surgery. Further studies in surgical patients should help to substantiate the safety and clinical benefits of this new concept.

COMMENTS

Background

Preoperative fasting has been applied before elective surgery to prevent the risk of aspiration of stomach contents during anesthesia, but no study has examined whether there are negative effects of a high calorie diet (HCD) on the effects of duration of fasting and preoperative feeding on metabolic changes and anesthesia in children.

Research frontiers

Children can tolerate the stress of surgery when they are fed with normal liquid food (NLF) and HCD until 2 h before surgery, because there was no difference regarding stomach residue and metabolic changes among patients that underwent surgery after fasting for short and long periods.

Innovations and breakthroughs

For many years, overnight fasting has been recommended before elective surgery. This fasting period is applied to reduce the risk of aspiration of stomach contents during anesthesia. The routine use of perioperative oral dietary supplements in patients about to undergo gastrointestinal surgery confers no clinical or functional benefit. However, this routine is now being questioned, because fasting causes discomfort and unnecessary problems with routine oral medication. It is novel to evaluate metabolic changes in children fed NLF and an HCD, depending on the duration of fasting before surgery. In the present study, blood glucose levels increased and stomach residue liquids were at a tolerable level in all patients fed NLF and an HCD. These results indicated that children can tolerate the stress of surgery when they are fed until 2 h before surgery, because there was no difference regarding stomach residue and metabolic changes among patients that underwent surgery after fasting for short and long periods.

Applications

The study results suggest that there is no need for more than 2 h of fasting before inguinoscrotal region surgery in preventing the risk of aspiration of stomach contents during anesthesia.

Terminology

Glucose infusions minimize the endocrine stress response. Prealbumin (transthyretin) level is used as an indicator of protein status because of its relatively short half-life, high tryptophan content, high proportion of essential to nonessential amino acids, and small pool size. Cortisol preserves the completeness of the cell membrane, inhibits increased capillary permeability, and has an anti-inflammatory effect.

Peer review

This is an interesting paper, well-written and well-documented. The analysis of data has been done very carefully and the results are convincing.

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S- Editor Tian L L- Editor Kerr C E- Editor Lin YP

APRI as a predictor of early viral response in chronic hepatitis C patients

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Received: August 18, 2009 Revised: September 1, 2009

Accepted: September 8, 2009

Published online: October 21, 2009

Abstract

AIM: To evaluate the aspartate aminotransferase (AST) to platelet ratio index (APRI) as a predictive factor of early viral response in chronic hepatitis C naive patients.

METHODS: We performed an ambispective case-control study. We enrolled chronic hepatitis C naive patients who were evaluated to start therapy with PEGylated interferon α -2b (1.5 μ g/kg per week) and ribavirin (> 75 kg: 1200 mg and < 75 kg: 1000 mg). Patients were allocated into two groups, group 1: Hepatitis C patients with early viral response (EVR), group 2: Patients without EVR. Odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the relationship between each risk factor and the EVR in both groups.

RESULTS: During the study, 80 patients were analyzed, 45 retrospectively and 35 prospectively. The mean \pm SD age of our subjects was 42.9 ± 12

years; weight 70 kg (± 11.19), AST 64.6 IU/mL (± 48.74), alanine aminotransferase (ALT) 76.3 IU/mL (± 63.08) and platelets 209 000 mill/mm³ ($\pm 84 429$). Fifty-five (68.8%) were genotype 1 and 25 (31.3%) were genotype 2 or 3; the mean hepatitis C virus RNA viral load was 2269 061 IU/mL ($\pm 7220 266$). In the univariate analysis, APRI was not associated with EVR [OR 0.61 (95% CI 0.229-1.655, $P = 0.33$)], and the absence of EVR was only associated with genotype 1 [OR 0.28 (95% CI 0.08-0.94, $P = 0.034$)]. After adjustment in a logistic regression model, genotype 1 remains significant.

CONCLUSION: APRI was not a predictor of EVR in chronic hepatitis C; Genotype 1 was the only predictive factor associated with the absence of EVR in our patients.

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Key words: Hepatitis C virus viral load; Viral genotype; Hepatitis C; Aspartate aminotransferase to platelet ratio index; Early viral response

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INTRODUCTION

According with the World Health Organization, 123 million individuals are infected with hepatitis C virus (HCV) worldwide, representing a prevalence of 2%^[1]. HCV can result in progressive hepatic injury and fibrosis, culminating in cirrhosis and end-stage liver disease. Chronic hepatitis C is a major indication for liver transplantation and increases the incidence of hepatocellular carcinoma^[2]. PEG-interferon and ribavirin

therapy during 24 to 48 wk leads to a sustained viral response (SVR, a sustained loss of serum HCV RNA)^[3].

There are factors identified as predictors of SVR among patients who received PEG-interferon and ribavirin, including HCV genotype other than 1, viral load less than 600 000 IU/mL, age of 40 years or less, and body weight of 75 kg or less. Sixty five percent of patients with early viral response (EVR) subsequently have an SVR^[4,5]. In addition, the absence of bridging fibrosis/cirrhosis has been significantly associated with SVR^[6,7]. Infection with genotype 2 or 3, low viral load, and absence of advanced hepatic fibrosis have consistently been identified as independent predictors of SVR^[8].

Percutaneous liver biopsy has been the gold standard for grading and staging liver disease; recently however, non-invasive methods have been developed to determine hepatic fibrosis, such as transient elastography, fibrotest, and the aspartate aminotransferase (AST) to platelet ratio index (APRI)^[9-11]. APRI is one of several markers that have been proposed to measure liver fibrosis. It is an inexpensive widely available tool^[11] that can predict accurately mild fibrosis in patients with a value < 0.42 and those with a value > 1.2 are diagnosed a significant grade of fibrosis. APRI establishes a 90% negative predictive value for the absence of fibrosis and a 91% of positive predictive value for its presence^[12-14]. Previous studies have not explored the usefulness of noninvasive tests to assess liver fibrosis for the prediction of viral response in hepatitis C naive patients. The purpose of this study was to evaluate APRI as predictive factor of EVR in hepatitis C chronic naive patients.

MATERIALS AND METHODS

Study design

We performed an ambispective case-control study with enrollment of APRI as a predictor of EVR in chronic hepatitis C naive patients prospectively from July 2006 to February 2008. We also reviewed retrospectively medical records of chronic hepatitis C naive patients from January 2004 to June 2006 who were evaluated to start therapy with PEG-interferon α -2b (1.5 μ g/kg per week) and ribavirin (> 75 kg: 1200 mg and < 75 kg: 1000 mg) at Hospital de Infectología "La Raza" National Medical Center at Mexico City. After the subjects were treated for 12 wk, they were allocated into two groups: patients with EVR and patients without EVR.

Study patients

Patients were eligible for the study if they were between the ages of 18 and 75 years old, had chronic HCV infection, had no history of treatment of HCV infection and had HCV RNA positive serum (at least > 50 IU/mL) according to a Real Time RT-PCR (Cobas Amplicor HCV v2.0, Roche Molecular Systems), serum aminotransferase levels and platelets measures. Patients were excluded if they had poorly controlled psychiatric

Table 1 Patient's baseline characteristics (*n* = 80)

Variable	mean \pm SD	Range
Age (yr)	42.9 \pm 12	19-74
Weight (kg)	70 \pm 11.19	47-100
Hemoglobin (g/dL)	14.8 \pm 1.92	8.1-19
Platelets (No/ μ L)	209 000 \pm 84 429	57 300-444 000
Leucocytes (cells/ μ L)	5.8 \pm 1.87	2.9-13.2
Glucose (mg/dL)	101 \pm 18.94	73-172
Creatinine (mg/dL)	0.96 \pm 0.26	0.6-1.2
Albumin (g/dL)	4.4 \pm 0.51	2.9-5.5
AST (IU/dL)	64.6 \pm 48.74	17-253
ALT (IU/dL)	76.3 \pm 63.08	14-249
LDH (IU/dL)	272.6 \pm 90.96	116-704
AP (IU/dL)	106.4 \pm 40.37	51-240
Baseline viral load (IU/mL)	2269 061 \pm 7 220 266	1200-56 000 000
APRI	1.61 \pm 1.90	0.17-9.0

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; AP: Alkaline phosphatase; APRI: AST to platelet ratio index.

disease, a solid organ transplant, an autoimmune condition, thyroid disease, a hemoglobin level of 12 g/dL or lower, a neutrophils count of 1500 per cubic millimeter or lower, a platelet count of 75 000 per cubic millimeter or lower, drug abuse within the previous 12 mo, or alcohol abuse within the previous 6 mo, and an HBV or HIV coinfection.

The study was conducted in conformance with the principles of the Declaration of Helsinki. The institutional review board of the hospital approved the protocol and consent forms. All participants in the prospective way provided written informed consent.

Clinical assessments

Medical history was recorded for all patients (general attributes, medical problems, and medications), physical exam (height and weight), and laboratory results (blood cell count, liver function test, HCV RNA, and viral genotype).

We calculated the APRI using the following formula: $APRI = \frac{AST \text{ levels (} \times \text{ULN)}}{\text{platelets count (} 10^3/\text{L)}} \times 100$, where ULN means the upper limit of normal.

Fibrosis was established if the APRI was ≥ 1.2 . The primary efficacy end point was EVR, defined as ≥ 2 log reduction of the HCV RNA level compared to the baseline HCV RNA level (partial EVR) or HCV RNA negative at treatment week 12 (complete EVR). To evaluate APRI as a predictor of EVR, we stratified between genotype 1 and genotype other than 1 (genotypes 2 or 3).

Statistical analysis

Statistical analysis was performed with SPSS for Windows (version 12.0; SPSS Inc., Chicago, Ill). Categorical variables were compared using the Pearson's χ^2 or Fisher's exact test. Odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the relationship between each risk factor. To adjust for the effects of potential confounders, we used logistic regression models. $P \leq 0.05$ were considered significant.

Table 2 Clinical characteristics in EVR and non-EVR patients (*n* = 80)

Variable	EVR patients (<i>n</i> = 54)	Non-EVR patients (<i>n</i> = 26)	<i>P</i> value
Age (yr)	43.61 ± 11.92	41.65 ± 12.90	0.505
Weight (kg)	71.09 ± 11.77	68.69 ± 9.88	0.372
WBC (cells/mm ³)	5,824 ± 1.901	5,040 ± 1.850	0.820
Hemoglobin (g/dL)	14.86 ± 2.05	14.77 ± 1.74	0.855
Platelets (No/μL)	212,162 ± 79,956	207,815 ± 94,603	0.781
Glucose (mg/dL)	101.16 ± 18.94	99.96 ± 17.13	0.697
Creatinine (mg/dL)	0.97 ± 0.31	0.93 ± 0.11	0.552
AST (IU/mL)	62.07 ± 46.52	69.84 ± 53.64	0.508
ALT (IU/mL)	72.81 ± 62.15	83.61 ± 65.62	0.477
LDH (IU/mL)	276.96 ± 99.77	263.65 ± 70.12	0.543
HCV RNA viral load (copies/mL)	2243191 ± 7664170	2322791 ± 6263297	0.964
APRI	1.47 ± 1.63	1.90 ± 2.37	0.353

EVR: Early viral response; HCV: Hepatitis C virus.

Table 3 Factors associated with EVR (Univariate analysis)

Variable	OR	95% CI	<i>P</i> value
Sex	1.079	0.797-1.491	0.624
Age > 40 years old	1.570	0.609-4.090	0.346
Weight > 75 kg	1.299	0.491-3.437	0.598
Platelets < 150 000/μL	0.776	0.264-2.280	0.644
Albumin < 3.5g/dL	1.471	0.144-14.863	1.0
ALT > 28 IU/dL	1.320	0.421-4.135	0.633
Viral load > 600 000 IU/mL	0.944	0.352-2.533	0.910
Genotype 1 <i>vs</i> genotype other than 1	0.286	0.086-0.834	0.034 ^a
APRI > 1.2	0.615	0.229-1.655	0.334

Chi square. ^a*P* < 0.05.

RESULTS

Patients

During the study, 84 patients were assigned to treatment; 80 patients completed the first 12 wk of therapy, three patients withdrew because of adverse events related to PEG-interferon, and one patient was lost during this period. Forty-five (56%) were evaluated retrospectively and 35 (34%) prospectively. Forty-one (51%) were female and 39 (49%) were male. The mean (± SD) age of our subjects was 42.9 ± 12 years. Fifty-five (68.8%) had genotype 1 and 25 (31.3%) had genotype 2 or 3 (Tables 1 and 2).

Virologic response and APRI findings

Analysis revealed EVR in 54 (67.5%) patients; 40 of them reached a complete EVR and 14 showed a partial EVR.

The APRI was calculated in all patients; 25 (31%) patients were ≥ 1.2; the mean ± SD APRI was 1.61 ± 0.090.

Independent factors associated with EVR

To identify predictors of EVR, in an univariate analysis, we observed the following: sex (OR 1.079, 95% CI 0.797-1.491, *P* = 0.624), age > 40 years (OR 1.57, 95% CI 0.609-4.090, *P* = 0.346), body weight > 75 kg (OR 1.29, 95% CI 0.491-3.437, *P* = 0.598), viral load > 600 000 IU/mL (OR 0.944, 95% CI 0.352-2.533, *P* = 0.910), genotype 1 *vs* genotype other than 1 (genotype 2 or 3) (OR 0.286, 95% CI 0.086-0.834, *P* = 0.034) and

Table 4 Factors associated with EVR (multivariate analysis)

Variable	EVR	95% CI	<i>P</i> value
Age > 40 years old	1.420	0.489-4.166	0.515
Weight > 75 kg	1.214	0.435-3.384	0.711
ALT > 28 IU/dL	1.645	0.469-5.773	0.437
Viral load > 600 000 IU/mL	0.946	0.328-2.729	0.919
Genotype 1 <i>vs</i> genotype other than 1	0.304	0.087-0.946	0.041
APRI > 1.2	0.469	0.151-1.457	0.190

Logistic regression model.

APRI > 1.2 (OR 0.615, 95% CI 0.229-1.655, *P* = 0.334), (Table 3). Final multiple logistic-regression model, including the following factors, was entered in the final stepwise regression analysis: sex, age (< 40 years *vs* > 40 years), body weight (< 75 kg *vs* > 75 kg), pretreatment viral load (< 600 000 copies/mL *vs* > 600 000 IU/mL), pretreatment alanine aminotransferase quotient (> 3 *vs* < 3), pretreatment APRI score (< 1.2 *vs* > 1.2), and HCV genotype (1 *vs* non-1). Only one factor increased the odds of achieving an EVR independently and significantly: HCV genotype other than 1 (OR 0.304, 95% CI 0.087-0.946; *P* = 0.041, Table 4).

Among patients with genotype 1, APRI score (< 1.2 *vs* > 1.2) was evaluated and showed an OR of 0.65 (95% CI 0.20-2.09, *P* = 0.475). For patients with genotype other than 1 we found an OR of 0.40 (95% CI 0.045-3.57, *P* = 0.575).

DISCUSSION

Our results suggested that the APRI is not a predictor of EVR. We found no association when APRI was more than 1.2. In addition, when we stratified between genotype 1 and genotype other than 1 to evaluate APRI as a predictor of EVR, we found no association. Nevertheless, we found one predictor of absence of EVR (genotype 1 *vs* genotype other than 1) in patients who were treated with PEG-interferon α-2b and ribavirin.

A reasonable interpretation of these results is that, despite the possibility that biopsy could predict treatment response in HCV infected patients; an indirect measure of fibrosis with APRI is not an option to pre-

dict viral response.

We also found that in this Mexican population sex, age, weight, HCV viral load, and aminotransferase serum levels were not associated with viral response. One possible explanation for our results is that we adjusted ribavirin and PEG-interferon α -2b doses according to patient's weight.

Our findings are comparable with previous reports that evaluate predictors of sustained viral response.

Though our results might seem to differ with those of Fried *et al*^[4], who found age and weight as predictors of viral response, most of the patients in their study were Caucasians, and race has been shown to be a factor in the response to therapy for hepatitis C virus infection^[4,15].

In their study, Akuta and colleagues concluded that hepatocytes steatosis is a factor associated with virological non-response, however they measured liver steatosis by obtaining liver biopsy percutaneously, and did not use a noninvasive assessment of liver fibrosis^[16].

Our results are consistent with those of Nagaki *et al*^[17], who found no association among age, weight, and HCV viral load with sustained viral response in patients with chronic hepatitis C genotype 1b. Finally, similar to all prospective treatment studies, we found genotype as the strongest predictor of response^[5,18-20].

This was an ambispective study, and it has several limitations, more than half of patients were analyzed in a retrospectively way. In addition, rapid virological response was not measured in this group of patients, which now is a very important predictor of sustained viral response. In addition, these results could have been affected by the small sample size.

Another limitation in our study is that some of the patients are still undergoing treatment, for this reason, we still do not have the results for sustained viral response, which is the goal of treatment in patients with HCV infection. Thus, although EVR is a predictor of SVR in approximately 60%-80%, SVR is necessary to gain full results.

Previous studies have not explored the utility of non-invasive tests to assess liver fibrosis for the prediction of viral response in hepatitis C naive patients.

Despite the limitation of our study, we believe our results show that APRI is not a predictor of early viral response in HCV naive patients.

It is necessary to perform a study including sustained viral response as the final goal of treatment. In addition, other indirect liver damage measures (fibrotest or transient elastography) should be used to improve recognition, diagnosis, and management. Finally, future prospective research to study noninvasive assessment of liver fibrosis to predict sustained viral response should be developed.

biopsy has been significantly associated with sustained viral response (SVR). Despite its widespread use, performing a liver biopsy is not without due cautions and concern for physicians and patients alike. Significant complications, defined as requiring hospital admission or prolonged hospital stay, occurs in 1% to 5% of patients. AST to platelet ratio index (APRI) is an easy and non-invasive method to evaluate hepatic fibrosis. APRI as a predictive factor of early viral response in hepatitis C chronic naive patients has not been evaluated.

Research frontiers

The APRI is one of several markers that have been proposed to measure liver fibrosis; it is an inexpensive widely available tool; however it has not been used as a predictive factor in chronic hepatitis C. Further studies across more diverse cohorts of liver disease will be necessary, and further refinement will likely enhance accuracy. In this study, the authors demonstrated that APRI is not a predictive factor for early viral response (EVR) in patients with chronic hepatitis C.

Innovations and breakthroughs

Recent studies have shown the importance of this test to decrease the number of liver biopsies, particularly because patients with an APRI of less than 0.40 have very little chance of having significant fibrosis. This is the first study to evaluate the APRI as a predictive factor of early viral response in patients with chronic hepatitis C.

Applications

APRI is a good estimator of hepatic fibrosis. It could be used to decrease the number of liver biopsies, however it is not useful to predict EVR in chronic hepatitis C patients.

Terminology

The APRI is one of several markers that have been proposed to measure liver fibrosis; this index accurately predicts mild fibrosis in patients with a value < 0.42 and those with a value > 1.2 are diagnosed as a significant grade of fibrosis. APRI establishes a 90% negative predictive value for the absence of fibrosis and a 91% of positive predictive value for its presence. We calculated APRI with the following formula: $APRI = \frac{AST \text{ levels } (\times ULN)}{\text{platelets counts } (10^3/L)} \times 100$.

Peer review

It is necessary to investigate the utility of APRI as a predictor of sustained viral response.

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COMMENTS

Background

Viral response in patients with chronic hepatitis C virus infection is more likely to be observed in some patients. The absence of bridging fibrosis/cirrhosis on

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S- Editor Li LF L-Editor Stewart GJ E- Editor Yin DH



BRIEF ARTICLE

***Helicobacter pylori* infection and gastropathy: A comparison between Indonesian and Japanese patients**

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Received: August 5, 2009 Revised: August 31, 2009

Accepted: September 7, 2009

Published online: October 21, 2009

Key words: *Helicobacter pylori*; Gastritis; Gastric cancer; Intestinal metaplasia; Atrophy

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Abdullah M, Ohtsuka H, Rani AA, Sato T, Syam AF, Fujino MA. *Helicobacter pylori* infection and gastropathy: A comparison between Indonesian and Japanese patients. *World J Gastroenterol* 2009; 15(39): 4928-4931 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4928.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4928>

Abstract

AIM: To compare the effects of *Helicobacter pylori* (*H. pylori*) infection on gastropathy between Indonesian and Japanese patients.

METHODS: Biopsy specimens were obtained during upper gastrointestinal endoscopy from 167 subjects (125 Indonesians and 42 Japanese) with uninvestigated symptoms of dyspepsia. The specimens were analyzed for the presence of *H. pylori* using urease analysis, histopathology, and cell culture. The grade and activity of gastritis was assessed using the updated Sydney system.

RESULTS: The percentages of Indonesian and Japanese patients who were *H. pylori*-positive at the antrum or body of the stomach were similar (68% and 59.5%, respectively; $P = 0.316$). Of those who were *H. pylori*-positive, more Japanese patients than Indonesian patients had high levels of polymorphonuclear cells ($P = 0.001$), mononuclear cells ($P = 0.013$), glandular atrophy ($P = 0.000$), and intestinal metaplasia ($P = 0.011$) in both the antrum and body of the stomach.

CONCLUSION: The grade of gastritis and prevalence of mucosal atrophy and intestinal metaplasia were higher in Japanese patients. The difference between Indonesian and Japanese patients was significant.

INTRODUCTION

There is abundant evidence of an association between *Helicobacter pylori* (*H. pylori*) chronic infection and gastric cancer^[1-5]. Some population-based studies show that high levels of *H. pylori* infection are not necessarily accompanied by high mortality from gastric cancer, the so-called African^[6-8] and Asian enigmas^[9].

Indonesia and Japan have a similar prevalence of *H. pylori* infection, but the incidence of gastric cancer is much higher in Japan than Indonesia^[10]. We conducted this cross-sectional study to compare the effects of *H. pylori* infection on gastropathy between Indonesian and Japanese patients.

We consider the old concept of a cascade of mucosal changes that develop from acute/chronic gastritis to atrophic gastritis with intestinal metaplasia, and finally to dysplasia and gastric cancer, as proposed by P. Correa before the discovery of *H. pylori* in the stomach^[11]. This is relevant because a difference in the pattern of *H. pylori*-associated gastritis may explain the difference in the incidence of gastric cancer between Indonesia and Japan.

MATERIALS AND METHODS

Selection of patients

Patients were eligible to participate in the study if they were aged 18 years or older and had experienced uninvestigated symptoms of dyspepsia for at least 3 mo before enrollment. We defined dyspepsia as epigastric pain or discomfort perceived to originate in

Table 1 Baseline characteristics of patients *n* (%)

Characteristic	Indonesian (<i>n</i> = 125)	Japanese (<i>n</i> = 42)	Total (<i>n</i> = 167)	<i>P</i>
Age (yr)	50.30 (18-82)	57 (20-79)		0.060
Sex				
Male	58 (46.4)	25 (59.5)	83 (49.7)	0.141
Female	67 (53.6)	17 (40.5)	84 (50.3)	
<i>Helicobacter pylori</i> (<i>H pylori</i>)-positive				
Antrum and/or body	85 (68)	25 (59.5)	110 (65.9)	0.316
Antrum	85 (68)	22 (52.4)	107 (64.1)	0.068
Body	5 (4)	20 (47.6)	25 (15)	0.000

the upper gastrointestinal tract, including heartburn, acid regurgitation, excessive belching, abdominal bloating, nausea, a perception of abnormal or slow digestion, and early satiety^[12,13]. Patients who experienced only heartburn and/or regurgitation were considered to have gastroesophageal reflux disease and were excluded. We also excluded patients who underwent upper gastrointestinal endoscopy and/or barium radiography less than 6 mo before the study or underwent these procedures on more than two separate occasions within the preceding 10 years, and patients who received eradication therapy for *H pylori* less than 6 mo before the study. We excluded patients who had undergone gastric surgery and those who had a documented history of ulcer disease, esophagitis, irritable bowel syndrome, or clinically significant abnormal results on laboratory analyses. None of the patients who enrolled in the study received treatment with nonsteroidal anti-inflammatory drugs, aspirin (> 325 mg/d), antibiotics, H2-receptor antagonists, proton pump inhibitors, misoprostol, sucralfate, prokinetic agents, or a bismuth compound within 14 d of the study.

From 1998 to 1999, 42 Japanese patients at Yamanashi Medical University Hospital, Koufu and 125 Indonesian patients at Metropolitan Medical Centre Hospital, Jakarta were diagnosed as having dyspepsia and were consecutively enrolled in the study. Informed consents were obtained from all of the patients. This study was reviewed and approved by the ethics committee of Faculty of Medicine University of Indonesia, who approved the protocol.

Intervention and measurements

All patients underwent an upper gastrointestinal endoscopy procedure to identify lesions and to obtain biopsy specimens from the antrum and body of the stomach. The grade and activity of gastritis was assessed using the updated Sydney system^[14]. The presence of *H pylori* in Indonesian patients was determined using histopathology, culture, and rapid urease test (RUT)^[15,16]. The presence of *H pylori* in Japanese patients was determined using urease analysis, histopathology, cell culture, and RUT.

Statistical analysis

SPSS for Windows version 14 software (SPSS Inc., Chicago, Illinois) was used to analyze data. The prevalence of *H pylori*, polymorphonuclear cells, mononuclear cells, glandular atrophy, and intestinal metaplasia in the antrum

Table 2 Comparison of the effects of *H pylori* infection on gastropathy in Indonesian and Japanese patients who were positive for *H pylori* at the antrum and/or body of the stomach *n* (%)

Characteristic	None or mild	Moderate or severe	<i>P</i>
Polymorphonuclear cells			
Indonesian (<i>n</i> = 85)	71 (83.53)	14 (16.47)	0.001
Japanese (<i>n</i> = 25)	12 (48)	13 (52)	
Mononuclear cells			
Indonesian (<i>n</i> = 85)	31 (36.47)	54 (63.53)	0.013
Japanese (<i>n</i> = 25)	2 (8)	23 (92)	
Glandular atrophy			
Indonesian (<i>n</i> = 85)	84 (98.82)	1 (1.18)	0.000
Japanese (<i>n</i> = 25)	16 (64)	9 (36)	
Intestinal metaplasia			
Indonesian (<i>n</i> = 85)	85 (100)	0 (0)	0.011
Japanese (<i>n</i> = 25)	22 (88)	3 (12)	

and body of the stomach was analyzed using the χ^2 test or Fisher's exact test, as appropriate.

RESULTS

Baseline characteristics of patients

We studied 125 Indonesian patients and 42 Japanese patients (males, 49.7%; females, 50.3%), of whom 110 (65.9%) tested positive for *H pylori* at the antrum and/or body of the stomach. The percentages of Indonesian and Japanese patients who were *H pylori*-positive at the antrum or body of the stomach were similar (68% and 59.5%, respectively; *P* = 0.316). The percentage of Japanese patients (52.4%) positive for *H pylori* at the antrum of the stomach was similar to that of Indonesian patients (68%) (*P* = 0.068) but more Japanese patients were positive for *H pylori* at the body of the stomach (47.6%) than Indonesian patients (4%) (*P* < 0.000) (Table 1).

Comparison of the effects of *H pylori* infection on gastropathy in Indonesian and Japanese patients who were positive for *H pylori* at the antrum and/or body of the stomach

Of patients who were *H pylori*-positive at the antrum and/or body of the stomach, more Japanese patients had high numbers of polymorphonuclear cells (*P* = 0.001) and mononuclear cells (*P* = 0.013), glandular atrophy (*P* = 0.000), and intestinal metaplasia (*P* = 0.011) than Indonesian patients (Table 2).

DISCUSSION

The development of gastric cancer is a long process and caused by many factors. Although *H pylori* is an important risk factor for gastric cancer, it is not the only factor that is involved in the etiology of gastric cancer. The association between gastric cancer and *H pylori* infection should be considered from the perspective of the multi-agent compound etiological theory^[17]. The *H pylori* infection rate is very high both in Indonesian and Japanese populations. The reason why Indonesians with *H pylori* infection do not develop gastric cancer to

the same extent as their Japanese counterparts remains unknown.

The limitation of this study was that samples were obtained from a hospital population. Consequently, the results may not represent the community. However, this is the first study in which the effects of *H pylori* infection on gastropathy between Indonesian and Japanese patients were compared.

This study showed that the grade and activity of gastritis was higher among Japanese *H pylori*-positive patients. The prevalence of mucosal atrophy was also greater in Japanese *H pylori*-positive patients than in Indonesians. In addition, more Japanese *H pylori*-positive patients had severe atrophic gastritis and intestinal metaplasia than Indonesians. The difference between those two groups was significant statistically.

Japanese *H pylori*-positive patients had higher grade and activity of gastritis than Indonesian *H pylori*-positive patients. This finding was consistent with the study by Kang *et al*^[18] which observed differences in gastritis among Chinese, Malays, and Indians. This indicates that risk factors other than *H pylori*, such as genetic factors, are associated with the development of gastric cancer in Japanese patients. Moreover, atrophic gastritis in Japanese subjects originating from autoimmune gastritis may explain the high grade and activity of gastritis in these patients.

Another important risk factor is a dietary factor because this affects the rate of development of gastric cancer^[19,20]. Japanese dietary habits such as high consumption of nitrite-rich, salty pickled vegetables and dried fish, and alcohol, are associated with atrophic gastritis and gastric cancer^[21-24]. A low consumption of fresh fruit and raw vegetables may increase this risk. Such dietary habits may act in synergy with *H pylori* in causing gastric cancer^[25]. Migrants to countries with low gastric cancer rates have a diminished risk of gastric cancer^[26].

In conclusion, although the prevalence of *H pylori* infection was similar in Indonesian and Japanese patients, the grade and activity of gastritis was higher in Japanese *H pylori*-positive patients. Moreover, the prevalence of mucosal atrophy and intestinal metaplasia was also higher in Japanese group. The difference between Indonesian and Japanese *H pylori*-positive patients was found to be significant. That difference may be responsible for the disparity in the incidence of gastric cancer in Indonesia and Japan. Further studies will be needed.

COMMENTS

Background

Although *Helicobacter pylori* (*H pylori*) has been classified as a class I (or definite) carcinogen by WHO, the controversy as to why only a minority of infected patients develop distal adenocarcinoma still remains. Moreover, in Asian countries such as Indonesia, Japan, China, and Thailand, where the *H pylori* infection rates are similar, there is a significant difference regarding the outcome of gastric cancer. In the present study, the authors evaluated the transformation of gastric mucosa that is induced by *H pylori* infection prior to gastric cancer. The authors also compared the transformation between Indonesian and Japanese patients, the two countries that represent an "Asian paradox".

Innovations and breakthroughs

By means of this study, the authors revealed that there was a significant

difference in the grade and activity of gastritis and the prevalence of mucosal atrophy and intestinal metaplasia between Indonesian and Japanese *H pylori*-positive patients. These findings may be responsible for the difference in the incidence of gastric cancer in Indonesia and Japan.

Applications

This study indicates that there is a difference in host response between Indonesian and Japanese patients regarding *H pylori* infection.

Peer review

The study is a good idea. Patients were enrolled consecutively with no selection bias. There was no difference in patients' clinical features. The endoscopic features were assessed using OMED Database of Digestive Endoscopy. The histological differences were assessed using the Updated Sydney System by two observers with good interobserver degree of agreement.

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S- Editor Tian L L- Editor Logan S E- Editor Ma WH

BRIEF ARTICLE

Community-based cross-sectional seroprevalence study of hepatitis A in Bangladesh

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Received: June 10, 2009 Revised: September 17, 2009

Accepted: September 24, 2009

Published online: October 21, 2009

group, anti-HAV seroprevalence was 32.3% in subjects < 10 years and 51.7% in those aged 11-20 years. Until now Bangladesh has been deemed to have high endemicity for HAV.

CONCLUSION: The transition from high to intermediate HAV endemicity may be underway; high SES adolescents and adults remain particularly at risk of symptomatic illness. Preventive measures need consideration.

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Key words: Age groups; Hepatitis A virus; Hepatitis A virus seroprevalence; Anti-hepatitis A virus antibodies; Socioeconomic groups

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Abstract

AIM: To elucidate the age-distribution of anti-hepatitis A virus (HAV) seroprevalence across different socio-economic status (SES) categories in Bangladesh which, despite scarce data, is generally deemed to have high endemicity.

METHODS: Blood samples of 818 subjects from a stratified sample of schools and hospitals, comprising different age categories and SES were collected. They were assayed for total anti-HAV antibodies. Social and medical history data were obtained using a questionnaire.

RESULTS: Overall anti-HAV seroprevalence was 69.6%, increasing with age from 1-5 years (40.4%) to > 30 years (98.4%). Seroprevalence was lowest (49.8%) in the high SES group and highest (96.5%) in the rural lower-middle SES group. Among subjects aged 6-20 years, anti-HAV seroprevalence was lowest in urban private school children (43.0%), followed by urban government school children (76.2%) and rural school children (96.5%) ($P < 0.01$). Within the high SES

INTRODUCTION

Hepatitis A virus (HAV) is the most common cause of acute viral hepatitis worldwide^[1]. Globally it is responsible for at least 1.4 million new infections each year^[2]. Although infection with HAV is often mild and asymptomatic in young children, the disease can be severe in adults^[3]. The distribution and prevalence of HAV infection is very closely related to local hygiene and sanitation conditions, and consequently may vary across countries depending on the socioeconomic status (SES) of the population^[4]. The highest incidence of hepatitis A infection has previously been reported in developing countries of Africa, Central and South America and South-East Asia^[5,6]. In these countries, virtually all children experience asymptomatic infection with hepatitis A before the age of 5 years. As the majority of children in these countries experience early exposure to infection,

the symptomatic disease that occurs in a small minority may still pose a significant disease burden^[7-9].

Ironically, with improving socioeconomic conditions, these countries are undergoing a transition phase in which the virus is still prevalent in the population but the improving hygiene and socioeconomic conditions delay the average age of infection^[10]. From a public health perspective, it is important to identify such epidemiological shifts, because the greater severity of the disease in older age groups can lead to an increased community disease burden^[11].

Bangladesh is considered to be a country where hepatitis A infection is hyperendemic with 100% of children ≤ 6 years of age exposed and immune to HAV^[3,12]. However, rapid improvements in hygiene and socioeconomic conditions, specifically in sections of the urban population, are taking place. This indicates a possible changing trend of hepatitis A epidemiology in Bangladesh. Neighbouring developing countries, such as India, Pakistan, Sri Lanka and Thailand, have passed, or are currently passing through a similar transition^[13-16]. Although there is some data indicating that 90% of the patients admitted with acute hepatitis are school children^[17], there is no data on seroprevalence of HAV in Bangladesh, which may be important in guiding the development of HAV prevention strategies.

This prospective, cross-sectional study aimed to determine the seroprevalence of HAV antibodies in a range of age groups, with different SES, living in urban and rural areas of Bangladesh.

MATERIALS AND METHODS

Study design and subjects

This hospital and school-based, cross-sectional seroprevalence study was conducted between October 2005 and December 2006 in Bangladesh in a number of sites selected at convenience. Healthy subjects aged > 1 year from different socioeconomic and educational backgrounds were enrolled in the study. To avoid information bias that might have arisen from the use of self-reported family income, we stratified according to age group and SES by choosing study sites where the study was feasible but which varied in terms of type of medical facility, type of school and residential locality, thereby allowing for recruitment of subjects from distinct SES. This methodology of using proxy markers for SES has been used in the past in Bangladesh^[18].

In the high SES group, subjects were recruited from the Popular Diagnostic Centre (a more costly private diagnostic centre in Dhaka city) and expensive urban private schools, with high tuition fees. In the lower SES group, subjects were recruited from Dhaka Shishu Hospital, a hospital which provides free treatment. Subjects in the lower-middle SES group were recruited from an urban government school, and subjects in the rural lower-middle SES groups were recruited from rural schools. This additional distinction between urban government and rural schools within the lower-middle SES group was deemed important because water supply

and sanitation in rural areas are often not of the same standard as in urban areas.

For analysis, enrolled subjects were stratified into five age groups: 1-5 years, 6-10 years, 11-20 years, 21-30 years and > 30 years. Different centres enrolled subjects of different age groups. Subjects between the ages of 1 and 5 years and > 20 years were enrolled from Dhaka Shishu Hospital and the Popular Diagnostic Centre. Subjects between the ages of 6 and 20 years were enrolled from urban private schools, urban government schools and rural schools.

Ethics

The study was conducted according to Good Clinical Practice of the International Council of Harmonization, and conforms to provisions of the Declaration of Helsinki. The research protocol was approved by the Ethical Review Committee of Dhaka Shishu Hospital.

Written informed consent was obtained from the subjects or (in the case of minors) from their parents/guardians, prior to conducting any study-specific procedure.

Laboratory assays

Two to three milliliters of blood was collected from each subject enrolled in the study. All the blood samples were assayed for total antibodies against HAV (anti-HAV IgG and IgM) at the Department of Microbiology of Dhaka Shishu Hospital using a commercial anti-HAV microparticle enzyme immunoassay (Abbott Laboratories, Illinois, USA). A sample optical density of 1.000 was considered as the assay cut-off value.

Data collection

All enrolled subjects were medically examined and checked for pre-defined inclusion/exclusion criteria. Subjects were excluded from participating in the study if they were suffering from hepatitis or if they had previously been vaccinated against hepatitis A. Information on age, height, weight, vaccination history, medical history, gender and various socioeconomic factors were collected on case report forms specifically designed for this study.

Sample size determination

The sample size was computed using nQuery by considering confidence interval (CI) for proportions using normal approximation with 95% CI level. Based on the various expected prevalences (30.0% for the age group 1-5 years, 31.0% for the age group 6-10 years, 64.0% for the age group 11-20 years, 89.0% for the age group 21-30 years and 90.0% for the age group > 30 years) of anti-HAV antibodies with the error margin at 0.1 (10.0%) among different age groups and SES, it was planned to enrol a total of 818 subjects in this study. The estimates considered for computing the sample size were published previously^[13,19]. The number of subjects planned for each stratum (combination of SES by age groups) is shown in Table 1.

Table 1 Group definition based on age and socioeconomic status classification

Socioeconomic status	Definition	Age group (yr)					Overall (n)
		1-5	6-10	11-20	21-30	> 30	
High	High socio-economic group of subjects from Popular Diagnostic Centre	81	NA	NA	38	32	151
High	High socio-economic group of subjects from urban private schools	NA	83	89	NA	NA	172
Lower middle	Lower middle socio-economic group of subjects from urban government schools	NA	83	89	NA	NA	172
Rural lower middle	Lower middle socio-economic group of subjects from rural schools	NA	78	94	NA	NA	172
Low	Low socio-economic group of subjects from Dhaka Shishu Hospital	80	NA	NA	39	32	151
Total		161	244	272	77	64	818

NA: Not available; n: Total number of subjects.

Table 2 Summary of demographic characteristics by socioeconomic status for the age groups 1-5 yr, 21-30 yr, > 30 yr and overall (n = 302)

Age group (yr)	Variables or categories	Value (%)		
		High (n = 151)	Low (n = 151)	Total (n = 302)
1-5	n	81	80	161
	mean ± SD	2.8 ± 1.1	2.4 ± 1.3	2.6 ± 1.2
	Female	29 (35.8)	32 (40.0)	61 (37.9)
	Male	52 (64.2)	48 (60.0)	100 (62.1)
21-30	n	38	39	77
	mean ± SD	24.0 ± 2.7	23.9 ± 2.8	24.0 ± 2.7
	Female	10 (26.3)	14 (35.9)	24 (31.2)
	Male	28 (73.7)	25 (64.1)	53 (68.8)
> 30	n	32	32	64
	mean ± SD	45.6 ± 11.4	40.3 ± 6.7	43.0 ± 9.7
	Female	13 (40.6)	8 (25.0)	21 (32.8)
	Male	19 (59.4)	24 (75.0)	43 (67.2)
Overall	n	151	151	302
	mean ± SD	17.2 ± 18.0	16.0 ± 15.9	16.6 ± 17.0
	Female	52 (34.4)	54 (35.8)	106 (35.1)
	Male	99 (65.6)	97 (64.2)	196 (64.9)

n: Total number of subjects.

Statistical analysis

All analyses were performed on subjects whose demographic and serological data were available and who abided with the protocol-defined procedures. Fisher's exact test was used to compare groups in the anti-HAV results between the SES groups for each age group. The exact 95% CI for the proportions of subjects seropositive for anti-HAV antibodies was used. All statistical analyses were performed using Statistical and Analysis Software version 8.2.

RESULTS

Study population

A total of 818 subjects were enrolled from different sites. Anti-HAV antibody status was studied in all 818 subjects. There were 161 (19.7%) subjects in the age group 1-5 years (mean: 2.6 ± 1.2 years) from the Popular Diagnostic Centre and Dhaka Shishu Hospital, 244 (29.8%) in the age group 6-10 years (mean: 7.7 ± 1.4 years) from urban private schools, urban government schools and rural schools, 272 (33.3%) subjects in the age group 11-20 years (mean: 13.7 ± 2.3 years) from

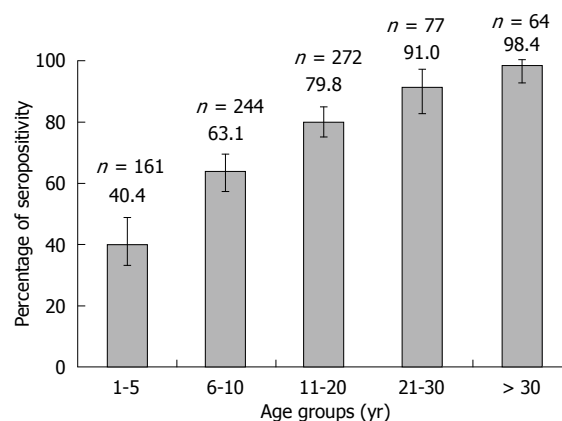


Figure 1 Overall seropositivity of anti-HAV antibodies by age group (n = 818). n: Total number of subjects with results. Percentage of seropositivity (anti-HAV antibodies) with 95% CI was calculated. The assay cut-off was sample optical density of 1.00.

urban private schools, urban government schools and rural schools, 77 (9.4%) in the age group 21-30 years (mean: 24.0 ± 2.7 years) from the Popular Diagnostic Centre and 64 (7.8%) subjects in the age group > 30 years (mean: 43.0 ± 9.7 years) (Table 1).

There were more males than females in the age groups 1-5 years, 21-30 years and > 30 years and more females than males in the age group 11-20 years. The male to female proportions were similar in the 6-10 years age group (Tables 2 and 3).

HAV seroprevalence

Overall anti-HAV seropositivity was 69.6% (569/818) with the 95% CI (66.28-72.70). Anti-HAV seropositivity increased considerably from lower age groups to older age groups, demonstrating a consistent trend (Figure 1).

Anti-HAV seropositivity was highest at 96.5% (166/172) with 95% CI (92.56-98.71) in the rural lower-middle group and lowest at 49.8% (161/323) in the high SES group.

Age groups and SES

Seropositivity for anti-HAV increased with age independently of the SES of subjects. Among the children aged 1-5 years, the seroprevalence was significantly high (50.0%) in the low SES group than in the high (31.0%) SES group ($P = 0.016$). Similarly, 64.0% and 88.0% of

Table 3 Summary of demographic characteristics by socioeconomic status for the age groups 6-10 yr, 11-20 yr and overall (*n* = 516)

Age group (yr)	Variables or categories	Value (%)			
		High (<i>n</i> = 172)	Lower middle (<i>n</i> = 172)	Rural lower middle (<i>n</i> = 172)	Total (<i>n</i> = 516)
6-10	<i>n</i>	83	83	78	244
	mean \pm SD	8.4 \pm 1.6	7.2 \pm 1.1	7.5 \pm 1.2	7.7 \pm 1.4
	Female	43 (51.8)	41 (49.4)	37 (47.4)	121 (49.6)
	Male	40 (48.2)	42 (50.6)	41 (52.6)	123 (50.4)
11-20	<i>n</i>	89	89	94	272
	mean \pm SD	13.9 \pm 2.0	14.9 \pm 2.7	12.4 \pm 1.4	13.7 \pm 2.3
	Female	68 (76.4)	46 (51.7)	63 (67.0)	177 (65.1)
	Male	21 (23.6)	43 (48.3)	31 (33.0)	95 (34.9)
Overall	<i>n</i>	172	172	172	516
	mean \pm SD	11.2 \pm 3.3	11.2 \pm 4.4	10.2 \pm 2.8	10.9 \pm 3.6
	Female	111 (64.5)	87 (50.6)	100 (58.1)	298 (57.8)
	Male	61 (35.5)	85 (49.4)	72 (41.9)	218 (42.2)

n: Total number of subjects.

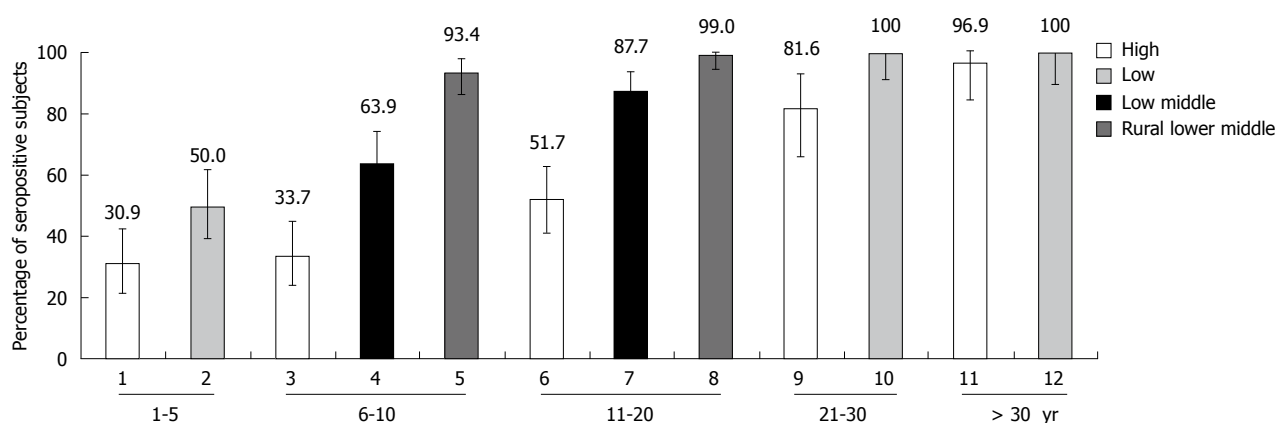


Figure 2 Percentage of anti-HAV seropositivity assessed by age groups 1-5, 6-10, 11-20, 21-30 and > 30 years and SES (*n* = 818). 1: *n* = 81; 2: *n* = 80; 3: *n* = 83; 4: *n* = 83; 5: *n* = 78; 6: *n* = 89; 7: *n* = 89; 8: *n* = 94; 9: *n* = 38; 10: *n* = 39; 11: *n* = 32; 12: *n* = 32.

subjects were positive for HAV antibody in the urban lower-middle class group compared to 34.0% and 52.0% in the urban high SES group aged 6-10 years and 11-20 years, respectively. There was also a significant difference in the rate of seropositivity in the urban lower-middle class group (64.0% and 88.0%), compared with 93.0% and 99.0% of the rural population of the same SES in the 6-10 years and 11-20 years age groups ($P < 0.01$), respectively (Figure 2).

In subjects aged between 6-20 years, the anti-HAV seropositivity rates were lowest in the high SES group (49.8%), progressively increasing in the lower-middle group followed by the rural lower-middle group (Figure 2). A statistically significant difference was observed in anti-HAV seropositivity between the high and the rural lower-middle SES groups among both the young (6-10 years) and the adolescent age groups (11-20 years) ($P < 0.01$). A statistically significant difference in anti-HAV seropositivity was also observed between the urban private school (high SES group) and urban government schools (lower-middle SES group) among the age groups 6-20 years ($P < 0.01$).

A statistically significant difference in anti-HAV seropositivity was observed between the high and low SES groups for the age groups 1-5 years ($P = 0.016$) and 21-30 years (Figure 2).

DISCUSSION

Three epidemiological patterns of HAV endemicity are commonly observed worldwide: low, intermediate and high. Each pattern is different with respect to seroprevalence in different age groups, transmission mode and disease burden. Most developing countries have high endemicity, in contrast to the generally low seroprevalence found in the developed world. Furthermore, contrasting endemicity may exist in the same country, based on regional or local differences in SES, water supply and hygiene.

This study demonstrated a clear trend in anti-HAV seroconversion with age, where seropositivity progressively increased from 40.4% in the 1-5 years age group to over 98.0% in the > 30 years age group. As expected, there was also an inverse correlation between increased anti-HAV seropositivity and low SES; the exception was the low SES group which had lower seropositivity than the urban and rural lower-middle socioeconomic groups, a finding which can be explained partly by chance and partly by the fact that the majority of the subjects in this group were aged 1-5 years.

The very low anti-HAV seroprevalence of about 30.0% observed among children below the age of 10 years

in the high SES group is not surprising given that these children have grown up in environments where they are likely to have good access to clean water and sanitation. However, a similar pattern was observed in the low socioeconomic group, where almost half the population in the 1-5 years age group and 36.0% of the population in the 6-10 years age group continued to be susceptible to HAV infections. This finding reflects the effects of improvements in water supply and sanitation that have accompanied economic progress, with benefits for the general population in Bangladesh. This is in marked contrast to previous studies which have shown 100% seropositivity in children aged 6 years or less^[3,12].

Amid the very positive improvements in child health that have accompanied economic progress in Bangladesh, nearly 60.0% of children in the 1-5 years age group enrolled in our study and over one third of children aged 6-10 years remain at risk of developing HAV infection, with the risk of symptomatic disease and complications increasing with age. Nevertheless, our study suggests that rural Bangladesh, where the majority of the population resides, can still be considered highly endemic for HAV.

In the high SES group (especially in urban areas), anti-HAV seroprevalence remained low among adolescents aged 11-20 years (51.7%) and relatively low (when compared to rates traditionally observed in hyperendemic countries) among adults aged 21-30 years (81.6%). In other words, in the high SES group, one in two adolescents and one in five adults remains at risk of hepatitis A infection.

During the transition from high (anti-HAV seroprevalence > 90% by age 5 years) to low endemicity, populations undergo a phase of intermediate endemicity for hepatitis A, when more people are susceptible to HAV infection due to lack of exposure in early childhood and adolescence. Circulating HAV in the community means this group are at risk of symptomatic and potentially clinically severe acute hepatitis^[11,19]. Symptomatic disease could lead to significant illness, prolonged debility and is likely to require time off from studies or work during the active and economically productive years of adolescence and early adulthood.

Only in the population aged > 30 years did we observe a high anti-HAV seroprevalence rate (98.4%), regardless of SES. This was expected and reflects the cohort effect of a population that was exposed to HAV early in life due to the poor water quality and improper sanitation facilities that would have prevailed during their childhood^[11].

A limitation of our design was that the subjects enrolled in the study were from hospital-based and school-based sub-populations. Although not a population-based study, the complete coverage of age and SES made it reasonable to extrapolate these findings to the general population. Our findings that HAV seroprevalence is associated with age and SES are in line with HAV epidemiology documented in developing countries and previously described in the Indian subcontinent. Studies conducted in other countries in the region (India, Pakistan, Thailand, Sri Lanka, Korea, Philippines and Malaysia)^[20,21], have likewise shown higher seroprevalence rates in rural populations when compared to urban populations and

demonstrated strong evidence of epidemiological shifts, especially in the upper socio-economic groups^[14,15,22,23].

The most important mode of hepatitis A control is by improvements in hygiene, sanitation and drinking water supply, which accompany socioeconomic progress. However, the World Health Organization position paper on hepatitis A vaccines states that in countries under the category of intermediate endemicity, childhood vaccination occasionally targeted to specific risk groups may be considered in addition to health education and improved sanitation to contain HAV infections^[24]. Effective hepatitis A vaccination programs have helped to control the disease with proven social and economical benefits. Regional experts have also called for wider use of hepatitis A vaccines^[25]. Vaccines against hepatitis A have been available since 2001 in the Bangladeshi private market, but are not widely used currently and are not a part of the universal expanded programme of immunization in Bangladesh.

In conclusion, although disparities exist in anti-HAV seroprevalence between the upper and lower socioeconomic classes in Bangladesh, in conjunction with previous studies we found some evidence of a possible epidemiological transition taking place, especially among the upper classes. In view of these findings and as suggested by regional experts, the introduction of safe and effective vaccination against hepatitis A (with broader coverage), along with improvements in water supply, sanitation and hygienic conditions in Bangladesh may have the potential to reduce and hopefully prevent the severity of future HAV infection in older children and adolescents, with accompanying public health benefits.

ACKNOWLEDGMENTS

The authors are grateful to Farah Mecci for medical writing, Roselynn F Tien and Ming Tung Lim for their assistance in the preparation of this manuscript, and Fakrudeen A Shafi for providing statistical input [All are employees of GlaxoSmithKline (GSK)]. Authors are also grateful to Mr. Hassan Al-Emran and Mr. Belal Hossain for technical assistance in analysing the specimens. This study was supported by GSK Biologicals, which was the funding source of this study and was involved in all stages of the study, and in the data analysis. GSK Biologicals also covered all costs associated with the development and publishing of this manuscript.

COMMENTS

Background

Hepatitis A virus (HAV) is the most common cause of acute viral hepatitis in children worldwide. Although, HAV infection is often mild and asymptomatic in young children, the disease can be severe in adults. As the distribution and prevalence of HAV infection is closely associated with local hygiene and sanitation conditions, it was important to explore the impact of HAV infection in Bangladesh, which previously was considered hyperendemic. However, rapid improvements in hygiene and socioeconomic conditions, specifically in sections of the urban population have taken place. A research group in Bangladesh has predicted that a probable transition from hyperendemicity to intermediate HAV endemicity may be underway. In the high SES group, particularly adolescents

and adults are particularly at a higher risk of acquiring symptomatic illness, indicating the need for immunization against HAV, especially in this high risk group.

Research frontiers

Bangladesh is considered to be hyperendemic for HAV. However, rapid improvements in hygiene and socioeconomic conditions, specifically in sections of the urban population, are taking place. This indicates a possible changing trend of hepatitis A epidemiology in Bangladesh as has previously been observed in neighbouring countries, such as India, Pakistan, Sri Lanka and Thailand. During this transition from high to low endemicity, populations undergo a phase of intermediate endemicity for hepatitis A when more people are susceptible to HAV infection due to lack of exposure in early childhood and adolescence, despite circulating HAV in the community, and are consequently at risk of symptomatic and potentially clinically severe acute hepatitis. Prior to this study, no data were available on the seroprevalence of HAV in Bangladesh in different age groups and social classes, which are important parameters that guide the development of local HAV prevention strategies.

Innovations and breakthroughs

This is the first study to assess the entire population spectrum of Bangladesh for hepatitis A seroprevalence. The study demonstrates that a possible epidemiological transition is in progress among those of high socioeconomic status (SES). However, more than half of children aged < 10 years and almost half of individuals aged 11-20 years were still at higher risk of symptomatic hepatitis. Surprisingly, there are also some indications that an early epidemiological transition may be occurring in the lower SES groups.

Applications

The study results may help decision making on public health policies and vaccination strategies for the control of hepatitis A in Bangladesh.

Terminology

HAV, HAV seroprevalence, anti-HAV antibodies, Socioeconomic groups, Age groups.

Peer review

The authors described an epidemiological study investigating the anti-HAV seroprevalence of 818 subjects from hospital- and school-based subpopulations in Bangladesh, and found that anti-HAV seroprevalence was correlated with increasing age and inversely correlated with higher SES. They concluded that vaccination against hepatitis A should be considered to reduce the future burden of symptomatic HAV infection in this country. There are few reports on the seroprevalence of anti-HAV in Bangladesh where hepatitis A infection is hyperendemic and hence, this manuscript is worthy of publication.

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S- Editor Tian L L- Editor Webster JR E- Editor Zheng XM



BRIEF ARTICLE

Clinical outcomes in patients with ICU-related pancreatitis

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Received: July 17, 2009 Revised: August 24, 2009

Accepted: August 31, 2009

Published online: October 21, 2009

was comparable to that in ICU patients without pancreatitis by case-control methodology ($P = 0.544$). Multivariate logistic regression analysis identified low $\text{PaO}_2/\text{FiO}_2$ (OR: 1.032, 95% CI: 1.006-1.059, $P = 0.016$) as an independent risk factor for mortality in patients with ventilator-related pancreatitis. The mortality rate in patients with ventilator-related pancreatitis was lower than that in patients with acute pancreatitis-related respiratory failure ($P < 0.001$).

CONCLUSION: We found that low $\text{PaO}_2/\text{FiO}_2$ was an independent clinical parameter predictive of ICU mortality in patients with ventilator-related pancreatitis.

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Key words: Acute pancreatitis; Hyperamylasemia; Hyperlipasemia; Mechanical ventilation; Respiratory failure

Peer reviewer: Chris E Forsmark, Professor, Division of Gastroenterology and Hepatology, University of Florida, FL, 32610-0214, United States

Tseng CC, Fang WF, Chung YH, Wang YH, Douglas IS, Lin MC. Clinical outcomes in patients with ICU-related pancreatitis. *World J Gastroenterol* 2009; 15(39): 4938-4944 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4938.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4938>

Abstract

AIM: To identify risk factors predictive of intensive care unit (ICU) mortality in patients with ventilator-related pancreatitis. The clinical outcomes of patients with ventilator-related pancreatitis were compared with those of patients with pancreatitis-related respiratory failure as well as controls.

METHODS: One hundred and forty-eight patients with respiratory failure requiring mechanical ventilation and concomitant acute pancreatitis were identified from a prospectively collected dataset of 9108 consecutive patients admitted with respiratory failure over a period of five years. Sixty patients met the criteria for ventilator-related pancreatitis, and 88 (control patients), for pancreatitis-related respiratory failure.

RESULTS: Mortality rate in ventilator-related pancreatitis

INTRODUCTION

Mechanical ventilation is an important method for maintaining gas exchange in patients with respiratory failure until the underlying disorders are corrected. However, it is also associated with numerous organ-system disorders that can significantly affect the outcome of critically ill patients^[1]. Possible mechanisms include injurious ventilatory strategies, high pressure with hypovolemic status, and sympathetic stimulation. Injurious ventilatory strategies may induce the release of proinflammatory cytokines. High intrathoracic pressure with hypovolemic status can cause splanchnic hypoperfusion^[2]. Sympathetic stimulation can promote the release of catecholamines^[3] and result in organ ischemia. All of these mechanisms can result in a systemic inflammatory response and multiple organ dysfunction. Acute pancreatitis may also be induced during mechanical ventilation *via* these mechanisms.

Moreover, ischemia or organ hypoperfusion alone is considered an important common mechanism for the induction of pancreatic injury^[4,5]. Among the several mechanisms suggested to explain how mechanical ventilation can cause acute pancreatitis, splanchnic hypoperfusion appears to be of particular importance^[1].

Acute pancreatitis is an inflammatory process that usually occurs in a previously normal pancreas and is diagnosed mainly by acute abdominal pain associated with a concomitant increase in serum amylase and lipase concentrations^[6,7]. Alcoholism and gallstones are established as the most frequent causes of acute pancreatitis. Other risk factors - drugs, hypertriglyceridemia, hypercalcemia, viral infection, and connective tissue disease - are also common^[8-10]. Many studies have examined the clinical spectrum of lung injury associated with acute pancreatitis. Pulmonary dysfunction ranging from hypoxemia to acute respiratory distress syndrome (ARDS) is one of the most important systemic manifestations of severe acute pancreatitis^[11]. Patients with severe pancreatitis are frequently associated with acute respiratory failure that subsequently develops into ARDS^[12,13]. The development of ARDS is associated with a high mortality and is highly correlated with hypoxemic status^[14].

Acute lung injury and respiratory failure are frequent and potentially fatal complications of acute pancreatitis^[15,16]. Importantly, the institution of positive pressure mechanical ventilation can itself induce acute pancreatitis by exacerbating splanchnic hypoperfusion^[1,17]. Recent studies have reported that elevated serum lipase levels are frequently encountered in critically ill patients, and hypoperfusion and inflammatory processes associated with multiple-organ failure appear to result in pancreatitis. However, the incidence, natural history, and outcomes of ventilator-related pancreatitis (VRP) have not been characterized in humans.

The object of this study was to determine the risk factors predictive of clinical outcomes and intensive care unit (ICU) mortality in patients with VRP. We also compared the outcomes of patients who developed pancreatitis during mechanical ventilation (VRP) in patients admitted with equal physiological scoring severity without pancreatitis and those patients with a primary diagnosis of acute pancreatitis complicated by respiratory failure (PRRF) requiring mechanical ventilation. We sought to determine whether patients with VRP had a poorer prognosis than patients with PRRF.

MATERIALS AND METHODS

Study population and design

Nine thousand one hundred and eight patients with acute respiratory failure admitted to the medical intensive care units (ICUs) at Chang Gung Memorial Hospital (CGMH), a tertiary care hospital in Kaohsiung, were identified from a prospectively collected dataset over a period of five years. This study was approved by our hospital's institutional review board and was also in

compliance with the Helsinki Declaration.

In this study, acute pancreatitis was diagnosed in patients meeting at least two of the following three criteria as previously described^[18]: (1) acute abdominal pain and tenderness in the upper abdomen; (2) elevated levels of pancreatic enzyme (serum lipase and/or amylase) in the blood, urine, or ascitic fluid; and (3) abnormal imaging findings for the pancreas associated with acute pancreatitis. A search of medical records using a combination of the two diagnostic categories of acute respiratory failure and acute pancreatitis identified 163 patients. Among these, 75 patients were diagnosed with VRP. Fifteen patients with a history of alcoholism (alcohol consumption > 60 g daily for 10 years), gallstones (by hepatic sonography), hypertriglyceridemia (triglyceride > 500 U/L within 1 year), or the use of drugs that have been well documented to cause pancreatitis (such as propofol) were excluded to avoid confounding. We excluded other well-known etiologies of pancreatitis except for mechanical ventilation. Thus, 60 patients met the criteria for VRP and 88 patients were classified as having PRRF (Figure 1A). In the patients with PRRF, the principal cause of acute respiratory failure requiring intubation was acute respiratory distress syndrome (ARDS), as a complication of acute pancreatitis.

Due to the wishes of the patients' families and the medical culture in Taiwan, most patients were not receiving sedation. All patients with VRP complained of abdominal pain. The serum amylase and lipase levels were examined one day after the patients complained of abdominal pain following mechanical ventilation. Acute pancreatitis was confirmed by an elevated serum lipase level, three times the upper limit (> 570 U/L) with or without elevated serum amylase. Confirmatory imaging studies were not routinely used in this study. Among the VRP patients, only 28 (46.7%) underwent an imaging study for the diagnosis of pancreatitis. Fourteen patients (23.3%) underwent imaging studies but had indeterminate findings due to patient agitation or excessive bowel or respiratory distress. Eighteen patients (30%) underwent no imaging studies due to their critical condition.

Patient demographic characteristics, comorbidities, presence of ARDS, lowest PaO₂/FiO₂ ratio, acute renal failure, systemic inflammatory response syndrome (SIRS), vasopressor use, renal replacement therapy, serum amylase, lipase level, and ICU mortality were recorded. ARDS was diagnosed according to the criteria of the American-European Consensus Conference Committee^[19]. Acute renal failure was defined as normal renal function prior to admission that was impaired along with disease progression occurring before or after acute respiratory failure, and defined with a cutoff value of creatinine ≥ 1.5 mg/dL. SIRS was defined as a temperature ≥ 38°C or ≤ 36°C, heart rate ≥ 90 beats/min, respiratory rate ≥ 20/min, and a white blood cell (WBC) count ≥ 12000/mL or ≤ 4000/mL or > 10% immature neutrophils without a definite infection source and we noted this sign after pancreatitis had been diagnosed.

For the outcome analysis, each VRP subject was matched with 3 subjects from the cohort of 9108 pa-

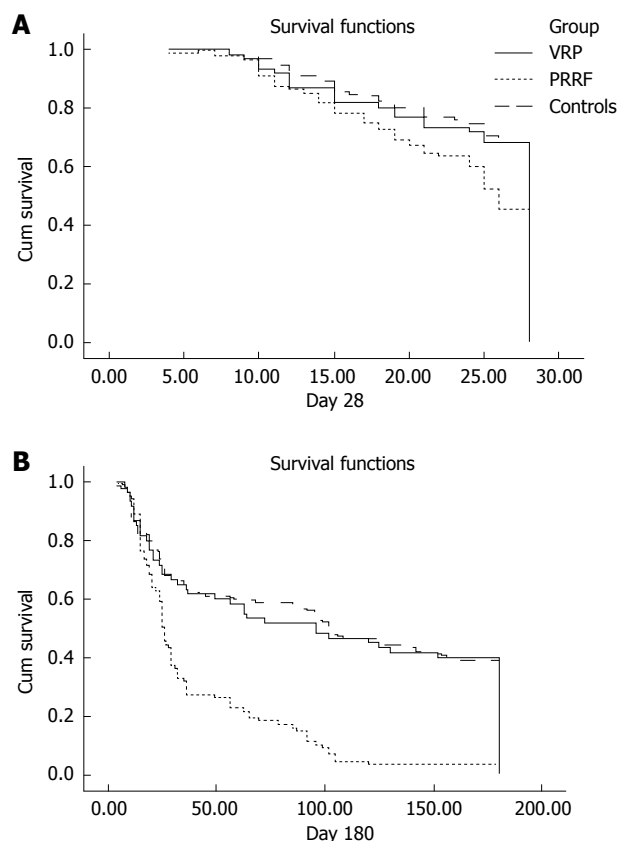


Figure 1 Kaplan-Meier estimates of short-term (A: 28 d) and long-term (B: 6 mo) survival of groups. A: $P = 0.007$ between VRP and PRRF, $P = 0.567$ between VRP and controls; B: $P < 0.001$ between VRP and PRRF, $P = 0.498$ between VRP and controls. VRP: Ventilator-related pancreatitis; PRRF: Pancreatitis-related respiratory failure.

tients with respiratory failure without pancreatitis by an investigator blinded to the outcomes. Matching was based on similarities in severity of illness as manifested by the Acute Physiology and Chronic Health Evaluation (APACHE) II score (± 2 points) at admission to ICU within 24 h, diagnostic category, status of lowest $\text{PaO}_2/\text{FiO}_2$, and diagnosis of ARDS. Among the matched cases, all patients had no abdominal pain described in their chart during admission to the ICU or if abdominal pain was noted, there was no elevation in serum amylase and lipase level.

Statistical analysis

Continuous variables were summarized using means and standard deviations (mean \pm SD), while categorical variables were summarized using counts and percentages.

Index cases and controls were compared using the Student's t -test or the Mann-Whitney U test, as appropriate for continuous variables, or the χ^2 test for categorical variables. Categorical variables were compared with the Pearson χ^2 test and, if appropriate, Fisher's exact test.

In patients with VRP, clinical parameters thought to be contributory to ICU mortality were analyzed by univariate regression analysis. Factors found to be statistically significant ($P < 0.1$) by univariate regression analysis were then retained for a multivariate logistic regression model to determine whether they remained predictive

for ICU mortality.

To assess the outcome between groups, survival time up to 6 mo post-admission was recorded, and Kaplan-Meier survival curves were constructed; comparison between the groups was by the log-rank test.

All testing was two-tailed and $P < 0.05$ were considered to be statistically significant.

RESULTS

Clinical characteristics associated with mortality in patients with ventilator-related pancreatitis

Among 60 patients with VRP, 35 patients (58.3%) were ICU survivors and 25 were ICU non-survivors (41.7%). The mean number of days to pancreatitis after mechanical ventilation in patients with VRP was 11.90 ± 7.22 d (range from 3 to 31 d). Of the 60 patients with VRP, amylase and lipase levels normalized and abdominal pain subsided in 51. The other 9 patients died before further blood sampling for amylase and lipase could be performed. The mean number of days to recovery from pancreatitis (duration from the onset of abdominal pain to the date of normalized amylase and lipase levels) was 7.08 ± 3.48 d (range from 2 to 15 d). Among the VRP patients, only 28 (46.7%) underwent an imaging study for the diagnosis of pancreatitis. Of those patients, all underwent an abdominal echo study, and 3 received both an abdominal echo and a CT study. After reviewing the images, we found that all cases showed mild-to-moderate swelling of the pancreas, and there were no cases of pancreatic necrosis or fluid retention in the abdominal cavity. High APACHE II score ($P = 0.001$), low $\text{PaO}_2/\text{FiO}_2$ level ($P < 0.001$), ARDS status ($P = 0.002$), SIRS occurrence ($P = 0.002$), acute renal failure status ($P = 0.005$), requirement for renal replacement therapy ($P = 0.003$), and male gender ($P = 0.040$) were significantly more frequent in ICU non-survivors than in survivors. High serum amylase ($P = 0.079$) and lipase level ($P = 0.072$) were not correlated with a poor prognosis. Interestingly, an underlying history of congestive heart failure ($P = 0.017$) was more frequent in survivors (Table 1). On univariate regression analysis, acute renal failure ($P = 0.006$), APACHE II score ($P = 0.003$), lowest $\text{PaO}_2/\text{FiO}_2$ ($P < 0.001$), ARDS status ($P = 0.003$), SIRS occurrence ($P = 0.003$), renal replacement therapy ($P = 0.008$), and male gender ($P = 0.044$) were predictors of death (Table 2). On multivariate analysis, only the lowest $\text{PaO}_2/\text{FiO}_2$ (OR: 1.032, 95% CI: 1.006-1.059, $P = 0.016$) predicted death and was, therefore, an independent risk factor for mortality in patients with VRP (Table 3).

Characteristics and outcomes of patients

Table 2 details the baseline characteristics of the 60 cases with VRP, 88 cases with PRRF, and 180 controls without pancreatitis. There were no statistically significant differences between the cases with VRP and the controls in any of the analyzed parameters. Between the patients with VRP and PRRF, there were no statistically significant differences in age, gender, or requirement for renal replacement therapy. More

Table 1 Comparison of the differences between ICU survivors and ICU non-survivors in patients with ventilator-related pancreatitis (mean \pm SD) *n* (%)

Characteristics	ICU survivors <i>n</i> = 35 (58.3)	ICU non-survivors <i>n</i> = 25 (41.7)	<i>P</i>
Age	60.77 \pm 20.71	66.72 \pm 15.73	0.232
Male gender	19 (54.3)	17 (80)	0.004
Apache II score	24.69 \pm 6.09	29.88 \pm 5.15	0.001
Lowest PaO ₂ /FiO ₂	283.86 \pm 64.13	175.32 \pm 72.72	< 0.001
Lipase level	830.93 \pm 511.05	1374.88 \pm 1391.60	0.072
Amylase level	227.34 \pm 207.35	374.96 \pm 368.87	0.079
ARDS	5 (14.3)	13 (52)	0.002
SIRS	5 (17.2)	13 (52)	0.002
Vasopressor	12 (34.3)	9 (36)	0.891
Acute renal failure	11 (31.4)	17 (68)	0.005
RRT	2 (5.7)	9 (36)	0.003
CHF	26 (74.3)	11 (44)	0.017
CVA	12 (34.3)	10 (40)	0.651
Liver cirrhosis	8 (22.9)	7 (28)	0.650
COPD	12 (34.3)	8 (32)	0.853
Neoplastic disease	3 (8.6)	5 (20)	0.199
Diabetes mellitus	17 (48.6)	12 (48)	0.965
Hypertension	19 (54.3)	15 (60)	0.660

Variables are expressed as mean (standard deviation) and categorical data are expressed as number (percentage). Continuous variables were analyzed by Student's *t* test or Mann-Whitney *U* test, and categorical data by χ^2 test. ICU: Intensive care unit; Apache: Acute Physiology and Chronic Health Evaluation; ARDS: Acute respiratory distress syndrome; SIRS: Systemic inflammatory response syndrome; RRT: Renal replacement therapy; CHF: Congestive heart failure; CVA: Cerebrovascular disease; COPD: Chronic obstructive pulmonary disease.

subjects with VRP had congestive heart failure and liver cirrhosis than those with PRRF. PRRF patients were significantly sicker, with higher serum lipase ($P = 0.001$), serum amylase levels ($P < 0.001$), and APACHE II scores ($P < 0.001$), as well as ARDS status ($P < 0.001$), SIRS occurrence ($P < 0.001$), and vasopressor requirements ($P = 0.019$). PRRF was also associated with higher mortality than VRP ($P < 0.001$). However, there was no statistical difference in mortality rates between VRP patients and the control group ($P = 0.544$).

Short- and long-term outcomes

Short- and long-term outcomes were significantly better in patients with VRP. Patients with VRP were more likely to be alive at day 28 than patients with PRRF (68.3% *vs* 45.5%, $P = 0.007$), to be discharged from ICU (58.3% *vs* 26.2%, $P < 0.001$), and to have survived during the 6-mo follow-up period (40% *vs* 3.4%, $P < 0.001$). However, the 28 d survival ($P = 0.567$), ICU survival ($P = 0.544$), and 6 mo survival ($P = 0.498$) rates were comparable between patients with VRP and controls. Survival curves were constructed using the Kaplan-Meier method to explain the survival differences between the groups (Figure 1).

DISCUSSION

This retrospective analysis yielded three main findings. First, patients with respiratory failure needing ventilator support may develop acute pancreatitis. When patients

Table 2 Comparison of baseline characteristics, clinical features, comorbidities and ICU mortality in groups (mean \pm SD) *n* (%)

	Ventilator-related pancreatitis (<i>n</i> = 60)	Controls (<i>n</i> = 180)	Pancreatitis related respiratory failure (<i>n</i> = 88)
Age	63.3 \pm 18.9	65 \pm 15	59.3 \pm 18.7
Male gender	39 (65)	110 (61.1)	54 (61.4)
Serum lipase level	1057.3 \pm 1005.7	-	8274.4 \pm 17018.6 ^a
Serum amylase level	288.9 \pm 292.4	-	884.6 \pm 1247.0 ^a
Apache II score	26.9 \pm 6.2	26 \pm 6	32.0 \pm 6.8 ^a
Lowest PaO ₂ /FiO ₂	238.6 \pm 86.2	224.9 \pm 60.2	168.8 \pm 66.5 ^a
ARDS	18 (30)	62 (34.4)	60 (68.1) ^a
SIRS	18 (30)	66 (36.7)	47 (53.4) ^a
Vasopressor	21 (35)	64 (35.6)	49 (55.7) ^a
RRT	11 (18.3)	31 (17.2)	24 (27.2)
Coexisting illness			
Congestive heart failure	37 (61.7)	105 (58.3)	32 (36.4) ^a
Cerebrovascular disease	22 (36.7)	67 (37.8)	22 (25)
Acute renal failure	28 (46.7)	68 (37.8)	36 (40.9)
Liver cirrhosis	15 (25)	48 (26.7)	41 (46.6) ^a
Obstructive lung disease	20 (33.3)	71 (39.4)	20 (22.7)
Neoplastic disease	8 (13.3)	28 (15.6)	18 (20.5)
Diabetes mellitus	29 (48.3)	97 (53.9)	39 (44.3)
Hypertension	34 (56.7)	106 (58.9)	31 (35.2)
ICU mortality	25 (41.7)	67 (37.2)	65 (73.8) ^a

Continuous variables were analyzed by Student's *t* test or Mann-Whitney *U* test, and categorical data by χ^2 test. ^a $P < 0.01$ compared with ventilator-related pancreatitis.

Table 3 Predictors of ICU mortality in patients with ventilator-related pancreatitis by univariate and multivariate logistic regression analysis

Predictors	Odds Ratio (95% CI)	<i>P</i>
Univariate analysis		
Apache II score	0.856 (0.772-0.948)	0.003
Lowest PaO ₂ /FiO ₂	1.021 (1.011-1.032)	< 0.001
ARDS	6.500 (1.901-22.229)	0.003
SIRS	6.500 (1.901-22.229)	0.003
Acute renal failure	4.636 (1.540-13.963)	0.006
RRT	9.281 (1.792-48.057)	0.008
Male gender	3.368 (1.031-11.010)	0.044
Amylase level	0.998 (0.996-1.000)	0.080
Lipase level	0.999 (0.999-1.000)	0.070
Multivariate analysis		
Lowest PaO ₂ /FiO ₂	1.032 (1.006-1.059)	0.016

CI: Confidence interval.

were diagnosed with VRP, clinical parameters such as a high APACHE II score, low PaO₂/FiO₂, SIRS occurrence, ARDS status, acute renal failure, renal replacement therapy, and male gender predicted mortality. Multivariate logistic regression showed that low PaO₂/FiO₂ was an independent risk factor for mortality. Secondly, the short- and long-term outcomes in patients with VRP were not worse than those in non-pancreatitis patients with an equal severity score, and were better than those in patients with PRRF, although both groups had respiratory failure and acute pancreatitis. Thirdly, patients with PRRF

had higher APACHE II scores, more frequent ARDS, lower $\text{PaO}_2/\text{FiO}_2$ levels, greater frequency of SIRS, and pressor-requiring shock, as well as higher serum lipase and amylase levels than VRP patients.

Using these diagnostic criteria, the incidence of acute pancreatitis in patients requiring mechanical ventilation was lower than we had anticipated. A possible reason is that intubated and sedated patients are frequently unable to indicate and localize serious abdominal pain and this may be overlooked by clinical staff. We speculate that the actual incidence of acute pancreatitis in the ICU is higher than appreciated and this entity would be more readily detected if comprehensive physical examination was conducted and laboratory testing initiated in patients with abdominal pain.

Numerous potential mechanisms could account for VRP. Mechanical ventilation, frequently with high levels of positive end-expiratory pressure (PEEP), can increase intrathoracic pressure and result in decreased venous return^[20]. Reduced preload in the return could result in decreased cardiac output and hypotension. Splanchnic blood flow is decreased in these settings in parallel with PEEP-induced reductions in cardiac output^[21]. Mechanical ventilation with PEEP is also associated with increased renin-angiotensin-aldosterone activity and elevated catecholamine levels because of sympathetic activation^[3,22]. Elevation of serum catecholamines can contribute to splanchnic hypoperfusion due to vasoconstriction and redistribution of blood away from the splanchnic vascular bed^[23]. The adverse effects of mechanical ventilation under injurious ventilatory strategies suggest an important role of cytokines in the pathogenesis of multiple organ complications. Pro-inflammatory cytokines can affect many organs and induce a variety of physiological and biochemical responses to critical illness^[24]. They can lead to a series of intracellular signaling events *via* highly specific cell surface receptors that typically result in elaboration of other cytokines within the target cell. If these processes are not attenuated, excessive amplification of the inflammatory cascade and overproduction of pro-inflammatory mediators can occur with the uncontrolled activation of the immune system and cause target organ damage. All of these mechanisms may result in organ ischemia and failure. If ischemic injury to the pancreas or pancreatic inflammation related to systemic inflammatory mediators occurs, it could account for the increased serum lipase and amylase levels observed in critically ill patients^[4,5]. Among several mechanisms suggested to explain how mechanical ventilation unfavorably results in acute pancreatitis, splanchnic hypoperfusion appears to be particularly important^[1].

A mortality comparison demonstrated a lower survival rate in PRRF than in VRP. Acute lung injury and ARDS, which have high mortality rates, are the most common manifestations of extra-abdominal organ dysfunction in patients with severe acute pancreatitis. The pathophysiology of ARDS is described as increased pulmonary vasculature leaking protein-rich transudate into the alveolar space and decreased lung compliance

clinically manifested as refractory hypoxemia, and radiologically as diffuse infiltration in the lungs. In the pathogenesis of systemic complications of pancreatitis, the role of active enzymes in circulation, the liberation of proinflammatory cytokines, decreased normal defense mechanisms, and the increased production of nitric oxide have been studied^[25,26]. The mortality and severity of the disease appear to be influenced by events occurring subsequent to the pancreatic injury as a result of the release of cytokines and other mediators. Hypoxemia is the most common sign presenting in patients with respiratory insufficiency resulting from severe acute pancreatitis; however, its presentation was not related to the development of atelectasis, pleural effusion, or pulmonary consolidation during the course of the disease. Severe hypoxemia is also a factor that predicts a poor prognosis. A recent study has shown that a baseline hypoxemia of less than 60 mmHg was a significant risk factor for pulmonary consolidation and ARDS, and can be used as a marker of poor outcome^[14]. Sustained systemic inflammatory states with multiple organ failure are frequently encountered and have a high attributable mortality rate^[27]. Progression to a shock state requiring vasopressor use or acute renal failure requiring renal replacement therapy is particularly ominous^[28,29].

Our study demonstrated that a high APACHE II score, low $\text{PaO}_2/\text{FiO}_2$, ARDS status, presence of SIRS criteria, acute renal failure, and the need for renal replacement therapy were predictors of outcome in patients with a diagnosis of ventilator-related pancreatitis; only a low $\text{PaO}_2/\text{FiO}_2$ level was an independent predictive factor as determined by multivariate logistic regression analysis. Thus, as mentioned above, hypoxemia or disease progression to ARDS are poor signs not only in PRRF patients but also in VRP patients.

To date, no specific management strategy has been proposed for acute pancreatitis with multiple organ failure other than intensive supportive treatment. The evidence available indicates that patients with severe acute pancreatitis do not benefit from therapy with available antisecretory drugs or protease inhibitors. Supportive therapy, such as vigorous hydration, analgesia, correction of electrolytes and glycemic disorders, and pharmacological or mechanical support targeted at specific organs, are still the mainstay of therapy^[30]. However, severe acute pancreatitis is still characterized by rapidly progressive multiple organ failure and high mortality, and both surgical and conservative therapies yield poor outcomes^[31]. Thus, most emphasis is placed on preventing the progression to multiple organ failure^[32,33].

Congestive heart failure was more common in VRP patients than in PRRF patients and was a good prognostic predictor for ICU mortality in patients with VRP. Acute pulmonary edema related to congestive heart failure was a frequent cause of respiratory failure in VRP patients; in contrast, the most common indication for intubation in patients with PRRF was ARDS. Heart failure-related pulmonary edema can often be reversed with diuretics and renal replacement therapy if there is concomitant renal failure. In contrast, there are no effective pharma-

cologic treatments for non-cardiogenic pulmonary edema associated with ARDS other than the treatment of the underlying disease. Conservative treatments with protective ventilatory strategies and fluid management may help to improve the hypoxemic status of patients with ARDS, however, these strategies had no benefit on mortality^[34,35]. This may explain, in part, why VRP patients in our study had better outcomes than PRPF patients and VRP patients with congestive heart failure had a better prognosis than those without congestive heart failure.

There are some limitations in this study. First, as this was a retrospective study, not every patient in the ICUs with abdominal pain had blood sampling for amylase and lipase levels. This ascertainment bias may have resulted in an overestimation of the incidence of VRP. Secondly, the Ranson score is widely used in predicting outcomes from severe acute pancreatitis^[36,38]. However, Ranson's criteria were not systematically collected in our cohort. Thus, we did not include Ranson's criteria as part of our analysis, therefore potentially limiting external validity. Third, although radiological imaging, particularly computed tomography, is valuable for diagnosis, risk stratification, and outcome prediction of acute pancreatitis^[36,39], not every patient had an imaging study to confirm acute pancreatitis in our study.

In conclusion, our findings suggested that low PaO₂/FiO₂ was an independent clinical parameter predictive of ICU mortality in patients with VRP. We also demonstrated that VRP was not associated with a higher mortality rate when compared with ICU patients with comparable disease severity but without pancreatitis and was associated with better outcomes than PRPF.

COMMENTS

Background

Mechanical ventilation is an important method in rescuing patients with respiratory failure, but it is also associated with numerous organ-system disorders. Acute pancreatitis may also be caused by mechanical ventilation, for which splanchnic hypoperfusion is considered the most important mechanism. However, the risk factors predictive of clinical outcomes and intensive care unit (ICU) mortality in patients with ventilator-related pancreatitis are still unclear. Thus, we conducted this study to clarify the clinical outcomes in patients with ventilator-related pancreatitis.

Research frontiers

Patients with severe acute pancreatitis may develop acute respiratory failure resulting in poor clinical outcomes. Such a concept is well documented. However, the notion of ventilator-related pancreatitis is not well understood, even though pancreatitis truly occurs after mechanical ventilation. We conducted this study to illustrate the different clinical outcome in patients with ventilator-related pancreatitis, pancreatitis related respiratory failure and equal severity ICU patients without pancreatitis.

Innovations and breakthroughs

The authors clarify the risk factors for predicting mortality in patients with ventilator-related pancreatitis. Short- and long-term outcomes in patients with ventilator-related pancreatitis are also illustrated by a comparison with patients who had pancreatitis-related respiratory failure and ICU patients admitted with equal physiological severity scoring without pancreatitis.

Applications

By understanding the nature of ventilator-related pancreatitis, the authors found that low PaO₂/FiO₂ was an independent clinical parameter predictive of ICU mortality in patients with ventilator-related pancreatitis. Despite the simultaneous diagnosis of acute respiratory failure and acute pancreatitis, patients with ventilator-related pancreatitis had better outcomes than patients

with pancreatitis-related respiratory failure.

Terminology

Ventilator-related pancreatitis is a disease in which pancreatitis occurs after mechanical ventilation by numerous mechanisms. Possible mechanisms include injurious ventilatory strategies, high pressure with hypovolemic status, and sympathetic stimulation mechanisms resulting in conditions such as pancreatic ischemia or pancreatic hypoperfusion are thought to be the cause of pancreatic injury.

Peer review

This interesting paper looks at a group of patients with a condition about which we know very little. They have coined the terms "ICU-related pancreatitis" and "ventilator-related pancreatitis", which are not terms currently in use but are reasonable. The findings of this study make sense.

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S- Editor Cheng JX L- Editor Webster JR E- Editor Lin YP



Study of glue extrusion after endoscopic N-butyl-2-cyanoacrylate injection on gastric variceal bleeding

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Received: June 5, 2009 Revised: August 25, 2009

Accepted: September 1, 2009

Published online: October 21, 2009

Abstract

AIM: To investigate glue extrusion after endoscopic N-butyl-2-cyanoacrylate injection on gastric variceal bleeding and to evaluate the long-term efficacy and safety of this therapy.

METHODS: A total of 148 cirrhotic patients in our hospital with esophagogastric variceal bleeding (EGVB) were included in this study. N-butyl-2-cyanoacrylate was mixed with lipiodol in a 1:1 ratio and injected as a bolus of 1-3 mL according to variceal size. Patients underwent endoscopic follow-up the next week, fourth week, second month, fourth month, and seventh month after injection and then every 6 mo to determine the cast shape. An abdominal X-ray film and ultrasound or computed tomographic scan were also carried out in order to evaluate the time of variceal disappearance and complete extrusion of the cast. The average follow-up time was 13.1 mo.

RESULTS: The instantaneous hemostatic rate was 96.2%. Early re-bleeding after injection in 9 cases (6.2%) was estimated from rejection of adhesive. Late re-bleeding occurred in 12 patients (8.1%) at 2-18 mo. The glue cast was extruded into the lumen within one month in 86.1% of patients and eliminated within one year. Light erosion was seen at the injection position and mucosa edema in the second week. The glue casts were extruded in 18 patients (12.1%) after one week

and in 64 patients (42.8%) after two weeks. All kinds of glue clumping shapes and colors on endoscopic examination were observed in 127 patients (86.1%) within one month, including punctiform, globular, pillar and variform. Forty one patients (27.9%) had glue extrusion after 3 mo and 28 patients (28.9%) after six months. The extrusion time was not related to the injection volume of histoacryl. Obliteration was seen in 70.2% (104 cases) endoscopically. The main complication was re-bleeding resulting from extrusion. The prognosis of the patients depended on the severity of the underlying liver disease.

CONCLUSION: Endoscopic injection of cyanoacrylate is highly effective for gastric varices bleeding. The glue clump shape is correlated with anatomic structure of vessels. The time of extrusion was not related to dosage of the glue.

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Key words: Gastric variceal bleeding; Glue extruded; N-butyl-2-cyanoacrylate; Portal hypertension

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Wang YM, Cheng LF, Li N, Wu K, Zhai JS, Wang YW. Study of glue extrusion after endoscopic N-butyl-2-cyanoacrylate injection on gastric variceal bleeding. *World J Gastroenterol* 2009; 15(39): 4945-4951 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4945.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4945>

INTRODUCTION

Although the outcome of variceal hemorrhage has improved over the past two decades, variceal hemorrhage is still the most serious complication of portal hypertension and chronic liver disease^[1-3]. Gastric varices (GV) and their association with portal hypertension were first described in 1913^[4]. Since then, there have been reports on different aspects of gastric varices including prevalence, bleed tendency and treatment options. Gastric varices occur in 20% of patients with portal hypertension either in isolation or in combination

with esophageal varices (EV)^[5-6]. GV possibly bleed less frequently than EV, but GV bleeding is typically difficult to control, and is associated with a high risk for re-bleeding and high mortality. Fundal varices, large GV (> 5 mm), presence of a red spot, and Child's C liver status are associated with a high risk for bleeding^[7-8]. gastro-esophageal varices type 1 (GOV1) have a much lower risk for bleeding. A portosystemic pressure gradient of > 12 mmHg is not necessary for GV bleeding, probably related to the high frequency of spontaneous gastrosplenic shunts in these patients^[9]. There is no consensus for the optimum treatment of GV including drugs, endoscopy, and surgery. Optimal management of GV requires a multidisciplinary approach and close cooperation between gastroenterologists, interventional radiologists and the surgical team.

Histoacryl (N-butyl-cyanoacrylate) is the only endoscopic treatment that has been shown to be effective. Sohendra *et al*^[10] first reported in 1986 that bleeding from GV could be controlled by sclerotherapy using the tissue adhesive agent butyl cyanoacrylate. Since then several authors have used different sclerosing agents to achieve hemostasis in bleeding gastric varices, including N-butyl-2 cyanoacrylate (histoacryl)^[11,12], 2-octyl cyanoacrylate^[13], ethanolamine oleate injection^[14], gastric variceal banding^[15], thrombin^[1] and sodium tetradecyl sulfate^[16]. However, N-butyl 2 cyanoacrylate (NBC) is the only promising agent. Cyanoacrylate injection can achieve primary hemostasis in 70% to 95% of patients with acute GV bleeding, with an early re-bleeding rate ranging from 0% to 28% within 48 h^[17-19].

To date, there are many studies on the efficacy and hemostasis rate of NBC injection on gastric variceal bleeding. However, there are no reports on detailed gel extrusion after injection. In this study, we investigated the glue extrusion after endoscopic NBC injection for gastric variceal bleeding and evaluated the long-term efficacy and safety of this therapy to define its role in initial treatment.

MATERIALS AND METHODS

Materials preparation

A 1:1 (v/v) mixture of N-butyl-2-cyanoacrylate (Histoacryl blue; B. Braun-Melsungen, Germany, 0.5 mL per ampoule) and lipiodol (Laboratoire Guerbet, Aulnay-Sous-Bois, France) was prepared just before injection. An endoscopic injector (hemostasis catheter) with a 23-gauge needle (Optiflo, Boston Scientific, Boston, USA) was used.

Patients

From June 2007 to December 2008, total of 148 cirrhotic patients with or without concomitant hepatocellular carcinoma (HCC) who presented to our hospital with acute gastrointestinal bleeding, or who were already hospitalized and developed acute gastrointestinal bleeding, were subjected to injection of the mixture, except those with severe encephalopathy, severe hemodynamic instability, pregnancy, or those who refused treatment. Active hemorrhage was defined

as bleeding or oozing of blood from a gastric varix, a clot or blackish ulcer or rent over a gastric varix, or the presence of distinct large GV and absence of EV or other causes of gastrointestinal (GI) bleeding. Liver function was classified according to the Child-Pugh classification criteria. The most common cause of GV was viral hepatitis-related cirrhosis (74.5%). The modality and location of the GV were classified according to the system proposed by Hashizume *et al*^[19]; the modality was classified into 3 types: tortuous (F1), nodular (F2), and tumorous (F3); and location, 5 areas: anterior (La), posterior (Lp), lesser (Ll) and greater curvature (Lg) of the cardia, and the fundic area (Lf). Most of the GV were large. Associated EV were usually small or absent. The most common locations of these GV were the fundus (Lf) and the most proximal body on the posterior wall (Lp) (94.4%).

The location of GV was determined according to the classification described by Sarin *et al*^[20] and divided into gastroesophageal varices type 1 (GOV-1), GV continuing as an extension of EV on the lesser curve of the stomach), gastro-esophageal varices type 2 (GOV-2) on the greater curvature or fundal varices communicating with EV, isolated gastric varices type 1 (IGV-1) and fundal varices within a few centimeters of the gastric cardia, or isolated gastric varices type 2 (IGV-2) and isolated ectopic gastric varices.

NBC was mixed with lipiodol in a 1:1 ratio and injected as a bolus of 1 to 3 mL according to variceal size. Thirty patients had isolated GV, 38 had postoperative residual GV, and 72 had dominant GV. The actual volume of the injection was based on the variceal appearance. The injection was stopped when the varices became engorged. Lipiodol was used to flush the injection needle before and after the injection. The tip of the endoscope and the accessory channel were treated with silicone oil to prevent endoscopic damage. All patients received octreotide infusion (50 mg/h) at admission and was continued for 3 d. All patients were given a proton pump inhibitor, initially intravenously for 48 h and then orally for 4-6 wk.

Indices to evaluate the extrusion rule of glue

Indices to evaluate the extrusion rule of glue are as follows: (1) Information on each patient's gender, age, Child-Pugh classification, stage of GV, number of treatments, dosage of cyanoacrylate, outcome of GV, diameter of main portal tract, liver function and routine blood tests were collected; (2) An X-ray film was obtained three days after injection to determine the contour of the histoacryl cast. A chest X-ray was also obtained to ascertain whether any embolic material had migrated into the chest. Ultrasound and/or computed tomographic scan of the abdomen were carried out within one week; (3) Patients underwent endoscopic follow-up the next week, fourth week, second month, fourth month, and seventh month after injection and then every 6 mo to determine the cast shape. An abdominal X-ray film and ultrasound or computed tomographic scan were also obtained in order to

Table 1 Baseline characteristics of the patients

Variables at baseline	n (%)
Total number	148
Median age (yr)	50.13 ± 13.55, (14-78)
Sex ratio (M:F)	108:40
Underlying cause	
Viral hepatitis B	87 (58.8)
Viral hepatitis C	7 (4.7)
Alcoholic cirrhosis	14 (9.4)
Primary biliary cirrhosis	6 (4.1)
Drug-induced liver cirrhosis	2 (1.3)
Budd-Chiari syndrome	3 (2.0)
CTPV	4 (2.7)
Wilson's disease	1 (0.67)
Unknown etiology	24 (16.2)
Association with other diseases	
Type-2 diabetes	16 (10.8)
Primary hepatic carcinoma	12 (8.1)
Hypertensive disease	9 (6.1)
Portal vein thrombogenesis	3 (2.0)
Clinical classification	
Isolated GV	30 (20.3)
EGV received EVS or EVL	72 (48.6)
Residual GV after EVS or EVL	20 (13.5)
GV bleeding after disconnection	18 (12.2)
Other condition	8 (5.4)
Sarin's category (%)	
GOV-1	68 (45.9)
GOV-2	49 (33.1)
IGV-1	30 (20.3)
IGV-2	1 (0.67)
Child-Pugh class	
A/B/C	42/73/33

GV: Gastric varices; EGV: Esophagogastric variceal; GOV: Gastroesophageal varices; IGV: Isolated gastric varices; CTPV: Cavernous transformation of the portal vein.

evaluate the time of variceal disappearance and complete extrusion of the cast; (4) The instant hemostasis rate, obliteration rate and re-bleeding rate were analyzed. Initial hemostasis was defined as a condition in which the vital signs were stable and no recurrent bleeding was noted for 48 h after the first injection. The definition of re-bleeding from GV included the following endoscopic conditions: spurting or oozing bleeding from GV or blood pools in the stomach accompanied by a fibrin cap on the GV. Primary success was defined as the absence of recurrent bleeding after the first histoacryl injection and during the entire follow-up period. Secondary success was defined as the absence of recurrent bleeding after the reinjection of histoacryl for recurrent bleeding. Definitive hemostasis included both primary and secondary success. Treatment failure was defined as failure to obtain definitive hemostasis.

Complications

Major complications included fever, chest pain, esophageal stricture, septicemia, mediastinum inflammation, pulmonary embolism, cerebral infarction, portal vein embolization, aspirated pneumonia and re-bleeding. The average follow-up time was 13.1 mo.

Statistical analysis

Statistical interpretation of data was performed using

Statistical Program for Social Sciences (SPSS) version 13. Results were expressed as mean ± SD, median (MD) for all continuous variables (e.g. age, gender, hospital stay, units of packed cells *etc*) and numbers (percentage) for categorical data (e.g. gender, Child's class, *etc*). Analysis was performed using the independent *t*-test, χ^2 test and Fisher's exact test wherever appropriate. *P* < 0.05 was considered statistically significant.

RESULTS

A total of 148 patients with liver cirrhosis and EGV at our hospital from June 2007 to December 2008 were subjected to the injection. This was a retrospective review of their records.

Clinical condition

According to Sarin's classification of GV, GOV-1 was detected in 68 patients (45.9%), GOV-2 in 49 (33.1%), IGV-1 in 30 (20.3%) and IGV-2 in 1 (0.67%). The common cause of GOV was liver cirrhosis and was segmental portal hypertension without liver disease in IGV. The majority of injections in this series were executed in selective cases, with the exception of 25 cases with acute bleeding who received emergent injections. The primary hemostatic rate was 96.2% (142/148). Among the 148 patients, 6 patients did not achieve hemostasis. The average number of sessions required to eradicate the GV was 2.9 ± 1.2 . It was observed that at follow-up (1-18 mo) the injected glue was rejected from GV, resulting in eradication. Early re-bleeding after injection in 9 cases (6.2%) was estimated from rejection of the adhesive. Late re-bleeding occurred in 12 patients (8.1%) at 2-18 mo. Some patients were treated with a second injection to successfully control the bleeding (Tables 1 and 2).

Vascular structure

Three-dimensional computed tomography was used in ten patients after injection in order to understand the vascular structure of GV. There were two types: one was composed of one blood vessel without obvious branches. The diameter of the influent vein and effluent vein were almost identical. The other was composed of many blood vessels with complex branches. Endoscopic feature of GV were related to their vascular structure. The former was common in regional GV (86%) and the latter in GV with a diffuse pattern (91%).

Glue extrusion

At follow-up (1-18 mo), it was observed that the injected glue was rejected from GV. The cast of glue was extruded into the lumen after one or two weeks, generally without resultant hemorrhage. An X-ray film was obtained three days after the injection to determine the contour of the histoacryl cast. It showed that all the GV were full of the histoacryl and lipiodol mixture, and 13 patients had mixture in the inferior segment of the esophagus. There were all kinds of glue clumping shapes including globular, trabs and flower shapes. The

Table 2 Results of histoacryl injection

Variables	n = 148 (%)
Number of bleeding times	
One time	49 (33.1)
Two times	38 (25.6)
Three times	18 (12.2)
Four times	9 (6.1)
Five times	6 (4.1)
≥ six times	17 (11.5)
Unidentified	11 (7.4)
Emergency endoscopy	25 (16.9)
Active gastric variceal bleeding	21 (14.2)
Rescue endotherapy	16 (10.8)
Number of injections	164
Volume of the first glue injection (mL)	
1.0	43 (29.1)
2.0	59 (39.8)
3.0	23 (15.5)
4.0	18 (12.2)
5.0	4 (2.7)
6.0	1 (0.7)
Location of the first injection	
Inferior esophagus	8 (5.4)
Cardia of stomach	36 (24.3)
Fundus of stomach	103 (69.6)
Descending segment of duodenum	1 (0.7)
Multiple injections required	15 (10.1)
Primary hemostasis rate	142 (96.2)
Re-bleeding rate	21 (14.1)
Obliteration rate	104 (70.2)

Table 3 Extrusion time of glue in 148 patients

Time of extrusion	Cumulative glue extrusion rate in numbers (%)
One week	18 (12.2)
Two weeks	64 (43.2)
One month	127 (85.8)
Three months	41 (27.7)
Six months	28 (18.9)

Table 4 The glue clumping shape on X ray examination and endoscopic examination in the fourth week

The glue clumping shape	n (%)
X ray examination	
Globular	28 (18.9)
Cord	96 (64.8)
Flower	24 (16.3)
Endoscopy examination	
Punctiform	36 (24.3)
Globular	88 (59.4)
Cord	24 (16.3)
The color of extrusion in endoscopy	
Black	23 (15.5)
Yellow	77 (52.1)
Brown	48 (32.4)

X ray examination (Figure 1).

One hundred and twenty eight patients underwent endoscopic follow-up in the second week, fourth week, second month, fourth month, and seventh month after injection and every 6 mo thereafter to determine the cast shape. Light erosion was seen at the injection position and mucosa edema in the second week. The glue casts were extruded in 18 patients (12.1%) after one week and in 64 patients (42.8%) after two weeks. All kinds of glue clumping shapes and colors on endoscopic examination were observed in 127 patients (86.1%) within one month, including punctiform, globular, pillar and variform. Forty one patients (27.9%) had glue extrusion after three months and 28 patients (28.9%) after six months. The extrusion time was not related to injection volume of histoacryl (Tables 3 and 4, Figures 2 and 3).

Prognosis

Twenty one patients (14.1%) had re-bleeding. Five patients died from re-bleeding, hepatic failure or HCC aggravation. Lipiodol was seen flowing into lung vessels during injection in 1 with no serious cough or signs of ectopic embolism. The obliteration rate was 70.2% (104 cases) as shown on endoscopic examination. The determining factor for long-term survival was the underlying disease leading to portal hypertension.

DISCUSSION

The majority of patients with cirrhosis will develop varices during their lifetime. Once the diagnosis of cirrhosis has been made, the incidence of new varices is 5% per year and these will progress from small to large varices at a rate of 10 to 15% per year. Growth seems to be influenced by the progression of liver failure. At least

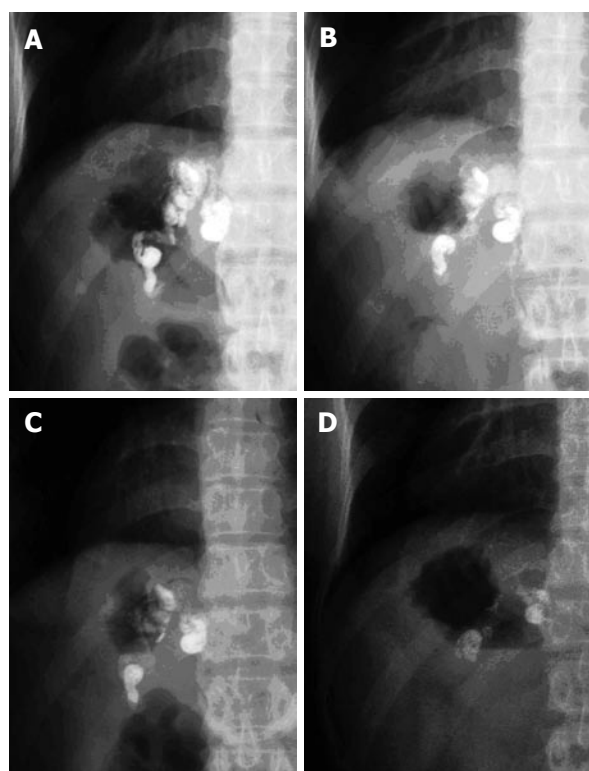


Figure 1 This 47-year-old female patient has isolated gastric varices. Expulsion of tissue glue on X-ray view. A: One day after injection; B: Two weeks after injection; C: One month after injection; D: Three months after injection. Lipiodol mass (intravascular) was in the stomach only and became smaller gradually. This indicated the extrusion process of glue.

contrast was smaller after one month in 132 patients and disappeared in 138 patients within one year as shown on

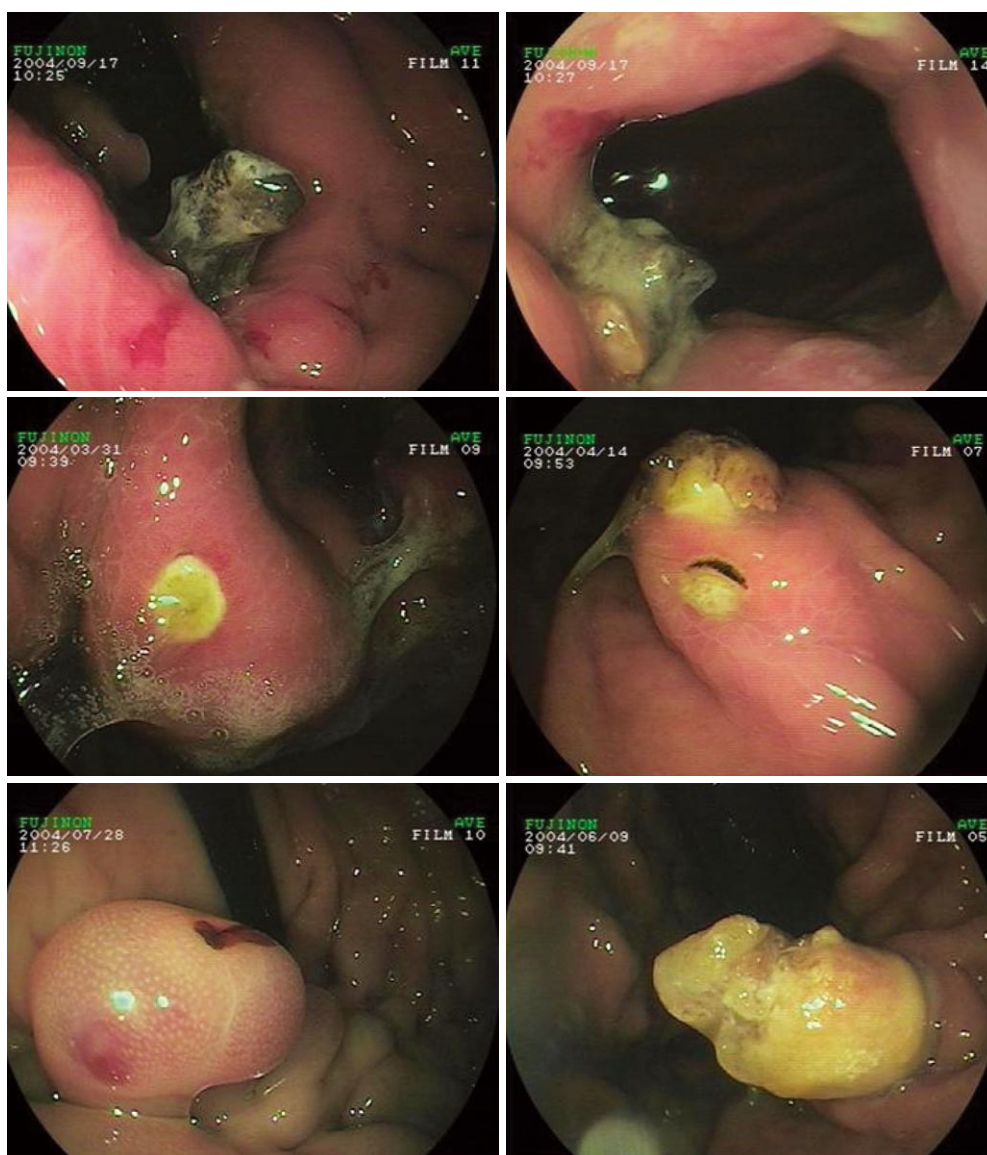


Figure 2 Different colors and shapes of contrast on endoscopic examination. A gastric mucosal defect can be seen with expulsion of the adhesive plug from the thrombosed gastric varix several weeks after endoscopic treatment with N-2-butyl-cyanoacrylate.

one-third of these patients will bleed from their varices and despite significant improvements in treatment and diagnosis, the mortality rate still remains high (30%).

China is one of the countries with the highest incidence of gastric variceal hemorrhage. Esophagogastric variceal bleeding (EGVB) is a serious and emergent condition and hemorrhage control in order to decrease death rate is important. Variceal bleeding results in considerable morbidity and mortality. Although GV bleeding occurs less frequently than EV bleeding^[20], whenever bleeding occurs it tends to be more severe and requires more red blood cell transfusions and has a higher mortality rate than EV bleeding^[21]; after control of acute bleeding, GV has a high re-bleeding rate of 34% to 89%^[22,23]. The principle of initial treatment is to achieve hemostasis and prevent bleeding-related complications such as renal failure, infection, and hepatic decompensation. Cyanoacrylate injection has been proved in large series to be a safe and effective therapy for gastric variceal bleeding.

A total 148 cirrhotic patients with EGV at our hospital from June 2007 to December 2008 were subjected to this treatment. Cyanoacrylate was injected intravariceally in a 1:1 mixture with lipiodal. Each patient underwent scheduled or emergent endoscopic injection of cyanoacrylate and were counterchecked on the 7th day, 1st month and 3rd month after the first treatment, respectively. Cyanoacrylate is a liquid with a consistency similar to water, which rapidly polymerizes on contact with blood. Although the adhesive is injected in a manner similar to sclerosants, extra precautions must be taken before its use to prevent damaging the endoscope. Silicone oil, oil-based contrast agents, simethicone or even olive oil should be used to flush the tip of the endoscope as well as the entire biopsy channel^[24,25]. The glue was rejected into the gastric cavity after one or two weeks without re-bleeding. The cast of glue was extruded into the lumen as a foreign body. Glue was extruded in 86.1% of patients within one month with all kinds of shapes and colors of contrast.

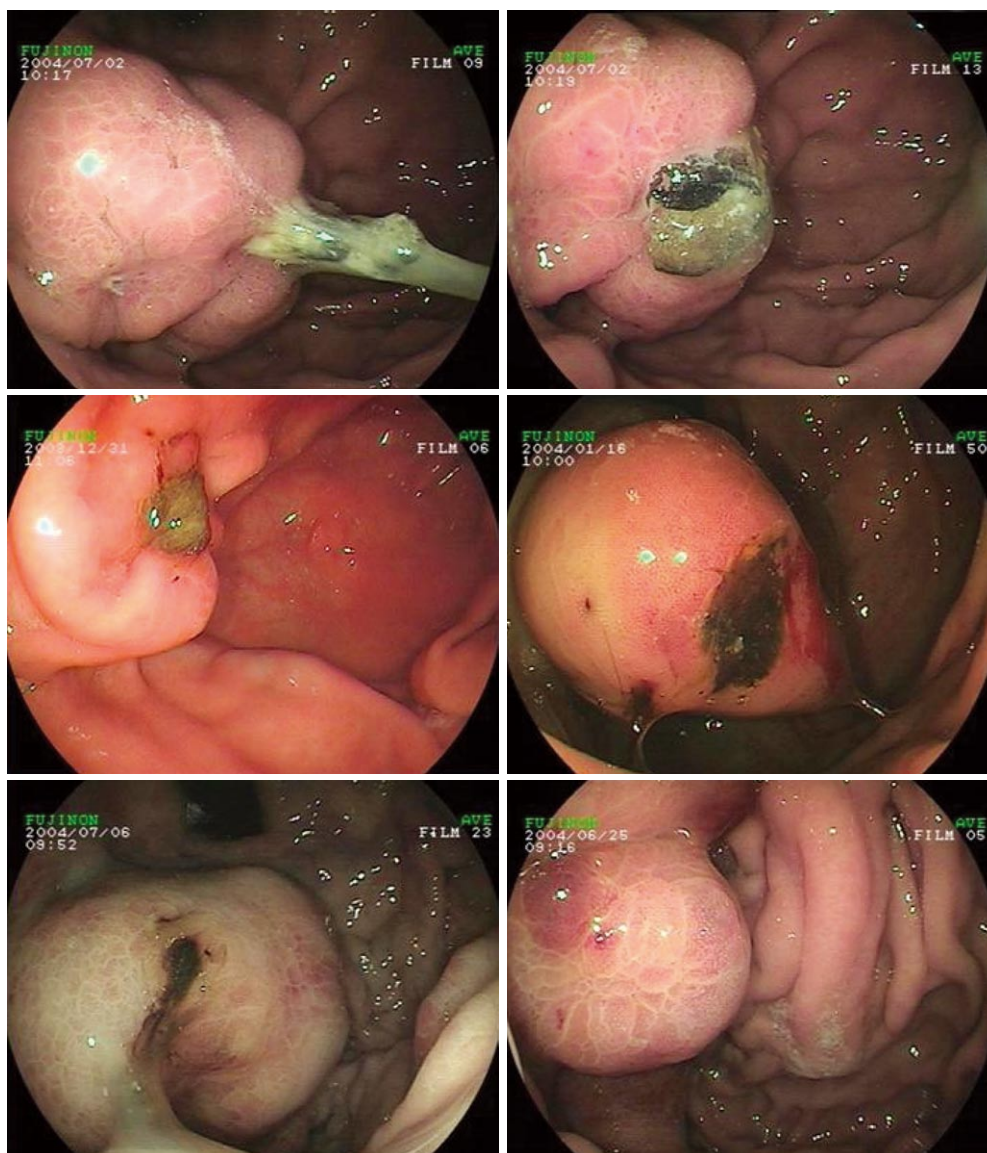


Figure 3 Different colors and shapes of contrast on endoscopic examination.

The rejection had no adverse effects. The glue was extruded in 18 patients (12.1%) within one week and in 64 patients (42.8%) within two weeks. All kinds of glue clumping shapes and colors were seen on endoscopic examination in 127 patients (86.1%) within one month, including punctiform, globular, pillar and variform. Forty one patients (27.9%) had glue extrusion after three months and 28 patients (28.9%) after six months. The extrusion time was not related to the injection volume of histoacryl. We did not observe contrast due to glue in six patients as a result of mucosal crimple.

We found re-bleeding in 9 patients due to glue extrusion. These patients were examined by endoscopy and erosion and ulcer at the injection site were noted. However, the volume of blood was not large. Time to re-bleeding was about one or two months. We administered antacid drugs, and in some, endoscopy therapy. All the patients recovered. Both follow up and supplementary treatment are important in maintaining and improving long-term outcomes. Repeated sclerotherapy using NBC is effective for remnant GV. The formation of collateral

veins around the cardia region after obliteration of gastric short veins or the posterior gastric vein may lead to new EV, which can be managed by conventional endoscopic or surgical measures.

Endoscopic injection of cyanoacrylate is highly effective in the treatment of gastric variceal hemorrhage, with only a few acute and long-term complications. This treatment modality may be referred to as the first choice for bleeding GV.

COMMENTS

Background

Esophagogastric variceal bleeding (EGVB) is a serious and emergent condition of portal hypertension and results in considerable morbidity and mortality. Gastric varices (GV) occur in 20% of patients either in isolation or in combination with esophageal varices (EV). GV possibly bleed less frequently than EV, but GV bleeding is typically difficult to control, and is associated with a high risk for re-bleeding and high mortality. There is no consensus on the optimum treatment of GV including drugs, endoscopy, and surgery. Optimal management of GV requires a multidisciplinary approach and close cooperation between gastroenterologists, interventional radiologists and the surgical team.

Sohendra *et al* first reported in 1986 that bleeding from GV could be controlled by sclerotherapy using the tissue adhesive agent butyl cyanoacrylate.

Research frontiers

Cyanoacrylate injection can achieve primary hemostasis in 70% to 95% of patients with acute GV bleeding, with an early re-bleeding rate ranging from 0% to 28% within 48 h. To date, there are many studies on the efficacy and hemostasis rate of N-butyl-2-cyanoacrylate injection on gastric variceal bleeding. However, there are no reports on detailed gel extrusion after injection.

Innovations and breakthroughs

Gastric variceal bleeding can be challenging to the clinician. Tissue adhesives can control acute bleeding in over 80% of patients, with re-bleeding rates of 20%-30%, and should be the first-line therapy where available. In this study, we investigated the glue extrusion after endoscopic N-butyl-2-cyanoacrylate injection for gastric variceal bleeding and evaluated the long-term efficacy and safety of this therapy to define its role in the initial treatment.

Applications

By understanding how the glue is extruded and the long-term efficacy and safety of endoscopic N-butyl-2-cyanoacrylate injection, this study may represent a better therapeutic intervention in the treatment of patients with gastric variceal bleeding.

Terminology

Gastroesophageal varices type 1 (GOV-1) are GV continuing as an extension of EV on the lesser curve of the stomach, gastro-esophageal varices type 2 (GOV-2) are found on the greater curvature or fundal varices communicating with EV. Isolated gastric varices type 1 (IGV-1) and fundal varices are found within a few centimeters of the gastric cardia, or isolated gastric varices type 2 (IGV-2) and isolated ectopic gastric varices.

Peer review

Looking at the rate of glue extrusion and its association with re-bleeding is quite a novel way of reporting the success of an old technique in arresting gastric variceal bleeding.

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BRIEF ARTICLE

Inhibitory effects of genistein on metastasis of human hepatocellular carcinoma

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Author contributions: Gu Y and Zhu CF contributed equally to this work; Gu Y and Dai YL designed the research; Zhu CF, Zhong Q and Sun B performed the research; Gu Y and Zhu CF analyzed the data and wrote the paper; Dai YL corrected the manuscript.

Supported by Grants From Shanghai Municipal Health Bureau, No. 054052 and Shanghai Municipal Commission of Science and Technology, No. 02JC14001

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Received: August 2, 2009 Revised: August 31, 2009

Accepted: September 7, 2009

Published online: October 21, 2009

xenograft transplantation of hepatocellular carcinoma was also significantly suppressed by genistein. The number of pulmonary micrometastatic foci in the genistein group was significantly lower compared with the control group (12.3 ± 1.8 vs 16.6 ± 2.6 , $P < 0.05$).

CONCLUSION: Genistein appears to be a promising agent in the inhibition of metastasis of hepatocellular carcinoma.

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Key words: Human hepatocellular carcinoma; Genistein; Metastasis

Peer reviewer: Samuel B Ho, Professor, Gastroenterology, VA San Diego Healthcare System, San Diego 92161, United States

Gu Y, Zhu CF, Dai YL, Zhong Q, Sun B. Inhibitory effects of genistein on metastasis of human hepatocellular carcinoma. *World J Gastroenterol* 2009; 15(39): 4952-4957 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4952.asp>
DOI: <http://dx.doi.org/10.3748/wjg.15.4952>

Abstract

AIM: To investigate the inhibitory effects of genistein on metastasis of MHCC97-H hepatocellular carcinoma cells and to explore the underlying mechanism.

METHODS: MHCC97-H hepatocellular carcinoma cells were exposed to genistein. A cell attachment assay was carried out in a microculture well pre-coated with fibronectin. The invasive activity of tumor cells was assayed in a transwell cell culture chamber, and cell cycle and apoptosis were evaluated by a functional assay. In addition, the expression and phosphorylation of FAK were detected by Western blotting. *In situ* xenograft transplantation of hepatocellular carcinoma was performed in 12 nude mice and lung metastasis of hepatocellular carcinoma was observed.

RESULTS: Genistein significantly inhibited the growth of MHCC97-H cells *in vitro*. Adhesion and invasiveness of MHCC97-H cells were inhibited in a concentration-dependent fashion, and the inhibitory effect of genistein was more potent in the 10 μ g/mL and 20 μ g/mL genistein-treated groups. Genistein caused G₀/G₁ cell cycle arrest, an S phase decrease, and increased apoptosis. The expression and phosphorylation of FAK in MHCC-97H cells were significantly decreased. *In situ*

INTRODUCTION

As a common malignancy, hepatocellular carcinoma (HCC) is chemoresistant to most currently available chemotherapeutic agents, and is the leading cause of cancer related deaths in the world, with increasing incidence in many countries^[1]. In China, primary liver cancer, of which more than 90% is HCC, remains the second leading cancer killer. HCC mainly affects middle-aged people, those in the prime of their most productive years^[2]. The high incidence of metastasis accounts for the poor overall survival in HCC patients. Research into interventions for liver cancer metastasis has special priority in the anti-cancer campaign. The MHCC97-H hepatocellular carcinoma cell line has high metastatic potential and was established from a subcutaneous tumor in a high-metastatic-potential model of human HCC cells in BALB/c nu/nu mice (LCI-D20). Lung is the preferential metastasis target of MHCC97-H cells^[3].

In this study, we investigated the inhibitory effects of genistein on metastasis of human HCC cells and explored the underlying mechanism.

MATERIALS AND METHODS

Cell culture and genistein

The human HCC cell line, MHCC97-H, was obtained from the Liver Cancer Institute of Fudan University in Shanghai. The cells were cultured at 37°C in 50 mL/L CO₂ air in high glucose Dulbecco's Modified Essential Medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA). Genistein (5,7,4'-trihydroxyisoflavone) purchased from Sigma Chemical Co. (St. Louis, MO, USA) was suspended in dimethyl sulfoxide (DMSO) for the experiments.

In vitro assays of MHCC97-H cell growth

A 96-well plate was incubated with exponentially growing cells at a density of 1×10^4 per well. Following incubation of MHCC97-H cells with or without genistein in different columns of 96-well microtiter plates, on day 1-6 methyl thiazol terazolium (MTT) was added to each well and incubated at 37°C for another 4 h before spectrophotometric detection (A_{595}). Each assay was performed in quadruplicate.

The inhibitory rate of tumor cell growth was calculated as: (average A_{595} value of control group-average A_{595} value of genistein group)/average A_{595} value of control group^[4].

In vitro assays of MHCC97-H cell adhesion and invasion

Pre-coating of 96-well microtiter plates was performed by incubating wells with 20 mg/L fibronectin at 4°C overnight. The wells were then blocked with 2% bovine serum albumin (BSA) for 45 min at 37°C. Cells were cultured in medium (DMEM with 2% FBS) for 24 h, then harvested at about 70% confluence, resuspended in serum-free DMEM medium supplemented with 0.1% BSA and distributed in the wells (8×10^4 cells/well). The cells were incubated at 37°C in a 50 mL/L CO₂ atmosphere for 20, 40, 60 and 90 min with or without genistein. The wells were washed 3 times with phosphate buffered saline (PBS) to remove unattached cells, and the attached cells were then incubated with MTT and the A_{595} was measured.

The invasive activity of MHCC97-H cells was assayed in Transwell cell chambers (Corning Inc., Corning, NY, USA), according to the method reported by Kido *et al*^[5]. Polyvinylpyrrolidone-free polycarbonate filters with an 8.0 µm pore size were pre-coated with 5 µg of fibronectin in a volume of 50 µL on the lower surface. The Matrigel was diluted to 100 µg/mL with cold PBS, applied to the upper surface of the filters (5 µg/filter), and dried overnight under a hood at room temperature. Log-phase cell cultures of MHCC97-H cells were harvested and washed 3 times with serum-free DMEM, then resuspended at a final concentration of 2×10^6 cells/mL in DMEM with 0.1% BSA. Cell suspensions (100 µL) with or without genistein were added to the upper compartment and incubated for 20 h at 37°C in a 50 mL/L CO₂ atmosphere. The filters were fixed with methanol and stained with Giemsa. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells invading the lower surface of the filter through the Matrigel and

the filter were manually counted under a microscope at a magnification of $\times 400$. Each assay was performed in triplicate. The inhibitory rate of adhesion and invasion were calculated^[4].

Cell cycle analysis

MHCC97-H cells were seeded at a density of 5×10^5 cells/well in six-well dishes. After 24 h the cells were treated with or without genistein for 72 h and harvested by trypsinization. The cells were then centrifuged at 300 *g* for 10 min, washed in PBS, and resuspended in cold 70% ethanol. The cells were then subjected to flow cytometric analysis on a FACScan cytofluorimeter (Becton Dickinson, Franklin Lakes, NJ, USA) after propidium iodide labeling.

Western blotting analysis

Control and genistein-treated cell extracts were prepared and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis using a FAK monoclonal antibody (1:1000; SC-713; Cell signaling, USA) and pFAK polyclonal antibody (1:500; SC-6243; Cell signaling, USA) as primary antibodies. Anti-rabbit/mouse immunoglobulin G (IgG)-HRP (Beyotime Biotech, China) was used as a secondary antibody, followed by the detection of chemiluminescence using chemiluminescence kits (Beyotime Biotech, China). The optical density was determined using a scanning densitometer and analyzed using Quantity One software (Bio-Rad). House-keeping gene β -actin was used as an internal standard.

In vivo experiments

Male athymic BALB/c nu/nu mice (46-wk-old), were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science and maintained in specific pathogen-free (SPF) conditions and fed with sterilized MF pellets and distilled water. All studies on the mice were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals". The mouse study protocol was approved by the Shanghai Medical Experimental Animal Care Commission.

MHCC97-H cells (5×10^6) in 0.2 mL of serum-free culture medium were injected subcutaneously into the upper flank region of nude mice, and the mice were observed for tumor growth. When a subcutaneous tumor had reached approximately 1.5 cm in diameter, a small piece was removed and cut into pieces of approximately 1 mm \times 1 mm \times 1 mm which were subsequently implanted into the livers of 12 new recipient nude mice by a method described previously^[6]. Of the 12 recipient nude mice, bearing an orthotopic tumor implant, 6 were randomly selected for treatment with genistein and the remaining 6, which did not receive genistein treatment, served as controls. Genistein (50 mg/kg) was administered intraperitoneally to each mouse in the genistein group daily for 20 d, while control animals were administered the same vehicle. The mice were observed for 35 d, and then killed by cervical dislocation. The liver and lungs were excised during autopsy, lungs were fixed and embedded in paraffin and coronal sections were cut.

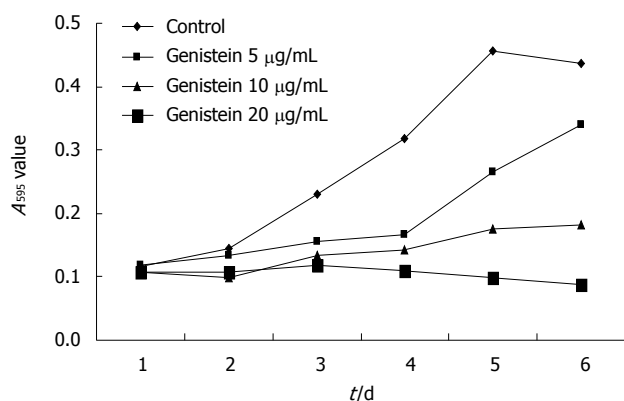


Figure 1 Inhibitory effects of genistein on MHCC97-H cell proliferation. MHCC97-H cells were treated with various concentrations of genistein for 6 d, control cells were left untreated, A_{595} values were determined daily.

Table 1 Adhesion of MHCC97-H cells to fibronectin coated microtiter plates

	20 min	40 min	60 min	90 min
Control	0.309 ± 0.16	0.432 ± 0.17	0.548 ± 0.12	0.717 ± 0.06
5 µg/mL genistein	0.181 ± 0.04 ^a	0.267 ± 0.06 ^a	0.341 ± 0.02	0.341 ± 0.11 ^b
10 µg/mL genistein	0.108 ± 0.02 ^b	0.219 ± 0.01 ^b	0.257 ± 0.02 ^a	0.367 ± 0.01 ^b
20 µg/mL genistein	0.142 ± 0.07 ^a	0.201 ± 0.04 ^b	0.225 ± 0.04 ^a	0.298 ± 0.09 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control. The A_{595} for MHCC97-H cells treated with or without genistein in fibronectin pre-coated 96-well microtiter plates was measured at 20, 40, 60 and 90 min. Values are A_{595} mean ± SD from experiments.

After hematoxylin and eosin staining, micrometastatic foci were counted microscopically.

Statistical analysis

All data are the mean ± SD. The statistical significance of differences between the treated and control groups were determined by applying the one-way ANOVA and χ^2 test. The statistical analysis software package Stata 6.0 was used for the tests, and $P < 0.05$ was considered statistically significant.

RESULTS

Effects of genistein on MHCC97-H cell growth

Genistein significantly inhibited MHCC97-H cell growth over the 6-d experimental period. The inhibitory rate of tumor cell growth in the 5, 10 and 20 µg/mL genistein groups was 22.3%, 58.2%, and 80.1%, respectively. The inhibitory rate of MHCC97-H cell growth in the 10 and 20 µg/mL genistein groups was significantly higher than the 5 µg/mL genistein group ($P < 0.05$). The concentration-dependent effects of genistein on MHCC97-H cell growth are shown in Figure 1.

In vitro effects of genistein on adhesion and invasion of MHCC97-H cells

The A_{595} for MHCC97-H cells treated with or without

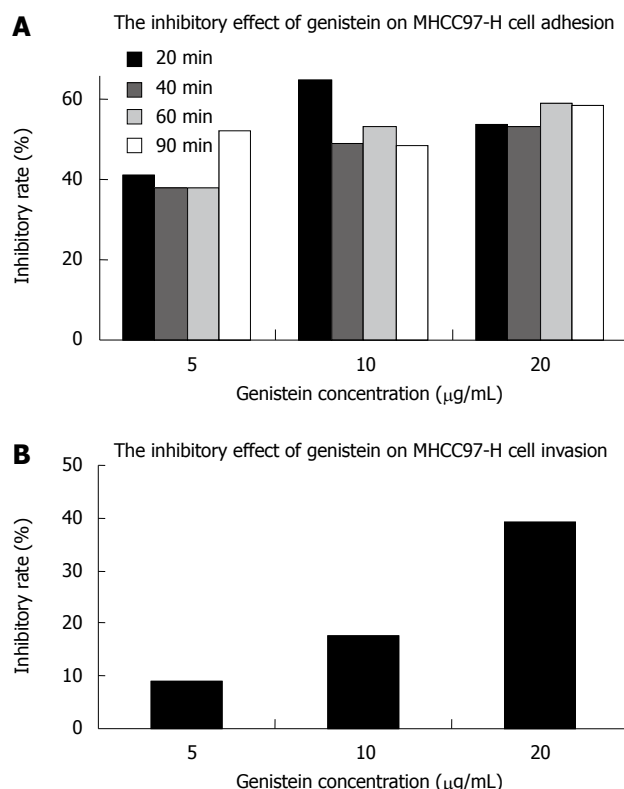


Figure 2 Inhibitory effects of genistein on adhesion and invasion of MHCC97-H cells. A: MHCC97-H cells were incubated for 20, 40, 60 and 90 min with various concentrations of genistein; B: MHCC97-H cells were incubated for 20 h with various concentrations of genistein.

genistein in fibronectin pre-coated 96-well microtiter plates at 20, 40, 60 and 90 min is shown in Table 1. Our results showed that genistein significantly inhibited tumor cell adhesion to fibronectin-coated substrates in a concentration-dependent fashion ($P < 0.05$), and the inhibitory effect of genistein on adhesion was more potent in the 10 and 20 µg/mL genistein groups. The inhibitory rate of genistein on MHCC97-H cell adhesion is shown in Figure 2.

We also investigated the capability of metastatic tumor cells to migrate through reconstituted basement membrane (Matrigel). The cells invading the lower surface of the filter through Matrigel in the control group, 5, 10, and 20 µg/mL genistein groups were 234.20 ± 12.36 /field, 213.60 ± 14.98 /field, 193.80 ± 19.92 /field, and 142.80 ± 23.66 /field, respectively. The inhibitory rate of invasion is shown in Figure 2. Our results showed that genistein inhibited the *in vitro* invasion of MHCC97-H cells. The inhibitory effect on invasion of MHCC97-H cells in the 20 µg/mL genistein group was more significant than that in the 5 and 10 µg/mL genistein groups ($P < 0.05$).

In vitro effects of genistein on cell cycle progression

The effects of genistein on the cell cycle in MHCC97-H cells were determined by flow cytometry. After treatment with genistein, the number of MHCC97-H cells in the G₀/G₁ phase increased significantly compared with control cells ($P < 0.05$). The number of cells in the G₂/M phase was decreased, but there was no statistical

Table 2 Effects of genistein on cell cycle progression in the MHCC97-H cell line

Treatment ($\mu\text{g/mL}$ genistein)	Cell cycle phase			Apoptosis (%)
	G ₀ /G ₁ (%)	G ₂ /M (%)	S (%)	
Control	59.12 \pm 3.89	10.95 \pm 6.41	29.93 \pm 2.52	1.18 \pm 0.01
5	67.86 \pm 2.36 ^a	4.08 \pm 1.82 ^a	28.06 \pm 0.55	3.92 \pm 1.63 ^a
10	69.53 \pm 2.13 ^a	6.80 \pm 0.48	23.65 \pm 1.76 ^b	7.84 \pm 1.72 ^b
20	71.85 \pm 3.60 ^a	8.84 \pm 4.07	19.31 \pm 1.51 ^b	18.90 \pm 4.98 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control. Cell cycle and apoptosis were evaluated by flow cytometric analysis on a FACScan cytofluorimeter. Values are mean \pm SD from experiments.

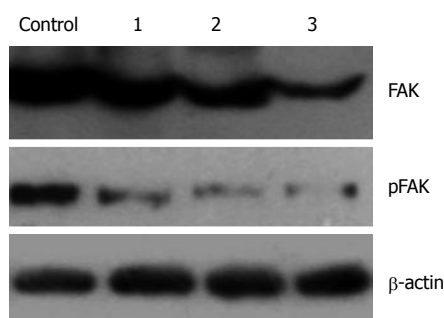


Figure 3 Western blotting analysis of protein expression and phosphorylation of FAK in MHCC97-H cells following genistein treatment. Lane 1: Genistein 5 $\mu\text{g/mL}$; Lane 2: Genistein 10 $\mu\text{g/mL}$; Lane 3: Genistein 20 $\mu\text{g/mL}$.

significance between the control group and the 10 and 20 $\mu\text{g/mL}$ genistein-treated groups ($P > 0.05$). S phase fractions decreased significantly in cells treated with 10 and 20 $\mu\text{g/mL}$ genistein ($P < 0.01$). The percentage of apoptotic cells in the genistein-treated groups increased significantly compared with the control group ($P < 0.05$). The results of the cell cycle analysis of MHCC97-H cells by flow cytometry are presented in Table 2.

Effects of genistein on protein expression and phosphorylation of FAK in MHCC97-H cells

The effect of genistein on expression and phosphorylation of FAK in MHCC97-H cells was determined using Western blotting. After treatment with genistein, the expression level of total FAK was decreased when the genistein concentration increased ($P < 0.05$). The optical density in the 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ genistein groups were significantly lower than that in the control group ($P < 0.05$). More interestingly, at the same time phosphorylated FAK also decreased. The 5 $\mu\text{g/mL}$ genistein treatment inhibited about 40% of FAK phosphorylation. These parameters were further significantly inhibited in the presence of genistein in a concentration-dependent fashion ($P < 0.05$). Data are shown in Figures 3 and 4.

Effects of genistein on tumor growth and metastasis in vivo

The anti-tumor activity of genistein was evaluated in nude mice bearing orthotopic tumor implants (Figure 5A). Treatment with genistein significantly inhibited local tumor growth, compared with the control

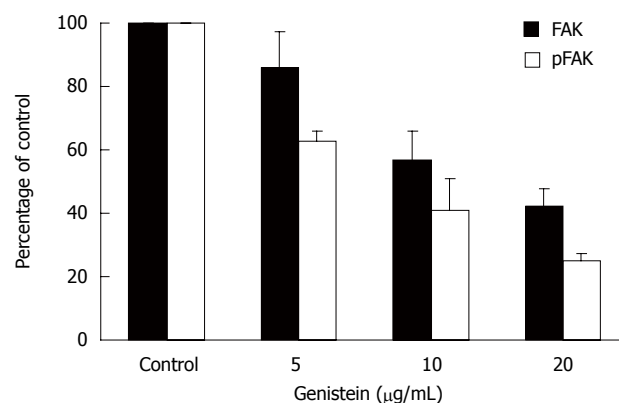


Figure 4 Effects of genistein on protein expression and phosphorylation of FAK. Black columns, protein expression of FAK; Open columns, phosphorylation of FAK. Values are mean \pm SD from experiments.

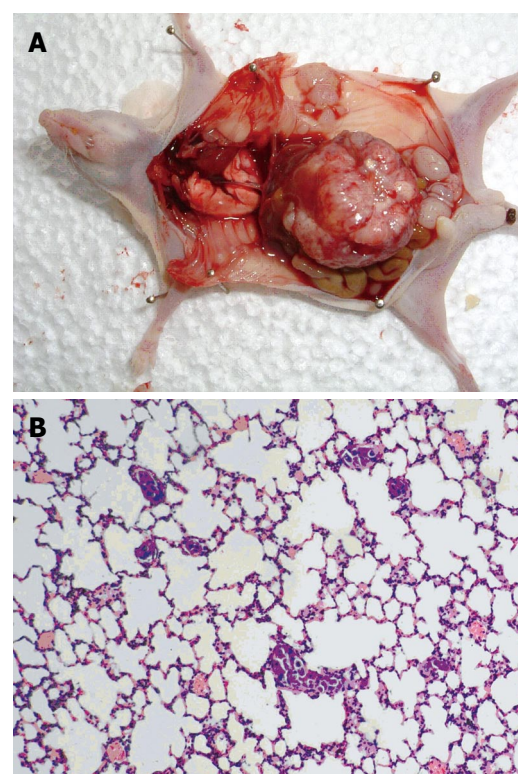


Figure 5 Autopsy and microphotographic appearance after orthotopic implantation. A: Autopsy of mice bearing orthotopic implantations. Rapid tumor growth occurred. Thirty-five days after tumor tissue implantation into the liver; B: Microphotography of lung metastasis by MHCC97-H cells. Thirty-five days after orthotopic implantation, MHCC97-H cells metastasized to the lungs, forming pulmonary metastatic lesions. HE, $\times 200$.

group. At the end of treatment, the tumor weight in the genistein group was significantly less than that in the control group (1.99 ± 2.07 g vs 2.63 ± 1.31 g, $P < 0.05$). Tumors in mice treated with genistein were reduced in weight by 24.3% compared with the control group.

After orthotopic implantation of tumor tissues, pulmonary metastases occurred in the recipient mice by day 35 (Figure 5B). Microscopy showed that the number of micrometastatic foci in the genistein group (12.3 ± 1.8) was significantly lower than that in the control group (12.3 ± 1.8 vs 16.6 ± 2.6 , $P < 0.05$).

DISCUSSION

Metastasis is a fundamental characteristic of cancer and the ultimate cause of most cancer mortality. Advances in surgical techniques and adjuvant therapies have proven useful in the treatment of primary tumors^[7]. However, metastasis remains a major cause of poor prognosis and death in cancer patients even after curative resection. The process of metastasis consists of sequential steps that include detachment, motility, invasion of the extracellular matrix, intravasation, circulation, adhesion, extravasation into the organ parenchyma and growth^[8]. The ability of cancer cells to form metastases depends on a set of unique biological properties that enable the malignant cells to complete all these steps of the metastatic process. Currently, only a few chemotherapeutic drugs are effective for the treatment of patients with malignant tumor metastasis, and there is a clear need to identify new anti-metastatic drugs^[9].

Genistein, an isoflavonoid abundant in soy beans, is a planar molecule with an aromatic A ring, has a second oxygen atom from that in the A ring, and has a molecular mass similar to those of the steroidal estrogens^[9-11]. Reports from epidemiological and experimental studies show that it plays an important role in the inhibition of tumors including breast cancer, prostate cancer, colon cancer, leukemia and melanoma^[12,13]. Genistein has a wide range of biological actions that suggest it may be of use in cancer treatment. Its molecular actions include: an inhibitory effect on protein tyrosine kinases, DNA topoisomerase I and II, and ribosomal S6 kinase; anti-estrogenicity; antioxidant activity; anti-angiogenesis activity; suppression of cell proliferation; induction of differentiation and modulation of apoptosis^[13-15]. However, few data are available on the effects of genistein on metastasis of HCC. The purpose of this study was to investigate the metastatic potential of genistein *in vitro* and *in vivo* in HCC and to gain primary insight into the underlying mechanism mediating the effects of genistein.

Several human and animal HCC cell clones have been established, but few of these have been suitable for the study of human HCC metastasis^[3,16,17]. However, most showed tumorigenicity when inoculated into experimental animals, and rarely did they demonstrate the full potential for distant metastases, as seen so frequently in patients. The metastatic HCC cell line MHCC97-H is characterized by high pulmonary metastasis potential and is a suitable cell line for the study of liver cancer metastasis^[3,18]. In this study, we found that genistein significantly inhibited MHCC97-H cell growth both *in vitro* and *in vivo*. The effect of genistein on MHCC97-H cells was concentration dependent; the *in vitro* inhibitory rates of tumor cell growth in the 10 µg/mL and 20 µg/mL genistein groups were about 58% and 80%, respectively. *In vivo*, tumor weight was significantly reduced by 24% in the genistein-treated group compared with the control group. We also found that genistein induced cell cycle progression arrest at the G₀/G₁ and G₂/M phases. These data are

in accordance with other reports which showed that genistein induced cell cycle progression arrest at the G₂/M phase and that the number of S phase cells decreased in a progressive way as the genistein incubation time was increased^[15,19,20]. Although the exact mechanisms of action of genistein have yet to be fully elucidated, induction of apoptosis may be partly responsible. In our studies, the percentage of MHCC97-H cells undergoing apoptosis was significantly higher in the genistein group than in the control group. This finding is consistent with apoptosis studies in breast, prostate and gastric cancer cell lines treated with genistein^[21,22].

The adhesion and invasiveness of tumor cells represents one of the several important steps necessary for the formation of metastases. Cell migration depends on adhesion between cells which is partly adjusted by integrin^[23]. We investigated the effect of genistein on the adhesive properties of MHCC97-H cells and found that genistein significantly inhibited MHCC97-H cell adhesion to fibronectin-coated substrates. The inhibition rate reached approximately 58% in the 20 µg/mL genistein group. This demonstrated that the reduction in cell adhesion caused by genistein may account for the ability of MHCC97-H cells to cross normal tissue boundaries and disperse to adjacent sites. Cell invasion was studied both *in vitro* and *in vivo* in our study. MHCC97-H cells which invaded the Matrigel to the lower surface of the Transwell filter were significantly inhibited in the genistein-treated groups compared with the control group. The inhibitory effect on invasion in the 20 µg/mL genistein group was 39%, which was significantly higher than that in the other groups. Our experiments with mice bearing orthotopic tumor implants further confirmed that treatment with genistein also significantly inhibited or halted lung metastasis of HCC, which correlated with the biological behavior *in vitro*.

FAK is a cytoplasmic tyrosine kinase which plays an important role in integrin-mediated signal transduction pathways closely related to cell adhesion, motility and growth. Upregulation of FAK expression is associated with oncogenesis, and a decrease in FAK is associated with loss of cell attachment, decreased migration and induction of apoptosis^[24]. We have reported that FAK is overexpressed in HCC, where the expression of FAK in invasive or metastatic HCC is significantly higher than that in non-invasive or non-metastatic HCC^[25]. Therefore, FAK seems to be an important pharmacologic target site. In this study, a significant downregulation of expression and phosphorylation of FAK after genistein treatment was observed, suggesting that genistein may serve as a potentially important anticancer agent for HCC progression by blocking the FAK signaling process, which plays a crucial role in the invasion and metastasis of HCC.

In conclusion, both *in vitro* and *in vivo* studies showed that genistein is a promising agent for inhibiting the metastatic potential of HCC. It may affect HCC progression as a result of its effects on cell cycle progression and apoptosis. However, because its precise mechanism of activity and its targets remain unclear, further in-depth studies of the molecular mechanism are

needed to establish the scientific basis for the possible use of genistein in the treatment of HCC metastasis.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related deaths in the world. A high incidence of metastasis accounts for the poor survival of HCC patients, and research into interventions for liver cancer metastasis has special priority in the anti-cancer campaign.

Research frontiers

Genistein, an isoflavonoid abundant in soy beans, has a wide range of biological actions in the inhibition of tumors including breast cancer, prostate cancer, colon cancer, leukemia and melanoma. However, few data are available on the effects of genistein on metastasis of HCC. In this study, the authors demonstrate that genistein inhibited the metastasis of HCC *in vitro* and *in vivo*. Genistein appears to be a promising agent for inhibiting the metastatic potential of HCC.

Innovations and breakthroughs

In this study, genistein was found to significantly inhibit the growth, attachment and invasion of MHCC97-H cells *in vitro* in a concentration-dependent fashion. The expression and phosphorylation of FAK in MHCC-97H cells were also decreased by genistein. In addition, our study on nude mice bearing orthotopic tumor implants showed that genistein significantly inhibited lung metastasis of MHCC97-H cells.

Applications

The findings from this study support the idea that genistein may serve as a potentially important anticancer agent for HCC progression by blocking the FAK signaling process.

Terminology

Genistein is a planar molecule with an aromatic A ring, has a second oxygen atom from that in the A ring, and has a molecular mass similar to those of the steroidal estrogens. It binds to and inhibits protein tyrosine kinase, thereby disrupting signal transduction and inducing cell apoptosis and differentiation.

Peer review

This paper reports the effect of genistein exposure on the growth, adhesion, migration and metastases of a highly metastatic HCC cell line MHCC97-H. They observed that genistein exposure significantly inhibited growth, adhesion to fibronectin, and invasion of the cell line. The paper is well written and has novel data.

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S- Editor Wang YR L- Editor Webster JR E- Editor Ma WH



BRIEF ARTICLE

A new polymorphism in the *GRP78* is not associated with HBV invasion

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Supported by The grant from Ministry of Science and Technology of China, No. 2006CB910104; the Foundation of Guangzhou Science and Technology Bureau, the People's Republic of China, No. 2005Z1-E0131

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Received: July 27, 2009 Revised: August 28, 2009

Accepted: September 4, 2009

Published online: October 21, 2009

HBV-DNA were associated with the allele/genotype variation in HBV infected individuals.

CONCLUSION: -86 bp T > A polymorphism in *GRP78* gene is not related to the clinical risk and acute exacerbation of HBV invasion.

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Key words: Acute exacerbation; Glucose-regulated protein 78; Hepatitis B virus; Single-nucleotide polymorphism

Peer reviewer: Dr. Stefan Wirth, Professor, Children's Hospital, Heusnerstr. 40, Wuppertal 42349, Germany

Zhu X, Wang Y, Tao T, Li DP, Lan FF, Zhu W, Xie D, Kung HF. A new polymorphism in the *GRP78* is not associated with HBV invasion. *World J Gastroenterol* 2009; 15(39): 4958-4961 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4958.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4958>

INTRODUCTION

Hepatitis B virus (HBV) is one of the major infectious diseases worldwide and is responsible for significant morbidity and mortality, particularly in developing countries^[1]. Of patients with chronic HBV infection, a quarter develop chronic liver disease, which can be associated with hepatoma in some cases^[2,3]. Increasing evidence indicates that genetic factors influence the natural history of HBV infection. Recent studies have proposed that a number of polymorphisms influence the progression of patients with HBV infection^[4,5].

The glucose-regulated protein 78 (*GRP78*), also called heat shock 70 ku protein 5, is a stress-inducible endoplasmic reticulum (ER) calcium-binding chaperone^[6]. The *GRP78* pathway is one of the most important responders to disease-associated stress^[7]. An elevated *GRP78* level generally correlates with HBV infection^[8-10]. As a stress-associated gene, *GRP78* may be a contributing factor or marker of stress-associated diseases, such as hepatitis B. HBV invasion and other physiopathologic changes cause a large amount of unfolding or false-folding protein accumulation in the ER, which in turn induces stress in the ER and expression of *GRP78*^[6,11].

Abstract

AIM: To examine the association between -86 bp (T > A) in the *glucose-regulated protein 78* gene (*GRP78*) and hepatitis B virus (HBV) invasion.

METHODS: DNA was genotyped for the single-nucleotide polymorphism by polymerase chain reaction followed by sequencing in a sample of 382 unrelated HBV carriers and a total of 350 sex- and age-matched healthy controls. Serological markers for HBV infection were determined by enzyme-linked immunosorbent assay kits or clinical chemistry testing.

RESULTS: The distributions of allelotype and genotype in cases were not significantly different from those in controls. In addition, our findings suggested that neither alanine aminotransferase/hepatitis B e antigen nor

The aim of the present study was to elucidate the potential association of -86 bp T > A, a new single-nucleotide polymorphism (SNP) from the estimated translation start site of *GRP78*, as a host genetic factor with the risk and acute exacerbation of HBV infection in a Chinese population.

MATERIALS AND METHODS

Subjects

A total of 382 unrelated HBV carriers (233 males and 149 females) between 24 and 39 years old (average age 30.5 ± 7.2 years) and a total of 350 sex- and age-matched healthy volunteers (203 males and 147 females) aged between 22 and 36 years old (average age 27.8 ± 7.0 years) who had no history of HBV infection or other conspicuous diseases from the Affiliated Tumor Hospital of Guangzhou Medical College were included in this study. The two groups had a similar frequency of distribution in age and gender ($P > 0.05$) (Table 1). The diagnosis of HBV infection was based on the presence of hepatitis B surface antigen and hepatitis B e antigen (HBeAg) or e antibodies, together with the absence of anti-HBs, for at least 36 mo prior to enrolment. The present study was approved by the Ethics Committee of Sun Yat-sen University and adhered to the tenets of the Declaration of Helsinki. Informed consent was also obtained from each participant.

PCR and resequencing

Blood samples were obtained and stored at -80°C until DNA extraction. Genomic DNA was extracted from peripheral blood leukocytes using the QIAGEN QIAamp DNA Mini Blood Kit (Hilden, Germany). The primers for PCR and resequencing were designed based on the published *Homo sapiens GRP78* DNA sequence (GenBank access No. NT_008470.18). Oligonucleotide primers were synthesized on a oligonucleotide synthesizer (Applied Biosystem, ABI) by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

PCRs were performed in 50 μL reaction systems containing 200 ng sample DNA, 5 μL of $10 \times \text{Ex Taq}$ buffer (Mg^{2+} free; Takara, Japan), 2 mmol/L MgCl_2 , 20 pmol of each primer (forward primer, AAGGGAGAACAAGCAGTAG and reverse primer, TGTCCCTGGAATTGTAAGC), 0.2 mmol/L of each deoxynucleoside triphosphate, and 5 U *Ex Taq* polymerase (Takara, Shiga, Japan). The amplification was performed using initial denaturation at 94°C for 10 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 90 s on a GeneAmp 9700 thermal cycler (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA, USA).

Resequencing was performed with the primer ATC CGC AAC CCC ACT TAC C, *Taq* polymerase, ABI PRISM® BigDye™ terminators on an ABI 3730xl DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA).

Table 1 Characterization of the participants (mean \pm SD) *n* (%)

Characteristics	Cases	Controls	<i>P</i>
Age (yr)	30.5 \pm 7.2	27.8 \pm 7.0	0.426 ¹
Gender			
Females	149 (39.01)	147 (42.00)	0.410 ²
Males	233 (60.99)	203 (58.00)	
ALT (U/L)	125.5 \pm 81.7	28.2 \pm 10.9	
HBsAg (+)	382 (100)	0	
Anti-HBs	0	0	
HBeAg (+)	134 (35.07)	0	
Anti-HBe	248 (64.93)	0	

¹Mann-Whitney test; ²Pearson χ^2 test. ALT: Alanine aminotransferase.

Table 2 Allele and genotype frequencies of the new SNP (-86 bp T > A) in *GRP78* among cases and controls, and risk of HBV *n* (%)

Allele/genotype	Cases	Controls	OR ¹ (95% CI)	<i>P</i> ¹
T	749 (98.1)	689 (98.5)	Reference	
A	15 (1.9)	11 (1.5)	1.24 (0.56-2.78)	0.534
TT	367 (96.1)	339 (96.8)	Reference	
AT	15 (3.9)	11 (3.2)	1.25 (0.57-2.80)	0.529
AA	0	0		

¹The data were calculated by logistic regression with adjusted for age and gender. OR: Odds ratio; CI: Confidence interval; HBV: Hepatitis B virus; SNP: Single-nucleotide polymorphism.

Serological testing

Serological markers for HBV infection were determined by commercially available enzyme-linked immunosorbent assay kits (Sina-American Biotechnology Co., Ltd., China) or clinical chemistry testing. The normal range for serum alanine aminotransferase (ALT) is 0-40 IU/L. The extraction and quantification of serum HBV-DNA were carried out using a quantitative HBV PCR fluorescence diagnostic kit (Shenzhen PG Biotech., China) in the LightCycler Systems (Roche Diagnostic, Rotkreuz, Switzerland). HBV DNA levels were expressed as log copies/mL.

Statistical analysis

χ^2 test was used to determine whether there was a significant difference between cases and controls in terms of gender. Mann-Whitney *U*-test was used to test the difference among the age groups. Hardy-Weinberg equilibrium (HWE) of genotype distribution among cases and controls was carried out using the Pearson χ^2 test. A significant difference in polymorphism between cases and controls (or between different subgroup cases) was ascertained by unconditional logistic regression model adjusted for age and gender, in which the odds ratios (ORs) and 95% confidence intervals (CIs) were acquired synchronously. One way analysis of variance (ANOVA) and the Bonferroni test were used to evaluate the association between serum HBV DNA levels and alleles/genotypes in cases. All statistical tests were two-sided and *P* values less than 0.05 were considered statistically significant.

Table 3 ORs and 95% CIs calculated by logistic regression with adjustment for age and gender between different case subgroups according to the alleles and genotypes of the new SNP (-86 bp T > A) in all HBV carriers

Alleles/genotypes	Serum ALT				Serum HBeAg			
	> 40 IU/L (n)	≤ 40 IU/L (n)	OR (95% CI)	P ¹	+ (n)	- (n)	OR (95% CI)	P ²
T	194	555	Reference		266	483	Reference	
A	2	13	0.45 (0.10-1.99)	0.262	2	13	0.28 (0.06-1.25)	0.076
TT	96	271	Reference		132	235	Reference	
AT	2	13	0.44 (0.10-1.97)	0.254	2	13	0.29 (0.06-1.23)	0.070
AA	0	0			0	0		

¹P values for ALT > 40 IU/L cases vs ALT ≤ 40 IU/L cases; ²P values for HBeAg positive cases vs HBeAg negative cases. +: Positive; -: Negative.

RESULTS

The observed genotype frequencies conformed to the HWE in both cases and controls (data not shown). According to the logistic regression analysis with adjustment for age and gender, the distributions of allelotype and genotype in HBV cases were not significantly different from those in the controls ($P > 0.05$, respectively) (Table 2).

To assess the possible association between the polymorphism and acute exacerbation of HBV infection, the cases were divided into two groups based on a normal or abnormal ALT value, or the absence or presence of HBeAg. There were no significant differences in allele frequencies or genotype distributions of -86 bp (T > A) between ALT abnormal cases (ALT > 40 IU/L) and ALT normal cases (ALT ≤ 40 IU/L), or between HBeAg positive cases and HBeAg negative cases ($P > 0.05$, respectively) (Table 3). In addition, the viral load demonstrated no significant differences among different alleles (OR: 3.96, 95% CI: 3.34-4.61, $P = 0.165$ for allele A) or genotypes (OR: 3.95, 95% CI: 3.33-4.64, $P = 0.175$ for AT) compared with the respective reference groups (OR: 3.60, 95% CI: 3.50-3.68 for allele T; OR: 3.57, 95% CI: 3.46-3.72 for genotype TT) by ANOVA and Bonferroni testing (Figure 1).

DISCUSSION

The findings in this study indicate that the -86 bp variation is not susceptible to HBV invasion and acute exacerbation.

As some of the polymorphisms may reflect the risk of onset and the severity of disease, assessing the effect of variations on the risk and progression of HBV invasion is important^[12,13]. The role of *GRP78* as a predisposing gene in the pathogenesis of viral diseases such as HBV has not been previously studied. Although no association was noted with *GRP78* using the -86 bp (T > A) SNP marker, there are a number of other loss/gain-of-function SNPs and haplotypes in the *GRP78* gene that have not been included in this study, which need further evaluation to conclusively exclude *GRP78* as a susceptibility locus.

The data presented in this study also demonstrate that neither ALT/HBeAg nor HBV-DNA were associated with the allele/genotype variation in HBV infected individuals. Because ALT, HBeAg and HBV-DNA are markers for acute infection of HBV^[14,15], we

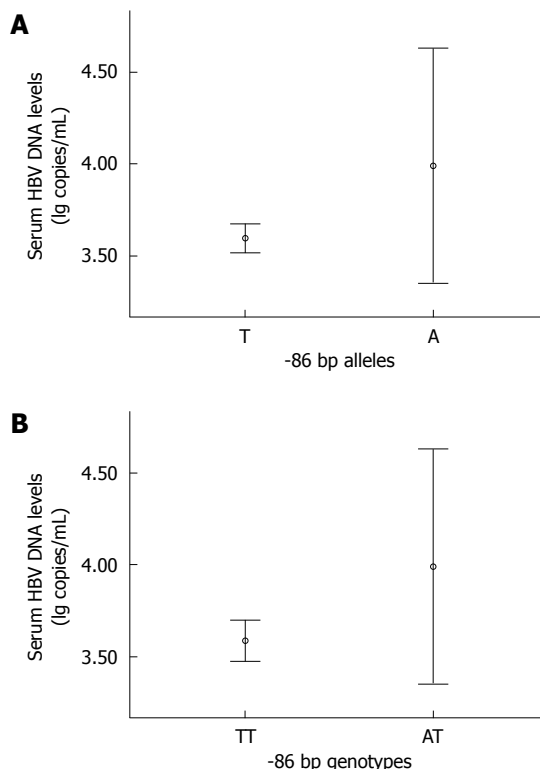


Figure 1 Association between serum HBV DNA levels and alleles and genotypes in the new SNP (-86 bp T > A) of *GRP78* gene. A: Compared with the reference group (allele T group, OR: 3.60, 95% CI: 3.50-3.68), allele A group (OR: 3.96, 95% CI: 3.34-4.61, $P = 0.165$) demonstrated no significant difference in HBV DNA levels; B: Compared with the reference group (genotype TT group, OR: 3.57, 95% CI: 3.46-3.72), genotype AT group (OR: 3.95, 95% CI: 3.33-4.64, $P = 0.175$) demonstrated no significant difference in HBV DNA levels.

can infer that -86 bp (T > A) is not associated with acute exacerbation of HBV invasion.

Moreover, a prospective study on the influence of polymorphisms on disease risk and progression mainly explored a statistical association between alleles/genotypes and clinical events^[16]. Therefore, the present study suggests a lack of association between the -86 bp and clinical risk as well as acute exacerbation. These data, however, do not exclude a possible physiopathological role of the *GRP78* in HBV progression.

To the best of our knowledge, this new found SNP in a Chinese Han population has not been deposited in a public database (<http://www.ncbi.nlm.nih.gov/SNP>). Although the functional effects of this polymorphism have not been elucidated, our data show that the specific

polymorphism evaluated in this study is not related to HBV susceptibility or acute exacerbation and suggest that, at least in this Chinese population, its role in HBV invasion could be less than expected. However, it must be underlined that this polymorphism should not be completely eliminated as studies with a larger sample size may demonstrate the small differences found in this study to be statistically significant.

ACKNOWLEDGMENTS

We greatly acknowledge the participants and their families, who took part in this study.

COMMENTS

Background

Highly variable disease outcomes in infected individuals cannot be fully explained by differences in viral or environmental factors but also involve host genetic susceptibility. Increasing data show that glucose-regulated protein 78 (GRP78) plays a crucial role in the proper folding of hepatitis B virus (HBV) protein. Therefore, we strongly believed that the influence of polymorphism on the risk and progression of HBV invasion should be studied so as to determine whether there is a statistical association between the -86 bp (T > A) genotypes and clinical events.

Research frontiers

In general, the present study is part of a series of research studies involving HBV infection and is aimed at mutational analysis of the *GRP78* gene among HBV carriers. To the best of our knowledge, this is the first study where an attempt has been made to correlate a new found gene mutation (-86 bp T > A) with HBV risk among Han Chinese.

Innovations and breakthroughs

The -86 bp T > A polymorphism in the *GRP78* gene is not related to the clinical risk and acute exacerbation of HBV invasion.

Applications

Genetic differences in the gene related to virus invasion may help us to predict an individual's susceptibility of developing serious disease and assist early detection which can substantially improve the cure rate. This study found that the -86 bp (T > A) is not a susceptible candidate locus, and future research should focus on its potential role in liver cancer risk.

Terminology

Human *GRP78* gene is located on 9q33-q34.1. GRP78 protein is a molecular chaperone that is critical to the folding, maturation and transport of proteins out of cells.

Peer review

The authors examined the association of a new polymorphism (-86 bp T > A) in the *GRP78* gene and risk of HBV infection. They did not find any association between this SNP and risk or acute exacerbation of HBV invasion. This study gives further evidence that *GRP78* is not directly involved in HBV infection.

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S- Editor Li LF L- Editor Webster JR E- Editor Zheng XM



BRIEF ARTICLE

Effect of neoadjuvant chemoradiotherapy on prognosis and surgery for esophageal carcinoma

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Received: August 1, 2009 Revised: September 1, 2009

Accepted: September 8, 2009

Published online: October 21, 2009

(0.89-2.48, $P = 0.503$) for all treatment mortality, 1.33 (0.94-1.88, $P = 0.04$) for the rate of adverse treatment, 1.38 (1.23-1.63, $P = 0.0002$) for local-regional cancer recurrence, 1.28 (0.85-1.58, $P = 0.60$) for distant cancer recurrence, and 1.27 (0.86-1.65, $P = 0.19$) for all cancer recurrence. A complete pathological response to chemoradiotherapy occurred in 10%-45.5% of patients. The 5-year survival benefit was most pronounced when chemotherapy and radiotherapy were given concurrently (OR: 1.45, 95% CI: 1.26-1.79, $P = 0.015$) instead of sequentially (OR: 0.85, 95% CI: 0.64-1.35, $P = 0.26$).

CONCLUSION: Compared with surgery alone, neoadjuvant chemoradiotherapy can improve the long-term survival and reduce local-regional cancer recurrence. Concurrent administration of neoadjuvant chemoradiotherapy was superior to sequential chemoradiotherapy.

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Key words: Esophageal neoplasms/surgery; Esophageal neoplasms/radiotherapy; Antineoplastic agents; Postoperative complications; Prospective studies; Randomized controlled trial; Meta-analysis

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Lv J, Cao XF, Zhu B, Ji L, Tao L, Wang DD. Effect of neoadjuvant chemoradiotherapy on prognosis and surgery for esophageal carcinoma. *World J Gastroenterol* 2009; 15(39): 4962-4968
Available from: URL: <http://www.wjgnet.com/1007-9327/15/4962.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4962>

Abstract

AIM: To investigate the role of neoadjuvant chemoradiotherapy in prognosis and surgery for esophageal carcinoma by a meta-analysis.

METHODS: PubMed and manual searches were done to identify all published randomized controlled trials (RCTs) that compared neoadjuvant chemoradiotherapy plus surgery (CRTS) with surgery alone (S) for esophageal cancer. According to the test of heterogeneity, a fixed-effect model or a random effect model was used and the odds ratio (OR) was the principal measure of effects.

RESULTS: Fourteen RCTs that included 1737 patients were selected with quality assessment ranging from A to C (Cochrane Reviewers' Handbook 4.2.2). OR (95% CI, P value), expressed as CRTS *vs* S (values > 1 favor CRTS arm), was 1.19 (0.94-1.48, $P = 0.28$) for 1-year survival, 1.33 (1.07-1.65, $P = 0.69$) for 2-year survival, 1.76 (1.42-2.19, $P = 0.11$) for 3-year survival, 1.41 (1.06-1.87, $P = 0.11$) for 4-year survival, 1.64 (1.28-2.12, $P = 0.40$) for 5-year survival, 0.82 (0.39-1.73, $P < 0.0001$) for rate of resection, 1.53 (1.33-2.84, $P = 0.007$) for rate of complete resection, 1.78 (1.14-2.78, $P = 0.79$) for operative mortality, 1.12

INTRODUCTION

Esophageal cancer is one of the most frequently occurring malignancies and the seventh leading cause of cancer-related deaths in the world^[1]. The majority of patients present with an advanced stage of disease and long-term survival is poor^[2]. Esophagectomy remains a standard treatment for patients with resectable esophageal cancer, however, the 5-year survival rate is only 10%-20% in patients with advanced esophageal carcinoma

treated with surgery alone^[3-5]. Treatment failure mainly results from recurrence or metastasis. Most patients with seemingly resectable esophageal cancer have little prospect for cure. The proximity of the esophagus to vital mediastinal structures often compromises the completeness of cancer resection, and micrometastatic systemic disease is often present at the time of initial cancer diagnosis. These two limitations of surgical therapy set the stage for cancer recurrence, both local-regional and systemic. Radiotherapy can control local-regional esophageal cancer and chemotherapy, usually including cisplatin and 5-fluorouracil, has both local and systemic antineoplastic activity. Several studies have also suggested improved long-term survival rates with combined chemotherapy, radiotherapy and surgery in patients with resectable esophageal cancer^[6-8].

In addition, esophageal cancer patients seem to tolerate preoperative (neoadjuvant) chemoradiotherapy better than postoperative (adjuvant) chemoradiotherapy. And based on these premises, many phase III trials of neoadjuvant chemoradiotherapy followed by surgery have been done. Although many trials have generated promising results, there is a lingering concern, especially among surgeons, that neoadjuvant chemoradiotherapy may cause an unacceptable increase in perioperative morbidity and mortality. Randomized controlled trials (RCTs) have been performed to address these issues, but the results are not consistent. Many of the RCTs enrolled small numbers of patients, thus limiting their power to detect a treatment benefit.

So we performed a meta-analysis of RCTs that compared chemoradiotherapy plus surgery (CRTS) with surgery alone (S) in patients with resectable esophageal carcinoma.

MATERIALS AND METHODS

PubMed and manual searches were done (independently and in duplicate) to identify all published (manuscripts and abstracts) RCTs that compared CRTS with S for resectable esophageal cancer. Trials were not excluded because of cancer histology (squamous or adenocarcinoma) or language of publication. The PubMed search was done on PubMed (available at: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). A set was created using the terms “esophageal neoplasms/surgery OR esophagectomy OR oesophagectomy OR esophageal cancer OR oesophageal cancer.” This yielded 37 604 citations (June 30, 2009). Another set was created using the terms “antineoplastic agents OR chemotherapy OR radiotherapy.” This yielded 2 448 725 citations. The two sets were combined using the Boolean operator “AND” to give 8974 documents on chemotherapy, radiotherapy, and surgery for esophageal cancer. This set was limited to “RCT” to yield 340 documents. These documents were reviewed to identify RCTs that compared CRTS with S. Fourteen studies were identified and retrieved^[9-22]. We did not attempt to identify unpublished RCTs. In total, 14 RCTs were found and these trials form the basis of the meta-analysis.

Given the limited number of RCTs, we designed the article selection process to be inclusive as opposed to exclusive. Meanwhile, trial validity assessment was done independently and in duplicate, and a trial quality assessment was assigned (A to C) according to the Cochrane Reviewers' Handbook 4.2.2^[23]. If reviewers disagreed on the quality assessment, discrepancies were identified and a consensus was reached. Trial data abstraction was also done independently and in duplicate, and any discrepancies in data abstraction were examined further and resolved by consensus.

Outcomes assessed by meta-analysis included 1-year survival, 2-year survival, 3-year survival, 4-year survival, 5-year survival, rate of resection, rate of complete (R0) resection, operative mortality, the rate of adverse treatment, all treatment mortality, local-regional cancer recurrence, distant cancer recurrence, and all cancer recurrence. The principle of treatment intention was used when calculating frequency of events, other than postoperative events (operative mortality, postoperative treatment complications). For all events, we used the most reliable data available. Raw data were considered the most reliable data, followed by percentages, and derivation of survival from graphically presented survival curves. Resection was defined as any resection, curative or palliative; esophageal bypass and exploratory surgery were not included. Complete resection was defined as a microscopically complete (R0) resection performed with curative intent. Most of the trials expressed operative mortality as a 30-d mortality, so a 30-d mortality was used for data analysis. Postoperative treatment complications included anastomotic leaks, pneumonia, respiratory failure, etc. All treatment mortality was obtained by adding preoperative deaths (usually secondary to chemoradiotherapy) and postoperative deaths. The most complete summation of these deaths was used from each individual trial. Local-regional cancer recurrence was defined as any local regional recurrence, as against isolated local-regional recurrence. Similarly, distant cancer recurrence was defined as any distant recurrence. All cancer recurrences were defined as any type (local, regional, distant), or combination of types, of cancer recurrence. Sensitivity analyses were performed on the 5-year survival data to identify the effects of cancer histology (squamous or adenocarcinoma) and scheduling of chemoradiotherapy (concurrent or sequential) on survival.

According to the test of heterogeneity, we selected a fixed effect meta-analysis model or a random effect model. This gives conservative confidence intervals and minimizes the risk of erroneously assigning benefit to the treatment group. Odds ratio (OR) was the principal measure of effect. ORs are presented as a point estimate with 95% confidence intervals (CI) and *P* values in parentheses. ORs are calculated as treatment (CRTS) vs control (S), and a number greater than one favors the CRTS group (higher frequency of desirable events). Funnel plot analysis did not suggest publication bias against negative trials^[24]. Review Manager 4.2 [Review Manager (Rev Man), (Computer program), Version 4.2 for Windows, Oxford, England: The Cochrane Collaboration, 2003] software was used.

Review: Impact of neoadjuvant chemoradiotherapy on prognosis and surgery for esophageal carcinoma

Comparison: 01 CRTS vs S

Outcome: 03 3 yr-survival rate

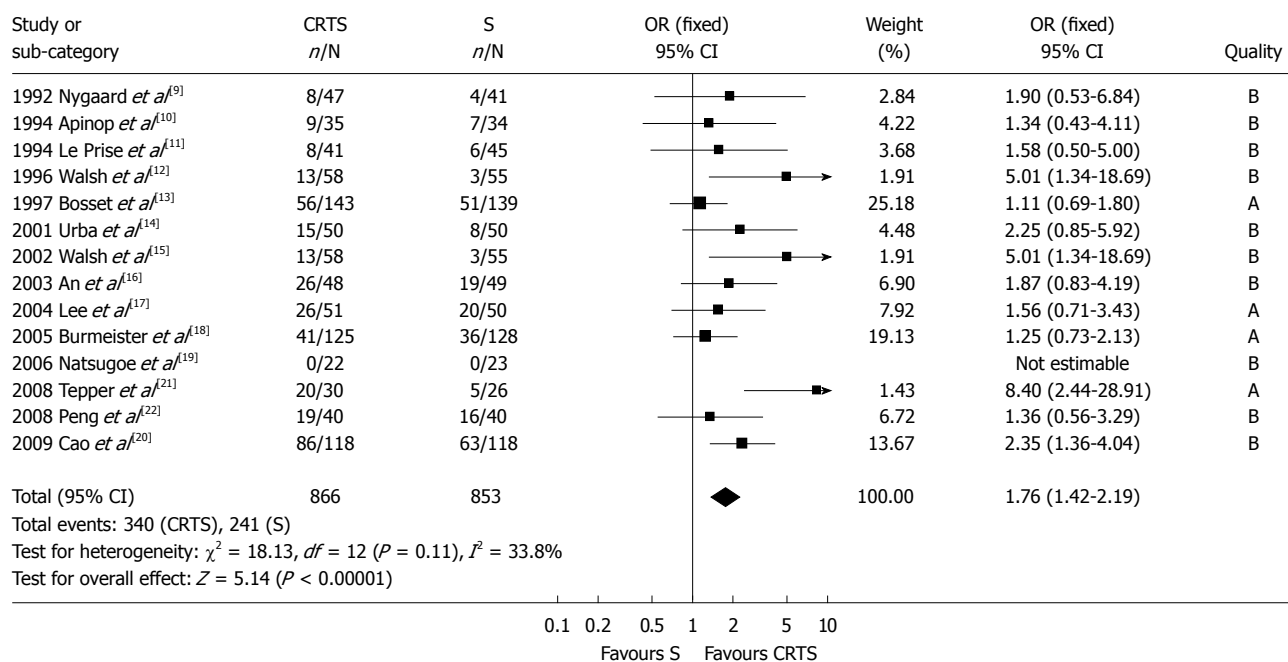


Figure 1 Three-year survival (OR: 1.76, 95% CI: 1.42-2.19, $P = 0.11$). OR: Odds ratio; CI: Confidence interval; CRTS: Neoadjuvant chemoradiotherapy; S: Surgery alone.

Median survival data could not be combined using meta-analysis methods because there was insufficient documentation of original patient data in many trials.

RESULTS

The two trial investigators agreed on the selection of fourteen RCTs^[9-22]. Combining these trials yielded data on 1737 patients. The RCT quality assessments included ten B, but four A, due to the inherent difficulty in blinding a treatment such as chemoradiotherapy. Survival of the two patient groups was similar at 1-year, but 2-year, 3-year, 4-year and 5-year-survival in CRTS group was superior to that in S group (Figures 1 and 2). OR (95% CI, P value), expressed as CRTS *vs* S (values > 1 favor CRTS arm), was 1.19 (0.94-1.48, $P = 0.28$) for 1-year survival, 1.33 (1.07-1.65, $P = 0.69$) for 2-year survival, 1.76 (1.42-2.19, $P = 0.11$) for 3-year survival, 1.41 (1.06-1.87, $P = 0.11$) for 4-year survival, and 1.64 (1.28-2.12, $P = 0.40$) for 5-year survival.

Five-year survival meta-analysis was repeated with RCTs separated according to the histology (squamous or adenocarcinoma) and chemoradiotherapy scheduling (concurrent or sequential). If only RCTs addressing squamous cancer were considered, the 5-year survival advantage of neoadjuvant chemoradiotherapy and surgery was similarly apparent (OR: 1.53, 95% CI: 1.12-2.1, $P = 0.40$). Restricting the analysis to RCTs of adenocarcinoma was not feasible since there were just two trials of this type^[12,15], moreover, only one study reported the 5-year survival. If meta-analysis was restricted to RCTs using concurrent chemoradiotherapy, the 5-year survival

strongly favored the combination of CRTS (OR: 1.45, 95% CI: 1.26-1.79, $P = 0.015$). Conversely, RCTs using sequential chemoradiotherapy did not demonstrate a survival benefit at 5 years (OR: 0.85, 95% CI: 0.64-1.35, $P = 0.26$).

Although the patients treated with surgery alone tended to undergo an esophageal resection than those treated with CRTS, there was no significance (OR: 0.82, 95% CI: 0.39-1.73, $P < 0.0001$). However, patients treated with CRTS had a higher rate of complete resection than those treated with S (OR: 1.53, 95% CI: 1.33-2.84, $P = 0.007$). Data analysis for the CRTS showed a complete pathological response in 10%-45.5% of patients. In regard to the extent and quality of surgical resection and lymphadenectomy, it was difficult to discriminate from the included studies.

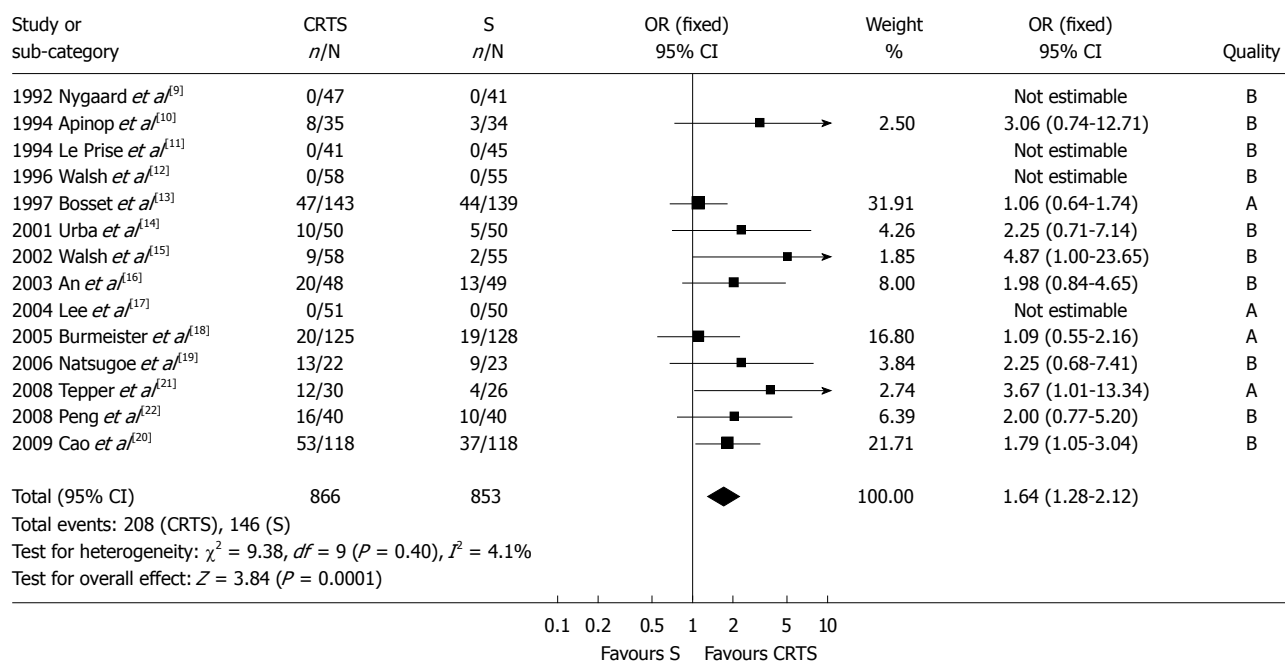
Moreover, the rate of adverse treatment events showed no significant difference between the two groups (OR: 1.33, 95% CI: 0.94-1.88, $P = 0.04$). However, there was a trend in favor of surgery alone for operative mortality (OR: 1.78, 95% CI: 1.14-2.78, $P = 0.79$; Figure 3) although there was no significant difference in all treatment mortality between CRTS and S groups (OR: 1.12, 95% CI: 0.89-2.48, $P = 0.503$).

As far as cancer recurrence is concerned, patients treated with CRTS had fewer local-regional cancer recurrences (OR: 1.38, 95% CI: 1.23-1.63, $P = 0.0002$). However, distant recurrence (OR: 1.28, 95% CI: 0.85-1.58, $P = 0.60$) and all cancer recurrence (OR: 1.27, 95% CI: 0.86-1.65, $P = 0.19$) were not statistically significant between the two groups of patients. A funnel plot, with regard to the publication bias of all analysis, showed the

Review: Impact of neoadjuvant chemoradiotherapy on prognosis and surgery for esophageal carcinoma

Comparison: 01 CRTS *vs* S

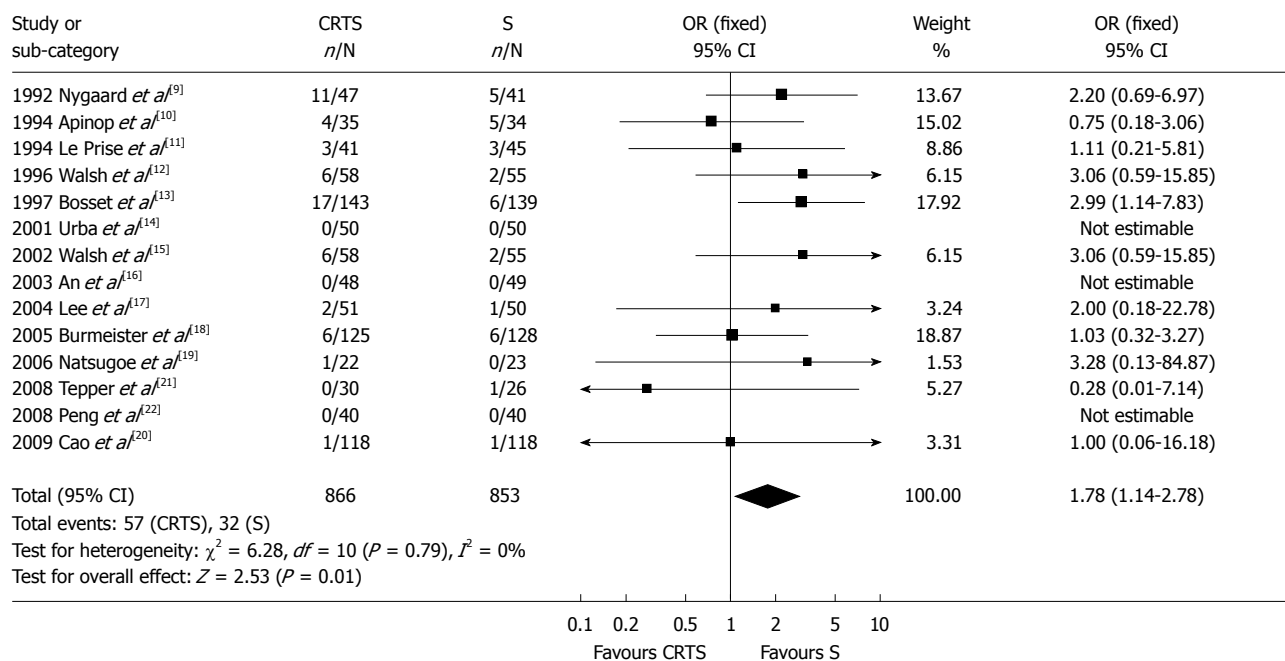
Outcome: 05 5 yr-survival rate

Figure 2 Five-year survival (OR: 1.64, 95% CI: 1.28-2.12, *P* = 0.40).

Review: Impact of neoadjuvant chemoradiotherapy on prognosis and surgery for esophageal carcinoma

Comparison: 01 CRTS *vs* S

Outcome: 06 Operative mortality

Figure 3 Operative mortality (OR: 1.78, 95% CI: 1.14-2.78, *P* = 0.79).

basic symmetrical and inverted funnel-shaped graphics (Figure 4).

DISCUSSION

Surgical resection is currently the preferred treatment for esophageal cancer if a patient is fit enough to un-

dergo major surgery and the tumor is considered to be resectable without evidence of distant metastases (cT₁₋₃ N₀₋₁ M₀). However, surgery as a solitary treatment modality for esophageal cancer remains dissatisfied. To date, assorted multimodality treatments have been investigated^[25-28]. Neither neoadjuvant radiotherapy and surgery, nor surgery and adjuvant radiotherapy, has

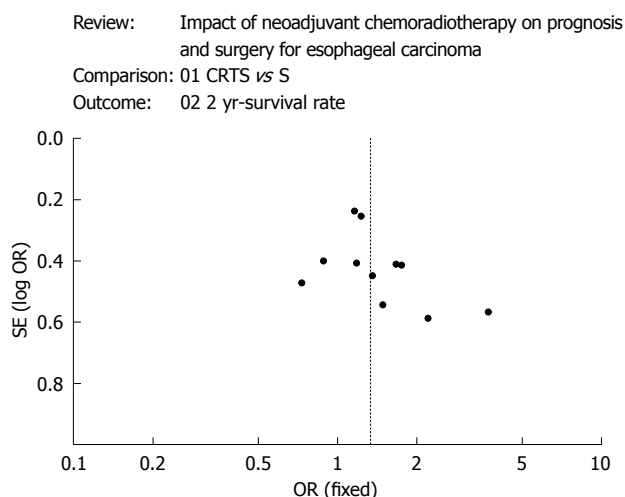


Figure 4 A funnel plot about 2-year survival with regard to the publication bias of all analysis, shows the basic symmetrical and inverted funnel-shaped graphics.

shown a significant survival advantage for these combinations of surgery and radiotherapy^[29,30]. Postoperative chemotherapy is frequently employed to prevent, delay or treat systemic metastases in patients with esophageal carcinoma, however, RCTs supporting the use of adjuvant chemotherapy are scarce, which showed no benefit for surgery followed by chemotherapy^[31,32]. In addition, some RCTs have compared neoadjuvant chemotherapy plus surgery with surgery alone^[33,34]. A complete pathological response after neoadjuvant chemotherapy was rare. Taken together, current neoadjuvant chemotherapy regimes remain not effective enough to improve the overall survival^[35,36].

The CRTS, an intuitively appealing treatment strategy, has brought about dramatic clinical and pathological responses in randomized esophageal cancer trials^[37,38]. Both chemotherapy and radiotherapy may be active against different tumor cell population, the chemotherapy may be effective against micrometastases while radiation is spatially cooperative. Neoadjuvant chemoradiotherapy can facilitate resection by down-staging tumors^[39,40]. However, whether or not CRTS actually increases long-term survival remains controversial, meanwhile CRTS seems to increase the morbidity and mortality of esophagectomy, so it is uncertain whether such a potential survival benefit outweighs the morbidity caused by such a treatment. A surgery alone is therefore still considered to be appropriate in randomized phase III studies for patients with esophageal cancer.

As yet only few meta-analyses examining the effectiveness of CRTS in patients with esophageal cancer has been published, and the previous analysis mainly focused on the 3-year survival^[55,36]. In contrast, our analysis pooled later survival data (up to 5-year survival) and more RCTs (up to 14 studies), which is more exhaustive. Our data contained the most cases (up to 1737 patients), meanwhile our funnel plot showed little publication bias. For all of these reasons, our findings may be more robust. Our meta-analysis of RCTs showed a long-term

survival benefit for the neoadjuvant chemoradiotherapy and surgery for resectable esophageal cancer. The survival benefit is involved in improved local-regional cancer control brought about by the neoadjuvant arm. Nevertheless, CRTS did not significantly reduce the incidence of distant recurrence. The sensitivity analysis of this regime showed the advantage of concurrent chemoradiotherapy, with maximal antineoplastic synergy between chemotherapeutic agents and radiation treatment, as compared with sequential chemoradiotherapy.

There was conspicuous difference between resection and complete resection rates for the two groups. More patients treated with S likely underwent esophageal resection, but more patients treated with CRTS likely underwent a complete resection. This indicates that CRTS downstages tumors and facilitates complete resection, which is also supported by the lower rate of local-regional cancer recurrence in the neoadjuvant chemoradiotherapy group. Although there was no significant difference for all treatment mortality between CRTS and S group (OR: 1.12, 95% CI: 0.89-2.48, $P = 0.503$) and adverse treatment events (OR: 1.33, 95% CI: 0.94-1.88, $P = 0.04$), our analysis showed a significant trend with respect to increased operative mortality (OR: 1.78, 95% CI: 1.14-2.78, $P = 0.79$) in the CRTS group. It is no doubt that surgeons will undertake a challenging esophagectomy resulting from operative difficulty and postoperative complications when performed after neoadjuvant chemoradiotherapy. For example, radiation might contribute to the failure of anastomotic leak and postoperative acute lung injury. Whether or not the survival benefit of neoadjuvant chemoradiotherapy can be negated by an increase in postoperative deaths has brought about extensive concerns.

In conclusion, this meta-analysis of RCTs that compared CRTS with S for resectable esophageal carcinoma showed a long-term survival benefit and reduced local-regional cancer recurrence for neoadjuvant chemoradiotherapy. Moreover, concurrent neoadjuvant chemoradiotherapy is more effective. CRTS has a higher rate of complete (R0) resection. There is no significant difference, but a trend of lowered rate of esophageal resection. In addition, it is concerned that this neoadjuvant approach is associated with increased mortality.

COMMENTS

Background

Esophagectomy is a standard treatment for resectable esophageal carcinoma but relatively few patients are cured. Combined neoadjuvant chemoradiotherapy with surgery may improve survival but there is concern about treatment morbidity.

Research frontiers

This meta-analysis investigated the survival data (up to 5-year survival) and RCTs (up to 14 studies).

Innovations and breakthroughs

Compared with surgery alone, neoadjuvant chemoradiotherapy and surgery improved the 3-year and 5-year survival and reduced local-regional cancer recurrence. It was associated with a lower rate of esophageal resection, but a higher rate of complete (R0) resection and operative mortality. There was a

nonsignificant trend toward the increased treatment mortality with neoadjuvant chemoradiotherapy. Concurrent administration of neoadjuvant chemotherapy and radiotherapy was superior to sequential chemoradiotherapy.

Applications

The study can be applied as a guidance of neoadjuvant chemoradiotherapy in prognosis and surgery for esophageal carcinoma.

Peer review

The manuscript gives results from a meta-analysis on the effects of neoadjuvant chemoradiotherapy on survival for esophageal carcinoma. The authors performed and reported a detailed literature search and analyzed 14 publications with regard to several survival end-points. The topic is interesting and statistical methods are appropriate. In the discussion, the authors describe that their meta-analysis is more detailed than previous ones which focus on the 3-year survival.

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S- Editor Tian L L- Editor Ma JY E- Editor Zheng XM

Management of venous stenosis in living donor liver transplant recipients

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Received: June 28, 2009 Revised: September 1, 2009

Accepted: September 8, 2009

Published online: October 21, 2009

Abstract

AIM: To retrospectively evaluate the management and outcome of venous obstruction after living donor liver transplantation (LDLT).

METHODS: From February 1999 to May 2009, 1 intraoperative hepatic vein (HV) tension induced HV obstruction and 5 postoperative HV anastomotic stenosis occurred in 6 adult male LDLT recipients. Postoperative portal vein (PV) anastomotic stenosis occurred in 1 pediatric left lobe LDLT. Patients ranged in age from 9 to 56 years (median, 44 years). An air balloon was used to correct the intraoperative HV tension. Emergent surgical reoperation, transjugular HV balloon dilatation with stent placement and transfemoral venous HV balloon dilatation was performed for HV stenosis on days 3, 15, 50, 55, and 270 after LDLT, respectively. Balloon dilatation followed with stent placement *via* superior mesenteric vein was performed for the pediatric PV stenosis 168 d after LDLT.

RESULTS: The intraoperative HV tension was corrected with an air balloon. The recipient who underwent emergent reoperation for hepatic stenosis died of hemorrhagic shock and renal failure 2 d later. HV balloon dilatation *via* the transjugular and transfemoral venous approach was technically

successful in all patients. The patient with early-onset HV stenosis receiving transjugular balloon dilatation and stent placement on the 15th postoperative day left hospital 1 wk later and disappeared, while the patient receiving the same interventional procedures on the 50th postoperative day died of graft failure and renal failure 2 wk later. Two patients with late-onset HV stenosis receiving balloon dilatation have survived for 8 and 4 mo without recurrent stenosis and ascites, respectively. Balloon dilatation and stent placement *via* the superior mesenteric venous approach was technically successful in the pediatric left lobe LDLT, and this patient has survived for 9 mo without recurrent PV stenosis and ascites.

CONCLUSION: Intraoperative balloon placement, emergent reoperation, proper interventional balloon dilatation and stent placement can be effective as a way to manage hepatic and PV stenosis during and after LDLT.

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Key words: Living donor liver transplantation; Venous obstruction; Anastomotic stenosis; Venoplasty; Stent

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Yang J, Xu MQ, Yan LN, Lu WS, Li X, Shi ZR, Li B, Wen TF, Wang WT, Yang JY. Management of venous stenosis in living donor liver transplant recipients. *World J Gastroenterol* 2009; 15(39): 4969-4973 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4969.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4969>

INTRODUCTION

Vascular complications after liver transplantation (LT) include occlusion or stenosis at the site of anastomosis in the hepatic artery, portal vein (PV) and hepatic vein (HV). Venous obstruction after LT, including HV outflow obstruction and PV stenosis, is a relatively uncommon but important complication after LT especially living donor liver transplantation (LDLT) and split liver transplantation (SLT). The incidence of HV

obstruction varies from 5.3% in a series of LDLTs^[1] to 12.9% in a series of reduced-size livers^[2]. It occurs more frequently in pediatric liver transplants where a small graft may twist around the HV anastomosis. When hepatic outflow obstruction occurs, hepatic congestion can cause massive ascites and hepatic dysfunction. With the increase in cases of LDLT and SLT, management of HV outflow obstruction has become an important issue^[3-8].

PV stenosis is a relatively rare complication after LT and it sometimes leads to life threatening events due to gastrointestinal bleeding or graft failure. PV stenosis affect 2%-14% of transplant recipients in children^[5,9-11]. With LDLT, left lobe segments are frequently used as grafts^[12]. During left lobe graft transplantation, the extrahepatic PV slants to the right posterior, and the PV rotates backward to the left anterior into the liver. Stenotic areas almost always exist near the flexion. As the liver graft grows, it may kink or compress the flexion.

Recently, interventional procedures including balloon dilatation and stent placement have been widely accepted as treatments of choice for the management of venous obstruction after LT. Since the first successful treatment using percutaneous balloon dilatation by Raby *et al*^[13], several methods of portal venoplasty have been reported for the treatment of PV stenosis using percutaneous transhepatic^[14], transjugular intrahepatic^[15], transfemoral venous^[13], or transileocolic venous^[16,17] approaches. Stent placement has also been widely accepted as a treatment of choice for the management of venous obstruction after LT^[18,19]. Metallic stents have been used to treat recurrent and elastic stenosis^[20].

Thus, the aim of this study was to evaluate retrospectively the management and outcome of HV and PV stenosis after LDLT in one single center.

MATERIALS AND METHODS

Patients

Between February 1999 and May 2009, 223 cases of LDLT were performed in West China Hospital. A total of 7 male recipients developed postoperative hepatic and/or PV obstruction/stenosis (Table 1). Patients ranged in age from 9 to 56 years (median, 44 years). HV obstruction/stenosis was diagnosed in 6 adult male patients after LDLT, including 1 intraoperative HV tension, 3 early-onset HV-vena cava anastomosis stenosis, and 2 late-onset HV-vena cava anastomosis stenosis after LDLT. One intraoperative HV tension was suggested by hepatic congestion and confirmed at the same time during LDLT. HV tension was induced by the small right lobe liver graft falling into the deep right upper abdomen. Three early-onset HV stenosis was confirmed 3, 14 and 15 d post LDLT, respectively. Two late-onset HV-vena cava anastomosis stenosis was diagnosed 51 and 180 d after LDLT, respectively. Extrahepatic PV anastomotic stenosis was diagnosed in 1 male pediatric patient who was treated with left lobe LDLT 140 d post LDLT.

Postoperative HV and PV stenosis was suggested by

intractable ascites and confirmed by means of Doppler ultrasonography (HV flow velocity < 10 cm/s; PV flow velocity < 12 cm/s) and percutaneous portography followed by interventional procedures.

Management of HV obstruction

The treatment procedures were shown in Table 1. One intraoperative HV obstruction was corrected with a balloon filled with 75 mL air during LDLT; the balloon was placed under the liver to underlay the graft. Emergent laparotomy was performed for 1 patient with abdominal bleeding 3 d post LDLT, and HV stenosis was diagnosed during the operation. Abdominal bleeding came from liver incision rupture, which was the result of severe hepatic congestion induced by HV stenosis. Liver rupture repair and HV-vena cava re-anastomosis were performed. Transjugular HV balloon dilatation and metallic stent placement were performed for the other two early-onset HV stenosis on the 15th and 50th d post LDLT, respectively. Transfemoral venous balloon dilatation was performed for the two late-onset HV stenosis on the 55th and 270th d post LDLT, respectively.

Management of PV stenosis

As shown in Table 1, the pediatric patient with PV anastomotic stenosis received PV balloon dilatation and plastic stent placement 168 d post LDLT. All procedures were performed under general anesthesia. A new approach was employed for balloon dilatation and stent placement: the approach *via* the superior mesenteric vein. A small incision was made in the middle upper abdomen. Balloon dilatation and a 10 mm × 30 mm intravascular plastic stent placement were performed *via* superior mesenteric venous puncture. The diameter of the venous stent was about 1.0 cm. Immediately after the procedure, the patient underwent systemic anticoagulation with heparin-sodium for 7 consecutive days to maintain a partial thromboplastin time and INR of 1.5 times higher than normal levels. Follow-up data were obtained with routine clinical examination and Doppler sonography surveillance on days 1, 2, 3, and 7, at 2 wk and at 1 and 2 mo post procedure.

RESULTS

The results are shown in Table 1. The balloon underlying the liver graft during LDLT eliminated postoperative HV tension induced venous obstruction, and the balloon was removed on the 12th postoperative day. There was no outflow obstruction detected by ultrasonography before and after removal of the balloon. This patient has survived for 1.5 mo without ascites and hepatic congestion now.

The patient who underwent emergent operation for liver graft fracture bleeding induced by severe hepatic congestion died of hemorrhagic shock and renal failure 2 d later. Transjugular and transfemoral venous HV balloon dilatation was technically successful in each of 2 patients. In these 4 patients, the patient receiving

Table 1 Management and outcome of HV and PV obstruction/stenosis during and post LDLT

Case	V-O	LDLT	Age (yr)	Diagnosis and management time	Management	Survival	Complication
1	HV T	RL	46	Intraoperative	Balloon underlaying	> 1.5 mo, alive	-
2	HVS	RL	45	3, 3 d post LDLT	Re-anastomosis	2 d, dead	H-S, RF
3	HVS	RL	44	14, 15 d post LDLT	Transjugular BD + stent	off the trail	Bloody ascites
4	HVS	RL	56	14, 50 d post LDLT	Transjugular BD + stent	2 wk, dead	LF, RF
5	HVS	RL	43	51, 55 d post LDLT	Transfemoral vein BD	> 8 mo, alive	-
6	HVS	RL	39	180, 270 d post LDLT	Transfemoral vein BD	> 4 mo, alive	-
7	PVS	LL	9	140, 168 d post LDLT	trans-superior mesenteric venous BD + stent	> 9 mo, alive	-

BD: Balloon dilatation; H-S: Hemorrhagic shock; HV-H: Hepatic vein hemorrhage; HVS: HV stenosis; HV T: HV tension; LF: Liver failure; LL: Left lobe; PVS: Portal vein stenosis; RF: Renal failure; RL: Right lobe; V-O: Venous obstruction. LDLT: Living donor liver transplantation.

transjugular balloon dilatation and stent placement on the 15th postoperative day left hospital because of bloody ascites 1 wk later and disappeared, the patient receiving the same interventional treatment on the 50th postoperative day died of graft failure and renal failure 2 wk later, and the other two patients with late-onset HV stenosis receiving transfemoral venous balloon dilatation on the 55th and 270th postoperative day have survived for more than 8 and 4 mo without recurrent stenosis, respectively.

Balloon dilatation and stent placement *via* the superior mesenteric venous approach was technically successful in the pediatric patient with PV anastomotic stenosis. This child has survived for more than 9 mo without recurrent PV stenosis and ascites now. The liver function is normal, and the diameter of the venous stent is about 0.9 cm.

DISCUSSION

Although it is a relatively uncommon complication, venous obstruction after LT, including HV and PV stenosis, can induce severe complications such as hepatic congestion, portal hypertension, massive ascites, hepatic necrosis and hemorrhage, hepatic dysfunction, and even liver failure and death. Thus venous obstruction after LT is a critical problem for the recipient. Intraoperative HV or PV obstruction can be corrected with re-anastomosis. Tissue expanders or Foley catheters can also be employed to reposition the graft to improve HV and PV outflows induced by graft malposition during LT^[21,22]. Key points in HV and PV outflow reconstruction include: (I) size of anastomotic orifice, (II) length and orientation of vessels, and (III) position of the graft. Excluding vessel anastomotic techniques, the main factor in intraoperative venous outflow blockade is graft malposition. Severe HV obstruction shortly after transplantation is a surgical emergency, and reoperation is usually necessary for correction^[1,21]. On the other hand, because of fibrotic changes around the anastomotic site, surgical correction is usually difficult for the late-onset HV outflow obstruction and PV stenosis. Thus interventional procedures were a treatment of choice for the management of venous obstruction in that situation^[3,8,13,23,24]. Since the first report of percutaneous balloon angioplasty by Raby *et al*^[13], it has been widely accepted as a treatment of choice for

the management of venous stenosis after LT^[25-31]. The reported initial technical success rate was between 76% and 100%^[4-6,14,30].

In our study, the incidence rates of HV and PV stenosis was 2.7% and 0.04%, respectively. Several types of modified vascular reconstruction technology were used to avoid HV anastomotic stenosis^[32,33]: (1) the HV-HV anastomosis should be performed by a triangular opening of the inferior vena cava (IVC); (2) size matching of the donor HV to that of the recipient. If the donor HV is smaller than that of the recipient, patching of the great saphenous vein (GSV) graft could be used for the donor HV plasty; (3) a technique of “end-to-side vertical anastomosis” between the right HV or the inferior right HV (IRHV). The triangle-shaped hole has better stability, so the rate of stenosis and obstruction was very low. This also ensured the long-term patency of the anastomotic stoma. However, 7 patients with venous obstruction occurred at different phases: during the LDLT operation, in the early post-LDLT period (< 1 mo) and the late post-LDLT period (> 1 mo), respectively. One intraoperative HV obstruction was corrected with a balloon filled with air during LDLT, and the balloon was removed 12 d later without recurrent obstruction and any complications. One HV obstruction occurred 3 d after LDLT induced liver graft incision fracture and massive bleeding; although emergent hepatic rupture mending and HV-vena cava re-anastomosis were performed, this patient died of hemorrhagic shock and renal failure 2 d later. Both transjugular HV balloon dilatation and stent placement procedures for HV stenosis occurring in early phase were technically successful. Although balloon angioplasty has been accepted as the safe and effective initial treatment to manage HV outflow abnormalities, it may induce rupture of the fresh anastomosis and may be ineffective for eliminating various etiologies of venous outflow obstruction in the early post-transplant period. Thus, the outcome of balloon dilatation and stent placement for the 2 early-onset HV stenosis were bad in our study. The early (15 d post LDLT) transjugular balloon dilatation and stent placement induced massive bloody ascites 1 wk later, and the HV anastomosis bleeding may have been the cause of bleeding ascites. This patient left hospital and disappeared. Although the transjugular balloon dilatation and stent placement on the 50th postoperative day

in the other one patient prevented HV anastomosis bleeding, the preexistence of massive ascites, liver injury and renal dysfunction induced by the early-onset HV stenosis eventually resulted in the patient's death 2 wk later. However, the outcome of balloon dilatation for the two late-onset HV stenosis patients was good. These 2 patients have survived for more than 8 and 4 mo without recurrent stenosis and venous anastomosis bleeding, respectively.

The percutaneous transhepatic approach is the traditional method of PV stenosis interventional management. Our approach of preference was the trans-superior mesenteric venous approach. The first reason for this was the child's noncooperation for the percutaneous transhepatic approach under local anesthesia. The second reason was the risk of injury to the liver, the intrahepatic bile duct and artery, which could lead to liver damage, bile leakage, and hemorrhage, respectively. The good exposure of the superior mesenteric vein was the third cause, usually because of nonsevere adhesions or scar tissue surrounding the superior mesenteric vein after LT. As the PV is the linear prolongation of the superior mesenteric vein, superior mesenteric vein puncture, sheath intervention and placement of the guidewire into the PV, balloon dilatation and stent placement were easily performed. As the superior mesenteric vein puncture hole was sutured with 5-00 blood vessel suture (Proline), venous injury was not present. Postoperative paralytic ileus reported in the previous study^[16] was not present in this child. This child has survived for 9 mo with normal liver function, without venous thrombosis and recurrent venous stenosis now. This effective result suggested that a plastic stent could be used to treat pediatric venous stenosis after LDLT.

In conclusion, the results in our study suggested that the use of balloon filled with air to improve HV tension in malposed liver allograft is a simple and effective method during LDLT. Although balloon dilatation and stent placement is a safe and effective treatment to manage HV stenosis in the late post-transplant period, it may induce rupture of the fresh anastomosis in the early post-transplant period. The trans-superior mesenteric venous approach is clinically feasible and it is a safe and effective approach for PV stenosis intervention management after LDLT in children unfit for percutaneous transhepatic approach.

COMMENTS

Background

Hepatic vein (HV) and portal vein (PV) obstruction or stenosis during and after living donor liver transplantation (LDLT) is uncommon but critical complications may result in graft loss if not properly treated. Due to the technical difficulties of surgical management, interventional procedures including balloon dilatation via percutaneous transhepatic, transjugular intrahepatic, transfemoral vein and transileocolic vein approaches have been used to manage these complications. However, the direct trans-superior mesenteric venous approach is not in common use. Balloon dilatation followed by stent placement also has been widely accepted as a treatment of choice for the management of venous stenosis after liver transplantation (LT). Metallic stents have been used for recurrent and elastic stenosis.

Research frontiers

As there is a relatively high recurrence rate, i.e. 28.6%-36.8%, following balloon angioplasty, subsequent stent placement has been used to manage recurrent HV and PV stenosis after LT. However, some authors prefer to perform primary stent placement rather than balloon angioplasty in the early posttransplantation period (< 1 mo).

Innovations and breakthroughs

In this study, management and outcome of intraoperative HV obstruction induced by venous tension, as well as post-LDLT venous anastomotic stenosis in one single center were retrospectively analyzed. A balloon filled with air was employed for repositioning the graft to improve HV outflow obstruction induced by intraoperative graft malposition. Transjugular and transfemoral venous balloon dilatation were performed for treatment of 2 postoperative HV anastomotic stenosis, and immediately followed by stent placement via transjugular approach in 2 recipients. Plastic stent placement after balloon dilatation via the superior mesenteric venous approach was administered to manage a pediatric PV stenosis post living left lobe LT, and a good outcome was achieved.

Applications

A balloon filled with air could be used for repositioning the graft to improve HV outflow induced by intraoperative graft malposition. Emergent reoperation should be performed for HV obstruction shortly after LDLT. Proper interventional balloon dilatation and stent placement can be effective to manage HV and PV stenosis after LDLT. Balloon dilatation and stent placement via the superior mesenteric venous approach can be employed for treatment of PV stenosis after LDLT in children who do not cooperate with performance of the percutaneous transhepatic approach under local anesthesia.

Peer review

The single center study by the authors evaluated management and outcome of HV and PV obstruction after LDLT. Although venous obstruction post LDLT is a rare complication, choosing the ideal management is crucial since this complication can be life-threatening. Though the authors only report on 6 patients with venous obstruction following LDLT, the results and the conclusions drawn from their observations are quite interesting and important.

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S- Editor Tian L L- Editor O'Neill M E- Editor Ma WH



CASE REPORT

Forgotten node: A case report

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Received: May 13, 2009 **Revised:** August 24, 2009

Accepted: August 31, 2009

Published online: October 21, 2009

CASE REPORT

A 41-year-old female visiting from the Caribbean, the island of St. Vincent, presented initially to my office with abdominal pain. The history revealed she had been treated with antibiotics for her abdominal pain. Concurrently, she was prescribed birth control for presumed endometriosis for 5 mo without change in symptoms. A pelvic ultrasound revealed a leiomyomatous uterus and a 2.3 cm vascular umbilical mass. All laboratory data was within normal limits, except an elevated amylase level of 155, and a CA 19-9 level of 172 (normal < 35); CEA and CA27-29 were also normal. Since abdominal pain increased over the next 2 d she was admitted to the hospital. The CT of the abdomen/pelvis on admission revealed a large mass at the ileocecal junction with intussusceptions into the cecum without evidence of bowel obstruction. Also present were multiple large exophytic intramural and subserosal leiomyomas. Gastroenterology, Gynecology and Oncology were consulted. Since an umbilical vascular mass could not be biopsied, a colonoscopy was performed. Colonoscopy did not reveal any intra-luminal mass, however there was a significant intussusception of the ileum into the cecum. No obvious mass could be biopsied. At this time, there was still no diagnosis. It was recommended that an operative intervention was needed to excise the umbilicus for tissue diagnosis and also address the obstruction in the ileo-cecal region.

Umbilical mass and cecal mass with intussusceptions all revealed endometriosis on all frozen sections. A right hemicolectomy was performed. Intra operatively there were diffuse patches of endometriosis involving both ovaries, the cul-de-sac, the serosa of the uterus, and peritoneum. These areas were cauterized using the Bovie.

No malignancy was found in this patient.

DISCUSSION

Cutaneous metastases localized to the umbilicus are named "Sister Mary Joseph nodules". The historical perspective of this node and its namesake is quite interesting. Sister Mary Joseph, born Julia Dempsey in 1856 was assigned to St. Mary's Hospital (now the Mayo Clinic) in 1889 at the age of 33 years. Within 3 years she was appointed nursing superintendent. She worked with William W Mayo and his two sons, Charles H Mayo and William J Mayo. As first surgical assistant to son, William J Mayo she noted an umbilical nodule on a patient who

Abstract

Sister Mary Joseph nodule or node refers to a palpable nodule bulging into the umbilicus and is usually a result of a malignant cancer in the pelvis or abdomen. Traditionally it has been considered a sign of ominous prognosis. Gastrointestinal malignancies, most commonly gastric, colon and pancreatic cancer account for about 52% of the underlying sources. Gynecological cancers, most commonly ovarian and uterine cancers account for about 28% of the sources.

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Key words: Umbilical node; Sister Mary Joseph node; Gastroenterology; Gastric malignancies; Endometriosis

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Fratellone PM, Holowecki MA. Forgotten node: A case report. *World J Gastroenterol* 2009; 15(39): 4974-4975 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4974.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4974>

INTRODUCTION

Umbilical metastases (Figure 1), also named Sister Mary Joseph node are well documented in the adult population and most usually associated with gastrointestinal or gynecologic malignancy. The appearance of this node generally indicates a grave prognosis for the patient. malignant process, this case presents a rare, but reported non-malignant lesion^[1].



Figure 1 Sister Mary Joseph node.

was having surgery for an abdominal malignancy. She reported this to William J Mayo whom in 1928 reported it in the Proceedings of the Staff Meetings. It was then termed “pants button umbilicus”. By coincidence, Sister Mary Joseph, Charles H Mayo and William J Mayo all died within 3 mo of each other in 1939. These three people worked closely in developing what is now the Mayo Clinic. It was not until 1949, that Hamilton Bailey in his textbook, *Physical Signs in Clinical Surgery*, gave the name Sister Mary Joseph nodule to the umbilical metastasis, which usually is predictive of incurable abdominal malignancies^[2,3].

Metastasis to the umbilicus is very uncommon^[4]. When it does occur, the umbilical nodules are usually malignant with the common site being an abdomino-pelvic tumor^[5-7]. In Shetty's review^[8] of all cases of malignant disease to the umbilicus only 42% originated from either the abdomen or from the pelvis, whereas in

Powell's review^[9] of umbilical nodules, 32% of the cases were benign neoplasms.

This case demonstrates a non-malignant (benign) lesion that caused a metastatic umbilical nodule. In our case this was endometriosis^[10]. Although rare, this nodule ranges in color from dusky red to blue to purplish as in our case. It may change in size during the menstrual cycle and may be tender, and even bleed. The ultrasound demonstrated a vascular umbilical mass. Other benign causes of umbilical nodules are fibroma, keloid, and epithelial inclusion cysts^[11].

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Zheng XM

CASE REPORT

Muscularis mucosae in desmoplastic stroma formation of early invasive rectal adenocarcinoma

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Received: July 14, 2009 Revised: September 7, 2009

Accepted: September 14, 2009

Published online: October 21, 2009

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Ban S, Shimizu M. Muscularis mucosae in desmoplastic stroma formation of early invasive rectal adenocarcinoma. *World J Gastroenterol* 2009; 15(39): 4976-4979 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4976.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4976>

Abstract

The origin of myofibroblasts or myofibroblastic cells in the desmoplastic stroma associated with carcinoma invasion has been controversial. In the early invasive area of a rectal adenocarcinoma reported here, an obvious transition between the muscularis mucosa and the bundles of eosinophilic stromal cells observed in the carcinomatous stroma was demonstrated both in morphology and in their cytoskeletal phenotype, which conceivably suggests that the smooth muscle cells of the muscularis mucosa could convert to the eosinophilic stromal cells, namely myofibroblasts. Moreover, type I procollagen was demonstrated in both protein and mRNA levels in the areas of eosinophilic stromal cells with a lesser degree of differentiated smooth muscle phenotype that showed a transition from the muscularis mucosa, implying that the myofibroblastic cells converted from smooth muscle cells of the muscularis mucosa could be responsible for type I collagen production. These findings suggest that the muscularis mucosae may not be a passive barrier through which colorectal carcinomas infiltrate into the submucosa, but may play an active role in the formation and remodeling of tumor stroma.

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Key words: Rectal adenocarcinoma; Muscularis mucosa; Myofibroblast; Cytoskeletal phenotype; Type I collagen

Peer reviewer: Jennifer D Black, PhD, Professor and Member,

INTRODUCTION

The invasion of some carcinomas is characterized by the induction of so-called desmoplastic stroma. The desmoplastic stroma is comprised of activated fibroblastic cells and the deposition of extracellular matrix (ECM) components^[1-3]. The activated fibroblastic cells commonly show some morphological and phenotypic features of smooth muscle cells, and are referred to as myofibroblasts or myofibroblastic cells^[4-6]. They would modulate the microenvironment and contribute to tumor invasion through production of extracellular matrices, proteases, and soluble factors^[1,3,7,8].

The origin of myofibroblasts or myofibroblastic cells has been controversial^[4,6]. With regard to myofibroblastic cells in the desmoplastic stroma, one experimental study of an organotypic assay using breast carcinoma cells and breast stromal cells stressed the local fibroblasts as the most frequent candidate for myofibroblasts, while it also observed the recruitment of vascular smooth muscle cells and pericytes^[9]. However, in contrast to those local resident precursors, bone marrow-derived circulating fibrocytes have recently been proposed to be myofibroblastic precursors^[10,11].

This case study of an early invasive rectal adenocarcinoma conceivably demonstrates that one of the precursors of myofibroblastic cells in the carcinomatous desmoplastic stroma is smooth muscle cells of the muscularis mucosa, and they are responsible for production of an ECM component around the invasive carcinomatous glands, reemphasizing the importance of local resident cells in the desmoplastic reaction, and giving a hint as to the implication and a role of the muscularis mucosa in the early invasive process of colorectal carcinomas.

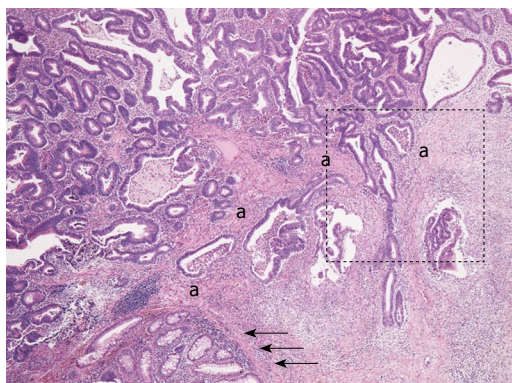


Figure 1 Low power view of an early invasive rectal adenocarcinoma with bundles of eosinophilic stromal cells (a), which are continuous with the muscularis mucosa (arrows, HE stain, original magnification $\times 40$).

CASE REPORT

A rectal tumor was transanally removed from a 59-year-old Japanese male patient. The tumor was a broad-based sessile polypoid lesion of 20 mm in the largest diameter, which was fixed in 10% formalin, embedded in paraffin blocks, and stained with haematoxylin and eosin (HE) routinely. Serial sections were also made for immunohistochemistry and *in situ* hybridization (ISH).

Immunohistochemical staining was performed by an established indirect method using the following monoclonal antibodies (clone; source; dilution in parenthesis): anti- α -smooth muscle actin; α -SMA (1A4; Dako, Glostrup, Denmark; 1:25), anti-desmin (D33; Immunotech, Marseilles, France; 1:25), anti-high molecular weight caldesmone; h-CD (h-CD; Dako; 1:50), and anti-type I procollagen (M58; Chemicon, Temecula, CA, USA; 1:500). Antigens were retrieved by autoclaving at 121°C for 5 min in a citrate buffer before immunostaining for desmin and h-CD, and by 1% trypsin digestion for 20 min at room temperature for procollagen I. Negative control sections were incubated without primary antibodies. Submucosal arteries and part of the proper muscle layer could be available for internal positive controls for α -SMA, desmin, and h-CD. As a positive control for procollagen I, we also immunostained scar tissues which were fixed in formalin and embedded in paraffin in the same way as the present case.

For ISH to detect type I procollagen mRNA, the tissue sections were deparaffinized, rehydrated, and treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Then they were treated with protease K (Dako, Glostrup, Denmark) for 60 min followed by depurination in 0.2 N HCl for 20 min both at room temperature, dehydrated, and air dried. Hybridization was performed using a cocktail of two synthetic DNA oligonucleotide probes^[12] labeled with digoxigenin at the 3'-end in a solution consisting of 50% formamide, 10 mmol/L Tris-HCl pH 7.6, 200 μ g/mL yeast tRNA, 100 μ g/mL sonicated salmon sperm DNA, 1X Denhardt's solution, 10% dextran sulfate, 600 mmol/L NaCl, 0.25% SDS, 1 mmol/L EDTA pH 8.0, and

10 μ g/mL of each probe, at room temperature overnight. After posthybridization washes (two brief washes in 2X SSC at 47°C, two washes in 1X SSC at 47°C for 30 min each, one wash in 0.5X SSC at 47°C for 30 min, one wash in 0.1X SSC at 47°C for 60 min, and one wash in 0.05 mol/L Tris-HCl pH 7.6 with 0.1% Tween 20 at room temperature for 5 min), incubation with horseradish peroxidase-conjugated anti-digoxigenin antibody was performed at room temperature for 30 min followed by detection with TSA[™] Biotin System (PerkinElmer, Wellesley, MA, USA). As controls, prehybridization digestion with RNase and hybridization using the hybridization solution with non-labeled probes or without probes were performed.

Histologically, the rectal tumor was a well-differentiated adenocarcinoma showing papillotubular growth, microscopically infiltrating into the submucosal layer beyond the muscularis mucosa. In the stroma of the invasive area, continuing to the muscularis mucosa of the adjacent normal mucosa, bundles of eosinophilic stromal cells were seen, and it was not easy to determine whether they were disarrayed muscularis mucosa remains or stromal cells simulating muscularis mucosa (Figure 1). When observed in detail, the bundles of eosinophilic stromal cells were not morphologically homogeneous but different in parts, namely, from those more similar to the smooth muscle cells of the muscularis mucosa to those composed of less eosinophilic spindle cells with plumper nuclei, showing a morphological transition from the former to the latter (Figure 2A and B).

Immunohistochemical examination of the cytoskeletal phenotype revealed that the former was more constantly positive for α -SMA, whilst the latter was positive for α -SMA less constantly or in a few cells (Figure 2C). The former was positive in a few cells or almost negative for h-CD and desmin, and the latter was negative for both (Figure 2D and E). The cytoskeletal feature of the muscularis mucosa was that of differentiated smooth muscle cells, namely, positive for α -SMA, desmin, and h-CD. Therefore, corresponding to the morphological transition, a continuous transition apart from the differentiated smooth muscle phenotype of the muscularis mucosa was revealed in the bundles of eosinophilic stromal cells. The presence of type I procollagen, both in protein level by immunohistochemistry and in mRNA level by *in situ* hybridization, was demonstrated in the less eosinophilic stromal cells with a lesser degree of differentiated smooth muscle phenotype, that is, less constantly positive for α -SMA and negative for desmin or h-CD (Figure 2F-H).

DISCUSSION

In our experience of routine histopathological diagnosis of early invasive colorectal adenocarcinomas, we commonly see bundles of eosinophilic stromal cells, which are confused with disarrayed muscularis mucosa remains around invasive carcinomatous glands. In the present case, an obvious transition between the

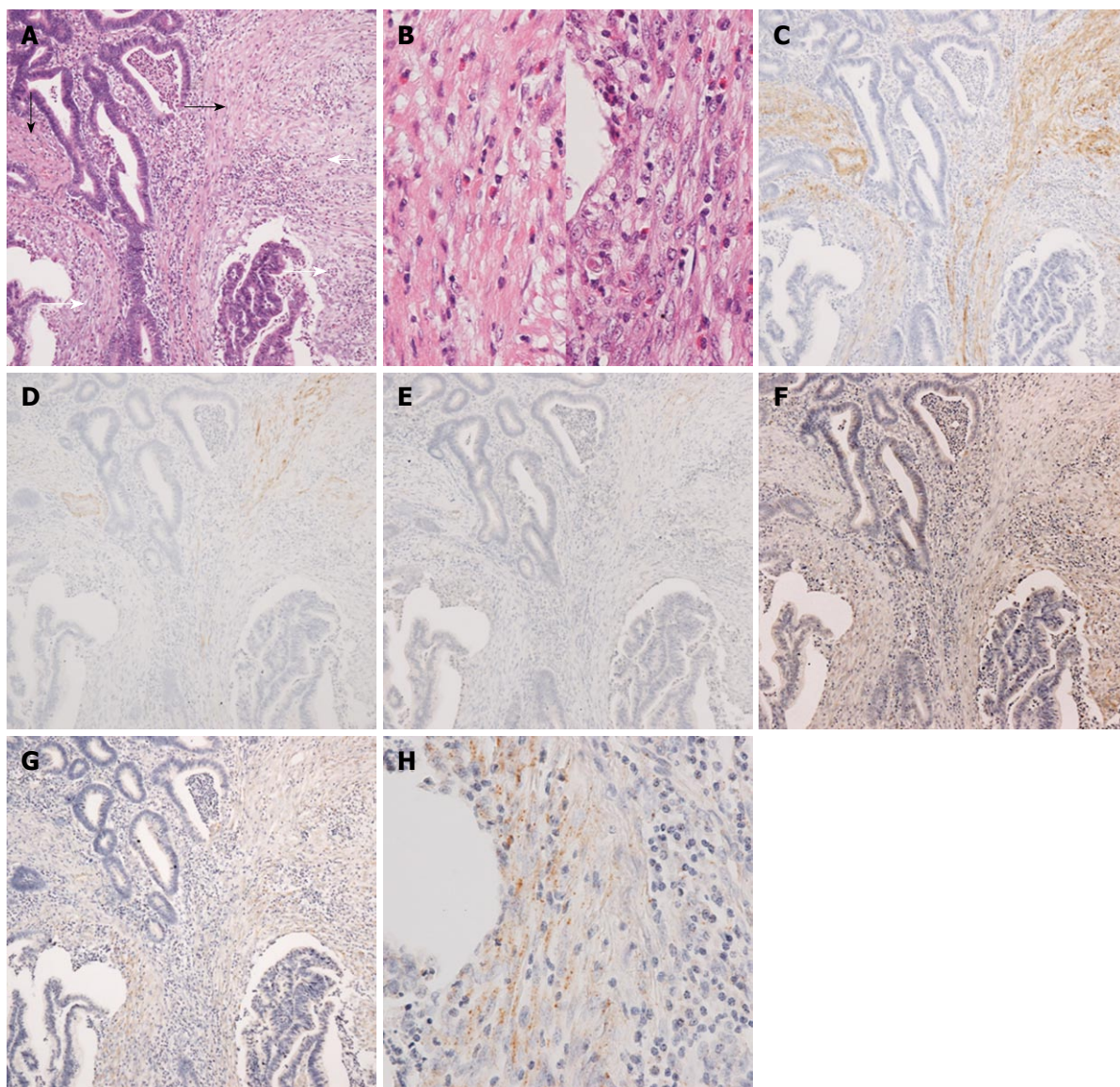


Figure 2 Middle power view of the invasive area (A) indicated by dotted box in Figure 1 shows a transition from bundles of eosinophilic stromal cells more similar to smooth muscle cells of the muscularis mucosa (black arrows) to those composed of less eosinophilic stromal cells with plumper nuclei (white arrows) (HE stain, $\times 100$). Higher magnification of the former eosinophilic stromal cells (left) and the latter ones (right) is shown in B (HE stain, $\times 400$). Immunohistochemistry of the serial sections demonstrates the former being more constantly positive for α -SMA compared to the latter (C), and h-CD is positive in a few cells in the former, and negative in the latter (D). Desmin is almost negative for both the former and the latter (E). Immunohistochemical expression of type I procollagen (F) and the presence of type I procollagen mRNA by *in situ* hybridization (G, H) are demonstrated in the latter area (original magnification of C to G, $\times 100$). Higher magnification of type I procollagen *in situ* hybridization is shown in H, which demonstrates positive signals in plump stromal cells (H, $\times 400$).

muscularis mucosa and the bundles of eosinophilic stromal cells (both in morphology and in their cytoskeletal phenotype) was demonstrated, which we think conceivably suggests that the smooth muscle cells of the muscularis mucosa could convert to the eosinophilic stromal cells in the stroma of invasive colorectal adenocarcinomas. Moreover, considering their eosinophilic and α -SMA positive features, the eosinophilic stromal cells could be referred to as myofibroblasts or myofibroblastic cells, so we could paraphrase that the muscularis mucosa could convert to myofibroblasts or myofibroblastic cells, making it one of the sources of myofibroblasts in the stroma of invasive colorectal adenocarcinomas. This concept is supported by an ultrastructural morphological study

that showed the smooth muscle cells of muscularis mucosae or muscularis propria as a possible source of myofibroblasts in the stroma of invasive colorectal adenocarcinomas^[13].

The stromal change associated with carcinoma invasion has been called desmoplasia or desmoplastic reaction, which is characterized by modifications in the composition of stromal cells and ECM components, the latter being an excessive deposition of ECM components such as collagen, fibronectin, and proteoglycan, and type I collagen is one of the ECM components which deposits in desmoplastic tumor stroma^[1-3]. In the present case, type I procollagen was demonstrated in both protein and mRNA expression in the areas of eosinophilic stromal cells that showed

a transition from the muscularis mucosa and a lesser degree of differentiated smooth muscle phenotype, implying that the myofibroblastic cells converted from smooth muscle cells of the muscularis mucosa could be responsible for type I collagen production and play a role in the formation of desmoplastic stroma. This notion is supported by an *in vitro* study that showed the induction of proteoglycan production of smooth muscle cells derived from the colonic wall by colon carcinoma cells^[14].

Two different sources of myofibroblasts or myofibroblastic cells have been proposed, i.e. bone marrow-derived circulating fibrocytes and local resident cells such as fibroblasts^[8], the former being stressed recently^[15]. However, the result of this case study seems to reemphasize roles of local resident cells in the desmoplastic reaction associated with carcinoma invasion. During the early invasive process of colorectal carcinomas, the muscularis mucosae may not be a passive barrier through which colorectal carcinomas infiltrate into the submucosa. They may, rather, play an active role in the formation and remodeling of tumor stroma and facilitate the invasive process of carcinoma cells.

ACKNOWLEDGMENTS

We thank Mr. Yoshiya Goto and Mr. Koichi Kamada for their technical help with immunohistochemistry and *in situ* hybridization.

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S- Editor Cheng JX L- Editor O'Neill M E- Editor Yin DH

CASE REPORT

Cystic Brunner's gland hamartoma in the duodenum: A case report

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Received: August 6, 2009 Revised: September 11, 2009

Accepted: September 18, 2009

Published online: October 21, 2009

World J Gastroenterol 2009; 15(39): 4980-4983 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4980.asp>
DOI: <http://dx.doi.org/10.3748/wjg.15.4980>

INTRODUCTION

Brunner's gland hamartoma is an infrequently encountered benign tumor in the proximal duodenum, accounting for approximately 5% of all duodenal masses^[1,2]. Fifty-seven percent of the tumors originate from the duodenal bulb and the incidence of the tumor decreases as the distance from the pyloric ring grows^[3].

Cystic lesions of the Brunner's glands are exceedingly rare. Only 12 cases have been documented in the literature under various terms such as Brunner's gland cyst, cystadenoma, mucocoele, and cystic Brunner's gland hamartoma^[4]. There has been no literature depicting predominant cystic lesions of Brunner's gland hamartoma in imaging modalities, except for two cases that were found at endoscopic ultrasonography. Although mild dilatation of the ducts may be present in Brunner's gland lesions, it is only detected in histologic test^[5,6].

Herein, we introduce a rare cystic Brunner's gland hamartoma with a long stalk arising from the duodenal third portion found on multidetector-row computed tomography (MDCT), magnetic resonance imaging (MRI), and a modified small bowel series, together with pathologic correlation.

CASE REPORT

A 30-year-old man with a 3-d history of aggravated episodes of nausea, vomiting, and epigastric pain was referred by a private clinic. He was healthy with no specific family or past medical history. Physical examination and laboratory evaluation revealed no abnormalities.

Upper tract endoscopy was not performed because endoscopic results reported by the private clinic were within the normal limits. MDCT showed a 4 cm × 4 cm polypoid mass with multifocal internal low densities in the proximal jejunum just below the Treitz ligament (Figure 1A), and the lesion's long stalk arising from the third portion of the duodenum (Figure 1B). Multifocal high-signal intensities on T2-weighted MRI reflected cystic components (Figure 1C), which were identical to the low densities on

Abstract

Cystic Brunner's gland hamartoma in the duodenum is exceedingly rare, although microscopic examination may sometimes reveal a Brunner's gland hamartoma containing dilated ducts in the duodenum. We present a case of large cystic Brunner's gland hamartoma in the duodenum with a long stalk, which is described in light of multidetector-row computed tomography, magnetic resonance imaging, and a modified small bowel series, together with pathologic correlation and differential diagnosis.

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Key words: Brunner's gland; Hamartoma; Duodenum; Multidetector-row computed tomography; Magnetic resonance imaging

Peer reviewers: Dr. Serdar Karakose, Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey; Kazuo Chijiwa, Profesor, Department of Surgery 1, Miyazaki University School of Medicine, Miyazaki, 889-1692, Japan

Park BJ, Kim MJ, Lee JH, Park SS, Sung DJ, Cho SB. Cystic Brunner's gland hamartoma in the duodenum: A case report.

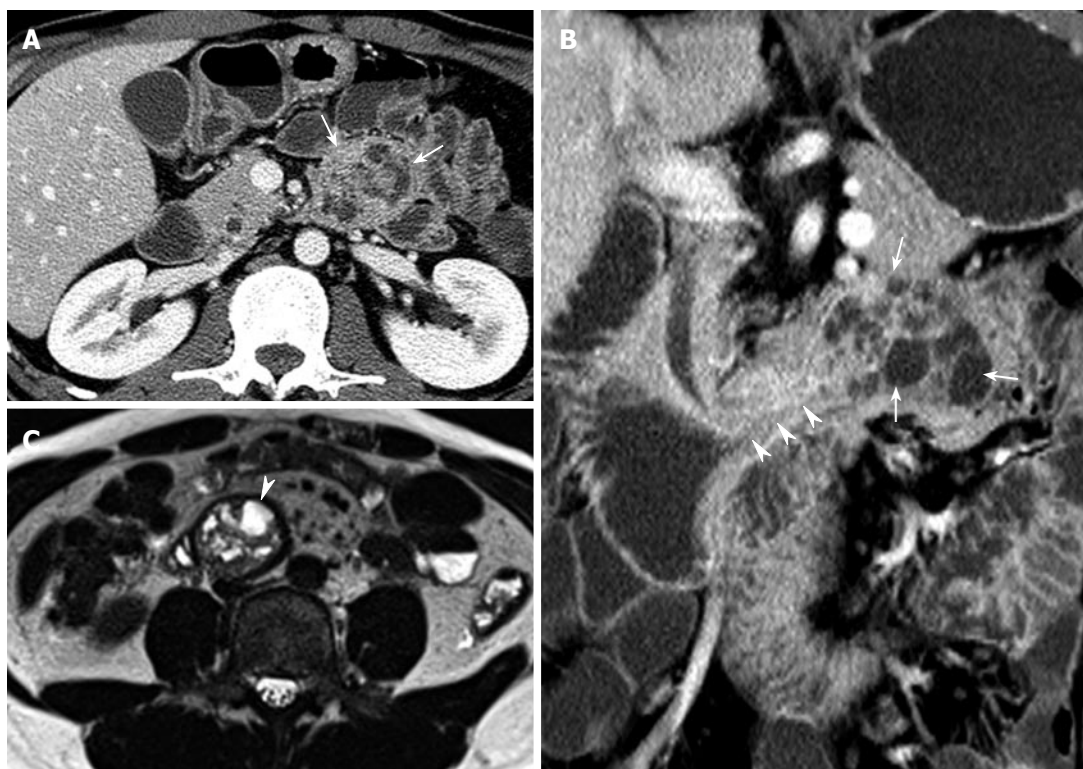


Figure 1 CT and MRI findings. A: Axial portal venous-phase CT scan showing an intraluminal round mass (arrows) with internal multifocal low densities in the proximal jejunum; B: Coronal reformatted portal venous-phase CT demonstrating an identical configuration to the modified small bowel series showing a long stalk (arrowheads) and a well-defined mass with multifocal cystic lesions (arrows); C: A large, well-defined multi-chambered cystic mass showing high signal intensities (arrowhead) with its moved location in the third portion of the duodenum on axial T2-weighted images.

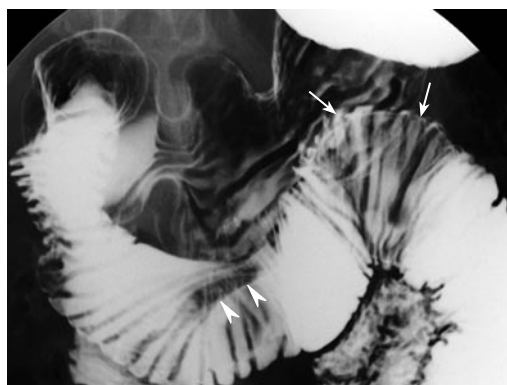


Figure 2 Modified small bowel series findings. A well-demarcated round filling defect (arrows) at the proximal jejunum just below the Treitz ligament and a tubular filling defect with a long stalk (arrowheads) originating from the proximal third portion of the duodenum.

MDCT (Figure 1A and B). The lesion's location moved to the third portion of the duodenum on MRI (Figure 1C). On the modified small bowel series, a mobile smooth lobular filling defect with a long stalk was visible in the proximal jejunum (Figure 2).

A pedunculated, well circumscribed mass was removed at laparoscopic enterotomy of the proximal jejunum, and the residual long remnant stalk was removed by post-operative endoscopy. The cut surface of the resected specimen revealed an approximately 4 cm × 3 cm whitish ovoid mass with multiple internal cystic spaces (Figure 3A). Histological examination of the specimen found that the

main body of the polyp contained a lobular collection of mature Brunner's glands with large multifocal areas of glandular cystic dilation (Figure 3B and C). There was no evidence of malignancy, and the lesion was diagnosed as a pedunculated cystic Brunner's gland hamartoma.

DISCUSSION

The etiology of Brunner's gland hamartoma has not been clearly elucidated. Brunner's gland hamartoma represents 5%-10% of all small bowel tumors and occurs most commonly in the fifth and sixth decades of life, with no gender or race predominance^[7]. Brunner's gland hamartoma is most commonly located on the posterior wall of the duodenum near the junction of the first and second portions. In a series of 27 patients described by Levine *et al*^[8], Brunner's gland hamartoma was located in the duodenal bulb, the second and third portions of the duodenum of 70%, 26%, and 4% of the cases, respectively. In the present case, the long stalk base was located in the proximal third portion of the duodenum.

Although Brunner's gland hamartoma is incidentally found, Levine *et al*^[8] reported that the most common presentations in symptomatic patients are gastrointestinal hemorrhage (37%) and obstructive symptoms (37%). Rarer presentations have been found in conjunction with recurrent pancreatitis^[9], obstructive jaundice^[10], and biliary fistula^[11]. Brunner's gland hamartoma is usually non-dysplastic and entirely benign. However, at least one recent report is available on a Brunner's gland hamartoma

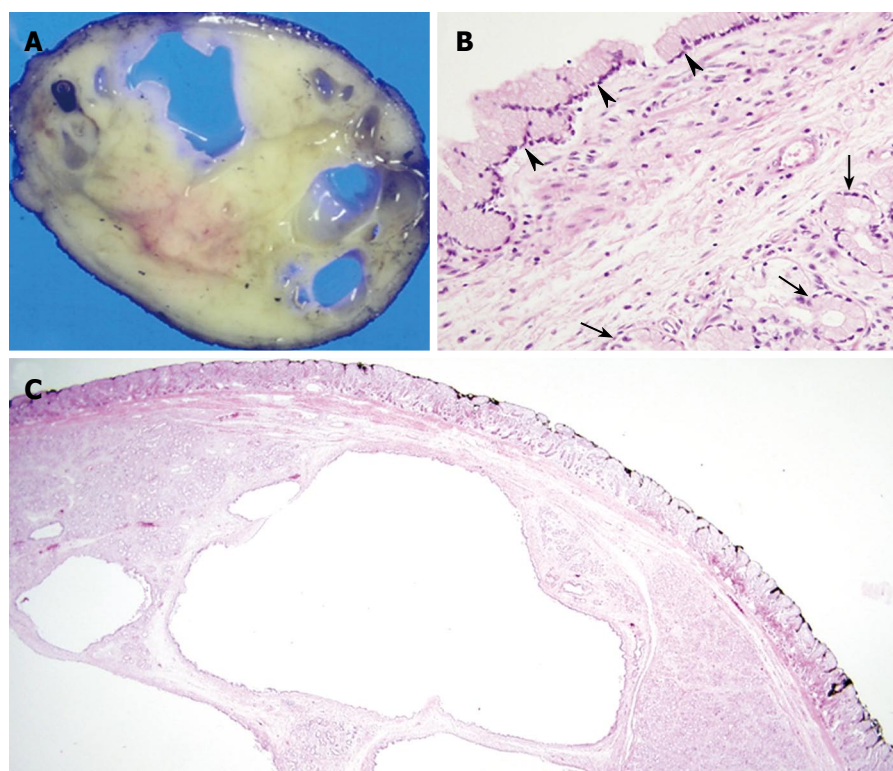


Figure 3 Histologic findings of the specimen. A: Cut surface of the gross specimen showing a multiloculated cystic appearance filled with mucin-like material; B: Aggregated glands (arrows) are lined with cuboidal to columnar cells with abundant pale cytoplasm and a basally-located oval nucleus, resembling a normal Brunner's gland in histopathologic findings of cystic Brunner's gland hamartoma (HE, $\times 200$) while the large cystic dilated gland showing the same lining (arrowheads) as a normal Brunner's gland; C: Cystic Brunner's gland hamartoma showing a well-demarcated nodular lesion, composed of a lobular collection of tubuloalveolar glands separated by fibrous septa, beneath the muscularis mucosa, in the low power field ($\times 12.5$), while some glands showing cystic dilatation.

with malignant potential^[12]. Brunner's gland hamartoma of the duodenum can be removed endoscopically because it is usually benign. However, we performed laparoscopic enterotomy because endoscopic results reported by the private clinic were within the normal limits. It has been suggested that endoscopic or surgical removal of Brunner's gland hamartoma can prevent the development of complications and endoscopic polypectomy represents the ideal approach depending on the site and size of Brunner's gland hamartoma and the presence of a peduncle^[13]. In the present case, considering its size and distal location, the endoscopist was reluctant to perform the procedure, hence laparoscopic enterotomy was performed as our next treatment option.

Patel *et al*^[14] have reported seven cases of Brunner's gland hamartoma on CT. In that report, two lesions with homogenous enhancement correlated with histologic findings of solid glandular proliferation, and the remainder were found with heterogeneous enhancement and hypodensities. The latter cases revealed smooth muscle proliferation and small cystic dilatation of the Brunner's glands. Up to now, to our knowledge, no study has described predominant cystic lesions of Brunner's gland hamartoma found on imaging modalities such as MDCT and MRI, except for two cases that were found on endoscopic ultrasonography^[5,6]. Endoscopic ultrasonography provides additional information about the internal components of the duodenal mass. Imaging modalities such as MDCT, MRI, and modified small bowel series were useful for tissue characterization and localization of the mass in our case, because the large pedunculated mass of the jejunum was difficult to approach by endoscopic ultrasonography.

In the present case, large cyst lesions in a pedunculated mass were revealed by characteristic multifocal high-signal

intensities on T2-weighted MRI images, and large hypodensities on enhanced MDCT correlated with large cystic dilatation of the glands. Mobility due to its long stalk was confirmed by a modified small bowel series and positional change during serial imaging studies.

Diagnosis of Brunner's gland hamartoma is not always easy at present because radiological findings are often nonspecific^[15]. CT is useful only for confirming the absence of extra-luminal extension of a tumor^[16]. Radiologic findings of Brunner's gland hamartoma are extremely similar to those of several other lesions, including adenomatous polyps, lymphoma, carcinoid tumors, and mesenchymal tumors such as gastrointestinal stromal tumor, leiomyoma, and neurogenic tumor. Since duodenal tumors depicted in a cystic configuration through radiologic modalities are rare, the recognition of a cystic component in a solitary duodenal mass may be useful for preoperative diagnosis of Brunner's gland hamartoma.

In conclusion, cystic configuration of a solitary duodenal mass should be generally considered the clue to the diagnosis of Brunner's gland hamartoma.

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S- Editor Wang YR L- Editor Wang XL E- Editor Ma WH

CASE REPORT

Travel of a mis-swallowed long spoon to the jejunum

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Received: July 7, 2009 Revised: August 14, 2009

Accepted: August 21, 2009

Published online: October 21, 2009

Abstract

Foreign-body ingestion is a relatively common presentation at emergency departments, but long metallic spoon swallowing is an infrequent occurrence. Unlike most cases of foreign-body ingestion, there have been no reported cases of long foreign bodies reaching the jejunum. We report a rare case of a coffee spoon that was swallowed accidentally and passed through the pylorus and duodenal loop and reached the jejunum, with no complications.

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Key words: Spoon; Foreign body; Jejunum

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Song Y, Guo H, Wu JY. Travel of a mis-swallowed long spoon to the jejunum. *World J Gastroenterol* 2009; 15(39): 4984-4985
Available from: URL: <http://www.wjgnet.com/1007-9327/15/4984.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4984>

INTRODUCTION

Foreign body ingestion is a frequent gastrointestinal emergency. However, long spoon swallowing is a rare

event. Most swallowed spoons have been found in the stomach^[1-4]. Previously, there has been no reported case of a long spoon reaching the jejunum. We present a rare case of a swallowed spoon with a length of 15 cm. The object passed through the pylorus and reached the jejunum, without clinical complications.

CASE REPORT

A 33-year-old woman presented to our emergency room after accidentally swallowing a spoon. She stated that she attempted to use the spoon to remove a fish bone, which was lodge in the pharynx 20 h previously. She had no abdominal pain, fever, vomiting, dysphagia, voice change, cough, or difficulty breathing. The patient was previously healthy and her past medical history was insignificant. General and abdominal examination was unremarkable. There was no abnormal finding in the laboratory tests.

An urgent plain abdominal radiograph revealed a metallic foreign body in the lower mid-abdomen (Figure 1). Inspection and removal was attempted by flexible gastroscopy. Gastroscopy found that a long spoon was resident in the stomach, together with a large volume of food (Figure 2). However, several attempts to remove the spoon failed because the patient could not tolerate the resultant nausea when the spoon was pulled to the cardia. There was a small amount of bleeding around the cardia. To follow-up the migration of the spoon, a second abdominal radiograph showed that the object position had changed (Figure 3). The second endoscopy confirmed that the foreign body disappeared from the stomach. She had to be taken to the operating room for an exploratory laparotomy, which disclosed that a 15-cm spoon had passed into the jejunum, following about 200 cm of the ligament of Treitz (Figure 4). Laparotomy demonstrated no free pus and fluid within the peritoneal cavity. Blood supply for the intestine was good. No bleeding and perforation was found in the stomach and intestine. After surgery, the patient was well and was discharged home on postoperative day 7.

DISCUSSION

Fortunately, most ingested foreign bodies pass spontaneously from the gastrointestinal tract. However, 10%-20% require nonoperative intervention, and ≤ 1% require surgery^[3,5,6]. Foreign bodies may cause serious complications such as impaction, obstruction, or perforation of the digestive or respiratory tracts. When complications occur, there is a relatively high rate of



Figure 1 Abdominal X-ray showing a radiopaque shadow in the lower mid-abdomen consistent with a spoon.

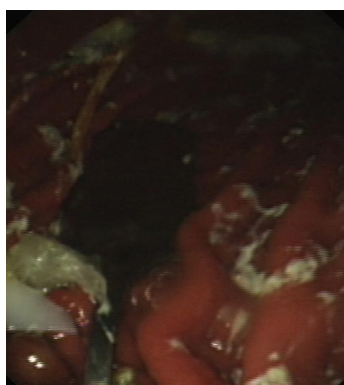


Figure 2 Laparotomy disclosing a metal spoon in the stomach.

mortality, with death rates as high as 1500 deaths per year in the United States^[7].

We tried to remove the spoon by endoscopy. However, our patient could not tolerate the discomfort associated with endoscopy, which had to be discontinued. Surprisingly, the spoon passed through the pylorus and duodenal loop several hours later. We had to remove the foreign body by surgery. In previous studies, long items such as forks or spoons, longer than 6-10 cm, have been unable to pass through the duodenal sweep^[5,6]. A Medline search indicated that other swallowed spoons have been found only in the esophagus and stomach. It has not been reported previously that a 15-cm spoon has passed through the pylorus and duodenal loop and reached the jejunum.

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Figure 3 The spoon had changed its position compared with that in Figure 1.

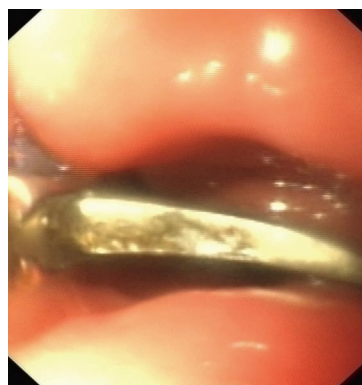


Figure 4 Laparotomy demonstrating that the spoon reached the intestine.

S- Editor Li LF L- Editor Kerr C E- Editor Yin DH



LETTERS TO THE EDITOR

Controversies about occult hepatitis B virus infection

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Received: July 13, 2009 Revised: August 31, 2009

Accepted: September 7, 2009

Published online: October 21, 2009

Abstract

We read with great interest the paper written by Shi *et al*, reviewing the molecular characteristics and stages of chronic hepatitis B virus (HBV) infection. We think that some points in the definition of occult HBV infection (OBI) and their conclusion about the management of OBI may need further considerations.

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Key words: Occult hepatitis B; Definition; Reactivation; Management

Peer reviewer: Jesus Prieto, Professor, Clinica Universitaria, University of Navarra, Pamplona 31080, Spain

Ozaslan E, Purnak T. Controversies about occult hepatitis B virus infection. *World J Gastroenterol* 2009; 15(39): 4986-4987 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4986.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4986>

TO THE EDITOR

We read with great interest the paper written by Shi *et al*^[1], reviewing the molecular characteristics and stages of chronic hepatitis B virus (HBV) infection. We think that some points in the definition of occult HBV infection (OBI) and their conclusion about the management of OBI may need further considerations.

First, they defined OBI as the existence of HBV DNA in serum, at a level < 20000 IU/mL. However, a recent meeting report^[2], clarified the confusion about the definition of OBI, describing it as the presence of

HBV DNA in the liver (with detectable or undetectable HBV DNA in serum) of individuals with negative HbsAg, and introduced a cutoff value for serum HBV DNA (< 200 IU/mL). So, cases whose serum HBV DNA levels are comparable to those in the different phases of serologically evident (overt) HBV infection are generally due to infection with escape mutants and should be labeled as “false” OBI^[2].

Second, Shi *et al*^[1] stated that OBI is a common and long-term consequence of acute hepatitis B resolution and termed it as secondary occult infection (SOI). Actually, SOI is the major clinical form of OBI that represents the tails of acute or chronic HBV infection^[2]. Cross-sectional studies across the spectrum of HBV infection have revealed a marked increase in OBI prevalence towards cirrhosis or hepatocellular carcinoma (HCC)^[3,4]. So, the majority of OBI cases are secondary to overt HBV infection and represent a residual low viremia level suppressed by strong immune response together with histological derangements occurred during acute or chronic HBV infection. Moreover, immune response to hepatocytes sustaining a low HBV replication level may contribute to chronic liver damage in the setting of OBI. Berasain *et al*^[5] showed that approximately 50% of patients with persistent hypertransaminasaemia of unknown etiology have chronic hepatitis or cirrhosis due to occult HBV or hepatitis C virus (HCV) infection.

Third, the authors concluded that no reports are available on the treatment of OBI. However, therapy should be considered during reactivation and cirrhosis settings. The reactivation of OBI in hemato-oncological malignancies (< 5%), although at a lower rate than that of HBsAg positive cases, carries a significant risk of mortality and morbidity^[6], which is much higher in the setting of stem cell transplantation^[7]. Many fatalities especially due to rituximab containing regimens have also been reported^[8-11]. Although a definitive conclusion cannot be reached at the moment, targeted therapy *via* HBV DNA monitoring or even routine pre-emptive nucleoside analogue prophylaxis was offered to all HBsAg negative/anti-HBc positive patients in recent consensus reports^[12,13]. Moreover, recent guidelines offer therapy for cirrhotic patients with a detectable HBV DNA level^[14].

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S- Editor Cheng JX L- Editor Wang XL E- Editor Zheng XM

ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Meetings

Events Calendar 2009

January 12-15, 2009

Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009

Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009

Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health
Disparities in Racial/Ethnic Minorities
and the Medically Underserved

February 7-10, 2009

Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009

Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A
Vital Partnership in Cancer Drug
Discovery and Development

February 13-16, 2009

Hong Kong Convention and
Exhibition Centre, Hong Kong, China
19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009

Orlando, Florida
AGAI/AASLD/ASGE/ACG Training
Directors' Workshop

February 27-Mar 1, 2009

Vienna, Austria
EASL/AASLD Monothematic:
Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009

Phoenix, Arizona
AGAI/AASLD Academic Skills
Workshop

March 20-24, 2009

Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on
Viral Hepatitis and Liver Disease

March 23-26, 2009

Glasgow, Scotland
British Society of Gastroenterology
(BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009

Silver Spring, Maryland
2009 Hepatotoxicity Special Interest
Group Meeting

April 18-22, 2009

Colorado Convention Center,
Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009

Copenhagen, Denmark
the 44th Annual Meeting of the
European Association for the Study
of the Liver (EASL)
<http://www.easl.ch/>

May 17-20, 2009

Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009

Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009

Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009

McCormick Place, Chicago, IL
DDW 2009
<http://www.ddw.org>

June 17-19, 2009

North Bethesda, MD
Accelerating Anticancer Agent
Development

June 20-26, 2009

Flims, Switzerland
Methods in Clinical Cancer Research
(Europe)

June 24-27 2009

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Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009

Beijing International Convention
Center (BICC), Beijing, China
World Conference on Interventional
Oncology
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009

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Pathobiology of Cancer: The Edward
A. Smuckler Memorial Workshop

July 17-24, 2009

Aspen, CO, United States
Molecular Biology in Clinical
Oncology

August 1-7, 2009

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CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009

Bell Harbor Conference Center,
Seattle, Washington, United States
Practical Solutions for Successful
Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009

Beijing International Convention
Center (BICC), Beijing, China
19th World Congress of the
International Association of
Surgeons, Gastroenterologists and
Oncologists(IASGO)
<http://iasgo2009.org/en/index.shtml>

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Taipei, China
Asian Pacific Digestive Week
<http://www.apdwcongress.org/2009/index.shtml>

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Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009

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and Marina, San Diego, CA, United
States
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Genetics, Biology, and Clinical
Applications

October 20-24, 2009

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on Tumor Microenvironment:
Progression, Therapy, and
Prevention

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Boston, MA, United States
The Liver Meeting

November 15-19, 2009

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Convention Center, Boston, MA,
United States
AACR-NCI-EORTC Molecular
Targets and Cancer Therapeutics

November 21-25, 2009

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Gastro 2009 UEGW/World Congress
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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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- 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

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- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G,** Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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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/eid.htm>

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- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Volume 15 Number 40
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World J Gastroenterol
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^[1]Passed away on October 20, 2007

^[2]Passed away on June 14, 2008



World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 15 Number 40
October 28, 2009



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PRINTING

Beijing Kexin Printing House

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PUBLICATION DATE

October 28, 2009

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Hepatitis C comorbidities affecting the course and response to therapy

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Received: April 22, 2009 Revised: September 5, 2009

Accepted: September 12, 2009

Published online: October 28, 2009

Abstract

Several studies have demonstrated that the outcome of chronic hepatitis C (CHC) infection is profoundly influenced by a variety of comorbidities. Many of these comorbidities have a significant influence on the response to antiviral therapy. These comorbidities negatively affect the course and outcome of liver disease, often reducing the chance of achieving a sustained virological response with PEGylated interferon and ribavirin treatments. Comorbidities affecting response to antiviral therapy reduce compliance and adherence to inadequate doses of therapy. The most important comorbidities affecting the course of CHC include hepatitis B virus coinfection, metabolic syndrome, and intestinal bacterial overgrowth. Comorbidities affecting the course and response to therapy include schistosomiasis, iron overload, alcohol abuse, and excessive smoking. Comorbidities affecting response to antiviral therapy include depression, anemia, cardiovascular disease, and renal failure.

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Key words: Comorbidities; Chronic hepatitis C; Response to therapy

Peer reviewer: Frank J Burczynski, Professor, Faculty of Pharmacy, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba, R3T 2N2, Canada

El-Zayadi AR. Hepatitis C comorbidities affecting the course and response to therapy. *World J Gastroenterol* 2009; 15(40): 4993-4999 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4993.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4993>

INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection varies throughout the world, with the highest number of infections reported in Egypt. The use of parenteral antischistosomal therapy in Egypt is thought to have contributed to a prevalence of antibodies against HCV in various regions ranging from 6% to 28% (mean, 22%)^[1]. An estimated 70% to 85% of infected patients are likely to develop chronic hepatitis, and up to 30% of these cases might progress to cirrhosis^[2].

When treating chronic hepatitis C (CHC), many clinicians do not take into consideration the presence of other comorbid conditions that lead to more progressive liver disease, such as cirrhosis and hepatocellular carcinoma (HCC)^[3-5]. In recent studies, it has been proved that such comorbidities might reduce the response rate to PEGylated interferon (PEG-IFN)/ribavirin (RBV) therapy in HCV patients^[6,7].

Eventually amelioration of these comorbidities before embarking on IFN-based therapy would improve the sustained virological response (SVR) and impair progression to cirrhosis and HCC.

COMORBIDITIES AFFECTING THE COURSE OF CHC

Hepatitis B virus (HBV) co-infection

Coexistent HCV infection has been estimated to be present in 10% to 15% of patients with chronic hepatitis B, and is more common among injecting drug users^[8]. Acute coinfection with HBV and HCV can shorten the duration of HBs antigenemia and lower the peak serum aminotransferase concentrations compared with acute HBV infection alone^[9,10]. However, acute coinfections of HCV and HBV, or acute HCV on preexisting chronic HBV, have also been reported to increase the risk of severe hepatitis and fulminant hepatic failure^[11].

However, combined chronic hepatitis B and C leads to more severe liver disease, an increased risk of hepatocellular carcinoma^[3,4] and lower response to IFN^[6]. Furthermore, co-infected patients represent a treatment challenge. No standard recommendations exist for treatment of viral hepatitis due to dual HBV/HCV infection, and therefore treatment must be individualized^[4].

Management: Treatment decisions should be based upon

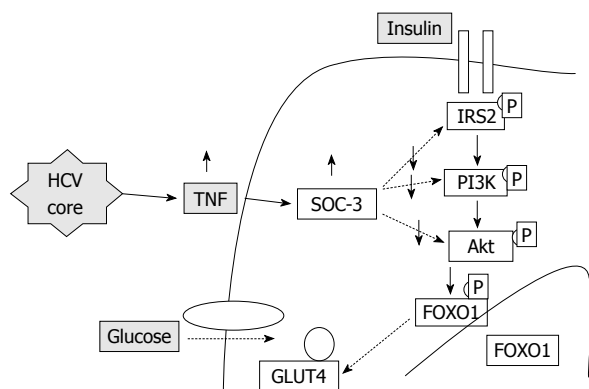


Figure 1 HCV-induced insulin resistance. Dotted lines represent inhibition, continuous lines represent activation. PI3K: Phosphatidylinositol 3 kinase.

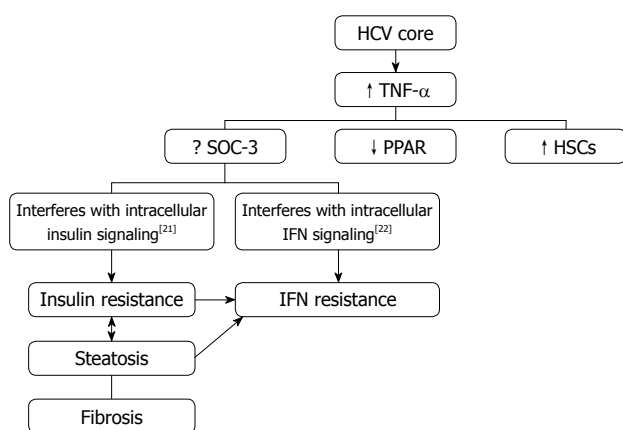


Figure 2 HCV-induced insulin resistance^[21-23]. SOC-3: Suppressor of cytokine-3; PPAR: Peroxisome proliferators activated receptor; HSCs: Hepatic stellate cells.

the determination of the "dominant" hepatitis virus. The more active virus should be treated using IFN plus RBV for hepatitis C and IFN plus Lamivudine for hepatitis B^[3,4]. Caution must be exercised in treating coinfecting patients, by observing reactivation of untreated virus as flares of the latter might occur.

Metabolic syndrome (MS)

MS is a cluster of abnormalities, including obesity, insulin resistance, type 2 diabetes mellitus, dyslipidemia, and hypertension. Moreover, patients with chronic HCV infection have increased prevalence of insulin resistance and of type 2 diabetes compared with age-, sex-, and liver disease-matched controls^[12].

It has been observed that overweight, insulin resistance, and liver steatosis have a negative impact on the course of CHC, being associated with more severe and progressive liver fibrosis^[5]. HCV proteins are associated with the dysfunction of mitochondria and endoplasmic reticulum that promote oxidative stress. The latter mediates signals that activate the expression of proinflammatory cytokines: tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, tumor growth factor- α , and the fas ligand..

Insulin resistance: HCV proteins activate the expression of TNF- α which inhibits the function of insulin receptor

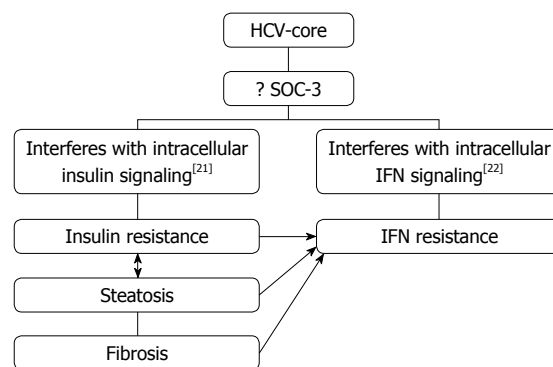


Figure 3 Core-protein induced insulin resistance^[21-23].

substrates (IRS) and decreases the expression of glucose transporter-4 and lipoprotein lipase in peripheral tissues, which are responsible for the promotion of insulin resistance (Figure 1).

Furthermore, reduced adiponectin levels, loss of adiponectin receptors, and decreased anti-inflammatory peroxisome proliferator-activated receptor α (PPAR- α) in the liver of HCV patients might contribute to reduced fatty acid oxidation, inflammation, and eventually, lipotoxicity^[12].

Insulin resistance has been clearly associated with steatosis, more severe and progressive fibrosis, and a reduced response to PEG-IFN and RBV therapy in HCV patients^[13]. Several recent studies have confirmed that SVR is impaired in patients with a high homeostasis model assessment index^[14].

Obesity/Hepatic steatosis: In hepatitis C infection, hepatic steatosis might be either metabolic [overweight & obesity (BMI ≥ 25), diabetes mellitus] or cytopathic due to the effect of the virus, as in genotype 3. However, genotype-4, which is predominant in Egypt, has no direct relation with the development of steatosis^[15]; however, it develops as a secondary metabolic effect as evidenced by HCV core protein promotes IR *via* TNF- α production^[16] (Figures 2 and 3). In addition, HCV non-genotype-3 induces insulin resistance through downregulation of IRS^[17-19]. HCV genotype-3 might induce a cytopathic effect and autoimmune aggression on β -cells of the pancreas^[20]. Furthermore, HCV produces necroinflammation of hepatocyte membranes with consequent malfunction of insulin receptors.

Several studies have recently confirmed that the SVR^[7] is impaired in patients with high body mass index and for those with hepatic steatosis. These data indicate that HCV carriers should avoid weight gain by diet and physical exercise before initiation of antiviral therapy, all efforts should be made to improve the metabolic steatosis of the patient.

Type 2 diabetes mellitus: Type 2 diabetes mellitus is frequently associated with hyperinsulinemia and fatty liver disease. The chronically elevated circulating insulin levels found in type 2 diabetic patients might be responsible for accumulation of fat in the liver by downregulating

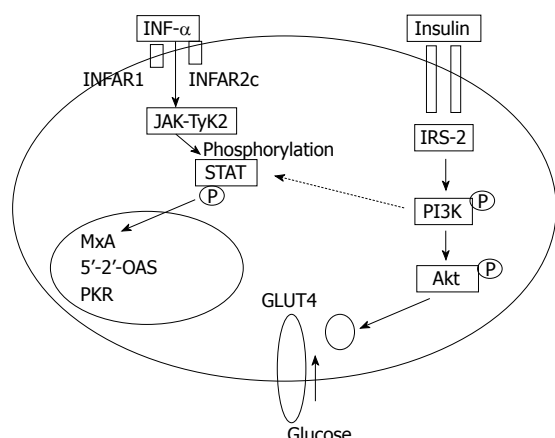


Figure 4 Insulin-induced IFN resistance. Interaction between insulin and the PEG-interferon α -signaling pathway. PI3K activated by insulin seems to be responsible for the block of STAT1 translocation that avoids the antiviral effect of interferon. Dotted lines represent inhibition, continuous lines represent activation. IRS-2: Insulin substrate-2; 2',5'-OAS: 2',5'-oligoadenylate synthetase; PKR: Protein kinase.

mitochondrial β -oxidation and blocking secretion of triglycerides from the liver. Diabetic individuals are at higher risk to develop non-alcoholic steatohepatitis, which might progress to cirrhosis in up to 5% of cases^[24,25].

Intestinal bacterial overgrowth (IBOG): Chronic liver disease is associated with slow, transient, altered gut permeability and translocation of intestinal bacteria and toxins to portal and systemic circulation^[26,27]. These toxins contribute to liver cell injury by induction of proinflammatory cytokines (TNF- α , IL-6, and IL-8) in the liver. Concomitant IBOG with HCV infection will aggravate the risk of severe hepatitis and progressive liver disease as a result of the immune response against HCV infection.

Management: Intestinal decontamination by metronidazole and probiotics to prevent endotoxin formation, open bowel by synthetic disaccharides (lactulose), and trigger peristalsis by prokinetics.

COMORBIDITIES AFFECTING THE COURSE AND RESPONSE TO THERAPY OF CHC

Schistosomiasis co-infection

Schistosoma mansoni has been the major risk factor of liver diseases in Egypt, especially in rural areas. In many patients, HCV infection is associated with schistosomiasis because of iv anti-schistosomal therapy^[28,29]. Schistosomiasis is an immunological disease, which suppress cellular immunity, triggering Th2 cytokine response favoring chronicity of hepatitis C infection.

Patients with CHC and concomitant schistosomiasis respond poorly to IFN therapy and have higher relapse rates compared to patients with HCV infection only. This might be due to HCV genotype 4 *per se*, or to the negative influence of schistosomiasis on the immune system,

leading to higher HCV RNA titers, and more severe liver damage with a higher incidence of cirrhosis. The latter is promoted by the longer duration of both infections^[30].

Diagnosis of schistosomiasis was based on a history of *Schistosoma* infection, detection of *S. mansoni* ova in stool, or a rectal biopsy^[31]. At baseline, patients with CHC and schistosomiasis had higher HCV RNA titers compared to patients without schistosomiasis^[30].

Management: Praziquantel 40 mg/kg single dose (can be repeated up to three weekly consecutive doses) before initiation of antiviral therapy.

MS

Insulin resistance has been clearly associated with steatosis, a more severe and progressive fibrosis, and a reduced response to PEG-IFN and RBV therapy in HCV patients^[13].

How does concomitant MS with HCV infection impair the response to interferon?

In obese patients, subcutaneous fat impairs absorption of interferon at the site of injection. In addition, hepatic steatosis decreases contact between interferon and hepatocytes receptors^[16]. Steatosis interferes with the signaling cascade of interferon [Janus Kinase (JAK) activate signal transduction and activator of transcription (STAT) which express *IFN* genes]^[32]. On the other hand, the HCV core protein promotes insulin resistance *via* TNF-production and insulin resistance induces steatosis, fibrogenesis, and IFN-resistance^[16,33,34]. In addition, HCV core protein and TNF- α upregulate suppressor of cytokines-3 which binds to JAK, inhibiting phosphorylation of STAT1^[35] and eventually interfering with IFN signaling^[33]. Furthermore, obesity in general is associated with a suppressed immune response^[36,37] (Figures 3 and 4).

Management of MS: Amelioration of metabolic factors before starting interferon therapy favors a good response to interferon therapy. HCV patients should avoid weight gain by life style modification (hypocaloric diet/exercise), use of insulin sensitizers, in the form of Metformin, to reduce hepatic gluconeogenesis, and pioglitazone to sensitize insulin receptors and mobilize visceral fat to subcutaneous tissues. Antioxidants (vitamin E, betaine, silymarin, and β -carotene), hepatoprotective drugs (UDCA) might add therapeutic benefit to inhibit the toxic effects of free radicals. Gut decontamination with metronidazole and probiotics to prevent gut endotoxin formation should be considered; the later induce proinflammatory cytokines in the liver that promote steatosis and steatohepatitis.

Iron overload

In hepatitis C patients, increased iron absorption in cirrhosis and difficulty in excreting iron from the body contribute to development of iron overload^[38]. There is growing evidence that iron overload enhances the amount of liver injury and progression to cirrhosis and HCC. In addition, it decreases the SVR to IFN/RBV treatment^[39,40].

Management: Venesection, restrict diets rich in iron, give antioxidants such as silymarin, betaine and vitamin E to nullify the toxic effects of free radicals.

Alcohol abuse

Alcohol abuse favors the development of alcoholic steatosis, steatohepatitis, and subsequently cirrhosis. It plays a role in resistance to interferon therapy through immunosuppression of CD4+ and NK cells^[41], by increased hepatic iron load, and by inhibiting the IFN- α -activated signals^[42,43].

Most studies found that alcohol decreased the response to interferon-based therapy and this effect is alcohol dose-dependent^[42-44]. Median daily alcohol use > 30 g/d is associated with failure to respond to PEG-IFN and RBV for treatment of hepatitis C. Past alcohol use should be evaluated when considering treatment for hepatitis C^[42].

Management: Stop alcohol abuse, intestinal antibiotics and probiotics to prevent gut endotoxin formation; and antioxidants as vitamin E, betaine and silymarin to block lipid peroxidation. Pentoxifylline is an oral phosphodiesterase inhibitor which decreases expression of TNF- α (and other proinflammatory cytokines) and which may inhibit apoptosis. When the full course is completed, the SVR is similar, regardless of alcohol intake^[45].

Excessive smoking

Heavy smoking increases the severity of hepatic inflammation and fibrosis when associated with hepatitis C infection^[46]. Heavy smoking induces resistance to interferon therapy by suppression of CD4+ and NK activity^[41] inducing apoptosis of T-cells^[47], and increasing hepatic iron load^[48].

Management: Stop smoking, venesection to reduce iron level, limit diet rich in iron and use of antioxidants as silymarin, vitamin E, betaine, β -carotene, lecithin and selenium.

COMORBIDITIES AFFECTING RESPONSE TO INTERFERON THERAPY

These comorbidities reduce compliance and adherence to inadequate PEG-IFN or RBV doses.

Depression

Depression is significantly more prevalent in chronically HCV-infected patients than in the general population^[49], which negatively affect patients' functional health, ability to work, self-perceived health, health-related quality of life (HRQL) and well being^[50].

The presence of mild/moderate depression at baseline is not considered an absolute contraindication to initiate antiviral therapy with PEG-IFN and RBV. However, this condition is certainly associated with a higher risk of developing severe depression during therapy that might lead to higher rates of treatment discontinuation in the absence of adequate antidepressant therapy^[51].

It has been reported that IFN- α downregulated glucocorticoid receptor (GR) and serotonin receptor 1A (5-HTR1A) levels in cell lines. These levels of GR and 5-HTR1A, following IFN- α -induced downregulation, recovered after withdrawal of IFN- α or addition of desipramine or fluoxetine. These data provide insights regarding the pathogenesis of IFN- α -induced depression^[52].

The pharmacokinetics of PEGylated IFN are different from those of standard IFN^[53] and the time of depression occurrence also differs between PEGylated and standard IFN^[54]; therefore, it will be important to determine if the mechanisms described by Cai *et al*^[52] on GR and 5-HTR1A receptors also apply to PEGylated IFN. In addition, anxiety and depression impair the level and activity of B cells, T cells, and NK cells^[55]. No differences in depression rates were observed by Neri *et al*^[56] comparing PEGylated- α 2a and PEGylated- α 2b, and this finding has recently been confirmed in the large randomized comparison trial IDEAL^[57].

Depression and anxiety of significant severity can adversely effect compliance and tolerance to medication. Although anemia and depression were associated with HRQL impairment, depression was the most consistent predictor^[50]. Several studies concluded that individuals who experience significant worsening of depressive symptoms during IFN therapy are less likely to achieve a virological response during therapy and an SVR after therapy withdrawal^[13].

Management: Therapeutic intervention has been shown to be effective in the management of IFN-induced depression in a controlled study^[58]. The most effective and well-studied antidepressants in this setting are those of the selective serotonin reuptake inhibitor class, in particular citalopram 25 mg/d^[59]. Importantly, all patients who received antidepressant treatment were able to complete the full course of IFN therapy; while discontinuation was necessitated in patients in the placebo arm^[60].

Anemia

Anemia that develops in a patient receiving HCV therapy often has multiple potential contributing factors, including RBV, interferon or PEG-interferon, underlying liver disease caused by HCV infection, and co-morbid conditions, such as HIV infection or chronic renal failure^[61]. The anemia associated with RBV most often occurs as dose-dependent hemolytic anemia, typically developing within the first 4 wk of therapy^[62,63]. At higher doses of RBV (1000-1200 mg/d), hemoglobin levels frequently decline by 2-3 g/dL. In addition to causing hemolysis, RBV can also downregulate the number of erythropoietin receptors^[64]. Interferon can also contribute to the development of anemia by suppressing bone marrow production of erythrocytes, but this process is generally slower and might account for the continued decline in hemoglobin concentration during the second and third months of treatment. Finally, patients developing anemia during HCV therapy often have inappropriately poor serum erythropoietin responses^[65], probably related to their underlying liver disease. It is often not possible to

pinpoint one particular drug as the primary cause of the anemia, because of the mixed nature of HCV treatment-associated anemia.

Management of anemia associated with HCV Therapy:

The conventional standard of care for managing anemia during HCV antiviral therapy has consisted of reducing the RBV dose by half if the hemoglobin level decreases to less than 10 g/dL, and to completely stop the RBV if the hemoglobin level drops below 8.5 g/dL. A decrease in RBV dose, especially in the first several months of treatment, can diminish response rates considerably. Furthermore, the strategy of decreasing the RBV dose will only partially correct the anemia. On average, the hemoglobin level will increase 1 g/dL with RBV dose reduction^[66]. Finally, some patients who have co-morbid conditions, such as diabetes mellitus, coronary artery disease, and chronic obstructive pulmonary disease, might poorly tolerate even mild levels of anemia.

The recombinant erythropoietin hormone, epoetin α , has emerged as an excellent option for improving HCV treatment-related anemia while supporting optimal treatment doses of RBV and interferon. Recombinant erythropoietin hormone acts by increasing the number of erythroid progenitor cells, and has demonstrated efficacy and safety in patients with chronic renal disease, those with malignancies receiving chemotherapy. Patients receiving once weekly epoetin α (4000 unites) had significantly higher hemoglobin levels at week 16^[67]. The major drawbacks of using any of the recombinant erythropoietin medications are high cost and slightly increased risk of thrombotic events.

Cardiovascular disease

HCV RNAs were found in the hearts of patients with cardiomyopathies, and negative strands of HCV RNA were also detected in the hearts, suggesting that HCV replicates in myocardial tissues^[68].

A major subset of CHC patients currently considered ineligible for PEG-IFN/RBV is represented by those with co-existing clinically significant heart disease. Durante-Mangoni *et al*^[69] prospectively evaluated safety and efficacy of PEG-IFN/RBV treatment in CHC patients with heart disease. They concluded that treatment with PEG-IFN/RBV might be safely offered to CHC patients with co-existing, clinically significant, heart disease. In qualified centers, CHC patients with overt heart disease should not be denied treatment, whenever indicated^[69].

However, some patients with coronary artery disease poorly tolerate even mild levels of anemia. Hence, doses should be reduced in more than 25% of patients for both PEG-IFN/RBV to avoid the serious adverse events on the sick heart.

Renal failure

CHC and chronic renal failure might occur together because of the association of HCV infection with cryoglobulinemia and membranoproliferative glomerulonephritis, or by infection of chronic renal failure patients

from exposure to HCV-contaminated blood or hemodialysis equipment.

Those with renal failure and chronic HCV infection might have significant liver disease. Although patients on dialysis tend to have milder liver disease and normal liver enzymes compared with patients with normal renal function^[70], patients with end-stage renal disease (ESRD) and CHC might have severe chronic hepatitis on liver biopsy^[71].

A liver biopsy should be performed in patients with CHC who are receiving hemodialysis^[72] and do not have major comorbidities. Among dialysis patients who are not candidates for renal transplantation, antiviral therapy is recommended in those with fibrotic disease for viral eradication and potential reduction of the stage of fibrosis.

In candidates for renal transplantation, cirrhosis is a contraindication to renal transplantation^[73]; a combined liver and kidney transplantation might be indicated in patients who progress to decompensated cirrhosis. In candidates for renal transplantation, treatment is appropriate in the pre-transplant setting^[73]. Interferon therapy is ineffective and has an unacceptably high risk of precipitating rejection after transplantation. Even in patients with mild liver disease, antiviral treatment is recommended to obtain a SVR before transplantation^[73], which will avoid the risk of progressive liver disease after transplantation.

Management of HCV infection associated with renal failure:

RBV is cleared by the kidneys and thus contraindicated in patients with renal failure^[74,75]. There is a risk of enhancement of the RBV-related hemolytic anemia, with a marked fall in hemoglobin levels. Interferon monotherapy at the standard dosing schedule of 3 million units subcutaneously three times weekly, or PEG-interferon monotherapy (α -2a or α -2b) injected once weekly, is used in patients with renal disease. The pharmacokinetics are similar to patients with renal disease down to a creatinine clearance of 20 mL/min. Trials of PEG-interferon in patients with ESRD have used either 135 mcg of PEG-interferon α -2a or 0.5-1.0 mcg/kg of PEG-interferon α -2b. In dialysis patients, the sustained virologic response achieved with interferon monotherapy is at least as good as in the general population^[76]. Adverse events leading to drug discontinuation, in decreasing order of frequency, include flu-like symptoms, neutropenia, depression, and neurological symptoms. Drop-out rates seen with interferon given at 3 million units three times weekly are between 20% and 30%. PEG-interferon monotherapy trials in patients with ESRD are currently under way.

CONCLUSION

In chronic hepatitis C, control or amelioration of comorbidities before embarking on antiviral therapy represents the milestone for higher post-antiviral therapy response.

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EDITORIAL

Achalasia: A review of Western and Iranian experiences

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Received: July 29, 2009 Revised: September 21, 2009

Accepted: September 28, 2009

Published online: October 28, 2009

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Key words: Achalasia; Esophagus; Motility; Treatment

Peer reviewer: Michele Cicala, Professor, Dipartimento di Malattie dell'Apparato Digerente, Università Campus Bio-Medico, Via Longoni, 83-00155 Rome, Italy

Mikaeli J, Islami F, Malekzadeh R. Achalasia: A review of Western and Iranian experiences. *World J Gastroenterol* 2009; 15(40): 5000-5009 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5000.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5000>

Abstract

Achalasia is a primary motor disorder of the esophagus, in which esophageal emptying is impaired. Diagnosis of achalasia is based on clinical findings. The diagnosis is confirmed by radiographic, endoscopic, and manometric evaluations. Several treatments for achalasia have been introduced. We searched the PubMed Database for original articles and meta-analyses about achalasia to summarize the current knowledge regarding this disease, with particular focus on different procedures that are used for treatment of achalasia. We also report the Iranian experience of treatment of this disease, since it could be considered as a model for medium-resource countries. Myotomy, particularly laparoscopic myotomy with fundoplication, is the most effective treatment for achalasia. Compared to other treatments, however, the initial cost of myotomy is usually higher and the recovery period is longer. When performing myotomy is not indicated or not possible, graded pneumatic dilation with slow rate of balloon inflation seems to be an effective and safe initial alternative. Injection of botulinum toxin into the lower esophageal sphincter before pneumatic dilation may increase remission rates. However, this needs to be confirmed in further studies. Due to lack of adequate information regarding the role of expandable stents in the treatment of achalasia, insertion of stents does not currently seem to be a recommended treatment. In summary, laparoscopic myotomy can be considered as the procedure of choice for treatment of achalasia. Graded pneumatic dilation is an effective alternative when the performance of myotomy is not possible for any reason.

INTRODUCTION

Achalasia is the most recognized primary motor disorder of the esophagus. In idiopathic achalasia, inhibitory ganglion cells in the myenteric plexuses of the esophagus undergo inflammatory degeneration. The term achalasia means “failure to relax”; the loss of ganglion cells leads to a defect in lower esophageal sphincter (LES) relaxation, which is the principal feature of idiopathic achalasia and causes functional obstruction of the esophagus, and reduced peristalsis in the esophageal body, which further impairs esophageal emptying. The functional obstruction in the LES is overcome only when the hydrostatic pressure of the retained material in the esophagus exceeds the LES pressure^[1]. Secondary achalasia may occur following several conditions, which are listed in Table 1.

The reported annual incidence of achalasia is approximately 1 per 100 000^[2]. Men and women are affected with equal frequency^[3]. Although achalasia is diagnosed mainly in people aged between 25 and 60 years, it may occur at any age^[2-4]. In general, achalasia has a subtle onset, and symptoms progress gradually. Therefore, patients experience symptoms for months or years before their disease is diagnosed. In a series of 87 patients with newly-diagnosed achalasia, the mean duration of symptoms was 4.7 years. The delay in diagnosis was due to misinterpretation of symptoms by physicians and atypical clinical manifestations^[5]. Many patients are treated for other disorders such as gastro-esophageal reflux disease (GERD) before the diagnosis of achalasia is made^[3]. The main symptom of achalasia is dysphagia^[3]. To relieve dysphagia and other symptoms of achalasia, several treatments have been introduced,

including medical therapy, myotomy, pneumatic dilation of the LES, and injection of botulinum toxin into the LES. Early treatment of achalasia may also reduce the reported increased risk of esophageal cancer among patients with achalasia^[6,7].

In this article, we report results of our review of medical literature on the subject of achalasia. Our aim is to summarize the current knowledge as regards this disease, with particular focus on different procedures that are used for treatment. Furthermore, several articles about achalasia have been published from Iran, mainly from the Digestive Diseases Research Center, Shariati Hospital of Tehran University of Medical Sciences, a nationwide referral center for the disease, where we provide follow-up care for 675 patients with achalasia. We present results of the Iranian studies, since our experience could be considered as a model for medium-resource countries.

PATHOPHYSIOLOGY

In achalasia, inhibitory ganglion cells in the myenteric plexuses of the esophagus, which produce nitric oxide, undergo inflammatory degeneration^[8-10]. The remaining ganglion cells are often surrounded by lymphocytes and, to a lesser extent, by eosinophils^[8,11]. Loss of inhibitory ganglion cells has two consequences: (i) basal LES pressure rises, which impairs normal relaxation of the sphincter and esophageal emptying, and (ii) the smooth muscle layer in the esophageal body loses normal peristalsis.

Several studies have suggested that a viral infection or some other environmental factor may initiate inflammation in the myenteric plexus. For example, a recent study suggested that the inflammatory reaction might be triggered by human herpes virus type 1^[12]. In certain individuals, the inflammation may lead to an autoimmune response against ganglion cells. Genetic susceptibility may play a role in this process^[13]. There are some reports of an association between some human leukocyte antigens and the presence of circulating antibodies to enteric neurons and the loss of ganglion cells^[14]. An hereditary association between achalasia and some other conditions such as adrenal glucocorticoid deficiency and alacrima has been reported^[15]. In our study on 25 children with achalasia, 2 cases (8%) had Achalasia, Alacrima, and Adrenal insufficiency (triple A syndrome or Allgrove syndrome) and 3 cases (12%) had Achalasia and Alacrima (double A syndrome). All of the 5 cases were siblings from two families^[16].

CLINICAL FEATURES

The prevalence of the main symptoms of achalasia is shown in Table 2. The most common symptom of achalasia is dysphagia to solid foods (over 99%), followed by dysphagia to liquids (90% to 95%)^[17]. Although dysphagia to liquids can occur in patients with other esophageal motility disorders (e.g. progressive systemic sclerosis), this symptom strongly suggests achalasia^[3].

Regurgitation, active or passive, occurs in 70% to

Table 1 Causes of secondary achalasia

Malignancy, especially carcinoma
Chagas' disease
Amyloidosis
Sarcoidosis
Neurofibromatosis
Eosinophilic gastroenteritis
Multiple Endocrine Neoplasia, type 2B
Juvenile Sjögren's syndrome with achalasia and gastric hypersecretion
Chronic idiopathic intestinal pseudo-obstruction
Anderson-Fabry disease

Table 2 Prevalence of symptoms in achalasia

Symptom	Percentage (%)
Dysphagia to solids	99
Dysphagia to liquids	93
Active regurgitation	84
Passive regurgitation	68
Weight loss	61
Chest pain	59
Nocturnal cough	45
Heartburn	35
Nocturnal dyspnea	20
Hiccup	8

80% of cases. Regurgitation can be troublesome; this may lead to aspiration when patients lie down^[2,3]. Weight loss, chest pain, and heartburn occur in approximately 40% to 60% of patients. Although severe weight loss can happen, the usual weight loss is 5 to 10 kg.

Occasionally, chest pain is the presenting symptom of achalasia. This is more common in younger patients, and tends to diminish with advancing age^[17]. In our study of 213 achalasia patients, chest pain was the only symptom whose prevalence between the sexes was significantly different, being more common among women than men (70.9% *vs* 54.5%, $P = 0.03$, respectively)^[18]. In both sexes, chest pain did not relate to the duration of symptom and the LES pressure. Chest pain was less frequently reported by patients over 56 years of age compared to those younger than 56 years ($P < 0.05$)^[18]. It seems that chest pain is a distinctive symptom of achalasia which is affected by sex as well as age. Although chest pain was not improved following pneumatic dilation in some studies^[17], others reported significant improvement after the procedure^[18].

Heartburn occurs frequently in achalasia. Patients with heartburn have lower LES pressures than those without this symptom^[19]. Heartburn may occur as a result of gastro-esophageal reflux or other causes, such as direct irritation of the esophageal mucosa by foods, pills, and the lactate produced by bacterial fermentation of retained carbohydrates^[19,20]. Hiccup is also a frequent symptom in achalasia, partly because of the obstruction of the distal esophagus^[21]. Functional and structural abnormalities of the lung, such as tracheo-bronchial compression and abnormalities on high-resolution CT-scan, may occur in half of the patients^[22]. The frequency

Table 3 Frequency of cardinal symptoms in achalasia

Symptom	Frequency (%)			
	Each meal	Daily	Weekly	None
Dysphagia to solids	84	13	2	1
Dysphagia to liquids	60	25	8	7
Active regurgitation	33	36	15	16
Passive regurgitation	Daily	Weekly	Monthly	None
	20	44	4	32
Chest pain	12	39	8	41

of cardinal symptoms in achalasia is shown in Table 3.

Since patients with achalasia may experience retro-sternal fullness following a meal, they may eat more slowly and induce regurgitation to relieve the feeling. They may perform some maneuvers to augment esophageal emptying, such as lifting the neck or throwing the shoulders back^[3].

Compared to the general population, patients with achalasia are at substantially increased risk, even as high as 33-fold, of developing esophageal cancer. The cancer typically is of squamous cell type^[6,7]. However, some series did not find any increase in the risk, particularly with early treatment of achalasia. We followed up 365 patients with achalasia for a mean duration of 43 mo; no case of esophageal cancer was identified. This may be related to the fact that our study participants were fairly young (mean age, 38 years) and the duration of follow-up was not very long^[23].

DIAGNOSIS

Diagnosis of achalasia is based on clinical findings. The diagnosis is confirmed by radiographic, endoscopic, and manometric evaluations.

Radiography

The usual findings on a plain chest X-ray are widening of the mediastinum, due to esophageal dilation, and absence of the normal gastric air. When achalasia is suspected, barium swallow is the primary screening test. Barium swallow typically shows a dilated esophagus that terminates in a beak-like narrowing as a result of contraction in the LES. When dilation is very severe, the esophagus may have a sigmoid shape^[3]. The overall sensitivity of barium swallow for diagnosis of achalasia is approximately 95%^[24], but in early stages of the disease it may be reported as normal. For example, in a prospective study achalasia was suggested by barium examination in only 21 out of 33 patients who eventually were diagnosed with achalasia^[2].

The timed barium esophagogram, which assesses esophageal emptying at 1, 3 and 5 min after swallowing of barium, can be more helpful than usual barium swallow. Vaezi *et al*^[25] reported that assessing both symptom improvement and objective improvement in esophageal emptying can better identify the response rate to pneumatic dilation and need for repeated dilations in the future. In their study of 37 patients, there was a significant

association ($P < 0.001$) between improvement in patient symptoms and barium height. In 38 out of 53 (72%) pneumatic dilations, the degree of symptom and barium height improvement was comparable. In 8 out of 26 (31%) patients, however, there was $< 50\%$ improvement in barium height despite near complete symptom resolution. Age was the only difference between the groups and patients with improvement in both symptoms and barium height, i.e. the first group, were significantly older than the second. They concluded that the timed barium esophagogram before and after dilation may identify a subset of patients with poor esophageal emptying but with good improvement in symptoms who may benefit from early repeated pneumatic dilation^[25]. Similarly, Chuah *et al*^[26] found in their study of 32 patients with achalasia who received pneumatic dilation that the timed barium esophagograms correlated with symptomatic improvement in up to 71% of patients, although seven patients who noted complete symptom resolution showed less than 50% improvement in barium column height and esophageal diameter. In a study of 52 patients, we also found that the volume of barium retention at 5 min could predict the LES pressure before and after balloon dilation in achalasia^[27], and in a study of 43 patients, surface area of barium retention at 5 min appeared to be an even better predictor for resting LES pressure^[28]. In a randomized clinical trial of 51 patients who underwent surgery or pneumatic dilation, results of the timed barium esophagogram also correlated well with outcome. Poor improvement in barium height following the treatments was associated with an increased risk of treatment failure^[29].

Manometry

Manometry is the most sensitive tool for diagnosis of achalasia. Elevated resting LES pressure (usually > 45 mmHg), incomplete LES relaxation, and aperistalsis in the smooth muscle portion of the body of the esophagus are three characteristic manometric features of achalasia^[30]. Swallows may be followed by either no esophageal contraction or simultaneous contractions. Simultaneous contractions may also occur spontaneously. Another common feature is that resting pressure in the body of the esophagus is slightly higher than in the stomach^[3].

In most patients the amplitude of esophageal contractions is low. On the other hand, in vigorous achalasia the simultaneous esophageal contractions have high amplitudes (e.g. > 60 mmHg). Some studies have suggested that vigorous achalasia may represent an early form of achalasia in which some inhibitory ganglion cells may not yet be destroyed^[8], and that patients with vigorous achalasia may benefit more from botulinum toxin injection than those with classic achalasia^[31]. At present, however, the distinction between vigorous and classic achalasia seems to have little clinical significance.

Endoscopy

Endoscopy in achalasia typically reveals a dilated

esophagus that often contains retained material. The esophageal mucosa usually appears normal, although inflammation and ulceration may result from chronic inflammation caused by retained food or pills. Endoscopy may be reported as normal if it is carried out in the early stages of the disease or it is not performed by experienced endoscopists^[2]. Achalasia at early stages may also be misdiagnosed as GERD. Food stasis and GERD are main factors contributing to esophageal mucosal inflammation in achalasia. The association between endoscopic food stasis and histological inflammation is significant, but endoscopic signs of esophagitis and histological inflammation are poorly associated. Because of low sensitivity of endoscopy to detect inflammation, surveillance endoscopy with biopsy sampling and assessment of stasis is warranted to detect early neoplastic changes^[32]. The stasis may predispose the esophagus to *Candida* infection.

Although the LES in achalasia is contracted, the endoscope can usually be traversed easily into the stomach aided by gentle pressure on the scope^[3].

Endoscopic ultrasonography (EUS) may show widening of the mean longitudinal and circular smooth muscle layers of the LES; however, this finding is not specific for achalasia^[33]. We compared the esophageal muscularis propria thickness in achalasia patients with a control group using EUS and assessed the relationship between EUS findings and demographic features in both groups. The esophageal muscular layer was significantly thicker in patients with achalasia compared to control group ($P < 0.05$). Among patients with achalasia, the thickness at 5 and 10 cm above the gastro-esophageal junction appeared to be correlated with age, being higher among older people^[34].

Clinical and endoscopic features of some other conditions, such as neoplasms, may be similar to those of achalasia. Since gastric adenocarcinoma is the most common neoplasm associated with pseudo-achalasia, the esophago-gastric junction and the gastric fundus should be carefully examined for any evidence of neoplasm. With certain features, malignancy is more likely: duration of symptoms less than 6 mo; presentation after the age of 60 years; excessive weight loss in spite of short duration of symptoms; difficult passage of the endoscope through the gastro-esophageal junction^[35]. In these cases, repeated evaluations and biopsies are recommended.

Symptomatic scoring

Several scoring system have been proposed to evaluate the severity of the symptoms in achalasia. One of the scoring systems is shown in Table 4. In this scoring system, scores for the following five symptoms; dysphagia to solids, dysphagia to liquids, passive regurgitation, active regurgitation, and chest pain are summed up to calculate the total score. In a study of 116 patients with achalasia, we found a good correlation between this score and LES pressure ($r = 0.29$, $P < 0.01$)^[36]. Among the main symptoms, active and passive regurgitation and dysphagia to liquids were significantly correlated to the

Table 4 Scoring system for evaluation of clinical symptoms

Symptom	Score by frequency of symptoms			
	Each meal	Daily	Weekly	None
Dysphagia to solids	3	2	1	0
Dysphagia to liquids	3	2	1	0
Active regurgitation	3	2	1	0
Passive regurgitation	Daily	Weekly	Monthly	None
	3	2	1	0
Chest pain	3	2	1	0

LES relaxation pressure ($P = 0.001$, 0.002 , and 0.046 , respectively)^[36].

TREATMENT

The mainstay of therapy is to reduce LES pressure in order to improve esophageal emptying by gravity. Several therapeutic modalities have been introduced to achieve this goal, including medical therapy, surgical myotomy (open or laparoscopic), pneumatic dilation of the LES, injection of botulinum toxin into the LES, and insertion of self-expanding stents. There are 3 recent meta-analyses of publications which have investigated different treatment approaches. One of these included 105 articles involving 7855 subjects, and investigated symptom relief, prevalence of gastro-esophageal reflux, and complications following treatments^[37]. Pneumatic dilation was more successful in symptom relief than botulinum toxin injection (68% *vs* 41%, $P = 0.02$, respectively). Symptom relief with laparoscopic myotomy plus an anti-reflux procedure was better (90%) than with all other treatments. Furthermore, complication rate was low with this method (6.3%)^[37]. Likewise, another meta-analysis of randomized and controlled treatment trials, which included 17 articles with 761 participants and investigated remission and relapse rates and complications, found a better remission rate and lower relapse rate for laparoscopic myotomy compared to other treatments. There was no difference between open and laparoscopic myotomy, in the only trial that compared these two methods, regarding remission and relapse rates. Remission rate following pneumatic dilation was higher than after botulinum toxin injection^[38]. A meta-analysis of controlled and uncontrolled studies in the Chinese literature (43 articles with 1791 participants) also showed that myotomy was associated with higher initial and long-term remission rates than pneumatic dilation or botulinum toxin injection. Only 2 studies compared open myotomy with laparoscopic myotomy; there was no difference in remission rate^[39]. Since results after any treatment may deteriorate over time, life-long follow-up and objective assessment of the results are recommended.

Medical therapy

Nitrates and calcium channel blockers (e.g. nifedipine) relax the smooth muscles of the LES^[40]. These medications are usually taken sublingually 10 to 30 min before meals. Pharmacotherapy for achalasia, however, is often ineffective and frequently associated with side

effects (e.g. headache, hypotension, and tachyphylaxis). Therefore, nitrates and calcium channel blockers are primarily used for patients who are unwilling to undergo or unable to tolerate more effective, invasive forms of therapy^[3].

Surgical myotomy

Surgical myotomy was first introduced by Ernst Heller in 1913. Nowadays, a modified technique is commonly used^[41]. The standard “open” myotomy can be performed using either an abdominal or, more commonly, a thoracic approach^[42,43]. More recently, laparoscopic and thoracoscopic techniques have been used to perform myotomy^[39,44-46].

The modified Heller approach results in good to excellent relief of symptoms in 70% to 90% of patients with few serious complications. The mortality rate (approximately 0.3%) is similar to that reported for pneumatic dilation^[42]. The major disadvantages of surgery are the high initial cost, long recovery period, and the frequent development of GERD. Reflux esophagitis develops in approximately 10% of patients treated by surgical myotomy^[42]; however, the efficacy of proton pump inhibitor treatment minimizes its clinical significance. There is a debate with regard to the need for additional fundoplication in open myotomy. In some studies, gastro-esophageal reflux was relatively frequent even after combining myotomy with anti-reflux procedures^[47,48].

There is increasing experience with Heller myotomy performed by minimally invasive techniques (laparoscopy or thoracoscopy), and these techniques have become the procedure of choice by many experienced surgeons for uncomplicated cases in Western countries. These approaches in several trials were more successful in symptom relief than other treatments^[37]. They are associated with few major complications^[49] and shorten the duration of hospitalization and recovery^[44,45]. Some pre- and postoperative findings may be helpful in predicting the outcome. In a study of 407 patients who underwent laparoscopic myotomy, high preoperative LES pressure (> 30 mmHg) was a predictor of a good response, while severe chest pain and the presence of a decompensated sigmoid esophagus (class IV) was associated with poor outcome^[50]. In a study of 200 patients who underwent laparoscopic or thoracoscopic myotomy plus a partial fundoplication, low LES pressure, presence of sigmoid esophagus, and longer duration of symptoms were associated with failure of treatment in long-term follow-up^[51]. Preoperative LES pressure over 35 mmHg was also a strong predictor of excellent postoperative relief in dysphagia in another study of 200 patients^[52].

Objective analyses have shown a high rate of gastro-esophageal reflux in laparoscopic myotomy without an anti-reflux procedure. In a study of 50 patients with achalasia who underwent laparoscopic Heller myotomy without anti-reflux procedures, significant heartburn was reported in 30% of cases. Twenty-four-hour pH monitoring revealed abnormal findings in 11 out of 22 patients tested^[53]. However, use of a fundoplication procedure with laparoscopic myotomy

reduced the rate of gastro-esophageal reflux (8.8% with a fundoplication *vs* 31.5% without a fundoplication, $P = 0.003$)^[37]. In a study of 20 patients who underwent laparoscopic myotomy and fundoplication, 24-h combined multichannel intra-luminal impedance and pH monitoring did not show any evidence of postoperative pathologic reflux in both upright and recumbent positions^[54]. Few randomized trials have investigated the efficacy of different fundoplication techniques used with laparoscopic myotomy in treatment of achalasia. In a randomized, controlled study of 144 patients who underwent laparoscopic myotomy, the outcome when using Dor *versus* Nissen fundoplication was investigated. Both techniques were successful in long-term control of gastro-esophageal reflux, but the recurrence rate of dysphagia was higher with the Nissen method^[55]. Based on the published literature, Dor fundoplication seems to be performed more commonly as the anti-reflux procedure during laparoscopic myotomy than other fundoplication techniques.

Pneumatic dilation

Although some studies reported similar short- and long-term efficacy for myotomy and pneumatic dilation, particularly with graded dilation (1-3 dilations with progressively larger balloons)^[56,57], as mentioned earlier, myotomy has been shown to be a more effective treatment for achalasia in several trials^[37,38]. However, pneumatic dilation is less expensive than myotomy and still improves symptoms in a substantial number of patients^[58-60]. In a meta-analysis of uncontrolled studies, a single pneumatic dilation was found to be effective in 72% of patients during a mean follow-up of 4.9 years^[61]. In a study of 150 patients with achalasia, pneumatic dilations were performed until remission was achieved or symptoms recurred, using an “on-demand strategy” based on symptom recurrence, and a long-term remission was achieved in nearly all patients^[60]. Pneumatic dilation can also be applied for some patients in whom dysphagia persists after surgery^[62,63]. In a study of 27 patients with recurrent dysphagia following surgery, pneumatic dilation improved symptoms in 76% of the patients^[63]. If pneumatic dilation fails, laparoscopic myotomy with fundoplication can be performed; the outcome is not affected by previous pneumatic dilation^[50,64].

A number of different balloon dilators have been used over the years. A systematic review of the treatment of achalasia compared the results of using different dilators; pneumatic dilation was performed in 2418 patients with “old” dilators, in 234 patients with the “new” Witzel dilator, and in 359 patients with the Rigiflex dilator^[65]. Using old dilators and Witzel dilators, two-thirds of patients had good to excellent improvement after one or more dilations during a mean follow-up of 4.6 years and one year, respectively. Using Rigiflex dilators, an equivalent improvement was achieved in up to 90% of patients, depending upon the diameter of the dilator used (74% for 3.0 cm, 86% for 3.5 cm, and 90% for 4.0 cm)^[65]. At present, the most popular pneumatic dilator is the Rigiflex balloon, which is passed over a guidewire and positioned

fluoroscopically or endoscopically in the LES. This balloon is available in three different sizes (3.0, 3.5, and 4.0 cm). The thinnest balloon is typically used in the first dilating session. The standard approach to balloon dilation is one dilatation per session; further need for dilation is based on the symptomatic response. Patients are usually referred to a surgeon if three consecutive dilations over a few months do not provide clinical remission. In long-term follow-ups, more than three sessions can be applied if symptoms recur.

In our center we perform all dilations with the Rigidflex balloon dilator. After a clear liquid diet for 24 h and an overnight fast, patients receive intravenous diazepam (5-10 mg) and meperidine (25-50 mg). A guidewire into the stomach is placed under endoscopic visualization. In the first dilating session, a 3.0 cm balloon dilator is passed over the guidewire under endoscopic guidance. The midpoint of the balloon is positioned at the LES. The balloon is gradually inflated to 6 pounds per square inch (psi) over 20 s and then to 8 psi for the next 20 s and finally to 10 psi for 60 s. Then the balloon is deflated and removed along with the guidewire. Patients are discharged after a 6-h observation period. If severe or sustained chest pain occurs, a gastrografin swallow is performed to rule out perforation.

Using the above method we conducted a study of 99 patients to assess therapeutic outcome after pneumatic dilation. Initially, all symptomatic patients underwent pneumatic dilation with a 3.0 cm balloon. If symptoms recurred, dilation was repeated with a 3.5 cm balloon. In the case of further relapse, a third dilation was carried out with a 4 cm balloon. The patients were followed for an average length of 47 (range, 18 to 60) mo. Dilation was repeated in 35 patients; only 6 of them required a third dilation. After the third dilation two patients did not display improvement and underwent myotomy. Over the study period, cumulative remission rate was 65% without re-dilation and 94% with re-dilation. The mean remission period was 44.7 mo^[66].

To address the optimal method for performing pneumatic dilation, regarding the amount and rate of inflation pressure and balloon diameter, we conducted a large long-term prospective study and enrolled 262 achalasia patients over 10 years. In the first 62 patients (group A), dilation was done using a 3.5 cm balloon, which was inflated to the pressure of 10 psi over 10 s. In group B (200 patients), we initially used a 3.0 cm balloon with inflation pressure of 10 psi in 30 s. We used a Rigidflex balloon and maintained pressure for 60 s after inflation in both groups. If symptoms recurred, dilation was repeated with incrementally larger balloons (for second dilation, 4.0 cm in group A and 3.5 cm in group B; for third dilation, 4.0 cm in both groups). The cumulative proportional remission rates with single dilation after 6 mo were 83% and 75% in groups A and B, respectively; the corresponding rates decreased to 60% and 57%, respectively, after 30 mo. The difference between the 2 methods was not statistically significant. The remission rate following re-dilation was good; 1 year after the second dilation, it was 88% in group A and 89% in group B,

and 2 years after the second dilation, it was 70% in both groups. All perforations ($n = 3$) occurred in group A at the first dilation (62 dilations) with rapid inflation of balloon (10 psi over 10 s); while there was no perforation in group B (296 dilations), in which gradual increasing of pressure (10 psi over 30 s) and graded dilation method was used^[67].

In another Iranian study, 45 patients who underwent pneumatic dilation were compared with 19 patients who underwent open myotomy. Good to excellent relief was achieved in 68% of patients with myotomy and 80% of patients with pneumatic dilation. After over 2 years of follow-up, relapse rates in both groups were not significantly different (39% in surgery group and 25% in pneumatic dilation group). The mean length of hospital stay and days off from work were significantly lower in the pneumatic dilation group; these were discovered to be 9 and 39 d in the myotomy group and 1 and 2 d in the pneumatic dilation group, respectively^[57].

Some predictors for the outcome of pneumatic dilation have been suggested, including age of patients^[68,69] and a decrease in LES pressure following dilation^[69]. In a study of 111 patients, short- and long-term remission rates were good (98% and 75% at months 24 and 60, respectively), but young age (≤ 37.5 years), high esophageal body pressure, and high LES pressure (≥ 17.5 mmHg) following first dilation were negative predictive factors. Young patients who required more than 2 dilations seemed not to benefit from this kind of treatment^[70]. We did not find any significant association between age, gender, previous treatment, or severity of initial symptoms and the outcome of pneumatic dilation ($P > 0.4$)^[66].

Esophageal perforation is the most important complication of pneumatic dilation. It occurs in approximately 3% to 5% of patients in most series, although the range varies from 0% to 21%^[3,71]. Patients with esophageal perforation usually present in the first hours after dilation. A high index of suspicion should be maintained in patients complaining of sustained pain or discomfort after the procedure. Some patients respond to conservative treatment with antibiotics and parenteral nutrition but others need a surgical repair. Other complications of pneumatic dilation include development of intramural hematomas, esophageal mucosal tears, and diverticula at the gastric cardia^[72]. Severe transient and intermittent post-procedural chest pain has been reported in approximately 15% of patients during the 24-48 h after dilation^[73,74]. Although this symptom is disturbing, it is not harmful.

Botulinum toxin injection

Botulinum toxin is a potent inhibitor of acetylcholine release from nerve endings. The toxin theoretically relaxes the LES by decreasing unopposed cholinergic stimulation of the LES^[75]. Although the effect of botulinum toxin injection generally is shorter than some other procedures, it can be useful under certain conditions. In patients with multiple medical problems who are poor candidates for more invasive procedures, as well as those unwilling to undergo either surgery or pneumatic dilation, botulinum toxin injection is the preferred approach. Older patients

and those who suffer from vigorous achalasia may benefit more from botulinum toxin^[76]. A multicenter randomized study suggested that dose of botulinum toxin may be a predictor of outcome: the higher the dose, the better the response^[77].

The use of botulinum toxin in achalasia was first introduced by Pasricha *et al*^[78]. Several studies indicate that 65% to 90% of patients respond to a single injection within 1 mo. The effect of botulinum toxin lasts from 3 mo to more than one year^[79-82]. Those who respond to the injection may do equally well after a second or even a third injection. In one series, for example, symptom relief was achieved in 75% of 57 patients who received repeated injections, as needed, during up to 2 years follow-up^[31]. However, the effect decreases over time; some studies reported clinical remission rates of 50% and 30% at 6 and 12 mo, respectively, following botulinum injection^[83]. Although a few studies reported that botulinum toxin injection could provide an efficacy equal to that of pneumatic dilation over a one year period^[84], several randomized clinical trials have shown that while initial symptomatic remission rates by pneumatic dilation and botulinum injection may be similar in some cases, pneumatic dilation is associated with a significantly higher long-term remission rate^[85,86]. In some studies, only pneumatic dilation was associated with improvement in objective measures of esophageal function, including esophageal manometry and barium studies^[85,87]. In our trial of 40 patients, pneumatic dilation was more efficient than botulinum injection in providing sustained symptomatic relief over a 12 mo period. The remission rates for pneumatic dilation and botulinum injection after 12 mo were 52% and 15%, respectively^[88].

Several formulations of botulinum toxin are available. A comparison between 100 U of Botox and 250 U of Dysport showed a similar efficacy for up to 6 mo of follow-up^[89]. In the most common method for injection therapy, 1 mL aliquots (20 to 25 units/mL) of the toxin are injected into each of four quadrants, approximately 1 cm above the Z line, using a standard sclerotherapy needle. We use and recommend the following method. After an overnight fast, patients are sedated with intravenous diazepam (5-10 mg) and meperidine (25-50 mg). The LES is identified by visualization of the sphincter rosette at the squamo-columnar junction during upper gastrointestinal endoscopy. Four hundred units of Dysport are diluted in 4 mL normal saline. Two 50-unit aliquots (0.5 mL) of Dysport are injected through a 5 mm sclerotherapy needle into each quadrant of the LES. Patients are discharged when routine post-sedation care is completed and allowed to eat later on the same day. Improvement in symptoms is usually observed after only 24 h; peak effects occur even later in some patients.

Reported complications after botulinum toxin are not major and include post-procedural transient chest pain (25%) and heartburn (5%)^[90]. Transient chest pain, the main complication, can be controlled by sedatives. Neutralizing antibodies have been detected in approximately 5% of patients treated chronically with botulinum toxin for skeletal muscle conditions; however,

their significance in relapse of dysphagia in achalasia is uncertain. These antibodies, however, might be a possible cause of the rapid relapse of dysphagia following botulinum toxin injection^[91]. Surgical treatment of achalasia in patients who previously received botulinum toxin may encounter some technical problems, but no significant difference in the outcome between patients with and without previous use of the toxin has been reported^[92].

Pneumatic dilation after botulinum toxin injection

Only a few studies have investigated the effect of botulinum toxin injection on the outcome of pneumatic dilation. In a retrospective study of the effect of the combined therapy, we studied 12 patients who underwent dilation following botulinum toxin injection and 12 patients with achalasia who underwent only pneumatic dilation (control group). With combined therapy, only one of the patients relapsed 30 mo after dilation, while all the others were in remission for an average of 25.6 mo. In the control group, all the patients relapsed after a mean period of 12.6 mo and needed further dilation. The cumulative remission rate was significantly higher in the combined therapy group than in the control group ($P < 0.01$). One month after dilation, the mean symptom score decreased by 76% in the combined therapy group and by 53% in the control group. Age, sex, duration and severity of symptoms were not correlated with response to treatment^[93].

We also conducted a prospective trial. Twenty seven patients were randomly assigned to receive botulinum toxin 1 mo before pneumatic dilation and 27 patients were assigned to undergo pneumatic dilation alone. One-year remission rates of patients in the botulinum toxin-pneumatic dilation group and the pneumatic dilation group were 77% and 62%, respectively ($P = 0.1$). In the pneumatic dilation group, the esophageal barium height significantly decreased at 1 mo ($P < 0.001$), but this reduction did not persist over 1-year follow-up. The botulinum toxin-pneumatic dilation group showed a significant reduction in barium height at both 1 mo and 1 year after treatment ($P < 0.001$). In the botulinum toxin-pneumatic dilation group, 91% of patients older than 40 years were in remission at 1 year, compared with only 55% of this age group in the pneumatic dilation group ($P = 0.07$)^[94].

We found an abstract in English that reported a series of 9 patients with achalasia who were treated with application of 250 IU Dysport into the LES and balloon dilation 7 d later. Two patients underwent myotomy because of poor relief of symptoms. Seven other patients, however, were in good symptomatic remission after one year. The remission was even observed for as long as 36 mo, which was the longest follow-up period^[95].

Insertion of stents

Only a few studies have investigated the role of expandable stents in treatment of achalasia^[96-99]. The results are controversial. Therefore, insertion of stents does not seem to be a currently recommended treatment for achalasia.

CONCLUSION

First stages of achalasia may be misdiagnosed as other diseases, such as GERD. Myotomy, particularly laparoscopic myotomy with fundoplication, is the most effective treatment for achalasia and can be considered as the procedure of choice. Compared to other treatments, however, the initial cost of myotomy is usually higher and the recovery period, particularly following open myotomy, is generally longer. When performing myotomy is not possible for any reason, e.g. medical contraindication, patient's unwillingness, when patients cannot afford surgery, or experienced centers for surgery or post-operative care are not easily accessible (situations that may not be rare particularly in some low- or medium-resource countries), graded pneumatic dilation (using 3.0 cm balloons initially) with slow rate of balloon inflation seems to be an effective and safe initial alternative. The duration of remission can be extended by repeated dilation with larger-sized balloons. Injection of botulinum toxin into the LES before pneumatic dilation seems to increase remission rates. However, this needs to be confirmed in further studies. The timed esophagogram may be used as a non-invasive objective tool for initial and post-operative or post-dilation assessment.

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S- Editor Tian L L- Editor Logan S E- Editor Ma WH



REVIEW

Treatment modalities for hypersplenism in liver transplant recipients with recurrent hepatitis C

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Received: March 13, 2009 Revised: September 23, 2009

Accepted: September 30, 2009

Published online: October 28, 2009

2009; 15(40): 5010-5013 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5010.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5010>

INTRODUCTION

Liver disease caused by chronic hepatitis C virus (HCV) infection is the most common indication for liver transplantation in the United States. Unfortunately, HCV is universally recurrent in the transplanted liver and is a major cause of graft failure and decreased patient survival^[1]. 10%-30% of patients who have recurrent infection develop advanced fibrosis or cirrhosis within the first 5 years post-transplantation^[2,3].

The combination therapy of interferon and ribavirin has been shown to be the most effective therapy for HCV recurrence after liver transplantation with sustained virologic response rates between 20%-40%^[4]. Further studies are needed to determine whether treatment should be started preemptively, at the time of acute hepatitis, or at the early stages of chronic hepatitis in the graft. The ability to treat patients with adequate doses of interferon and ribavirin or even to initiate treatment is often limited by leucopenia, anemia, and thrombocytopenia. Chang *et al*^[5] reported in their study that almost 50% of patients (109 out of 216) had a platelet count below 50 000/ μ L before liver transplantation. At one year of follow-up, 21% of transplanted patients (45 out of 216) continued to have moderate to severe thrombocytopenia. Clinical factors associated with sustained thrombocytopenia were pretransplant severe thrombocytopenia ($< 50\,000/\mu\text{L}$) and pretransplant large spleen volume ($> 2000\text{ mm}^3$).

Splenectomy and partial splenic embolization represent two interventional therapies to improve thrombocytopenia which could potentially allow treatment of HCV. In this review we examine the effectiveness and risks of these approaches in liver transplant patients.

SPLENECTOMY

Splenectomy has been performed routinely in the past in liver and kidney transplant patients for immunologic reasons. It allowed patients to tolerate azathioprine therapy for episodes of rejection^[6]. This practice continued

Abstract

Hepatitis C is the most common indication for orthotopic liver transplantation in the United States. Unfortunately, hepatitis C recurs universally in the transplanted liver and is the major cause of decreased graft and patient survival. The combination therapy of interferon and ribavirin has been shown to be the most effective therapy for recurrent hepatitis C. However, pre- and post-transplant hypersplenism often precludes patients from receiving the antiviral therapy. Splenectomy and partial splenic embolization are the two invasive modalities that can correct the cytopenia associated with hypersplenism. In this report we review the two treatment options, their associated outcomes and complications.

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Key words: Hypersplenism; Leukopenia; Recurrent hepatitis C; Thrombocytopenia; Liver transplant

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Sibulesky L, Nguyen JH, Paz-Fumagalli R, Taner CB, Dickson RC. Treatment modalities for hypersplenism in liver transplant recipients with recurrent hepatitis C. *World J Gastroenterol*

until the early 1980s when cyclosporine was introduced. Current accepted indications for post-transplant splenectomy include recurrent ascites, splenic infarction, large aneurysms of the splenic artery, thrombocytopenia secondary to hypersplenism prior to or after liver transplantation, and small-for-size syndrome in recipients of living donors with associated thrombocytopenia and impaired liver function. Splenectomy was also performed at the time of liver transplantation for ABO incompatibility and preemptive HCV treatment with interferon and ribavirin in thrombocytopenic patients^[7-9]. Tashiro *et al*^[10] advocated performing concurrent splenectomy with liver transplantation in all patients who had a pre-transplant platelet count of less than 60000 mm³ so that this group of patients could tolerate preemptive administration of combination therapy in the post-transplant period.

Although successful results have been reported, splenectomy is potentially associated with multiple complications. It is an invasive procedure that can be technically difficult, with a high risk of bleeding in patients with portal hypertension, varices, and enlarged spleen. Portal vein thrombosis and pancreatic leaks requiring surgical reexploration have been described as complications^[9,11]. However, in our opinion, the risk of infection post-splenectomy is the most serious and potentially life-threatening complication in the immunosuppressed population. Troisi *et al*^[12] reported that 4 out of 10 liver transplant patients who underwent splenectomy developed sepsis, which led to their demise. Samimi *et al*^[13] reported 17.5% *vs* 2.7% one-month and 30% *vs* 11.5% one-year sepsis-related mortality in patients who underwent concomitant splenectomy with liver transplantation *vs* those who underwent liver transplantation alone. Neumann *et al*^[14] reported an increased risk for opportunistic pneumonia in patients who underwent simultaneous splenectomy and liver transplant. Splenectomy also places patients at risk for overwhelming post-splenectomy sepsis syndrome (OPSS), usually due to encapsulated organisms. It is recommended by the Center for Diseases Control to immunize patients prior to splenectomy to decrease the risk of OPSS from *Streptococcus pneumoniae*, *Haemophilus influenzae* type B, and *Neisseria meningitidis*^[15]. According to guidelines issued by the American Society of Transplantation in 2004, these vaccines are administered to all candidates prior to liver transplantation^[16]. Unfortunately, the response rate is only 40%-80%^[17,18].

Despite vaccinations, fulminant bacterial sepsis carries a high risk of morbidity and mortality, especially in immunocompromised patients. The risk is greatest in the early months and years after splenectomy, but a period of as long as 45 years after the procedure has been reported in the literature^[19].

invasive treatment of hypersplenism. It is usually performed *via* a percutaneous femoral artery approach. The embolization catheter is advanced into the splenic hilum as far as possible in order to avoid injury to the pancreatic circulation. Gelatin sponge slurry suspended in an antibiotic solution, coils, microspheres, and polyvinyl alcohol particles are used for embolization of approximate 60%-70% of spleen parenchyma. Splenic embolization procedures date back to 1973, when the entire spleen parenchyma was ablated. At that time the procedure was associated with high rates of complications, including splenic abscesses, rupture, and pancreatic infarction, resulting in a high mortality rate^[20]. Subsequently, this procedure became more successful with selective ablation of the spleen. In 1984, Mozes *et al*^[21] showed in a prospective randomized trial that partial splenic embolization was as effective as splenectomy for treatment in renal transplant candidates on hemodialysis with a low platelet count prior to administration of immunosuppression. In this study excessive infarction of the spleen was avoided with a mean of 65% of spleen parenchyma ablated. Partial splenic embolization is an effective method to reduce the platelet pool and improve platelet count and is greatly dependent on the infarcted splenic volume. Hayashi *et al*^[22] reported that splenic volume was the best predictive factor for increase in platelet count at one month and one year in patients with liver cirrhosis and hypersplenism.

In several reports in the literature, partial splenic embolization has been described in patients after liver transplantation. It has been successful in patients with thrombocytopenia and recurrent HCV who were able to undergo treatment with interferon and ribavirin as a result of ablation^[23-25].

Most patients develop post-embolization syndrome, including symptoms of fever, left upper quadrant pain, pleural effusion, pneumonia, and atelectasis. Splenic abscesses and rupture are infrequent and are more commonly encountered and less tolerated by immunocompromised cirrhotic patients with a greater area of embolization^[26]. The risk is greatly reduced with aseptic technique, antibiotic prophylaxis, and careful control of pain. Extent of embolization is important as well, with more complications following greater than 70% area of ablation. In partial splenic embolization, achieving the intended target embolization area remains challenging. Graded partial splenic embolization at several settings has been entertained in order to avoid excessive embolization and severe complications associated with it^[27].

DISCUSSION

Hepatitis C is the most common indication for liver transplantation in the United States and Europe, but unfortunately the virus almost always recurs with up to a third of patients developing cirrhosis within the first 5 years. Interferon and ribavirin therapy has been widely accepted as the treatment for recurrent disease. Cytopenia, including thrombocytopenia, which often

PARTIAL SPLENIC ARTERY EMBOLIZATION

Partial splenic embolization is a non-surgical, less

afflicts liver transplant patients, leads to failure to initiate this antiviral regimen, dose reduction, or discontinuation of therapy, which ultimately decreases the likelihood of sustained virological response^[28].

Although, hemolytic growth factors, such as erythropoietin and growth colony stimulating factors, are used to counter the anemia and neutropenia associated with interferon and ribavirin treatment, there is no approved therapy for low platelet count in HCV infected patients.

A new group of synthetic thrombopoietic agents, including romiplostim and eltrombopag, have been found to be effective in stimulating platelet production^[29]. In 2008, romiplostim was approved by the FDA for the treatment of thrombocytopenia in patients with chronic idiopathic thrombocytopenic purpura (ITP). These agents are now being investigated in clinical trials for the treatment of thrombocytopenia in cirrhotic patients with hepatitis C infection^[30,31].

CONCLUSION

Currently, splenectomy is the more popular choice of treatment for hypersplenism and thrombocytopenia. The question is whether it is the optimal choice. Partial splenic embolization is an alternative option that is often overlooked. It is less invasive and potentially carries fewer infectious complications since there is a remnant of functional splenic parenchyma remaining after the procedure. Although it diminishes with time, the risk of OPSS in asplenic patients is life-long. It carries a high mortality rate and therefore, we feel, other options should be seriously considered. Thus, further prospective studies are needed to investigate both modalities in this select group of patients.

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S- Editor Li LF **L- Editor** Webster JR **E- Editor** Lin YP



REVIEW

Steatosis and insulin resistance in hepatitis C: A way out for the virus?

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Author contributions: Del Campo JA and Romero-Gómez M performed the research and drafted the article.

Supported by A Grant, PAI-CTS-532, from Junta de Andalucía, Andalucía, Spain. CIBEREHD was Funded by Instituto de Salud Carlos III

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Received: June 1, 2009 Revised: September 27, 2009

Accepted: October 4, 2009

Published online: October 28, 2009

Abstract

The hepatitis C virus (HCV) induces lipid accumulation *in vitro* and *in vivo*. The pathogenesis of steatosis is due to both viral and host factors. Viral steatosis is mostly reported in patients with genotype 3a, whereas metabolic steatosis is often associated with genotype 1 and metabolic syndrome. Several molecular mechanisms responsible for steatosis have been associated with the HCV core protein, which is able to induce gene expression and activity of sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor γ (PPAR γ), increasing the transcription of genes involved in hepatic fatty acid synthesis. Steatosis has been also implicated in viral replication. In infected cells, HCV core protein is targeted to lipid droplets which serve as intracellular storage organelles. These studies have shown that lipid droplets are essential for virus assembly. Thus, HCV promotes steatosis as an efficient mechanism for stable viral replication. Chronic HCV infection can also induce insulin resistance. In patients with HCV, insulin resistance is more strongly associated with viral load than visceral obesity. HCV seems to lead to insulin resistance through interference of intracellular insulin signalling by HCV proteins, mainly, the serine phosphorylation of insulin receptor-1 (IRS-1) and impairment of the downstream Akt signalling pathway. The HCV core protein interferes with *in vitro* insulin signalling by genotype-specific mechanisms, where the role of suppressor of cytokine signal 7 (SOCS-7) in genotype 3a

and mammalian target of rapamycin (mTOR) in genotype 1 in IRS-1 downregulation play key roles. Steatosis and insulin resistance have been associated with fibrosis progression and a reduced rate of sustained response to peginterferon plus ribavirin.

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Key words: Steatosis; Insulin resistance; Hepatitis C virus

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Del Campo JA, Romero-Gómez M. Steatosis and insulin resistance in hepatitis C: A way out for the virus? *World J Gastroenterol* 2009; 15(40): 5014-5019 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5014.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5014>

INTRODUCTION

Hepatocyte steatosis, defined as accumulation of fat droplets in hepatocytes, is a histological feature of a group of liver diseases including not only metabolic or alcoholic disorders but also chronic hepatitis C virus (HCV) and drug-induced liver disease^[1,2]. Steatosis is a very common lesion in chronic HCV, seen in more than half of patients, with a prevalence of 40% to 86% according to the genotype. The majority of patients show simple steatosis, but features of non-alcoholic steatohepatitis have been found in approximately 10% of patients with chronic HCV^[3]. Two main types of steatosis have been defined in HCV: metabolic steatosis found in patients infected by genotype 1 and associated with metabolic syndrome, and viral steatosis reported in patients with genotype 3a, without other known steatogenic cofactors and directly linked to a cytopathic effect of the virus. In the same way, chronic HCV infection can also induce insulin resistance^[4,5]. Epidemiological data support an association between HCV infection and a risk for the development of type 2 diabetes mellitus, both in cross-sectional and longitudinal cohorts. The majority of

transgenic mice expressing the HCV core protein developed insulin resistance and type 2 diabetes. Indeed, patients with HCV showed a higher insulin resistance index than healthy controls or patients with liver diseases other than HCV matched by sex, body mass index and age. Insulin resistance could be promoted by viral proteins and the intrinsic mechanism seems to be genotype-specific^[5]. In this sense, HCV replicons - genomic or subgenomic constructs expressing the viral replicase complex and capable of autonomous viral replication - constitute a powerful tool to investigate the molecular mechanism leading to steatosis and insulin resistance in host cells.

In this review, we aim to analyse the role of HCV in the pathogenesis of steatosis and insulin resistance as a possible “way out” mechanism of the virus, together with the impact of both metabolic abnormalities on the clinical course of the disease.

PATHOGENESIS OF STEATOSIS IN HEPATITIS C

HCV infection is characterized by a high rate of progression to fibrosis, chronic hepatitis, leading to cirrhosis and ultimately to hepatocellular carcinoma. In addition, various observations suggest that hepatic steatosis is a common histological feature of chronic HCV infection. Furthermore, increasing evidence indicates that hepatic steatosis is a more vulnerable factor that leads to liver inflammation and fibrosis. These suggest that HCV has a direct role in the development of steatosis and/or that the presence of steatosis affects the progression of HCV-related liver disease. The core protein component of HCV is known to contribute to hepatic steatosis^[6], hepatic fibrosis, and hepatic carcinogenesis^[7]. Some studies suggest that HCV core protein causes hepatic steatosis through inhibition of microsomal triglyceride transfer protein (MTP) activity and very low density lipoprotein (VLDL) secretion, and impairment of the expression and transcriptional activity of peroxisome proliferator-activated receptor (PPAR) α ^[8]. Sterol regulatory element binding protein 1s (SREBP1s) belong to the basic helix-loop-helix-leucine zipper family of transcription factors. A key role of two SREBP1 isoforms in regulating fatty acid synthesis in liver is suggested by study of transgenic mice overexpressing the constitutively active mature forms of SREBP1 isoforms^[9]. The transgenic mice study suggests that SREBP1s increase the transcription of genes involved in hepatic fatty acid synthesis [including fatty acid synthase (FAS); acetyl-CoA carboxylase (ACC); and stearoyl-CoA desaturase (SCD)], inducing massive hepatic steatosis through increased accumulation of triglycerides.

PPAR γ is a transcription factor, belonging to the nuclear receptor superfamily, is a master regulator of adipocyte differentiation, and is important in regulation of a number of genes involved in fatty acid and glucose metabolism^[10]. There is evidence suggesting that liver PPAR γ increases the transcription of genes involved in hepatic fatty acid synthesis (including FAS, ACC and SCD) and fatty acid uptake (including FAT/CD36 and

fatty acid translocase). Thus, liver PPAR γ contributes to regulation of lipid synthesis, transport and storage within hepatocytes, causing the development of hepatic steatosis.

Using a cell culture based model, Kim and coworkers^[11] have shown that HCV core protein is able to induce the gene expression and transcriptional activity of SREBP1, thereby causing the increase of fatty acid synthesis. They also observed that HCV core protein elevates PPAR γ activity, inducing the expression of fatty acid uptake-associated gene. These results suggest that SREBP1 and PPAR γ may represent a new potential therapeutic target in the pathogenesis in HCV infection.

Steatosis development is due to both viral and host factors. Viral steatosis is mostly reported in patients with genotype 3a, in whom fat accumulation correlates with HCV replication levels in serum and liver and disappears after successful antiviral therapy, strongly suggesting a direct role of specific viral products in the fat deposition. To address the role of specific HCV genotype on lipid accumulation in cells, Abid and coworkers^[12] developed an *in vitro* model to study the effect of the core protein belonging to several viral genotypes (1b, 2a, 3a, 3h, 4h and 5a). They concluded that the pattern observed in Huh7 cells upon expression of the six core proteins largely corroborated the phenotype seen *in vivo*. The genotype 3a-derived core protein was about three-fold more efficient than the corresponding protein from genotype 1b at inducing triglycerides accumulation in transfected cells, and is proposed as the ideal candidate to study the pathogenesis of HCV-induced steatosis. This group also reported the role of PPAR γ expression on triglyceride accumulation in Huh7 cells transfected with genotypes 1b and 3a core proteins^[13]. They found that the expression of HCV 3a core protein was associated with an increase in triglyceride accumulation and with a significant reduction of PPAR- γ mRNA compared with HCV 1b. Moreover, Fukasawa and coworkers^[14] showed that ACC1 and FAS, enzymes responsible for *de novo* lipid biosynthesis, are induced in Huh7 cells transfected with HCV core protein. Using microarray analysis Paziienza *et al*^[15] compared the gene expression profile of Huh7 cells transfected with the core protein of HCV genotype 1b and 3a, leading to the conclusion that several genes involving lipid transport and metabolism were up- or down-regulated in a genotype-specific manner. This fact could explain the variable disease expression associated with HCV infection. Taking all these observations together, we can conclude that there is a direct link between virus infection and steatosis development.

It has been shown that genotype 3 infected patients had lower expression of PPAR α or MTP mRNA in the liver and fat accumulation was three times higher in comparison with non-genotype 3 patients^[16]. Although the mechanism by which genotype 3a induces steatosis more efficiently than others genotypes is not completely understood, some differences in the amino acid sequence in the core protein could explain, at least in part, these differences. Indeed, a specific polymorphism in core protein from genotype 3 has been associated with

lipid accumulation in hepatocytes. Amino acid substitution at positions 182 and 186 caused intracellular lipid accumulation in hepatic cells and contributed to steatosis development^[17]. Indeed, single polymorphisms in the core gene promoting an amino acid change from tyrosine to phenylalanine (Y164F) has been associated with greater cumulative lipid droplet area in cultured cells than in cells producing the wild-type core protein^[18].

The immune response against HCV releases reactive oxygen species (ROS) from sequestered phagocytes and activated Kupffer cells in the liver. Increased oxidative stress caused by HCV may result in the activation of Kupffer cells^[19]. The change in H^+ concentration alters the balance in the Na^+/H^+ exchanger, causing the Kupffer cells to swell and eventually burst. This releases ROS and an arsenal of inflammatory mediators such as $TNF-\alpha$, $TGF-\beta$, IL-6 and IL-8. The rising concentrations of ROS induce lipid peroxidation and damage triglycerides. The process of lipid peroxidation disrupts cellular membranes and can induce mitochondrial dysfunction^[20].

Metabolic and viral steatosis share the same pathophysiological pathways, although metabolic abnormalities are more often seen in genotype 3a resulting in greater steatosis. A predominant but not exclusive metabolic or viral mechanism could be associated with each genotype, and steatosis could appear as a consequence of the interplay between both host and viral factors.

Recently, steatosis has been implicated in viral replication. The disruption of the association between core protein and fat droplets impairs viral fitness^[21]. In infected cells, core protein is targeted to lipid droplets, which serve as intracellular storage organelles. According to their results, two facts show the relationship between lipids droplets and viral replication: first, the change in the distribution of the core protein from wild sites juxtaposed to lipids droplets at an early stage, which later agreed with a peak production of virus. Secondly, JFH1_{DP}, a mutant strain obtained from JFH1, which did not give rise to virus progeny, expressed a core protein that was targeted to punctuate sites indistinguishable from those identified for the wild type protein at early times, but JFH1_{DP} core did not proceed to coat lipid droplets. In such cases, an association between core protein and lipid droplets would be essential for virus assembly. Furthermore, alteration of a phenylalanine in domain 2 of the core protein generates an unstable form of protein associated with reduced replication rates. Lastly, Shavinskaya *et al*^[22] identified the lipid droplet binding domain of HCV core as the major determinant for efficient virus production. They show that D2 in HCV core is a critical determinant for efficient virus assembly and that small numbers of variations (mutations) in this highly conserved domain can exert a significant effect on production of infectious HCV. Thus, HCV promotes steatosis as an efficient mechanism for stable viral replication.

From a clinical perspective, the negative impact of severe hepatic steatosis on graft dysfunction during the immediate post-transplant period has long been recognized^[23]. Donor livers containing greater than

30%-50% steatosis are at increased risk of developing primary nonfunction and delayed function and are associated with reduced graft and patient survival. Since chronic HCV is the most common indication for liver transplantation (LT), several studies have examined the impact of steatosis within the donor graft on the severity of recurrent HCV and/or survival following LT^[24,25]. From those studies, a direct relationship between marginal donors, graft steatosis and more frequent and earlier recurrence for HCV-related cirrhosis has been established^[25]. The putative relationship between steatosis and viral replication could explain this fact.

INSULIN RESISTANCE IN HEPATITIS C

Liver fibrosis has been considered for a long time to be responsible for the appearance of insulin resistance and type 2 diabetes in patients with chronic liver diseases. Hyperinsulinemia in liver cirrhosis has been reported to be due to diminished hepatic insulin extraction by liver dysfunction and not to pancreatic hypersecretion. C-peptide (a peptide resulting from the split of proinsulin into insulin and C-peptide) and insulin are secreted in equimolar quantities, and more than 50% of insulin is degraded in the liver at first pass, whereas C-peptide is degraded in the kidneys. Simultaneous measurements of C-peptide and insulin revealed that both insulin resistance and insulin secretion contribute to glucose intolerance in patients with chronic HCV^[26]. From a clinical point of view, the insulin resistance index was higher in patients with chronic HCV showing mild or no fibrosis than matched healthy controls. Moreover, insulin resistance was found to be higher in patients with chronic HCV than patients with other causes of chronic hepatitis matched by age, sex, body mass index, family history of type 2 diabetes and fibrosis staging^[27]. On the other hand, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) correlated with HCV RNA level and was found to be higher in patients with HCV than healthy controls in spite of a lower body mass index. Indeed, in patients with HCV, insulin resistance is more strongly associated with viral load than visceral obesity^[4]. Insulin resistance is a common metabolic disorder in the pre-diabetic state. Thus, if HCV promotes insulin resistance it should be linked to type 2 diabetes. Indeed, diabetes was more often seen in HCV than other liver diseases (20%-25% in patients with HCV and in 10% of those with hepatitis B)^[28]. Moreover, in a recent systematic review an increased risk of type 2 diabetes mellitus in patients with hepatitis C in comparison with non-infected people has been reported^[29].

HCV seems to cause metabolic syndrome, but the mechanisms by which HCV promotes insulin resistance have not been completely understood^[30]. HCV induces several complex mechanisms that lead to oxidative stress, insulin resistance, steatosis, fibrosis, apoptosis, altered gene expression and hepatocellular carcinoma. HCV seems to lead to insulin resistance through interference of intracellular insulin signalling by HCV proteins, mainly the serine phosphorylation of IRS-1

and impairment of the downstream Akt signalling pathway^[31].

Transgenic mice expressing core HCV protein developed insulin resistance, which does not occur in wild-type animals^[32]. During HCV replication, core protein has been found to localize to the outer mitochondrial membrane and is also associated with the endoplasmic reticulum (ER)^[33]. In the mitochondria, HCV core protein induces mitochondrial permeability transition, calcium accumulation, stimulation of electron transport and ROS production, as well as promoting glutathione depletion and release of cytochrome C^[34]. Moreover, HCV proteins are assembled and correctly folded by chaperones in the ER, but in some circumstances the ER fails to export synthesized proteins properly leading to an accumulation of misfolded proteins^[35]. The misfolded protein response causes ER dysfunction and promotes inflammation and ER stress^[36]. Of the HCV nonstructural proteins, NS3 and NS5A act as key mediators in the induction of oxidative stress and inflammation. The association of NS5A with the ER has been suggested to stimulate mitochondrial ROS production by releasing calcium from the ER^[37]. In addition, NS3 has been shown to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) that generates ROS^[38]. Nox2 can nitrosylate proteins within cells and can lead to a large number of pathological processes. Consequently, a role for NOX enzymes in hepatic fibrosis, characterized by hepatic stellate cell (HSC) proliferation and accumulation of extracellular matrix proteins, has been suggested^[39,40].

HCV core protein inhibits PPAR α and γ expressed in hepatocytes and adipocytes promoting IRS-1 degradation and insulin resistance^[13]. HCV core protein induces the over production of TNF α , responsible for phosphorylation of serine residues of IRS-1 and IRS-2 and down-regulation of glucose transporter gene expression. TNF correlates with the hyperinsulinemic state and the blockade of TNF production by anti-TNF drugs like infliximab inhibits the development of insulin resistance. Thus, TNF promotes hyperinsulinemia and hyperglycaemia and has been linked to an increased risk of diabetes development^[41]. Moreover, non-structural proteins such as NS3 and NS5 interact with the ER. NS3 enhances Nox2 activity and increases nitrosylated proteins and ROS^[42]. NS5A and NS5B proteins activate toll-like receptor-4 and the NF κ B pathway enhances TNF and IL-6 production and promotes insulin resistance^[43]. IL-6 is a cytokine that is secreted from Kupffer cells, adipocytes, B cells, and hepatocytes. HCV-infected patients are known to have elevated levels of IL-6 due to the virus-induced inflammatory state^[44]. Increased IL-6 derived from adipocytes leads to an ongoing acute-phase response that acts on hepatocytes and promotes hepatic insulin resistance. IL-6 is able to inhibit the expression of LPL in mice^[45]. Unlike TNF- α , IL-6 circulates at high levels in plasma, perhaps representing a hormonal role of IL-6 that may induce insulin resistance in other tissues besides liver.

The HCV core protein interferes with *in vitro* insulin

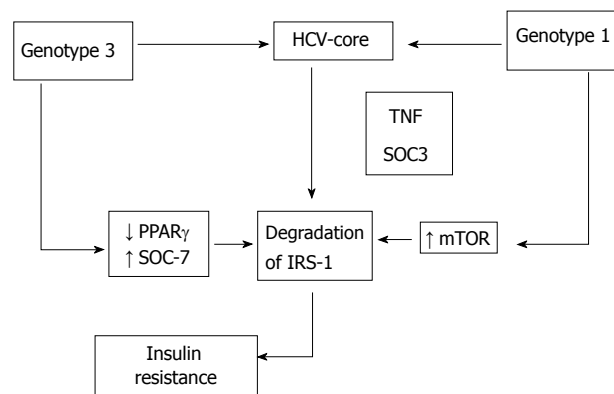


Figure 1 Hepatitis C core induces insulin resistance promoting proteasomal degradation of insulin receptor substrate-1 (IRS-1).

signalling by genotype-specific mechanisms (Figure 1). Pazienza *et al*^[46] used the previously described model of transient expression of the core protein of different genotypes of HCV to assess the interaction of HCV genotype 3a with the insulin signaling, using as comparison the genotype 1b. They found that Insulin Receptor-1 (IRS-1) protein level was significantly reduced in Huh-7 cells expressing the core protein of both genotypes 3a and 1b. However, while the core protein of genotype 3a promoted IRS-1 degradation through the down-regulation of PPAR γ and by up-regulating the suppressor of cytokine signal 7 (SOCS-7), the core protein of genotype 1b activated the mammalian target of rapamycin (mTOR), demonstrating that interaction between viral core protein and IRS-1 degradation is genotype-specific.

Enhanced SOCS production after HCV infection seems to play a crucial role in inducing interferon resistance by inhibiting interferon- α intracellular signalling^[47]. Moreover, SOCS inhibit the phosphorylation of Akt and phosphatidylinositol 3 kinase (PI3K), impairing intracellular insulin signalling, blocking the transactivation of GLUT-4 and avoiding glucose uptake by cells. Over-expression of SOCS-3 has also been linked to interferon and insulin resistance^[48]. Indeed, in transgenic mice unable to express SOCS-3 and expressing HCV core protein, insulin resistance did not appear in the absence of SOCS-3^[49]. Therefore, as previously reported in steatosis, patients with HCV could suffer from viral or metabolic insulin resistance. HCV itself induces insulin resistance by several factors also implicated in interferon resistance, allowing the virus to resist antiviral treatment and to promote fibrosis progression^[50]. However, some controversial results support the presence of additional mechanisms in the development of insulin resistance in patients with chronic HCV and metabolic or viral type insulin resistance. Pazienza and coworkers^[46] have shown that SOCS-1 and SOCS-3 mRNA levels did not change following transfection with both core proteins from genotypes 1b and 3a. However, SOCS-7 mRNA levels were found to be significantly higher in cells expressing the core protein 3a (but not in those transfected with core protein 1b). Their results were corroborated at the protein level by immunoblot. The role of SOCS-7 in IRS-1 downregulation in genotype 3a-transfected cells was con-

firmed using siRNA. They found that the mechanism of IRS-1 degradation by genotype 3a seems to be quite different from that possessed by genotype 1b. The latter is apparently mediated neither by PPAR γ or SOCS-7 nor by SOCS-1 or SOCS-3, as suggested by other authors.

In chronic HCV infection, steatosis up-regulates hepatocyte CD95/Fas and thus increases apoptosis, which facilitates inflammation and fibrosis^[51]. It has been recently shown that adiponectin reduced FFA-induced CD95/Fas expression and apoptosis of HepG2 hepatoma cells, which suggests a protective role for this hormone with promising therapeutic implications^[52].

There are still many molecular mechanisms and open questions to uncover on the HCV-host interaction for the development of effective drugs for the treatment of this disease. In this regard, cell culture based replication systems described so far will help in this task. The availability of systems for replication of all known HCV genotypes together with animal models is highly desirable, in order to find out the importance of the virus genome in disease development.

CONCLUSION

Steatosis development is linked to HCV infection. There is evidence for the accumulation of lipids in the infected cell that could play a determinant role for efficient virus assembly. Thus, HCV promotes steatosis as an efficient mechanism for stable viral replication. HCV itself induces insulin resistance by several factors also implicated in interferon resistance, allowing the virus to resist antiviral treatment and to promote fibrosis progression.

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S- Editor Tian L L- Editor O'Neill M E- Editor Ma WH

ORIGINAL ARTICLE

Do statins reduce hepatitis C RNA titers during routine clinical use?

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Supported by The Veterans Health Administration Research Career Development Award (DEK)

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Received: August 20, 2009

Revised: September 9, 2009

Accepted: September 16, 2009

Published online: October 28, 2009

For those subjects with longitudinal assessment of HCV viremia prior to and while on statins, there were no significant differences between pre- and post-HCV viral titers. Additionally, no differences in HCV titers were observed at any dose level of the most prescribed statin, simvastatin. However, hypertriglyceridemia independently correlated with HCV titers, and niacin exposure was associated with significantly lower viral titers ($P < 0.05$).

CONCLUSION: There was no apparent effect of statins on HCV viral replication in this analysis. Further investigation is warranted to explore the possible antiviral properties of triglyceride-lowering agents and their potential role as adjuncts to standard HCV therapy.

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Key words: Hepatitis C; 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor; Statins; Geranylgeranyl; Prenylation

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Abstract

AIM: To compare hepatitis C virus (HCV) titers in patients with chronic hepatitis C with and without exposure to 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins).

METHODS: Medical records were reviewed for 6463 patients with documented HCV infection at a single center between March 2004 and September 2006. Patients with confirmed viremia and meeting inclusion criteria were assigned to one of three groups: Group A ($n = 50$), dyslipidemic patients with statin usage during HCV RNA polymerase chain reaction (PCR) determination; Group B ($n = 49$), dyslipidemic patients with prior or future statin usage but not at the time of HCV RNA PCR determination; and Group C ($n = 102$), patients without statin usage during the study period. The primary analysis explored the effect of statin therapy on HCV viremia. Secondary analyses assessed class effect, dose response, and effect of other lipid-lowering therapies on HCV viral titers.

RESULTS: Median HCV RNA titers did not significantly differ among the three groups (Group A: 4550000 IU/mL, Group B: 2850000 IU/mL, Group C: 3055000 IU/mL).

Forde KA, Law C, O'Flynn R, Kaplan DE. Do statins reduce hepatitis C RNA titers during routine clinical use? *World J Gastroenterol* 2009; 15(40): 5020-5027 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5020.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5020>

INTRODUCTION

Hepatitis C virus (HCV) infection affects approximately 1.8% of the United States population^[1,2]. The burden of disease is markedly increased in the United States Veteran population with 4%-19% of veterans being seropositive for antibodies against the virus^[3-5]. Pegylated interferon combined with ribavirin is the current standard therapy for HCV and is curative in approximately 40%-50% of patients. However, the adverse effects and contraindications to therapy limit the applicability and utilization of this regimen in many infected persons^[6]. The potential sequelae of chronic HCV infection, the limitations of current therapy, and the large economic burden of this disease provide a critical impetus for the pursuit of novel therapeutic agents.

Recent studies using HCV replicon systems suggest a potential therapeutic role for the lipid-lowering agents 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, in chronic HCV infection. These studies are predicated on cardinal observations of the HCV life cycle. Firstly, HCV virions circulate in plasma in association with low density lipoprotein (LDL) particles. Secondly, the LDL receptor and the high density lipoprotein (HDL) scavenger receptor B1 putatively facilitate HCV entry into hepatocytes^[7]. Thirdly, and perhaps more importantly, HCV replication depends on the formation of a “membranous web” replication complex^[8,9]. Within this endoplasmic reticulum-based replication complex, host proteins are found to be closely associated with HCV nonstructural proteins. The process which links these host and HCV proteins, termed prenylation, appears to depend on two distinct host protein pools, farnesyl and geranylgeranyl, both protein products of the cholesterol synthesis pathway. Therefore, statins, agents which block the formation of the lipid precursors for prenylation, could theoretically interfere with viral replication^[10].

Indeed, *in vitro* studies using HCV replicon-bearing hepatoma cell lines do suggest that statins inhibit HCV replication by disrupting the formation of viral replication complexes, an effect that can be reversed by the addition of mevalonate or geranylgeraniol, synthetic proteins in the cholesterol pathway^[10-12]. Further, the combination of interferon- α and fluvastatin in experimental models exhibited strong synergistic inhibitory effects on HCV RNA replication suggesting that fluvastatin, in particular, but potentially other statins, could be useful as an adjunct to interferon- α based therapy^[11]. While the findings from these studies have been invaluable, the applicability to human use remains in question.

The effect of statins on HCV replication in human subjects has been prospectively addressed in two small studies with mixed results^[13,14]. O’Leary *et al.*^[13] found no reduction in HCV RNA titers at week 4 and week 12 relative to baseline levels. In contrast, another study identified a non-sustained, non-dose-related, reduction in HCV RNA titers in 50% of those treated^[14]. Neither study, however, explicitly addressed the efficacy of individual statin drugs, viral genotype, or controlled for exposure to non-statin lipid-lowering agents. In order to examine a larger number of exposed subjects, to assess the relative efficacy of individual statin formulations, to control for potential confounders, and to determine whether further prospective trials might be warranted, we performed a cross-sectional and longitudinal analysis of HCV RNA viral loads in chronic HCV patients who received a statin for therapy of dyslipidemia.

MATERIALS AND METHODS

Ethics

This study protocol was reviewed and approved by the Institutional Review Board of the Philadelphia Veterans Affairs Medical Center (VAMC).

Selection of patient groups

We performed a retrospective analysis of chronic HCV-infected patients who were seen at the Philadelphia VAMC from March 14, 2004 to September 14, 2006 and who had at least one quantitative HCV RNA polymerase chain reaction (PCR) test performed using the Taqman[®] assay (Roche Diagnostics, Indianapolis, IA). Screening for viral hepatitis is part of routine intake for all primary care clinic patients at the Philadelphia VAMC. Patient level data was extracted from the facility’s Hepatitis C Registry, an automated system that registers all patients with positive HCV antibody testing from clinical laboratory data and facilitates acquisition of additional clinical information from the Computerized Patient Record System (CPRS) and Veterans Health Information Systems and Technology Architecture databases. Charts of patients identified as HCV antibody-positive were queried as to the presence and type of confirmatory PCR testing. Those without confirmatory PCR testing and those without detectable viremia by quantitative Taqman[®] HCV PCR assay were excluded. Patients were also excluded if HIV antibody, HIV RNA and/or HBsAg were positive or if antiretroviral drugs were present in the medication profile. Patients with acute HCV, defined as seroconversion within 2 years of exposure, and patients with chronic kidney disease, defined as serum creatinine greater than 2 mg/dL, were also excluded.

Pharmacy records were then examined to identify the start and stop dates of HMG-CoA reductase inhibitors, niacin, clofibrate, ezetimibe, and interferon- α preparations. Interferon- α based therapy was confirmed through review of progress note documentation in the CPRS. HCV RNA titers obtained during interferon- α therapy were annotated. Utilizing refill data and chart review, patients in whom the HCV RNA determination date(s) occurred at least 30 d after initiation of HMG-CoA reductase inhibitor therapy, in whom exposure to the statin drug spanned at least 60 d, and in whom the duration of statin therapy included the date of HCV RNA determination were designated as Group A. Two control groups who also met the inclusion criteria were selected for comparison. Subjects with hypercholesterolemia for whom a statin medication was prescribed but either had HCV RNA titers drawn prior to the statin initiation date or in whom statin therapy was discontinued at least 60 d prior to the HCV RNA determination were selected as dyslipidemic controls Group B. Group C was chosen from a pool of HCV positive subjects without documented statin exposure during the evaluation period. Analysis of this group was performed with and without exclusion of subjects with total cholesterol levels greater than 200 mg/dL. Using the date of closest correlation to the HCV RNA titer obtained, laboratory data, including basic chemistries, liver associated enzymes, coagulation panels, and lipid profiles were extracted for each group. The longitudinal results of HCV RNA titers during ongoing statin therapy were also recorded for those patients in Group A with more than one HCV RNA determination within the study period.

Table 1 Patient characteristics

Variables	Median (range)			P-value		
	Group A (n = 50)	Group B (n = 49)	Group C (n = 102)	All	Group A vs Group B	Group A vs Group C
Gender (male/female)	50/0	49/0	100/2	0.38		
Race (white/black/other/unknown)	15/28/0/7	11/31/0/7	18/64/0/20	0.74		
Ethnicity (hispanic/non-hispanic/unknown)	2/44/4	1/38/3	3/78/21	0.15		
Prior interferon-alpha therapy (%)	14	8	3	0.042	0.32	0.015
HCV genotype (1/2/3/not-typed)	34/4/1/1	32/5/0/0	70/5/0/0	0.26		
Age (yr)	56 (47-87)	55 (42-80)	56 (41-83)	0.60		
Body mass index	29.1 (18.9-47.8)	28.1 (20.8-43.6)	26.8 (17.1-41.8)	0.0021	0.24	0.001
Albumin (g/dL)	4 (3-5)	4 (3-4)	4 (2-5)	0.37		
ALT (U/L)	46 (11-388)	40 (12-207)	53 (15-345)	0.09	0.19	0.035
AST (U/L)	37 (18-199)	36 (16-136)	53 (22-304)	< 0.0001	0.24	0.002
Alkaline phosphatase (U/L)	74 (28-215)	79 (44-162)	77 (40-1106)	0.23		
Total bilirubin (mg/dL)	0 (0-2)	0 (0-1)	0 (0-2)	0.01	0.40	0.060
Creatinine (mg/dL)	1 (0-1)	1 (0-1)	1 (0-2)	0.24		
International normalized ratio	1 (0-2)	0 (0-1)	1 (0-3)	0.48		
Platelets ($\times 1000/\text{mL}$)	242 (129-758)	242 (122-450)	208 (55-606)	0.020	0.97	0.024
Total cholesterol (mg/dL)	177 (110-304)	187 (71-277)	161 (75-260)	0.0002	0.21	0.038
HDL cholesterol (mg/dL)	40 (25-70)	42 (26-69)	41 (21-110)	0.16		
LDL cholesterol (mg/dL)	114 (45-222)	122 (64-187)	93 (22-173)	< 0.0001	0.29	0.006
Triglycerides (mg/dL)	120 (35-384)	114 (44-443)	105 (39-467)	0.31		

Group A: Cases (HCV PCR+ with concomitant statin exposure); Group B: Dyslipidemic control (HCV PCR+ with non-coincident statin exposure); Group C: Unexposed control (HCV PCR+, no statin exposure).

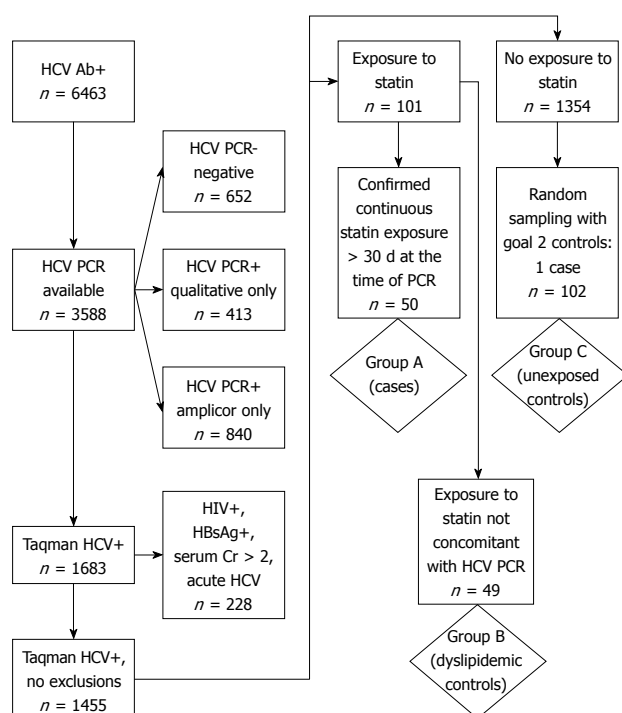


Figure 1 Patient assignment algorithm.

Statistical analysis

Comparisons of frequency data were performed with χ^2 or Fisher's exact testing as appropriate. All group-wise comparisons were performed using non-parametric tests including Kruskal-Wallis or Mann-Whitney *U* tests. Regression analysis was conducted to explore the effect of potential confounders on the primary study endpoint, HCV RNA titers. All analyses were conducted with JMP 7 software (SAS Institute, Cary, NC) and/or STATA 9.2

(College Station, TX). *P*-values < 0.05 were considered significant.

RESULTS

Patient selection and characteristics

A total of 6463 patients were found to be HCV antibody-positive (+). Fifty HCV-infected patients who met criteria for statin exposure with concomitant HCV RNA determination (Group A), 49 HCV-infected dyslipidemic patients not on a statin at the time of HCV RNA determination (dyslipidemic controls, Group B) and 102 statin-unexposed HCV-infected controls (Group C), were analyzed (Figure 1).

Patients in the three groups were similar in terms of age, gender, race, ethnicity, HCV genotype, serum albumin, alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin, creatinine, international normalized ratio, platelets, HDL cholesterol, triglyceride levels and exposure to non-statin lipid-lowering agents (Table 1). Among Group A, the median duration from first statin exposure to HCV RNA determination was 288 d (range 34-1435, data not shown). Group A, when compared to Group C, had a significantly higher median body mass index (BMI) (29.1 *vs* 26.8, *P* < 0.01), platelet count ($243 \times 1000/\text{mL}$ *vs* $208 \times 1000/\text{mL}$, *P* < 0.05), total cholesterol level (177 mg/dL *vs* 161 mg/dL, *P* < 0.01), and LDL levels (114.3 mg/dL *vs* 93.4 mg/dL, *P* < 0.01). However, there were no significant differences between Group A and Group B. Group C had a significantly higher median aspartate aminotransferase level (54 U/L *vs* 37 U/L, *P* < 0.01) and ALT level (53 U/L *vs* 46 U/L, *P* < 0.05) when directly compared with Group A.

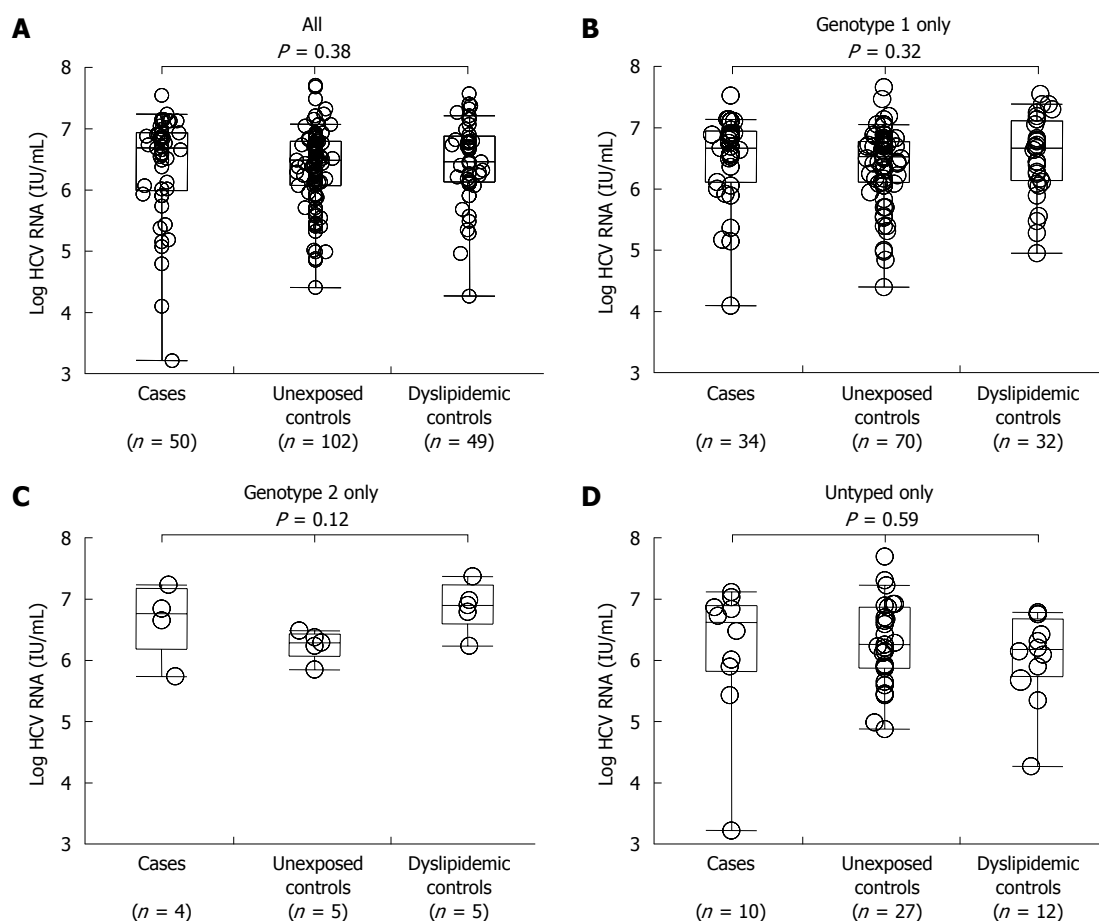


Figure 2 Hepatitis C RNA titers relative to HMG-coA reductase inhibitor exposure. Log HCV RNA viral load (IU/mL) in Group A, Group B, and Group C subjects. A: Entire cohort; B: Genotype 1 patients; C: Genotype 2 patients; D: Patients in which genotype was not available. Box plots indicate median, 25th and 75th percentiles (box), and 5th and 95th percentiles. *P*-value obtained by Kruskal-Wallis test.

HCV RNA titers and statin usage

Median HCV RNA titer in Group A was 4550000 IU/mL *vs* 2850000 IU/mL in Group B *vs* 3055000 IU/mL in Group C (Figure 2A). The similarity in serum titers suggest that in this cohort there was no evidence that as a class HMG-CoA reductase inhibitors exhibit antiviral properties *in vivo*. To confirm that there was no genotype-specific effect of HMG-CoA reductase inhibitors, we analyzed each known HCV genotype separately for genotype 1, genotype 2 and untyped subjects in all groups. We again found no significant differences between these groups for any genotype (Figure 2B-D).

Statin preparations, HCV RNA titers, and dose response

Among Group A, 42 (84%) received simvastatin, 5 (10%) lovastatin, 2 (4%) pravastatin and 1 (2%) fluvastatin. When comparing HCV RNA titers among patients receiving any of the four statin agents, there were no differences observed (Figure 3A). Furthermore, there was no apparent antiviral effect of simvastatin, the most prescribed statin in this cohort, at any dose level (Figure 3B). However, when comparing the two most commonly used agents in our cohort, there was a trend towards lower HCV RNA titers in subjects who received lovastatin relative to simvastatin. To further investigate whether or not the trend towards lower HCV RNA titers in lovastatin users might be significant, we compared

HCV RNA titers in active lovastatin users in Group A and former or future lovastatin users in Group B. However, no significant trend towards a lower HCV viral load could be identified (Figure 3C). Thus, there was no class effect of HMG-CoA reductase inhibitors and further conclusions regarding specific statin formulations cannot be made.

Serum total cholesterol and HCV RNA titers

The lack of apparent anti-viral effect of statins theoretically could have resulted from neutralization from a pro-viral effect of hypercholesterolemia rather than a lack of effect of the statin in individual patients. To control for this potential confounder, we correlated viral titers and total cholesterol for Group A (Figure 4A), Group B (Figure 4B), and Group C subjects (Figure 4C) and found no evidence of a relationship between total serum cholesterol and HCV RNA titers in any group. Further, there was no correlation of HCV RNA titers and HDL, LDL, or triglyceride levels (data not shown). Thus, hypercholesterolemia itself did not appear to mediate a pro-viral effect or to be related to cholesterol metabolism in a fashion that may negate any anti-viral effect of statin preparations.

BMI and HCV RNA titers

As noted above, Group A subjects had a significantly

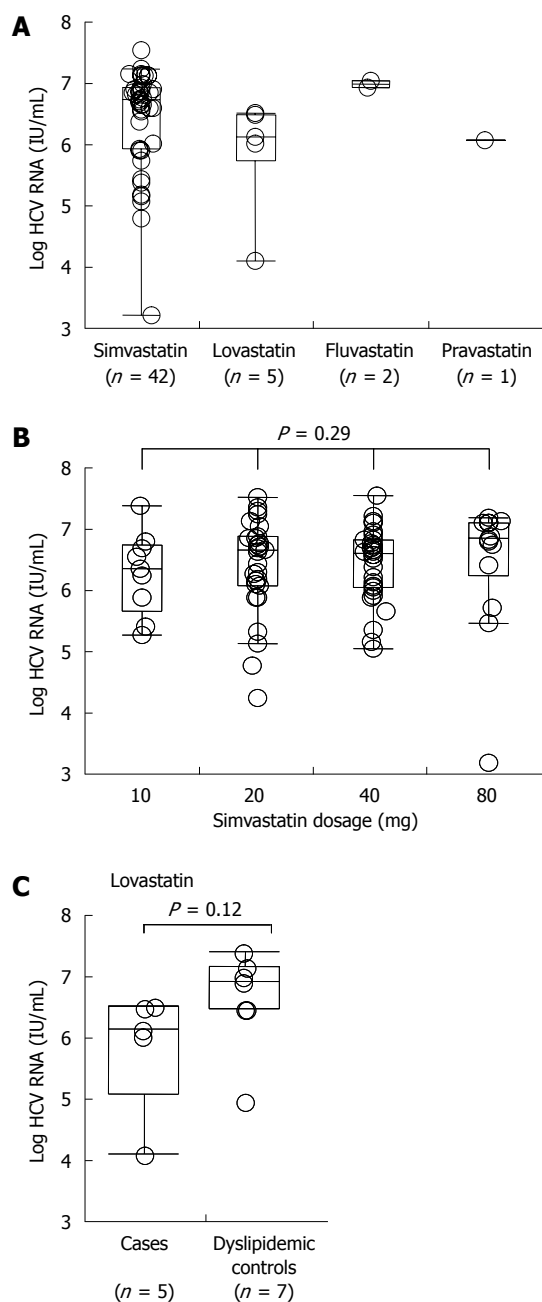


Figure 3 Antiviral effects of individual HMG-CoA reductase inhibitors. A: Log HCV RNA viral load (IU/mL) for Group A subjects who received simvastatin ($n = 42$), lovastatin ($n = 5$), fluvastatin ($n = 2$) and pravastatin ($n = 1$); B: Log HCV RNA viral load (IU/mL) for simvastatin-exposed subjects who were receiving 10 mg ($n = 4$), 20 mg ($n = 8$), 40 mg ($n = 20$), 80 mg ($n = 10$). P -value obtained by Kruskal-Wallis Test; C: Pairwise comparison of median HCV RNA titers in lovastatin-exposed Group A subjects and Group B subjects who received lovastatin but were not being treated at the time of the RNA determination. P -value from Wilcoxon sign rank test.

higher median BMI (29.1 *vs* 26.8, $P < 0.01$) than Group C, but no significant difference in BMI was found between Group A and B. To assess any potential interaction of BMI on HCV RNA titers, we performed regression analysis that demonstrated no association between BMI and HCV RNA titer in either Group A or Group C.

Hypertriglyceridemia, other lipid lowering agents and HCV RNA titers

In order to assess serum triglyceride levels as a confo-

ounding variable, we analyzed the association between serum triglycerides as well as triglyceride-directed therapy on HCV RNA titers. As shown in Table 1, 6/50 in Group A, 2/49 in Group B and 5/102 in Group C received non-statin lipid lowering therapy at the time of HCV RNA determination. Of these, a total of 10 exposures were to niacin (5/6 in Group A, 2/2 in Group B, and 3/5 in Group C). After excluding patients on niacin and gemfibrozil, the presence of triglyceride levels greater than 250 mg/dL was associated with a higher median HCV RNA titer (> 250 mg/dL: 6 760 000 IU/mL *vs* < 250 mg/dL: 3 130 000 IU/mL, $P < 0.05$, Figure 4D) and triglycerides were weakly but significantly correlated with HCV viral titers ($R^2 = 0.023$, $P < 0.05$, data not shown). After excluding two patients taking gemfibrozil, niacin-exposure, irrespective of statin exposure, was associated with lower median HCV RNA titers (exposed: median 835 000 IU/mL *vs* unexposed: 3 350 000 IU/mL, $P < 0.05$, Figure 4E). Compared to 13 patients with untreated hypertriglyceridemia (defined as triglycerides > 250 mg/dL), niacin-exposure was associated with a 1.16-log reduction in median HCV RNA titer ($P < 0.05$). Similar analyses for fibric acid derivatives or ezetimibe were not possible due to the limited number of exposed patients.

Longitudinal effect of statin therapy

Twenty-eight Group A subjects (23 simvastatin, one fluvastatin and four lovastatin) had more than one determination of HCV RNA titer, allowing for longitudinal assessment of changes in HCV viremia during the duration of HMG-CoA reductase exposure. In the vast majority of non-interferon-treated subjects, there was either a modest increase or no change in HCV RNA titers during such therapy (Figure 5). In 11 of these 28 subjects, an HCV RNA determination was made prior to and after the institution of statin therapy. Within this subgroup, there was no significant difference between pre- and post-statin levels of HCV viremia. In two of three lovastatin-exposed subjects with initial HCV RNA testing within 1 mo (range: -15 to +17 d) of initiation of interferon, HCV RNA titers declined by 0.7-1.1 log between 9-16 mo. Only two subjects with repeated assessments were exposed to interferon during the observation period. These data imply that statin exposure in this cohort was not associated with significant inhibition of viral replication for individuals with longitudinal follow-up.

DISCUSSION

The sustained virologic response rate of chronic hepatitis C in registration trials with currently approved interferon- α based antiviral therapy ranges from 44%-54%^[15,16], but varies widely by genotype, ethnicity and underlying histology. In practice, at centers similar to the study site, HCV treatment response rates range from 20% for genotype 1 to 43%-52% for genotypes 2-3^[17]. Suboptimal response and significant toxicity continue to spur the development of novel anti-HCV therapies.

The capacity of HMG-CoA reductase inhibitors

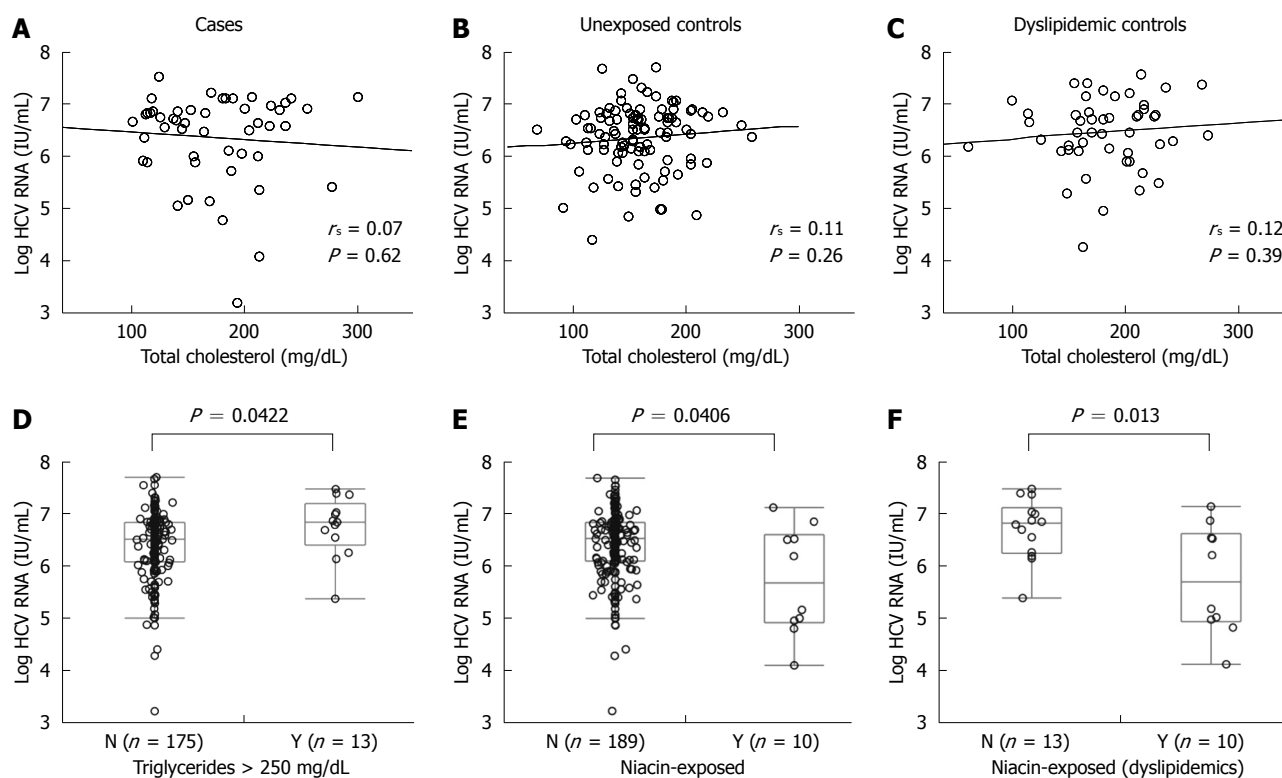


Figure 4 Lack of correlation between serum cholesterol and HCV RNA viral load, but apparent association with hypertriglyceridemia. Linear correlation of log HCV RNA titers (IU/mL) and serum total cholesterol (mg/dL) in Group A (A), Group B (B), and Group C (C). *P*-values obtained by Spearman correlation; D: Log HCV RNA titers for patients with serum triglycerides greater than or below 250 mg/dL excluding patients on gemfibrozil or niacin (> 250 mg/dL: median 6 760 000 IU/mL vs < 250 mg/dL: median 3 130 000 IU/mL, $P = 0.042$); E: Log HCV RNA titers for patients with or without exposure to niacin, excluding patients on gemfibrozil (exposed: median 835 000 IU/mL vs unexposed: median 3 350 000 IU/mL, $P = 0.041$); F: Log HCV RNA titer among patients with hypertriglyceridemia, excluding gemfibrozil therapy, with and without exposure to niacin shows lower HCV RNA viral load in niacin-treated patients (niacin-exposed: median 835 000 IU/mL vs unexposed: median 6 760 000 IU/mL, $P = 0.013$).

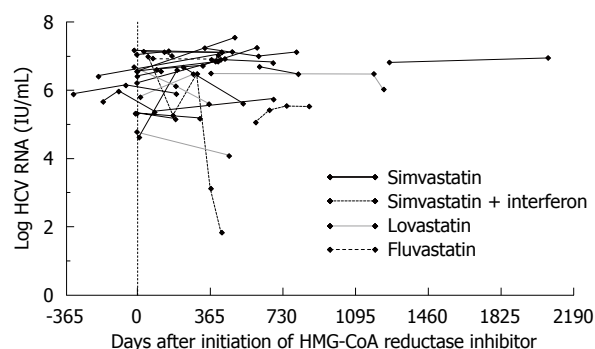


Figure 5 Longitudinal effect of statin therapy. Log HCV RNA titer plotted against days relative to the statin initiation date for simvastatin (black lines, $n = 21$), simvastatin plus interferon ($n = 2$), lovastatin (grey lines, $n = 4$), and fluvastatin (big dotted line, $n = 1$) for patients with more than one determination of HCV RNA titer. Eleven subjects had HCV RNA titers measured before and after the initiation of statin therapy.

to impede HCV replicon replication *in vitro* in a dose-dependent fashion raised hope that this commonly prescribed and acceptably safe class of medications could serve as an adjunct to standard interferon-based therapy. Data from such *in vitro* studies further demonstrated a hierarchy of statin-induced viral inhibition, with the greatest effect demonstrated with fluvastatin followed by atorvastatin, simvastatin, and lovastatin, respectively. These *in vitro* findings prompted a pilot study in which

10 subjects with chronic hepatitis C and laboratory evidence of dyslipidemia were treated with atorvastatin 20 mg daily. However, no significant changes in HCV RNA titers were demonstrated at the atorvastatin dose administered though such subjects did have a significant lowering of LDL and total cholesterol^[13]. This first study did not examine the effect of fluvastatin, the most potent statin identified in *in vitro* experiments. A more recent small, uncontrolled study of 22 patients given fluvastatin at doses ranging from 20-80 mg/d found transient, 0.5-log reductions in HCV RNA titers in 50% of treated subjects^[14], but did not correlate these responses with the lipid lowering therapeutic effect of statins or specifically explore viral genotypes and other medications used.

In order to validate the need for further prospective study of the effect of statins on HCV viral replication in clinical practice, we performed a cross-sectional study of a larger number of subjects and controlled for potential confounders such as viral genotype, cholesterol levels, triglyceride levels and exposure to other lipid lowering medications. We found exposure to different statin preparations, primarily simvastatin, during routine clinical use, was not associated with a change in HCV viral titers. In a limited number of subjects with longitudinal measures of HCV viral load pre- and post-initiation of statin therapy, our data suggested that there was no evidence of clinically significant change in HCV

RNA titers. Specifically for simvastatin, the statin for which we had the most data, we were unable to show a dose-dependent association with reduced HCV titers, contrasting with *in vitro* studies^[11]. Given the small number of fluvastatin-exposed subjects at our institution ($n = 2$), we were unfortunately not able to analyze the effect of exposure to the most potent *in vitro* inhibitor.

A plausible explanation for the discrepancy between our *in vivo* and others' *in vitro* results may rest with the pharmacokinetic properties of statins. There is a significant first pass effect for all statins with the exception of pravastatin^[18]. Intrahepatic concentrations of statins with routine use, however, have not been to our knowledge well documented in the literature. Serum levels of such agents after prolonged therapy are significantly lower than the statin concentrations that were used in replicon systems. For example, the maximal serum concentration of fluvastatin dosed at 40 mg daily is approximately 0.589 $\mu\text{mol/L}$ ^[19], approximately 10-fold lower than effective statin concentrations for inducing viral inhibition in the replicon systems^[10-12].

Additionally, replicon-bearing cell lines are highly adapted and behave quite differently from *in vivo* hepatocytes. For instance, Huh7 cells exposed to interferons are exquisitely responsive to the agent regardless of viral genotype^[20]. This is in direct contrast to the disparate rates of sustained virologic response observed clinically with interferon therapy. It is possible that the adaptations that confer interferon sensitivity also confer statin sensitivity, explaining the *in vitro* results. Alternatively, the specific dependence on prenylation of nonstructural proteins for establishment of the viral replication complex might be a feature of cell-culture models for which alternative pathways may be present *in vivo*. The HCV might also develop resistance mutations under selection pressure induced by statin therapy, an effect that could be demonstrated *via* sampling at regular intervals early after initial statin exposure for early viral load changes and sequence evolution. Lastly, pro-viral effects of statins might occur *via* induction of LDL-receptor expression which may paradoxically facilitate viral uptake into uninfected hepatocytes^[21].

Another possible explanation for the lack of difference between our study groups could be the presence of a significant confounding variable. For example, obesity, which is associated with non-response to interferon-based antiviral therapy^[22], and hypercholesterolemia, were significantly more prevalent in Groups A and B than C. We, however, did not find within any of the groups an association of HCV RNA titers with height, weight or body-mass index (data not shown) nor with total cholesterol; therefore we do not believe that differences in these variables could explain our negative findings. Since hypertriglyceridemia was directly associated with viral titer, the excess of hypertriglyceridemia cases in Group A could contribute to a type II error. However, when we controlled for triglycerides in the regression analysis, statin exposure remained insignificantly associated with HCV RNA titer.

While not powered for an analysis of non-statin lipid lowering medications and their effect on HCV

viral load, our analysis unexpectedly identified a possible direct association of triglycerides and viremia and a suggestion that niacin may have antiviral properties *in vivo*. Recent work with human serum^[23] and with primary hepatocytes^[24] suggests that HCV is co-secreted with VLDL, implicating triglyceride metabolism as an additional critical step in the viral lifecycle. Given these preliminary findings as well as a biologically plausible mechanism for the action of triglyceride lowering medications on HCV replication, these findings merit further investigation in a larger dataset and, if confirmed, in a prospective clinical trial.

There are several limitations of this analysis that we would like to acknowledge. First, the cross-sectional design inherently precludes determining causality and is inherently weaker than prospective evaluation. Further, given the limited number of subjects with measurements prior to and after the initiation of statin therapy, we cannot definitively rule out an association between statins and reduction in HCV viral replication. Secondly, the sample size remained relatively small after applying our exclusion criteria, raising the possibility of a type II error and specifically that of finding no association when one does indeed exist. Thirdly, there were no direct measurements of patient adherence with prescribed statin therapy, an important factor since poor adherence may make the medication in question appear to be less efficacious. Fourthly, the preferred statin agent on the formulary during the observation period was not the most highly active agent *in vitro*. Lastly, there is a small possibility that patients in Group B could have received a statin agent prescribed by a non-VA physician, which was not recorded in the VA electronic medical record. Since there is significant financial incentive for most veterans to obtain medications through the VA, we believe the impact of this factor is quite small.

In summary, in this single center, retrospective analysis, there was no evidence of an apparent effect of statins on HCV viral replication. Unexpectedly, we found that triglyceride lowering agents such as niacin may have HCV antiviral properties *in vivo*. Therefore, we suggest exploration of this result. Additionally, the potential antiviral efficacy of drugs such as niacin in chronic hepatitis C as adjuncts to interferon-based therapies merit further investigation.

ACKNOWLEDGMENTS

This material is based upon work supported in part by the Office of Research and Development, Department of Veterans Affairs and with the resources and the use of facilities at the Philadelphia VA Medical Center. We also thank Kyong-Mi Chang, MD for her review of the manuscript and for development of the hepatitis C registry at the Philadelphia VAMC.

COMMENTS

Background

Interferon and ribavirin are the mainstay of treatment for chronic hepatitis C

infection. Unfortunately, this combination is only effective in approximately 50% of all-comers. Therefore, novel therapies must be explored. Cardinal observations of the hepatitis C virus (HCV) life cycle have demonstrated that the lipid metabolism pathway is implicated in viral entry and replication within the hepatocyte. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, referred to commonly as statins, have been shown to not only disrupt lipid metabolism within the hepatocyte but also halt HCV viral replication in *in vitro* models of chronic HCV infection. Relatively few studies have explored the effect of statins on HCV viral replication *in vivo*.

Research frontiers

Though *in vitro* studies using HCV replicon-bearing hepatoma cell lines do suggest that statins inhibit HCV replication, the applicability of use in humans with chronic hepatitis C infection remains in question. In this study, the authors attempt to, but are unsuccessful in, demonstrating a significant reduction in HCV viral titers with routine use of statins in a cohort of patients infected with hepatitis C.

Innovations and breakthroughs

The results did not suggest an effect of statins on HCV viral replication. However, the investigators did find preliminarily that triglyceride lowering agents may in fact lower levels of HCV viremia. Though significant, this finding was noted in a small number of patients and given that the study was not powered for this question, this should be viewed as hypothesis generating.

Applications

By continuing to explore potential adjunct therapies to current HCV anti-viral therapies, it is likely that some highly effective adjunct(s) will be discovered. This study, while not demonstrating a significant effect of statins in this cohort, does provide some preliminary data to support further investigation of lipid altering medications and their potential effect on HCV.

Terminology

The process which links host and HCV proteins during intrahepatic viral replication is termed prenylation. Prenylation is dependent on two distinct host protein pools, farnesyl and geranylgeranyl, both protein products of the cholesterol synthesis pathway.

Peer review

This is a well written manuscript. The study suffers from the limitations of a retrospective cohort study and may have not detected a difference in HCV viral titers because longitudinal assessment was not made for all subjects prior to and after initiation of statin therapy. The authors need to comment on these limitations in the discussion.

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S- Editor Li LF L- Editor Logan S E- Editor Zheng XM



ORIGINAL ARTICLE

Interventional treatment for symptomatic acute-subacute portal and superior mesenteric vein thrombosis

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Supported by The National Natural Science Foundation, Project No. 30670606; Chinese army "Eleventh Five-Year Plan" Research Fund, Project No. 06MA263

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Received: May 5, 2009 Revised: August 13, 2009

Accepted: August 20, 2009

Published online: October 28, 2009

complications. In 1 patient, the thrombi did not respond to the interventional treatment and resulted in intestinal necrosis, which required surgical treatment. In 3 patients with interventional treatment, thrombi re-formed 1, 3 and 4 mo after treatment. In these 3 patients, indirect PV-SMV thrombolysis was performed again and was successful.

CONCLUSION: Interventional treatment, including direct or indirect PV-SMV thrombolysis, is a safe and effective method for patients with symptomatic acute-subacute PV-SMV thrombosis.

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Key words: Portal thrombosis; Superior mesenteric vein thrombosis; Thrombolysis; Interventional treatment

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Abstract

AIM: To summarize our methods and experience with interventional treatment for symptomatic acute-subacute portal vein and superior mesenteric vein thrombosis (PV-SMV) thrombosis.

METHODS: Forty-six patients (30 males, 16 females, aged 17-68 years) with symptomatic acute-subacute portal and superior mesenteric vein thrombosis were accurately diagnosed with Doppler ultrasound scans, computed tomography and magnetic resonance imaging. They were treated with interventional therapy, including direct thrombolysis (26 cases through a transjugular intrahepatic portosystemic shunt; 6 through percutaneous transhepatic portal vein cannulation) and indirect thrombolysis (10 through the femoral artery to superior mesenteric artery catheterization; 4 through the radial artery to superior mesenteric artery catheterization).

RESULTS: The blood reperfusion of PV-SMV was achieved completely or partially in 34 patients 3-13 d after thrombolysis. In 11 patients there was no PV-SMV blood reperfusion but the number of collateral vessels increased significantly. Symptoms in these 45 patients were improved dramatically without severe operational

Liu FY, Wang MQ, Fan QS, Duan F, Wang ZJ, Song P. Interventional treatment for symptomatic acute-subacute portal and superior mesenteric vein thrombosis. *World J Gastroenterol* 2009; 15(40): 5028-5034 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5028.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5028>

INTRODUCTION

With the development of imaging technology, the rate of diagnosis of portal and superior mesenteric vein thrombosis has gradually increased^[1]. When thrombosis occurs only in the portal vein, clinical symptoms such as abdominal pain, nausea, anorexia, and weight loss are usually not serious. When thrombosis involves the superior mesenteric vein, patients may have cramps in the upper middle or lower middle abdomen, nausea, anorexia, vomiting, diarrhea, and/or bloody stool^[2-4]. Conservative medical treatment is often unsatisfactory. Surgical treatment is accompanied by more tissue damage, more complications, and a high recurrence rate. Therefore, it is seldom used as a standard treatment unless intestinal necrosis occurs. Recently, interventional and minimally invasive techniques were introduced to treat portal vein and superior mesenteric vein thrombosis (PV-SMV) thrombosis. The results have been

excellent^[5,6]. Herein we summarize the interventional treatment of 46 patients with symptomatic acute-subacute PV-SMV thrombosis. Our goal was to describe how to choose a specific interventional treatment, and how to enhance the effect of the treatment and reduce complications.

MATERIALS AND METHODS

General information

We reviewed the case histories of 46 patients including 30 males and 16 females, with an average age of 48 years (range: 17-68 years). All patients had different degrees of abdominal pain, fullness, and anorexia. Other symptoms and signs included diarrhea (25 cases), vomiting (11 cases), and ascites (8 cases). There was no obvious abdominal rigidity and rebound tenderness. Causes of the disease were clearly defined in 42 patients, including 18 who had splenectomies within one year, 8 who had a recent history of pancreatitis, 14 who had liver cirrhosis with portal hypertension (6 of them accompanied by esophageal and gastric varices), 1 who had the condition secondary to duodenal surgical repair for traumatic injury within the last 24 h, and 1 who had appendicitis within the last month.

The period of the disease included 28 acute cases (thrombosis occurred within 1 wk of onset) and 18 subacute cases (1-3 wk from onset). The number of platelets was higher ($500-980 \times 10^9/L$) than standard scores in 28 patients. The number of white cells was higher ($12-20 \times 10^9/L$) than normal standards in 17 patients. Red cell number and hemoglobin levels were in the normal range. Liver function tests showed 42 with Child A, and 4 with Child B. Renal function and plasma electrolytes were in the normal range. All patients were clearly diagnosed as having PV-SMV thrombosis by Doppler ultrasound scan, computed tomography (CT) and magnetic resonance imaging (MRI). They were fast and gastrointestinal decompressed.

Interventional treatment

Direct PV-SMV thrombolysis: Twenty four patients were seen within 1 wk of the onset of disease and diagnosed as having acute PV-SMV thrombosis without obvious lateral branch angiogenesis by imaging. Eight patients were seen between 1 and 2 wk from the onset of disease and diagnosed as subacute PV-SMV thrombosis without obvious lateral branch angiogenesis by imaging.

Thrombolysis through transjugular intrahepatic portosystemic shunts (TIPS): In 26 patients, a 10F long sheath (RUPUS 100 system, COOK Corp, USA) was used to puncture the right internal jugular vein according to Seldinger's method and then advanced into the hepatic vein *via* the superior vena cava, right atrium, and inferior vena cava. A shell-type needle was introduced into the hepatic vein through the sheath and it was advanced into the right branch of the PV under X-ray guidance. When PV branches were filled with thrombi and blood flow was interrupted, it was not possible to determine whether PV was punctured or not using the aspirate puncture-cannula. Under such conditions, the contrast should be injected slowly *via*

the puncture-cannula while the puncture-cannula is withdrawn gradually. When the puncture was successful, the contrast remained in the PV branches and the shape of vessels could be seen clearly. An ultra-smooth guide wire was easily introduced into the PV-SMV system. Once it was in the right branch of the PV, a 4F Cobra Catheter (Cordis Corp. USA) or an inferior vena cava angiographic catheter (COOK Corp, USA) was advanced to the distal SMV for direct PV-SMV angiography.

Before thrombolysis, 30-50 mg sodium heparin was injected intravenously into patients without any contraindications. An 8F large thin-walled introducer catheter (90 cm Cordis Corp., USA, or a 100 cm Boston Scientific Corp. USA) was used to aspirate the thrombi and a 4F pigtail catheter (Cordis Corp. USA) was used for mashing the thrombi. A 4F inferior vena cava catheter with multiple lateral holes (COOK Corp. USA) was inserted into the PV-SMV and used to deliver sodium heparin and urokinase (average doses of 0.8 MIU, 0.5-1.5 MIU) intermittently for local thrombolysis. After most of the thrombi in the PV-SMV had been removed, the catheter was maintained for continuous thrombolysis with urokinase (0.5-1.5 MIU/d) and sodium heparin (30-200 mg/d) for 3-13 d. Clopidogrel (75-150 mg/d) or enteric-coated aspirin (100-150 mg/d) were administered to patients with platelet counts greater than $300 \times 10^9/L$. During treatment, thrombin time (TT) and activated partial thromboplastin time (APTT) were monitored to maintain TT at 1.5-2.5 times the normal range, and APTT at 2-2.5 times the normal range. In the meantime, levels of D-dimer were monitored^[7]. The duration of indwelling catheters, which depended on the improvement of symptoms and thrombolysis, usually lasted less than 2 wk. After the catheter was withdrawn, intravenous sodium heparin was continued for 2 additional weeks, and then changed to oral warfarin sodium (for not less than 1 year). Treatment with warfarin and heparin should overlap for 3 d. After being discharged from the hospital, patients were monitored with standard blood tests and we maintained the international normalized ratio (INR) at 2.0-3.0. Abdominal ultrasound scans were repeated every 1-3 mo and CT or MRI scans as necessary.

Thrombolysis through percutaneous transhepatic portal vein cannulation: In 6 patients, a percutaneous transhepatic puncture was performed with a 22-gauge Chiba needle (COOK Corp, USA) into the right branch of the PV, at the midline through the right armpit. Then an 8F artery sheath (Cordis Corp. USA) was introduced into the PV for thrombolysis. The following thrombolysis procedure was the same as the TIPS method. After treatment, the punctured tract was broken by a gelatin sponge and a steel ring (COOK Corp, USA) was used to prevent secondary liver bleeding.

Indirect PV-SMV thrombolysis

Patients who received indirect PV-SMV thrombolysis included: (a) 4 with acute PV-SMV thrombosis diagnosed by imaging tests within one week of the onset of the disease and who refused direct PV-SMV thrombolysis, (b) 4 with subacute PV-SMV thrombosis diagnosed within 1-2 wk,

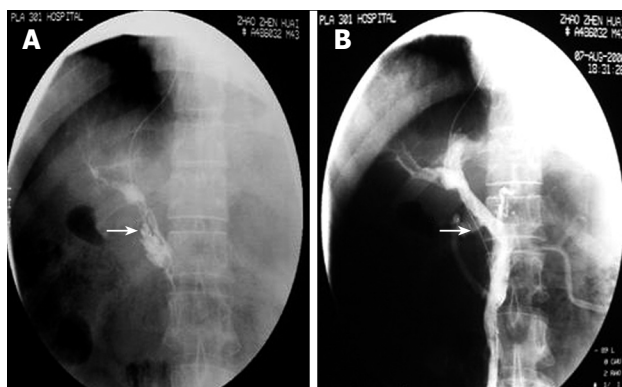


Figure 1 The figure shows a male, 43-year-old patient, 2 mo after splenectomy, with abdominal fullness for 6 d. He had thrombolysis through a transjugular intrahepatic portosystemic shunt (TIPS). A: Direct PV-SMV angiography showed extensive thrombosis in the PV and SMV. Contrast agent remained. No lateral branch angiogenesis (arrow); B: Mash and suck thrombi with intermittent injection of urokinase and heparin sodium. Repeated angiography 30 min after thrombolysis showed blood reperfusion in the PV-SMV and normal PV-SMV branches (arrow). Symptoms disappeared.

including 3 who refused and 1 who failed direct PV-SMV thrombolysis, (c) 6 with subacute PV-SMV thrombosis diagnosed within 2-3 wk, including 3 showing lateral branch angiogenesis around the PV-SMV system in the CT scan, suggesting a long duration of the disease.

Thrombolysis through the femoral artery to the superior mesenteric artery cannulation

In 10 patients, a 4F artery sheath (Terumo Corp. Japan) was used to puncture the right femoral artery according to a modification of Seldinger's technique, and then a 4F Cobra Catheter was inserted to perform celiac artery, superior mesenteric artery and indirect PV-SMV angiography. When angiography was completed, 3-6 holes were drilled at the front of the Cobra Catheter with a microdriller. An intensive dose of urokinase (0.2 MIU) was administered into the superior mesenteric artery through the catheter. Afterward, the catheter remained in the superior mesenteric artery in order to continue the thrombolysis with urokinase (0.75-1.5 MIU/d) and sodium heparin (30-200 mg/d) for 3-11 d. All treatments during and after indwelling catheters and review were the same as those performed in the TIPS method.

Thrombolysis through the radial artery to superior mesenteric artery catheterization

The left radial artery in 4 patients was punctured with a radial puncture system (COOK Corp, USA) using a modified Seldinger's method. A 5F artery sheath (COOK Corp, USA) was inserted and then an extended (120 cm) 5F Cobra Catheter (Terumo Corp. Japan) was introduced for celiac artery, superior mesenteric artery and indirect PV-SMV angiography. All other procedures were the same as those through the femoral artery to the superior mesenteric artery cannulation thrombolysis methods.

RESULTS

Direct PV-SMV thrombolysis was performed in 32 pa-

tients, taking 80-180 min each. In 22 of the 32 patients, direct PV-SMV angiography showed extensive thrombosis in the PV and SMV with contrast agent remaining in the vessels and without lateral branch angiogenesis (Figures 1A, 2B, 3C). Ten patients had thrombosis mainly in the PV, SMV and splenic vein. After thrombolysis, repeated angiography showed normal PV-SMV systems in 26 patients (Figure 1B) while thrombosis partially remained in the PV-SMV in 6 patients. Symptoms such as abdominal pain, fullness, and diarrhea disappeared or were significantly relieved. Hospital stays lasted from 2 wk to 2 mo. One patient whose thrombosis was secondary to duodenal surgical repair had PV-SMV reperfusion and symptomatic improvement but died because of an abdominal abscess and multiple organ failure. In 3 patients with interventional treatment, thrombosis recurred (at 1, 3 and 4 mo, respectively) after the treatment. For these 3 patients, indirect PV-SMV thrombolysis was performed again and repeated angiography showed increased lateral branch angiogenesis in the PV-SMV branches. Their symptoms were improved. All other patients had good PV-SMV circulation without abnormal symptoms. Ultrasound and CT scans did not show signs of recurrence.

In 14 patients treated with indirect PV-SMV thrombolysis, one patient with acute thrombosis and another one with subacute thrombosis had partial recovery of PV-SMV flow, partial thrombolysis, and complete symptomatic relief (Figure 2C). Eleven patients had intact thrombosis in the PV-SMV and obvious lateral branch angiogenesis. Their symptoms were dramatically improved with only slight abdominal pain and fullness, and less diarrhea remaining (Figure 3D). One patient with acute thrombosis had no improvement in symptoms 3 d after continuous thrombolysis and developed exudation around the remaining sheath. Ileum segmental necrosis was found on laparotomy 2 d after the catheter was withdrawn. Bowel resection was performed and treatment was continued with an anticoagulant treatment. Six patients with esophageal-gastric varices had obvious improvement confirmed by gastroscop examination after treatment. During follow-up, one patient had upper gastrointestinal bleeding 22 mo after the interventional treatment and was treated with endoscopic sclerotherapy (EIS). Other patients did not develop symptoms of PV-SMV thrombosis again.

DISCUSSION

PV-SMV thrombosis has a concealed onset without any specific symptoms and signs. The diagnosis is therefore, easily delayed. If the following conditions occur, the possibility of PV-SMV thrombosis should be considered in order to achieve early diagnosis and treatment. (1) unexplained abdominal pain, abdominal distension, especially with nausea, vomiting, and bloody stool; (2) intractable ascites; (3) unexplained bloody ascites; (4) unexplained portal hypertension; (5) unexplained upper gastrointestinal bleeding or progressive spleen en-

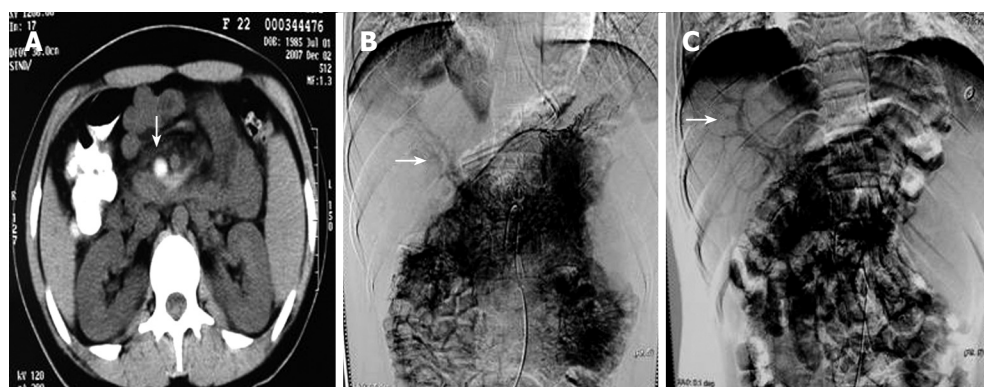


Figure 2 The figure shows a female, 22 year old patient who had abdominal pain for 17 d. A: A CT scan showed a high density of superior mesenteric vein thrombosis (characteristic of subacute thrombosis) (arrow); B: A Cobra catheter was inserted via the femoral artery into the superior mesenteric artery. Indirect angiography showed extensive thrombosis in the PV-SMV. Angiographic contrast agent remained (arrow); C: Indwelling catheters for 8 d. Angiography showed partial blood reperfusion in the PV-SMV. PV branches in the liver were intact (arrow). Symptoms were relieved.



Figure 3 A 24 year old male with abdominal pain for 8 d. A: The CT scan showed a low density SMV thrombosis (arrow); B: An enhanced CT scan still showed a low density of thrombosis (arrow). This fulfilled the symptoms and signs of acute thrombosis; C: An extended Cobra catheter was introduced into the superior mesenteric artery via the radial artery. The indirect angiography shows extensive thrombosis in the PV-SMV without lateral branch angiogenesis (arrow); D: Indwelling catheters for 10 d. The indirect angiography showed that the lateral vessels of the PV-SMV had significantly increased (arrow). Symptoms were relieved.

largement without apparent splenic hyperfunction; (6) unexplained paralytic intestinal obstruction, necrosis or peritonitis, *etc*^[8,9]. The diagnosis of PV-SMV thrombosis relies on imaging. Color Doppler ultrasound is very simple, noninvasive, and has a high negative predictive value. It should be chosen first. If a positive result is found, CT or MRI scans should be considered for further investigation. Accurate judgment of the PV-SMV thrombosis duration - acute, subacute, or chronic - is extremely important for disease management^[10]. According to our experience, thrombosis shown in a CT scan has a low density during the acute period (within 1 wk of the onset of the disease) (Figure 3A). It has a high density during the subacute period (1-3 wk after disease onset) with a CT value of 5-15 HU which is higher than values for the abdominal aorta and inferior vena cava (the so-called CT scan mesenteric vein angiographic phenomenon, an important piece of diagnostic evidence). It has a low density during the chronic period (> 3 wk) and is accompanied by lateral branch angiogenesis. The density of the thrombosis is not increased with contrast (Figure 3B)^[11]. In the MRI scan, PV-SMV thrombosis is shown as a T1WI low signal and a T2WI high signal during the acute period. During the sub-acute period, both T1WI

and T2WI signals are high. During the chronic period, T1WI gives mixed signals and T2WI gives low signals. After Gd-DTPA injection, the signal for thrombosis is not increased^[12].

Traditional PV-SMV thrombolysis treatment includes conservative internal treatment and surgical treatment. Medical treatments include thrombolysis and anticoagulation, and others, which can improve symptoms in some patients. But conservative treatment cannot directly remove the obstruction due to the thrombosis. Therefore, its efficiency is very limited and the mortality rate due to gastric bleeding is high^[13]. The application of surgical treatment is limited by tissue damage and additional complications^[14-17]. With the development of interventional radiology, minimally invasive technology has become one of the predominant means of treating acute-subacute PV-SMV thrombosis without obvious intestinal necrosis, perforation, and peritonitis^[18-26]. The method includes direct and indirect PV-SMV thrombolysis^[27]. In our study, 46 patients with acute-subacute PV-SMV thrombosis were treated interventionaly. The thrombolysis was effective without severe complication.

Our study showed that the effect of direct thrombolysis is better than indirect treatment. Injecting throm-

bolytic agents directly into a PV-SMV thrombus can dramatically increase the effect of thrombolysis, reduce the dose of thrombolytic agent, and reduce the complication of bleeding. Using a mechanical method such as aspirating and mashing to eliminate the thrombi, balloon expanding, stent implantation, etc. can result in reperfusion within a short time and recover blood circulation^[28]. However, when the disease duration is too long (> 2 wk) or angiography shows some lateral branch angiogenesis around the main vessels, there is no indication for TIPS and surgical treatment; or, if the treatment *via* TIPS fails, indirect PV-SMV thrombolysis is still an option^[24]. Evaluation of the efficacy of indirect PV-SMV thrombolysis should rely not only on reperfusion of the main vessels. The improvement in clinical symptoms and lateral branch angiogenesis with treatment are also important indications of efficacy. We found that indirect PV-SMV thrombolysis is simple and easy. When direct thrombolysis is difficult to perform, indirect thrombolysis can dissolve some thrombi, promote lateral branch angiogenesis and relieve symptoms for acute-subacute patients. With thrombolysis through the radial artery, one can achieve superior mesenteric artery indwelling catheters. Patients with such indwelling catheters do not need to rest in bed. Complications such as bleeding at the puncture point and infection are significantly reduced. Therefore, the procedure does not introduce any inconveniences into the patient's daily life and is easily accepted by patients.

Regarding the choice of pathways for direct PV-SMV thrombolysis, the way through TIPS does not pass through the intraperitoneal cavity. Hence, it is suitable for patients with existing ascites, coagulative dysfunction and catheters that have been indwelling for a long time^[22]. It has a specific advantage in prevention of bleeding, not only avoiding hepatic surface injury, but also reducing bleeding due to thrombosis being aspirated through the big vessel sheath (> 7F) because the cannula is inside the liver. Moreover, cannulation through TIPS can also divert portal blood flow and effectively relieve portal hypertension^[29,30]. Therefore, it is very suitable for patients requiring a portosystemic shunt for portal hypertension and embolism for esophageal-gastric varices. The disadvantages of TIPS are its complexity and difficulties in performing it^[31]. The procedure for percutaneous transhepatic portal vein cannulation is simpler, easier and cheaper than TIPS^[25,32]. It is suitable for patients without ascites and coagulative dysfunction^[33]. Recently, this procedure has been improved in several ways. Ultrasound was used to guide a fine needle to the puncture. A steel coil and a gelatin sponge were used to fill the puncture channel to reduce intraperitoneal haemorrhage^[25,34]. This method can be an alternative for cases of unsuccessful TIPS or cases that are unsuitable for TIPS but that require direct PV-SMV thrombolysis.

During the time that the catheter is indwelling, determining safe and effective doses of urokinase and heparin is fundamental for the success of the treatment. Our study suggests doses for urokinase (0.5-1.5 MIU/d) and heparin sodium (30-200 mg/d). Urokinase should be rapidly injected through the catheter within half an

hour, twice a day. Heparin sodium can be admitted *via* peripheral veins when urokinase treatment is performed *via* the catheter. It must be administered *via* the catheter to the thrombosis in the time gap between urokinase treatments. Patients with PV-SMV thrombosis usually have complications of chronic hepatic disease, clotting factor insufficiency, and generally low coagulation conditions but high coagulation conditions in the portal vein. The direct injection of urokinase and heparin into the PV is more effective. Clopidogrel or enterically-coated aspirin are administered for patients with platelet counts more than $300 \times 10^9/L$. In our study, one patient had a platelet count of $600-970 \times 10^9/L$ while they had indwelling catheters after splenectomy and was treated with hydroxycarbamide to inhibit platelet formation.

Urokinase is a plasminogen activator. Its half-life is short (15-20 min). Quickly administering urokinase can instantly lead to its penetration into the thrombosis and cause thrombolysis. In our study, urokinase was injected within 30 min. If the injection is too slow, the effect of thrombolysis becomes weak. Urokinase can also cause degeneration of some clotting factors such as fibrinogen (FIB). FIB is an important clotting factor and a reflective index for activation of the fibrinolysis system. It is also a fundamental factor for plasma viscosity and platelet aggregation. The elevation of plasma FIB can promote blood coagulation and form clots. Because the half-life of urokinase is very short, FIB will increase after 24 h of urokinase administration and return to previous levels after 48 h. If it is not combined with other anticoagulation treatments, it will increase the incidence of vascular thrombosis obstruction after thrombolysis. Therefore, anticoagulation treatment during thrombolysis is necessary.

Heparin sodium affects blood clotting by inhibiting synthesis of fibrous protein factors and the extension of existing clots within the blood. In our study, all patients had anticoagulation treatment with a combination of urokinase and heparin sodium. The prevalence of hemorrhage is high (23%-28%)^[21,35-38]. Therefore, an emergency treatment including ECG monitoring and blood pressure control for bleeding must be prepared. All indexes for coagulation and anticoagulation should be monitored. Presently, most researchers suggest that maintaining TT at 1.5-2.5 times the normal range and APTT at 2-2.5 times the normal range can not only achieve the best treatment efficacy, but also avoid severe bleeding. In our study, only three patients had exudation around the catheter sheath. Their symptoms were relieved with a compression bandage without any severe organic bleeding. This is related to understanding the time window of urokinase and heparin, and monitoring the dose for both medications according to all factors involved in coagulation and anticoagulation.

COMMENTS

Background

With the development of imaging technology, the rate of diagnosis of portal and superior mesenteric vein thrombosis has gradually increased. Thrombosis of

portal vein and superior mesenteric vein thrombosis (PV-SMV) is a severe disease. The consequences of these thromboses can be severe, including mesenteric ischemia and variceal bleeding, with high mortality rate. There are no uniform protocols for the effective treatment of PV-SMV thrombosis. Conservative medical treatment is often unsatisfactory. Surgical treatment is accompanied by more tissue damage, more complications, and a high recurrence rate.

Research frontiers

The treatment of symptomatic acute thrombosis of the PV and SMV is controversial due to unsatisfactory results obtained in some cases with medical treatment, as well as the difficulty in performing surgical procedures in some cases. Recently, interventional and minimally invasive techniques were introduced to treat PV-SMV thrombosis.

Innovations and breakthroughs

The authors summarize the interventional treatment of 46 patients with symptomatic acute-subacute PV-SMV thrombosis, which demonstrated the feasibility of this method in the management of this challenging illness. Compared to conservative medical and surgical treatment, interventional treatment has the least tissue damage, complications, invasive and a high success rate.

Applications

Interventional endovascular thrombectomy with direct or indirect thrombolysis can offer a non-surgical alternative for the treatment of symptomatic acute-subacute PV-SMV thrombosis. This technique can be performed in patients who do not present with bowel ischemia and infarction, or who are not at risk for bleeding, and have persistent symptoms or worsening of symptoms despite anticoagulation.

Terminology

PV-SMV: Portal vein and superior mesenteric vein thrombosis. TIPS: Transjugular intrahepatic portosystemic shunts. TT: Thrombin time. APTT: Activated partial thromboplastin time. INR: International normalized ratio.

Peer review

This study summarizes the interventional treatment of 46 patients with symptomatic acute-subacute PV-SMV thrombosis.

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S- Editor Tian L L- Editor O'Neill M E- Editor Ma WH

Expression of γ -synuclein in colorectal cancer tissues and its role on colorectal cancer cell line HCT116

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Received: August 7, 2009 Revised: September 9, 2009

Accepted: September 15, 2009

Published online: October 28, 2009

Abstract

AIM: To investigate the expression pattern of γ -synuclein in colorectal cancer (CRC) tissues, and to study the effects of γ -synuclein on CRC cell line HCT116 biological features *in vitro*.

METHODS: The expression pattern of γ -synuclein was determined in 54 CRC tissues and 30 tumor-matched nonneoplastic adjacent tissues (NNAT) 5 cm away from the tumor *via* real-time quantitative reverse transcription PCR (RT-PCR) and immunohistochemistry. The relationship between γ -synuclein protein expression and clinicopathological factors of CRC tissues was analyzed. Three small interfering RNA (siRNA) targeting γ -synuclein mRNA plasmids were constructed and transfected into the CRC cell line HCT116. The stable

cell lines were selected with G-418 for 28 d, and the biological features of these cells were examined by cell growth curve, soft agar assay, and cell migration and invasion assays *in vitro*.

RESULTS: The expression of γ -synuclein mRNA and protein was much higher in CRC tissue samples than in NNAT samples ($P = 0.02$, $P = 0.036$). There was a significant correlation between the γ -synuclein protein expression and clinical stage and lymph node involvement of CRC ($P = 0.02$, $P = 0.033$). In functional analysis we found that down-regulation of γ -synuclein expression in HCT116 cells could inhibit the growth, colony formation rate, and migration and invasion ability of HCT116 cells.

CONCLUSION: Increased expression of γ -synuclein in CRC tissues and the biological effects of reduced γ -synuclein expression on HCT116 cells suggest that γ -synuclein may play a positive role in the progression of CRC.

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Key words: γ -synuclein; Colorectal cancer; Expression; Cell proliferation; Colony formation; Migration; Invasion

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Ye Q, Feng B, Peng YF, Chen XH, Cai Q, Yu BQ, Li LH, Qiu MY, Liu BY, Zheng MH. Expression of γ -synuclein in colorectal cancer tissues and its role on colorectal cancer cell line HCT116. *World J Gastroenterol* 2009; 15(40): 5035-5043 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5035.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5035>

INTRODUCTION

The synucleins are a family of small, soluble, highly conserved neuronal proteins that consist of α -, β -, and γ -synuclein. They are a natively unfolded group of proteins that are characterized by 5-6 repeats of the amino acid motif (KTKGV), constituting most of the N-terminal half of the proteins^[1-3]. The synucleins

have attracted considerable attention due to their involvement in neurodegenerative diseases. α -synuclein is the major component of Lewy bodies in Parkinson's disease and has also been identified as the non-amyloid component of amyloid deposition, the hallmark of Alzheimer's disease^[4,5]. β - and γ -synuclein are assumed to have a neuroprotective role by inhibiting α -synuclein aggregation and toxicity^[6,7].

γ -synuclein gene [also referred to as breast carcinoma specific gene 1 (BCSG1)] initially was cloned from infiltrating breast carcinoma cells by using the expressed sequence tag-based differential cDNA sequencing approach^[8]. γ -synuclein maps to chromosome region 10q23, and is composed of five exons and transcribed into an mRNA of about 1 kb, coding 127 amino acids^[9]. γ -synuclein expression is usually highly tissue-specific and restricted to brain tissue and presynaptic terminals^[2]. However, the tissue-specificity appears to be lost, and γ -synuclein is abnormally expressed in a high percentage of advanced breast and ovarian cancers, but not in normal or benign tissues^[10]. Furthermore, overexpression of γ -synuclein can stimulate proliferation, and induce invasion and metastasis of breast cancer cells^[11]. γ -synuclein has also been shown to compromise normal mitotic checkpoint controls, resulting in multinucleation as well as faster breast cancer cell growth^[12,13]. Liu *et al*^[14] found that γ -synuclein protein was also abnormally expressed in a high percentage of tumor tissues of other cancer types, including liver, gastric, lung, prostate, cervical *etc.*, but rarely expressed in tumor-matched nonneoplastic adjacent tissues (NNAT). However, Zhou *et al*^[15] had an opposite conclusion in esophagus cancer, in which low expression levels of γ -synuclein in human esophageal squamous cell carcinoma (ESCC) and biological effects of γ -synuclein overexpression on ESCC 9706 cells suggested that γ -synuclein might play a role as a negative regulator in the development of human ESCC. Therefore, further study in cancer tissues and cell line culture is needed to understand the roles of γ -synuclein in the development of other human neoplastic diseases.

Recent reports demonstrate that colorectal cancer (CRC) has been the third most common malignancy and the third leading cause of cancer-related deaths worldwide^[16]. The conventional therapies involving surgery and adjuvant therapy seem to give rise to improvements in progression-free and overall survival; nevertheless about 50% of patients die within 5 years owing to metastasis or recurrent disease. Patients with early stage CRC have an estimated 5 year survival rate of 91%, compared to only 6% for those with later stage disease. Early detection remains the most important factor in improving long-term survival. Furthermore, tumor invasion and regional lymph node metastasis are important factors for determining CRC prognosis^[17-19].

To further determine whether aberrant expression of γ -synuclein is involved in the development of CRC, and identify a new biomarker or a potential target for diagnosis and treatment, we examined expression patterns of γ -synuclein in CRC tissues, analyzed the

relationship between γ -synuclein protein expression and clinicopathological factors of CRC, and then studied the effects of γ -synuclein down-regulation on colorectal cancer cell line HCT116 biological features *in vitro*.

MATERIALS AND METHODS

Tissue samples and cell lines

Fifty-four CRC samples and 30 NNAT samples 5 cm away from the tumor were obtained from patients undergoing CRC surgery between January 2005 and October 2008 at the Department of General Surgery, Ruijin Hospital, Shanghai, China. After washing with RNase-free 9 mL/L NaCl to remove blood after surgery, one half of each sample was snap-frozen in liquid nitrogen immediately and stored at -80°C for RNA extraction, and the other half was fixed in 40 g/L formalin for histological assessment. For tumor samples, non-tumor portions were trimmed off from the frozen tumor blocks and the selected areas had more than 80% tumor cells as shown by histological assessment. Tumors were staged using the TNM and World Health Organization classification systems. The ethics committee at Ruijin Hospital approved the use of these tissues for research purposes. The colorectal cancer cell line HCT116 (ATCC No. CCL-247) was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies Inc, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO, USA). The HCT116 cells were maintained in a humidified incubator at 37°C with 50 mL/L CO₂, fed every 3 d with complete medium, and subcultured when confluence was reached.

Total RNA extraction and real-time quantitative reverse transcription PCR (RT-PCR) of γ -synuclein mRNA

Cultured cells were washed twice with phosphate-buffered saline (PBS) and harvested, and tissues were ground into fine powder in liquid nitrogen before extraction of RNA. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis from 1 μ g of RNA was performed with a reverse transcription system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time quantitative PCR was carried out in 96-well polypropylene microplates on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBR Green Realtime PCR Master Mix (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions. Amplification was carried out with the following profile: 1 cycle at 95°C for 1 min, and 40 cycles each at 95°C for 15 s, 59°C for 15 s, 72°C for 45 s. All PCR reactions were performed in triplicate wells. Specificity of the resulting PCR products was confirmed by melting curves. H₂O was used as a negative control. Data were analyzed by using the comparative Ct (Δ Ct) method and the amount of γ -synuclein relative to GAPDH was expressed as 10000 \times 2^{-(Δ Ct)}. Table 1 provides the sequences of the primers used in these studies.

Table 1 Sequences of γ -synuclein gene-specific primers

Primer	Sequence (5'-3')	Product size (bp)
γ -synuclein-5'	GGAGGACTTGAGGCCATCTG	73 (339 to 411)
γ -synuclein-3'	CTCCTCTGCCACTTCCTCTTTC	
GAPDH-5'	GGACCTGACCTGCCGTCTAG	100 (831 to 930)
GAPDH-3'	GTAGCCCAGGATGCCCTTGA	

Immunohistochemistry (IHC) analysis

Unstained 4 mm sections were cut from the tissue paraffin-embedded block and deparaffinized in xylene, and the slides were bathed in 0.01 mol/L sodium citrate and heated in a microwave oven for 12 min. The sections were incubated with mouse anti- γ -synuclein monoclonal antibody (SantaCruz, CA, USA) at a dilution of 1:100 and kept at 4°C overnight. Negative control slides were treated with non-immunized mouse immunoglobulin fraction under equivalent conditions. For the secondary developing reagents, a labeled streptavidin-biotin kit (DAKO, CA, USA) was used. Slides were developed with diaminobenzaminidine and counterstained with hematoxylin. Positive cases were defined by the presence of intracellular staining with red/brown color in epithelial cells. Negative cases were defined by the absence of specific intracellular staining as seen in negative controls. Samples were evaluated under light microscopy independently by two pathologists without prior knowledge of the patients' clinical data.

Plasmid construction

The pGCsi-U6/neo/GFP plasmid (Shanghai GeneChem., Ltd, Shanghai, China), a siRNA expression vector containing a green fluorescent protein gene (GFP) under a separate promoter for tracking the transfection efficiency, was used for the cloning of small synthetic oligonucleotides that encode two complementary sequences of 19 nucleotides separated by a short spacer region of 9 nucleotides. Three sequences (as shown in Table 2) unique to the coding region of γ -synuclein were designed and inserted between the *Bam*H I and *Hind*III sites of the pGCsi-U6/neo/GFP plasmid. The positive clones were confirmed by sequencing.

siRNA transfection and selection of HCT116 stable transfectants

One day before transfection, HCT116 cells were plated in a six-well plate with 1×10^5 cells per well using culture medium without antibiotics. The cells were transfected with 3.0 μ g/well of pGCsi-U6/neo/GFP-vector and pGCsi-siRNA plasmids, respectively, using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells transfected with medium but lacking DNA served as controls. Fresh growth medium was replaced after 4 h of transfection. Cells were passaged at a 1:10 dilution at 24 h after transfection and cultured in medium supplemented with G418 (Promega, Madison, WI) at 1000 μ g/mL for 4 wk. Stably transfected clones were picked and maintained in medium containing 400 μ g/mL G418 for further study.

Western blotting analysis

Cells were harvested and lysed with mammalian protein extraction reagent (Pierce Rockford, IL, USA). Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Rockford, IL, USA). Samples containing 50 μ g of protein were mixed with $2 \times$ sodium dodecyl sulfate (SDS) gel-loading buffer (100 mmol/L Tris-CL, 200 mmol/L dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), boiled for 5 min, loaded onto each lane of 15% acrylamide gel in a minigel apparatus (Bio-Rad, Richmond, CA, USA), and separated by SDS-PAGE. The separated proteins were electrophoretically transferred to a Sequi-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After being incubated with mouse anti- γ -synuclein monoclonal antibody (SantaCruz, CA, USA) (1:500), and goat anti-mouse IgG-AP antibody (SantaCruz, CA, USA) (1:5000) respectively, immune complexes were detected using BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma, St. Louis, MO). GAPDH served as a loading control.

Cell proliferation analysis

Cells were seeded onto 96-well plates at a density of 2×10^3 cells per well in 100 μ L medium containing 10% FBS. The number of viable cells was determined daily with WST-8 cytotoxicity assay using the Cell Counting Kit-8 (Dojindo, Japan). Briefly, 10 μ L of the CCK-8 solution was added to each well of the microplate, and the absorbance at 490 nm was measured by a microplate reader (μ Quant, Bio-Tek, USA) after 4 h incubation.

Soft agar colony formation assay

Cells (1×10^3) were trypsinized to a single-cell suspension and then plated in triplicate onto six-well plates in complete culture medium containing 0.3% agar on top of 0.6% agar in the same medium. Cultures were maintained at 37°C in the 50 mL/L CO₂ incubator for 15 d. The colonies were fixed with 70% ethanol, and stained with 0.2% crystal violet. The colonies containing at least 50 cells were counted. Colony formation rates were calculated as the number of colonies relative to that of cells initially plated in a well (1×10^3), and expressed as mean \pm SD.

Cell migration and invasion assay

Boyden chambers with 8 μ m polycarbonate membranes in 24-well dishes (Nucleopore, Pleasanton, CA) were used for migration assay, and chambers coated with 4 mg/mL growth factor reduced Matrigel (50 μ g; Collaborative Biomedical, Becton Dickinson Labware) were used for the invasion assay. Cells (1×10^5) were resuspended in serum-free DMEM and added to the upper chamber in triplicate. Consecutively, DMEM with 10% FBS was added to the lower chamber. Chambers were incubated at 37°C in the 50 mL/L CO₂ incubator for 24 h. After incubation, the chambers were fixed with 70% ethanol, and stained with 0.2% crystal violet. Cells on the surface of the upper chamber were removed by

Table 2 Sequences of small synthetic oligonucleotides unique to the coding region of γ -synuclein

Oligonucleotides	Sequence (5'-3')
siRNA1: Target sequence	AAGACCAAGGAGAATGTTGTA
Sense strand	5'-GATCCGACCAAGGAGAATGTTGTATTCAAGAGATACAACATTCTCCTTGGTCTTTTTTGGAAA-3'
Antisense strand	5'-AGCTTTTCCAAAAAGACCAAGGAGAATGTTGTATCTCTTGAATACAACATTCTCCTTGGTCG-3'
siRNA2: Target sequence	AAGGAGAATGTTGTACAGAGC
Sense strand	5'-GATCCGAGAATGTTGTACAGAGCTTCAAGAGAGCTCTGTACAACATTCTCCTTTTTTGGAAA-3'
Antisense strand	5'-AGCTTTTCCAAAAAGGAGAATGTTGTACAGAGCTCTCTTGAAGCTCTGTACAACATTCTCCG-3'
siRNA3: Target sequence	AATGTTGTACAGAGCGTGACC
Sense strand	5'-GATCCGTGTTGTACAGAGCGTGACCTTCAAGAGAGGTCACGCTCTGTACAACATTTTTTGGAAA-3'
Antisense strand	5'-AGCTTTTCCAAAAATGTTGTACAGAGCGTGACCTCTCTTGAAGGTCACGCTCTGTACAACACG-3'

Table 3 The expression of γ -synuclein in CRC and NNAT

Group	n	γ -synuclein mRNA expression		P value	γ -synuclein protein expression		P value
		Median (Range)	mean \pm SD		Positive	Negative	
CRC	54	11.06 (38.24)	14.15 \pm 10.14	0.02 ¹	26	28	0.036 ²
NNAT	30	6.11 (29.4)	9.46 \pm 8.47		7	23	

¹Calculated by the Mann-Whitney *U*-test; ²Calculated by the Fisher's exact test. CRC: Colorectal cancer; NNAT: Nonneoplastic adjacent tissues.

Table 4 Correlation between γ -synuclein protein expression and clinicopathological factors of colorectal cancer patients

Variable	n	γ -synuclein protein expression		P value ¹
		Positive	Negative	
Age				
≥ 60	28	14	14	0.793
< 60	26	12	14	
Sex				
Male	32	14	18	0.580
Female	22	12	10	
Histological types				
Differentiated	47	21	26	0.243
Undifferentiated	7	5	2	
Stage				
I	13	2	11	0.020
II	11	5	6	
III	24	14	10	
IV	6	5	1	
Lymph node invasion				
Positive	29	18	11	0.033
Negative	25	8	17	
Distant metastasis				
Positive	6	5	1	0.095
Negative	48	21	27	

¹Statistical significance was determined with Fisher's exact test.

swiping with cotton swabs. The amount of migration and invasion cells in the lower chamber was determined under light microscopy. The data are means \pm SD of counting ten random fields of vision.

Statistical analysis

Statistical analyses were performed using SPSS11.0 software (Shanghai jiaotong University School of Medicine, Shanghai, China). Mann-Whitney *U*-test was used to analyze γ -synuclein mRNA expression in paired CRC and NNAT samples. The Fisher's exact test was used to test the significance of the difference in frequency of

γ -synuclein protein expression between CRC and NNAT samples, and to assess the relationship between the protein expression and clinicopathological characteristics of CRC. Two-way analysis of variance (ANOVA) was performed to detect the effects of γ -synuclein knockdown on cell proliferation, soft agar colony formation, cell migration and invasion, and Student-Newman-Keuls test was used to detect the difference between any two groups. *P* < 0.05 was selected as the statistically significant value.

RESULTS

Examination of γ -synuclein mRNA and protein expression in CRC and NNAT samples

γ -synuclein mRNA expression in 54 CRC and 30 NNAT samples was examined using Q-RT-PCR. Table 3 shows the results of Q-RT-PCR. γ -synuclein mRNA expression in CRC samples ranged from 1.12 to 39.36 with a median value of 11.06, while in matched NNAT samples it ranged from 0.81 to 30.21 with a median value of 6.11. The γ -synuclein mRNA expression levels in CRC samples were significantly higher than those in NNAT samples (*P* = 0.02).

The expression and subcellular localization of γ -synuclein protein were evaluated *via* IHC in 54 CRC and 30 NNAT samples. NNAT sections showed either no protein expression (*n* = 23) or relatively weak protein expression (*n* = 7) in the cytoplasm of epithelium cells (Figure 1A). Conversely, the immunoreactive patterns of γ -synuclein were predominantly positively identified in the cytoplasm, sometimes in the nucleus of cancer cells (Figure 1B) with a relatively high frequency of 48.1% (Table 3, *P* = 0.036).

In the analysis of γ -synuclein protein expression in CRC tissues and various CRC patients' clinicopathologic variables, the results clearly showed a close association of γ -synuclein staining with clinical stage and lymph

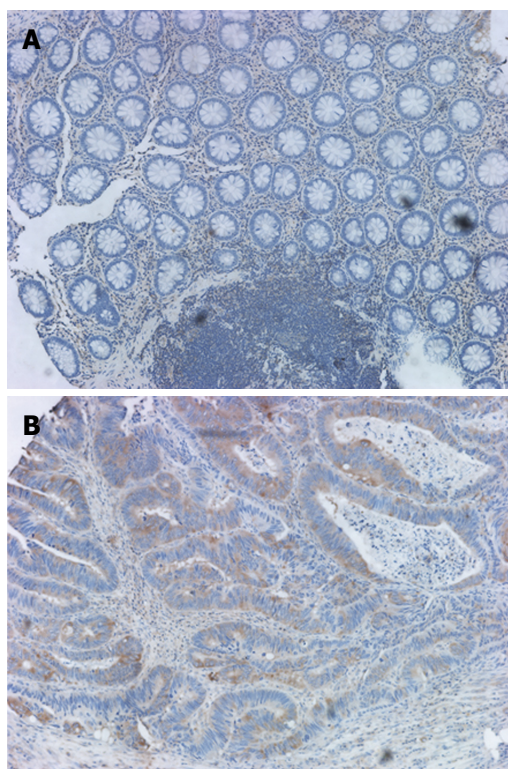


Figure 1 γ -synuclein protein expression detected by IHC. A: Negative γ -synuclein immunostaining in NNAT; B: Positive γ -synuclein immunostaining in the cytoplasm of CRC cells (original magnification $\times 200$). IHC: Immunohistochemistry; NNAT: Nonneoplastic adjacent tissues; CRC: Colorectal cancer.

node involvement (Table 4). The frequency of positive γ -synuclein staining was much higher in later stage tumors than in earlier stage tumors ($P = 0.02$), and was much higher in lymph node-positive tumors than in lymph node-negative ones ($P = 0.033$). However, there was no significant correlation between the γ -synuclein protein expression and other clinicopathologic characteristics.

Identification of the effective siRNA target sequence

We first investigated three recombinant γ -synuclein-specific siRNA plasmids, pGCsi-siRNA1, pGCsi-siRNA2, and pGCsi-siRNA3. These γ -synuclein-specific siRNA plasmids and pGCsi-U6/neo/GFP-vectors were transfected into HCT116 cells. After 24 h, these cells were examined for γ -synuclein expression by Q-RT-PCR. As shown in Figure 2A, γ -synuclein levels were different in the transfected HCT116 cells containing siRNA1, siRNA2, siRNA3 and the vector. There were no significant changes of γ -synuclein mRNA expression in pooled HCT116/vector, HCT116/siRNA1, and HCT116/siRNA3 cells. However, in HCT116/siRNA2 cells, γ -synuclein mRNA levels were significantly low, compared with parental HCT116 cells and HCT116/vector cells. Then stable transfected clones of HCT116/siRNA2 cells were selected with G418. After 4 wk of the selection, stable transfected clones were established (Figure 2B). These clones were examined for γ -synuclein expression by western blotting, the result of which suggested that pGCsi-siRNA2 plasmid could specifically

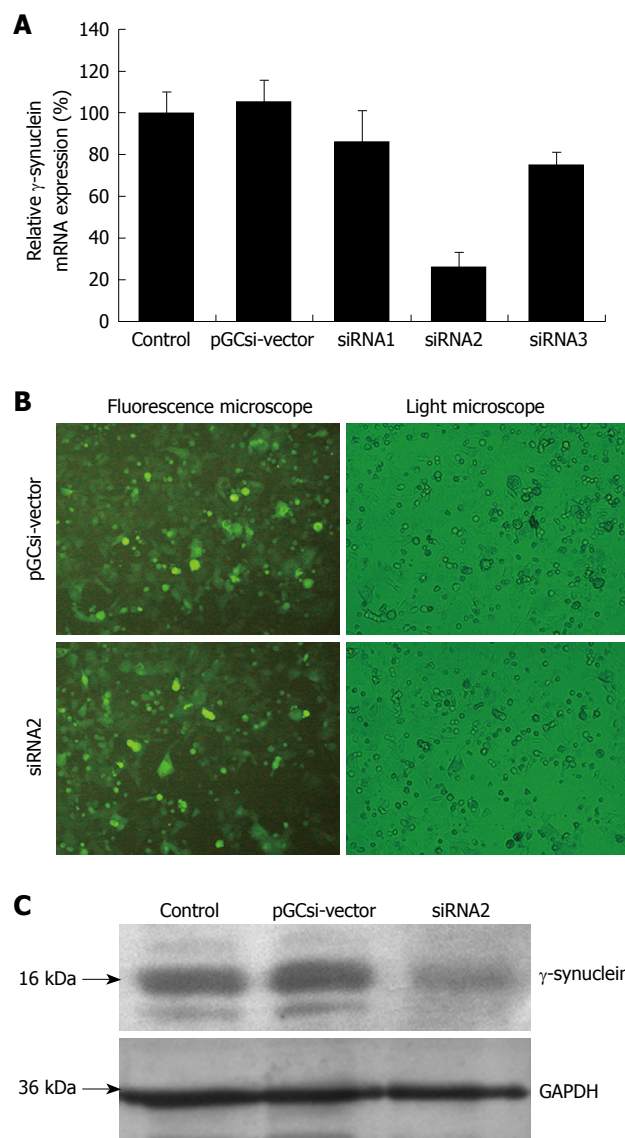


Figure 2 The siRNA plasmid can specifically knock down γ -synuclein expression in the HCT116 cells. A: γ -synuclein mRNA expression detected by Q-RT-PCR after transient transfection. Values were γ -synuclein/GAPDH expression of one group cells relative to that of parental HCT116 cells; B: The vision of HCT116 stable transfectants in fluorescence microscopy and light microscopy; C: γ -synuclein protein expression detected by western blotting after selection of stable transfectants. Control: Parental HCT116 cells; pGCsi-vector: HCT116/vector cells; siRNA1 (2,3): HCT116/siRNA1 (2,3) cells.

knock down γ -synuclein protein expression in the stable transfected HCT116 cells (Figure 2C).

Inhibition of cell proliferation and colony formation by γ -synuclein knockdown

In vitro cell proliferation tests, two clones derived from stable transfectants with control vector, siRNA2 plasmids, and parental HCT116 cells were chosen for further study. As shown in Figure 3A, γ -synuclein knockdown suppressed cancer cell growth significantly in regular medium. The number of pooled HCT116/siRNA2 cells was significantly reduced by 48, 72, 96, and 120 h after plating, respectively, compared with the HCT116/vector and parental HCT116 cells ($P < 0.05$).

Subsequent soft agar colony formation assay was

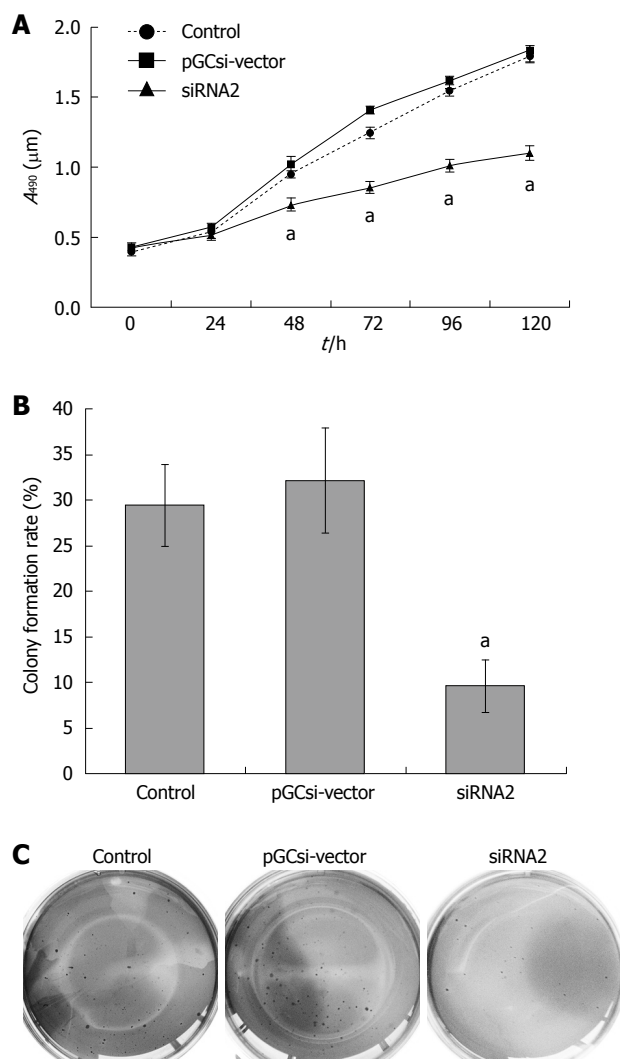


Figure 3 Inhibition of cell proliferation and colony formation by γ -synuclein knockdown. A: The role of γ -synuclein in regulating HCT116 cell proliferation was determined by CCK8 assay. Values were the mean \pm SD of absorbance at 490 nm for five independent experiments; ^a $P < 0.05$; B: The colony formation rates were analyzed by soft agar assay. Values were the mean \pm SD for three independent experiments; ^a $P < 0.05$; C: The colonies were stained with 0.2% crystal violet and photographed. Control: Parental HCT116 cells; pGCsi-vector: HCT116/vector cells; siRNA2: HCT116/siRNA2 cells.

done to evaluate the tumorigenicity of γ -synuclein down-regulated cells *in vitro*. Colony formation rates were $29.4\% \pm 4.5\%$, $32.1\% \pm 5.8\%$ and $9.6\% \pm 2.9\%$ in parental HCT116 cells, HCT116/vector and HCT116/siRNA2 cells (Figure 3B, $P < 0.05$). The size of colonies formed by HCT116/siRNA2 cells was much smaller than that of two control cells, and there were no significant differences between parental HCT116 cells and HCT116/vector cells colonies both in number and size (Figure 3C).

Inhibition of cell migration and invasion by γ -synuclein knockdown

The close correlation of γ -synuclein protein expression and CRC staging suggests that γ -synuclein might be involved in advanced stage tumor progression and metastasis. We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of γ -synuclein on cell migration and invasion

(Figure 4A). The results showed that the amount of migration HCT116/siRNA2 cells in the lower chamber was much less than that of parental HCT116 cells, and HCT116/vector cells (Figure 4B, $P < 0.05$). It was demonstrated that γ -synuclein down-expression led to suppression of cell motility in HCT116 cells. Similarly, we observed that γ -synuclein down-expression led to decreased cell invasion in HCT116 cells. HCT116/siRNA2 cells showed a significant decrease in the number of invasive cells compared to that of two control cells (Figure 4C, $P < 0.05$).

DISCUSSION

γ -synuclein belongs to the synuclein protein family, consisting of α -, β -, and γ -synuclein, which are abundantly expressed in nervous tissues^[9]. Although there has been a report that showed down-regulation of γ -synuclein in human ESCC, more studies support the statement of over-expression of γ -synuclein in more types of cancer^[14,15,20-22]. The loss of tissue-specificity raises questions about the involvement of γ -synuclein in the process of tumorigenesis and metastasis, and presents the possibility to use γ -synuclein as a potential target for early diagnosis and treatment. However, little is known about the expression and biological effects of γ -synuclein in CRC.

In the current study, we showed that γ -synuclein expression levels were higher in CRC tissues than those in matched NNAT. IHC analysis also confirmed that CRC tissues exhibited abundant γ -synuclein expression in the cytoplasm of cancer cells, in contrast to NNAT, which did not appear to exhibit γ -synuclein expression or exhibit faint expression.

To further investigate how γ -synuclein contributes to the biological behavior changes in CRC, we constructed specific γ -synuclein siRNA plasmids and established permanent transfected HCT116 cells to investigate the potential role of γ -synuclein in the progression of CRC. Consistent with our observation that the γ -synuclein expression levels were lower in NNAT than in CRC tissues, the cell growth and colony formation rate decreased in HCT116/siRNA2 cells with reduced expression of γ -synuclein, compared with parental HCT116 cells, and HCT116/vector cells, which gave evidence that γ -synuclein indeed had the ability to promote cell growth. Previous studies have demonstrated that ectopic expression of γ -synuclein increased breast cancer cell growth in anchorage dependent and independent conditions through interaction with BubR1, a mitotic checkpoint kinase, which led to inhibition of the mitotic checkpoint control^[12,13,23]. It has also been shown that γ -synuclein could constitutively activate ERK1/2, and increase ER- α transcriptional activity through an HSP-based multiprotein chaperone complex, which led to an increase in breast cancer or ovarian cell survival and proliferation^[24-28].

In addition to the effects on cell growth, γ -synuclein is associated with cell invasion and metastasis. In previous *in vitro* studies, retinoblastoma cell lines

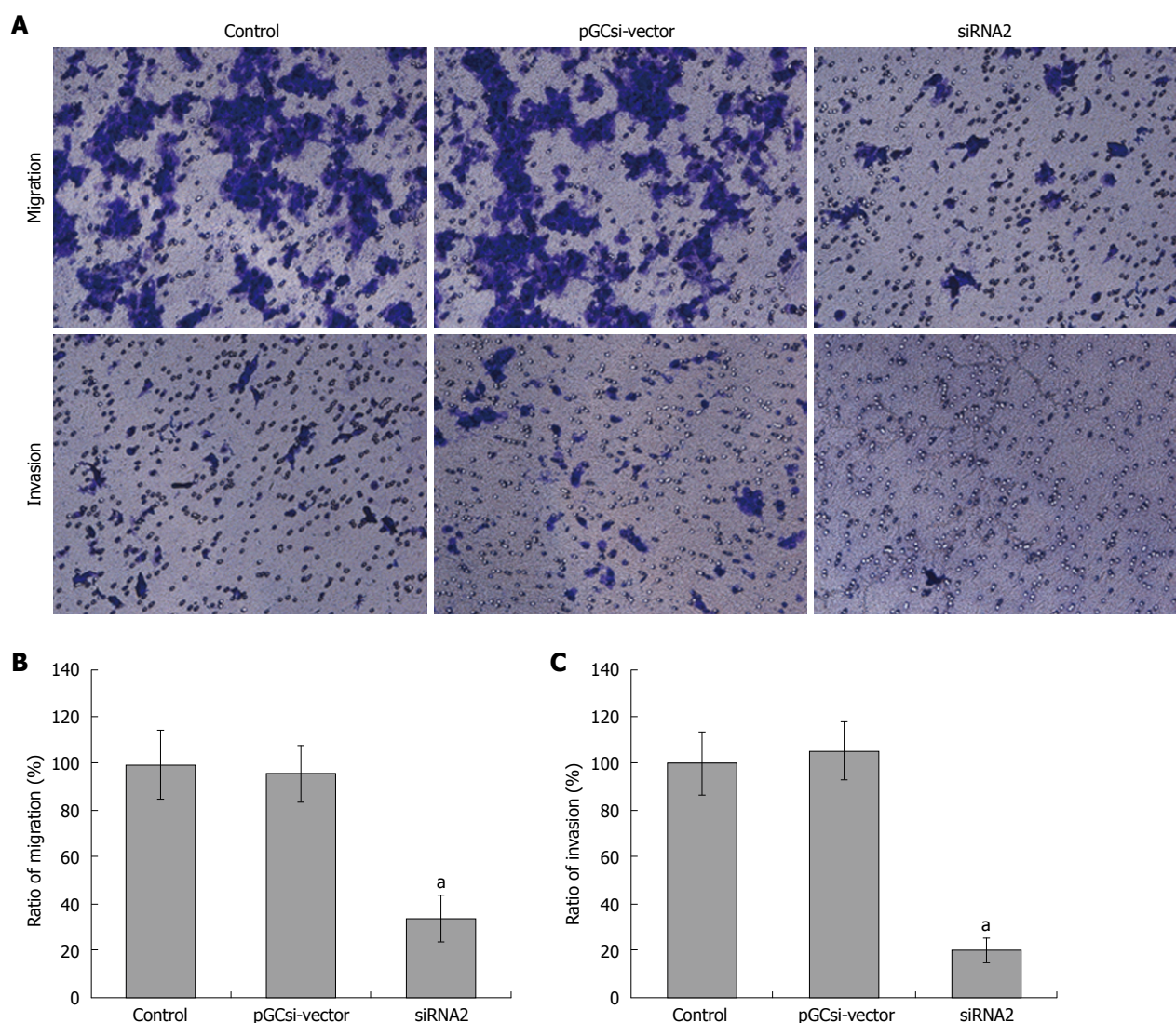


Figure 4 Inhibition of cell migration and invasion by γ -synuclein knockdown. A: Boyden chambers with 8 μ m polycarbonate membranes coated with or without 4 mg/mL growth factor-reduced Matrigel were used for migration or invasion assay. The chambers were stained with 0.2% crystal violet and analysed by photography, and the stained cells were migration or invasion cells in the lower chamber; B, C: The migration or invasion cells were counted in ten random fields of vision. Values were the number of cells relative to that of parental HCT116 cells, and expressed as mean \pm SD for three independent experiments; ^a $P < 0.05$. Control: Parental HCT116 cells; pGCSi-vector: HCT116/vector cells; siRNA2: HCT116/siRNA2 cells.

overexpressing γ -synuclein were shown to have higher MMP9 protein levels and activity, which were enhanced in cell motility and invasion^[29]. In *in vivo* studies, γ -synuclein was also shown to cause metastasis in nude mice on implanting γ -synuclein expressing MDA-MB 435 breast cancer cells in fat pads of these mice. IHC results showed mice given implants of γ -synuclein positive cells displayed an increase in tumor growth, and metastasis into axillary lymph nodes and lungs, compared with mice given control implants^[11]. In the current study, we presented the clinical evidence and experimental data to indicate that γ -synuclein played a key role in CRC invasion and metastasis. We analyzed the relationship between γ -synuclein protein expression in 54 CRC tissues and the clinicopathologic characteristics of patients with CRC, and found that the frequency of positive γ -synuclein staining was much higher in tumors with lymph node-positive or later stage than in lymph node-negative or earlier stage tumors ($P < 0.05$).

Our results also showed that there was a tendency for high γ -synuclein expression with metastasis. However, this difference is not significant ($P = 0.095$), possibly because of our relatively small sample size. Consistent with the clinical evidence, we also observed that reduced γ -synuclein expression led to decreased cell motility and invasion in HCT116 cells. All these results gave evidence that γ -synuclein may indeed function as key mediators of cancer cell growth and metastasis and will be a promising target for CRC treatment. Biological treatment targeting γ -synuclein has been studied in breast cancer, and Singh *et al.*^[30] have designed and characterized a γ -synuclein targeting peptide inhibitor, which associates with γ -synuclein and enhances sensitivity of breast cancer cells to antimicrotubule drugs.

In summary, we have shown that strong expression of γ -synuclein occurred in CRC tissues and correlated with the advancement of tumor stage and lymph node involvement. With a vector-based siRNA method, we

showed that stable down-regulation of γ -synuclein expression inhibited CRC cell growth, colony formation, motility and invasion. Therefore, γ -synuclein is likely to play an important role in the progression of CRC. Further study is needed to prove the value of γ -synuclein as a biomarker or molecular target for CRC diagnosis, prognosis evaluation and therapy. The following research may encompass: 1. Examination of γ -synuclein expression in serum or stool samples from patients with CRC; 2. Relationship between γ -synuclein expression and 5-year survival rate of CRC patient; 3. Delineation of the interaction between γ -synuclein and other proteins, and γ -synuclein targeting biotherapy in cell culture and animal model.

ACKNOWLEDGMENTS

We are grateful to Dr. Feng Zhang (Institute of Digestive Surgery, Ruijin Hospital, China) for excellent technical assistance and advice.

COMMENTS

Background

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related deaths worldwide. The conventional therapies involving surgery and adjuvant therapy do not significantly prolong the survival period. It is necessary to identify a reliable biomarker or a potential target for diagnosis and treatment.

Research frontiers

γ -synuclein, a member of synuclein protein family, is abundantly expressed in brain tissue and presynaptic terminals. However, the tissue specificity of γ -synuclein expression appears to be lost in some types of cancer. Particularly in breast cancers, γ -synuclein promotes malignancy of breast cancer cell lines in *in vitro* studies and animal models. However, little is known about γ -synuclein in colorectal cancer.

Innovations and breakthroughs

The results of this study provide strong evidence suggesting that γ -synuclein expression is up-regulated in CRC tissues, and is significantly correlated with clinical stage and lymph node involvement of CRC. The authors also constructed specific γ -synuclein siRNA plasmids and established a permanent transfected colorectal cancer cell line HCT116, and found that down-regulation of expression of γ -synuclein in HCT116 cells could inhibit the growth, colony formation rate, and migration and invasion ability of HCT116 cells.

Applications

These results demonstrate that γ -synuclein indeed may function as a key mediator of cancer cell growth and metastasis and will be a promising target for CRC diagnosis, prognosis evaluation and biotherapy.

Terminology

Mitotic checkpoint is a cellular inherent mechanism, which strictly controls the cell division cycle and makes sure there is faithful cell replication. G418, a kind of aminoglycoside antibiotic, is a most common resistance selection reagent, used for stable transfectants in molecular biology tests.

Peer review

The paper investigated expression pattern of γ -synuclein in CRC tissues, and the effects of γ -synuclein on CRC cell line HCT116 biological features also were studied *in vitro*. The study is well conducted and the results is clear.

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S- Editor Tian L **L- Editor** O'Neill M **E- Editor** Lin YP



ORIGINAL ARTICLE

Effects of LY294002 on the invasiveness of human gastric cancer *in vivo* in nude mice

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Supported by The Health Foundation of Jiangsu Province (H200719), the Institution of Higher Education Foundation of Jiangsu Province (08KJB320014) and the Natural Science Foundation of Jiangsu Province (BK2008168)

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Received: July 19, 2009 Revised: September 12, 2009

Accepted: September 19, 2009

Published online: October 28, 2009

Abstract

AIM: To investigate the effects of class I phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 on the invasiveness and related mechanisms of implanted tumors of SGC7901 human gastric carcinoma cells in nude mice.

METHODS: Nude mice were randomly divided into model control groups and LY294002 treatment groups. On days 5, 10 and 15 after treatment, the inhibitory rate of tumor growth, pathological changes in tumor specimens, expression levels of matrix metalloproteinase (MMP)-2, MMP-9, CD34 [representing microvessel density (MVD)] and vascular endothelial growth factor (VEGF), as well as apoptosis indexes in tumor samples were observed.

RESULTS: In this study, we showed that treating

the tumors with LY294002 could significantly inhibit carcinoma growth by 11.3%, 29.4% and 36.7%, after 5, 10 and 15 d, respectively, compared to the control group. Hematoxylin & eosin staining indicated that the rate of inhibition increased progressively ($23.51\% \pm 3.11\%$, $43.20\% \pm 3.27\%$ and $63.28\% \pm 2.10\%$ at 5, 10 and 15 d, respectively) along with apoptosis. The expression of MMP-2 was also downregulated (from $71.4\% \pm 1.6\%$ to $47.9\% \pm 0.7\%$, $31.9\% \pm 0.9\%$ and $7.9\% \pm 0.7\%$). The same effects were observed in MMP-9 protein expression (from $49.4\% \pm 1.5\%$ to $36.9\% \pm 0.4\%$, $23.5\% \pm 0.9\%$ and $7.7\% \pm 0.6\%$), the mean MVD (from $51.2\% \pm 3.1\%$ to $41.9\% \pm 1.5\%$, $30.9\% \pm 1.7\%$ and $14.9\% \pm 0.8\%$), and the expression of VEGF (from $47.2\% \pm 3.1\%$ to $25.9\% \pm 0.5\%$, $18.6\% \pm 1.2\%$ and $5.1\% \pm 0.9\%$) by immunohistochemical staining.

CONCLUSION: The class I PI3K inhibitor LY294002 could inhibit the invasiveness of gastric cancer cells by downregulating the expression of MMP-2, MMP-9, and VEGF, and reducing MVD.

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Key words: Phosphatidylinositol 3-kinase; LY294002; Gastric cancer; Neoplasm invasiveness

Peer reviewer: Dr. Wang-Xue Chen, Institute for Biological Sciences, National Research Council Canada, 100 Sussex Drive, Room 3100, Ottawa, Ontario K1A 0R6, Canada

Xing CG, Zhu BS, Fan XQ, Liu HH, Hou X, Zhao K, Qin ZH. Effects of LY294002 on the invasiveness of human gastric cancer *in vivo* in nude mice. *World J Gastroenterol* 2009; 15(40): 5044-5052 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5044.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5044>

INTRODUCTION

Gastric cancer is the fourth most frequently diagnosed malignancy worldwide, accounting for 12% of all cancer-related deaths. In Asia and parts of South America in particular, gastric cancer is the most common epithelial malignancy and a leading cause of cancer-related death^[1,2]. The major cause of death from gastric cancer

is metastasis that is usually resistant to conventional treatment.

Invasiveness and metastasis are the leading biological characteristics of a malignant tumor, and have a close relation with factors such as movement of tumor cells, apoptosis and metastasis-associated genes. Matrix metalloproteinase (MMP)-2, MMP-9, intratumoral microvessel density (MVD) and vascular endothelial growth factor (VEGF) are important angiogenic factors, which have a higher expression in tumor tissues, may induce angiogenesis in the tumor and play an important role in metastases, invasion and prognosis of gastric cancer^[3-7]. However, the underlying mechanism remains uncertain.

The phosphatidylinositol 3-kinase (PI3K) pathway plays a central role in the regulation of cell proliferation, growth, differentiation and survival^[8,9]. Dysregulation of this pathway is frequently observed in a variety of tumors, including brain tumors and breast, ovarian and other carcinomas^[10-12]. Therefore, inhibition of PI3K signaling is under investigation as a potentially useful approach for cancer treatment. However, the detailed mechanisms are poorly understood.

LY294002 is a specific inhibitor of class I PI3K. The antitumor activity of LY294002 may be related to the induction of apoptosis of tumor cells, but the precise mechanism of its antitumor activity is not well understood.

In the present study, we investigated whether the inhibition of class I PI3K by LY294002 restricted the growth and invasiveness of implanted tumors of SGC7901 cells in nude mice. Through this research, we would suggest that the class I PI3K inhibitor LY294002 could play an important role in the inhibition of progression of gastric carcinoma, and may be of value in effective clinical antitumor therapy.

MATERIALS AND METHODS

Materials

SG7901 cells and female Balb/c nude mice (4 wk old, 16-18 g) were purchased from the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium was purchased from Gibco (Rockville, MD, USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Material Co. (Hangzhou, China), anti-MMP-2 (sc-13595), anti-MMP-9 (sc-21733), anti-CD34 (sc-19621) and anti-VEGF (sc-57496) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA). SP kit was purchased from Maixin Biotechnology (Fuzhou, China).

Drug preparation

LY294002 was purchased from Cell Signaling Technology (Beverly, MA, USA) and diluted in phosphate buffered saline (PBS) to create a stock solution that was stored according to the manufacturer's instructions. The final concentration of the LY294002 solution used was 500 $\mu\text{mol/L}$. This concentration of LY294002 was selected on the basis of our experiments on implanted tumors of human SGC7901 cells in nude mice.

Cell culture

SGC7901 cells were maintained in RPMI-1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Hangzhou Sijiqing Biological Engineering Material Co.), 0.03% L-glutamine (Sigma) and incubated in a 5% CO₂ atmosphere at 37°C. Cells in the mid-log phase were used in the experiments.

Inhibitory rate of tumor growth

A transplanted tumor model was established by injecting 1×10^9 cells/mL human SGC7901 into the subcutaneous tissue in the armpit of nude mice. After 10 d, 25 nude mice were randomly divided into four groups, and 0.2 mL normal saline solution, LY294002 (500 $\mu\text{mol/L}$) was directly injected adjacent to the tumor, twice at a 2 d interval (5 d group), three times at 2 d intervals (10 and 15 d group). There were six nude mice given saline, 19 nude mice given LY294002 (six in the 5 d group, six in the 10 d group, seven in the 15 d group). Changes in tumor volume = $(\pi/6) \times abc$ (a: the length of the tumor, b: the width of the tumor and c: the depth of the tumor) were measured at 5, 10, 15 d after LY294002 treatment and the tumor inhibition rate of each group was calculated.

Inhibitory rate of tumor growth = $[C(V_1 - V_0) - T(V_1 - V_0)]/C(V_1 - V_0)$

Where C is the control group, T is the treated group, V_1 is the volume before treatment (mm^3), and V_0 is the volume after treatment (mm^3).

Hematoxylin and eosin (HE) and immunohistochemical staining

Tumor specimens were taken from areas next to the margin of the tumors as well as from more central areas. All the specimens were formalin-fixed, paraffin-embedded and pathologically diagnosed to be gastric carcinoma and evaluated using HE for conventional histological assessment. Histological characteristics were reviewed by two pathologists.

The tumor samples were cut into 4 μm thick slices and fixed in acetone. After washing in PBS, slices were incubated in 0.3% H₂O₂ solution at room temperature for 5 min. Slices were then incubated with anti-MMP-2 or anti-MMP-9 or anti-MVD or anti-VEGF monoclonal antibody at a 1:300 dilution at 4°C overnight. After washing in PBS, the second antibody, biotinylated anti-rat IgG, was added and the cells were incubated at room temperature for 1 h. After washing in PBS, avidin-biotin complex was added and then incubated at room temperature for 10 min. Diaminobenzidine was used as the chromogen. After 10 min, the brown color signifying the presence of antigen bound to antibodies was detected by light microscopy. Controls were prepared in the same manner as the experimental group, except for incubation with the primary antibody. The positive rate (PR) was calculated as follows: PR = (number of positive cells/total number of cells) \times 100%.

Immunohistochemical assessment

The cytoplasm with MMP-2, MMP-9 and VEGF

appeared as brown in color. Immunohistochemical staining was independently evaluated by two pathologists, who were blinded to the experimental data. Two hundred cells were chosen under the microscope to evaluate the stained cell number against the total cell number in the field. Based on the positive cell number, the criteria were set as follows: negative (-) having a positive cell number < 10%; (+) having 11%-50% positive cells; (++) having 51%-75% positive cells; and (+++) having > 75% positive cells. The results of staining of MMP-2, MMP-9 and VEGF were classified into negative (staining of \leq 10% of cells) or positive (staining of > 10% of cells).

Evaluation of MVD

MVD-CD34 of tumor tissues were evaluated at low power field ($\times 40$). The tissue sections were screened and five areas with the most intense neovascularization (hot spots) were selected. Microvessel counts of these areas were performed at high power field ($\times 200$). Cases with discrepancies in scores between the two investigators were evaluated jointly to obtain a common consensus score. Any positively stained endothelial cell or endothelial cell cluster that was clearly separated from the adjacent microvessels, tumor cells and connective elements was counted as one microvessel, irrespective of the presence of a vessel lumen. An automated microvessel count/field was computed in each hot spot and the mean microvessel count of the 5 most vascularized areas was taken as the MVD-CD34, which was expressed as the absolute number of microvessels per 0.74 mm^2 ($\times 200$ field). The microvessel count that was higher than the median of microvessel count was taken as high MVD-CD34 and the microvessel count that was lower than the median of microvessel count was taken as low MVD-CD34.

Statistical analysis

All data are presented as mean% \pm SD. Statistical analysis was carried out using ANOVA followed by Dunnett's *t*-test, with $P < 0.05$ taken to indicate significance.

RESULTS

Effect of the LY294002 on tumor growth

SGC7901 cells (1×10^5) were injected subcutaneously into the armpit of nude mice. Within 1 wk, visible tumors had developed at the injection sites. To determine the therapeutic effectiveness of LY294002, intratumoral injection of LY294002 was started after the volume of the implanted tumor reached 20 mm^3 , and was repeated every 2 d for a total of three times. As shown in Table 1, LY294002 markedly suppressed tumor growth compared with PBS alone ($P < 0.01$). No gross adverse effects, i.e. the loss of body weight, were observed during the experimental periods. Moreover, LY294002 inhibited the proliferation of the implanted tumor of human SGC7901 cells in nude mice in a time-dependent fashion. The inhibition rate of the tumors

Table 1 Comparison of different pathological indexes for volume of tumors (mean \pm SD)

Groups	Number of animals		Volume of tumors (mm^3)		Inhibition rate (%)
	Beginning	End	Beginning	End	
Control	5	5	20.6 \pm 1.1	536.3 \pm 12.4	
PBS	5	5	20.5 \pm 1.2	525.7 \pm 6.9	
LY294002	15	15			
5 d	5	5	20.9 \pm 1.4	476.2 \pm 6.7	11.3 \pm 1.13 ^a
10 d	5	5	20.8 \pm 1.6	435.9 \pm 7.4	29.4 \pm 1.47 ^a
15 d	5	5	20.4 \pm 1.7	379.2 \pm 9.3	36.7 \pm 2.12 ^a

^a $P < 0.05$ vs control.

was 11.31% \pm 13% on day 5, 29.4% \pm 1.47% on day 10 and reached 36.7% \pm 2.12% on day 15.

LY294002 reduced cell viability and induced apoptosis of transplanted SGC7901 tumor cells

Treatment with LY294002 for 5, 10 and 15 d in SGC7901 cells produced intensive HE staining indicating apoptosis. A significant increase in apoptosis was observed along with the time that tumors were treated (Figure 1). After 5 d, the rate of inhibition reached 23.51% \pm 3.11%. The rate of inhibition rose when the experimental time increased, reaching 43.20% \pm 3.27% on day 10 and 63.28% \pm 2.10% on day 15 after LY294002 treatment. All the results indicated that LY294002 induced apoptosis (Figure 1E-H).

Similarly, in the lymphatic vessels, the effect of the drug increased with time. The invasion rate of the tumor cells in the lymphatic vessels decreased from 75.69% \pm 3.5% to 63.78% \pm 4.3%, 45.62% \pm 3.1% and 28.17% \pm 2.7% at 5, 10 and 15 d, respectively, after treatment with LY294002 (Figure 1A-D).

LY294002 inhibited the expression of MMP-2 and MMP-9 proteins

Positive staining was distributed in the cell membrane and cytoplasm (Figures 2 and 3). The PR of MMP-2 protein downregulated was from 71.4% \pm 1.6% in the control group to 47.9% \pm 0.7%, 31.9% \pm 0.9% and 7.9% \pm 0.7% at 5, 10 and 15 d, respectively, after treatment with LY294002 for (Figure 2E-H). Significant differences in the expression were observed between the 500 $\mu\text{mol/L}$ LY294002 group and the control group at every time point ($P < 0.05$). Similarly, the expression of MMP-9 decreased from 49.4% \pm 1.5% to 36.9% \pm 0.4%, 23.5% \pm 0.9%, 7.7% \pm 0.6%, respectively (Figure 3E-H).

In the lymphatic vessels, we found that the expression of MMP-2 in the tumor cells also decreased from 59.23% \pm 6.2% to 45.46% \pm 7.3%, 32.14% \pm 1.9%, 14.01% \pm 3.9% at the various time points (Figure 2A-D). Interestingly, MMP-9 shared the same characteristics in expression as that of MMP-2, which fell from 39.95% \pm 5.7% to 25.32% \pm 6.5%, 12.84% \pm 2.8%, 4.01% \pm 5.9% (Figure 3A-D).

LY294002 decreased the expression of MVD and VEGF

Positively stained particles of MVD-CD34 were mainly distributed in the plasmalemma of cells, though some

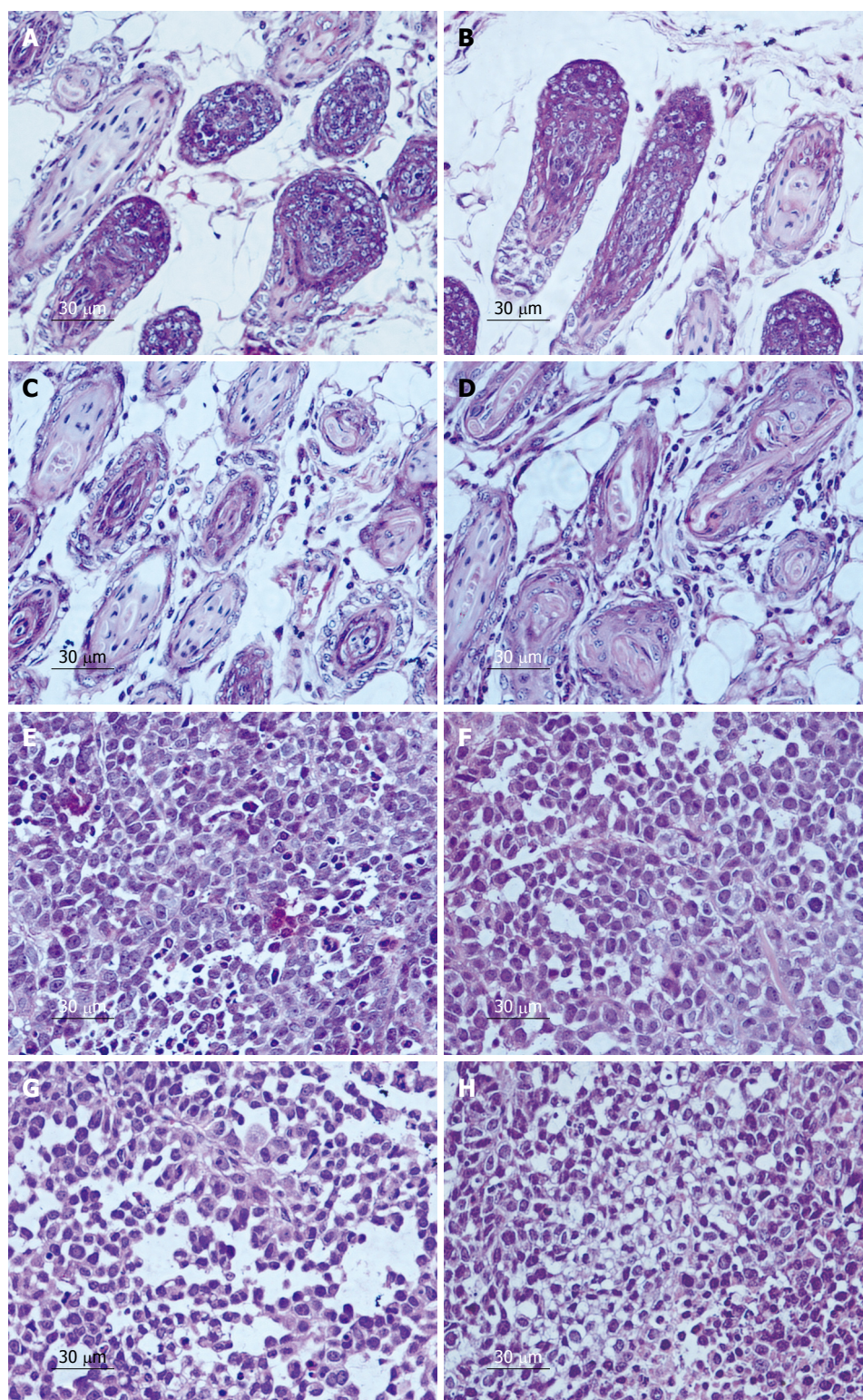


Figure 1 Pathological changes in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, $\times 200$) and tumor cells (E-H) (HE, $\times 100$); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

were expressed in the cytoplasm (Figure 4). The mean MVD was much lower in the tumors treated with LY294002 than in the control group.

According to the PR of MVD protein of the control group and the experimental group after treatment with LY294002 5, 10, 15 d, the expression of MVD was downregulated from $51.2\% \pm 3.1\%$ to $41.9\% \pm 1.5\%$, $30.9\% \pm 1.7\%$ and $14.9\% \pm 0.8\%$ after 5, 10 and 15 d, respectively (Figure 4E-H). A significant difference in positive expression was observed between the 500 $\mu\text{mol/L}$

LY294002 group and the control group at every time point ($P < 0.05$). Similarly, the expression of VEGF decreased from $47.2\% \pm 3.1\%$ to $25.9\% \pm 0.5\%$, $18.6\% \pm 1.2\%$ and $5.1\% \pm 0.9\%$, respectively (Figure 5E-H).

In the vascular area, we found that the effect of the agent increased with time, and the MVD of the tumor cells decreased from $37.65\% \pm 2.7\%$ to $27.24\% \pm 5.3\%$, $10.64\% \pm 3.5\%$, $5.26\% \pm 1.3\%$ at 5, 10 and 15 d, respectively (Figure 4A-D). The VEGF expression was also downregulated from $34.25\% \pm 1.7\%$ to $25.21\% \pm$

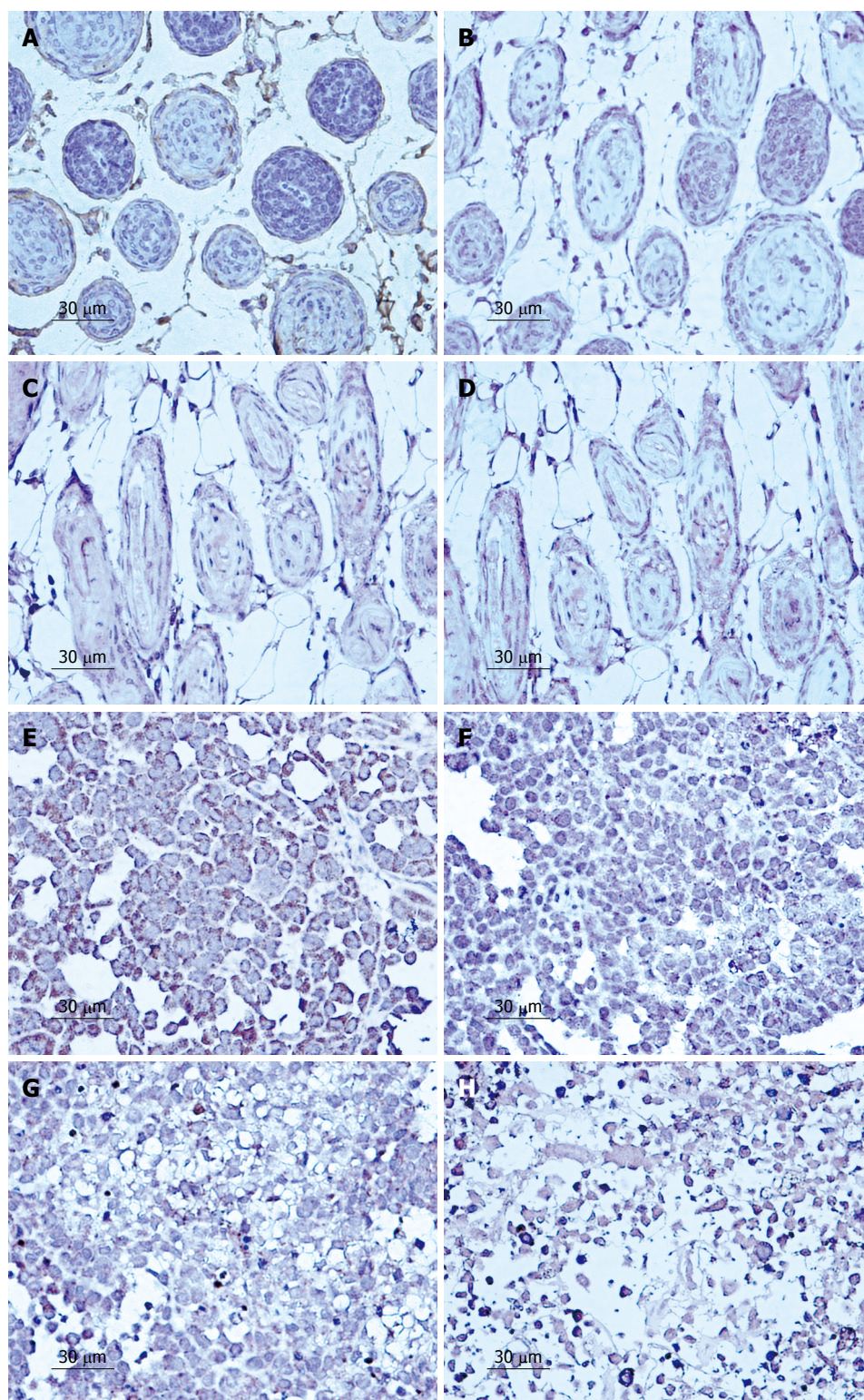


Figure 2 Pathological changes of the expression of MMP-2 in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, $\times 200$) and tumor cells (E-H) (HE, $\times 100$); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

2.3%, $11.34\% \pm 5.1\%$ and $3.26\% \pm 0.3\%$, respectively (Figure 5A-D).

DISCUSSION

Currently only a few chemotherapeutic drugs are effective for the treatment of human gastric carcinoma^[13] and there is an increasing interest in the use of drugs to prevent its occurrence or invasiveness. The lipid kinase PI3K is a proto-oncogene that generates 3'-phosphoinositides at the

cell membrane. The best-characterized inhibitors of PI3K are LY294002 and wortmannin. LY294002 effectively inhibits the growth of many types of tumor cells *in vitro* and *in vivo*, via the inhibition of PI3K and downstream components of the pathway^[14-17]. It is possible that LY294002 inhibits proliferation and induces apoptosis in SGC7901 cells through inhibiting class I PI3K. However, the downstream molecules involved in the apoptotic death of tumor cells following inhibition of PI3K/Akt by LY294002 remain to be identified^[1,18].

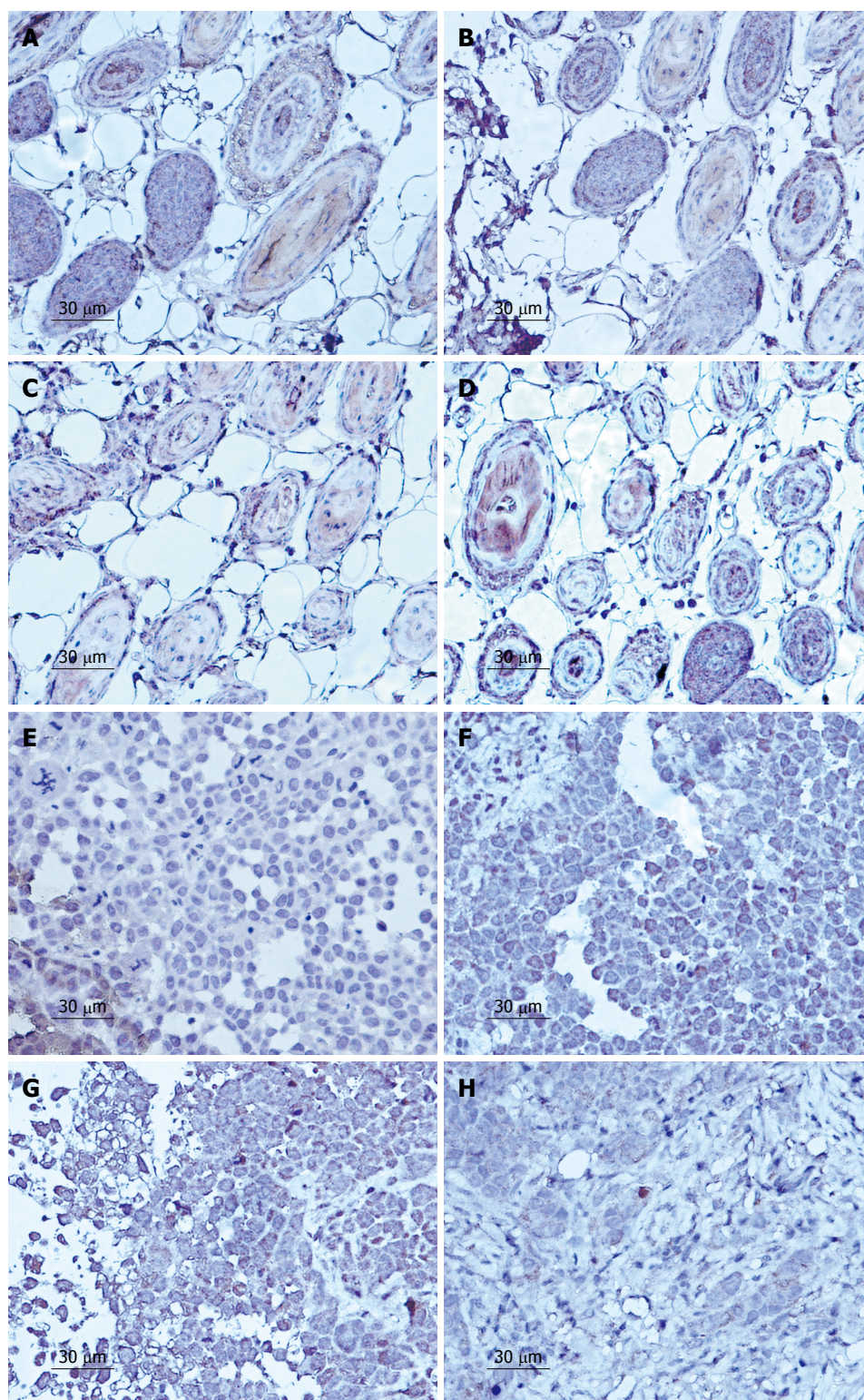


Figure 3 Pathological changes of the expression of MMP-9 in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, $\times 200$) and tumor cells (E-H) (HE, $\times 100$); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

Tumor metastasis involves a series of complex processes in which many gene products take part in regulating MMPs, which play an important role in breaking the extracellular matrix, are overexpressed in malignant tumors and are believed to contribute to tumor proliferation, invasion, and metastasis^[19]. Among the MMPs, MMP-2 and MMP-9 are closely related to the metastasis of tumors, and have been specifically considered to be important factors in facilitating lymphatic invasion and metastases in gastric carcinoma^[20-22].

MVD is a quantitative index for carcinoma angiogenesis, which indicates the regulation of the progress and metastasis of primary gastric carcinoma, and can therefore serve as a marker for carcinoma prognosis^[23-25]. VEGF is one of the agents accelerating the formation of blood vessels, and plays a vital part in tumor-associated microvascular invasion^[26-28]. It has been found that tumor metastasis is speeded up by VEGF, which is highly expressed in gastric carcinoma, thus may be used as an index for poor prognosis of gastric carcinoma^[29-31].

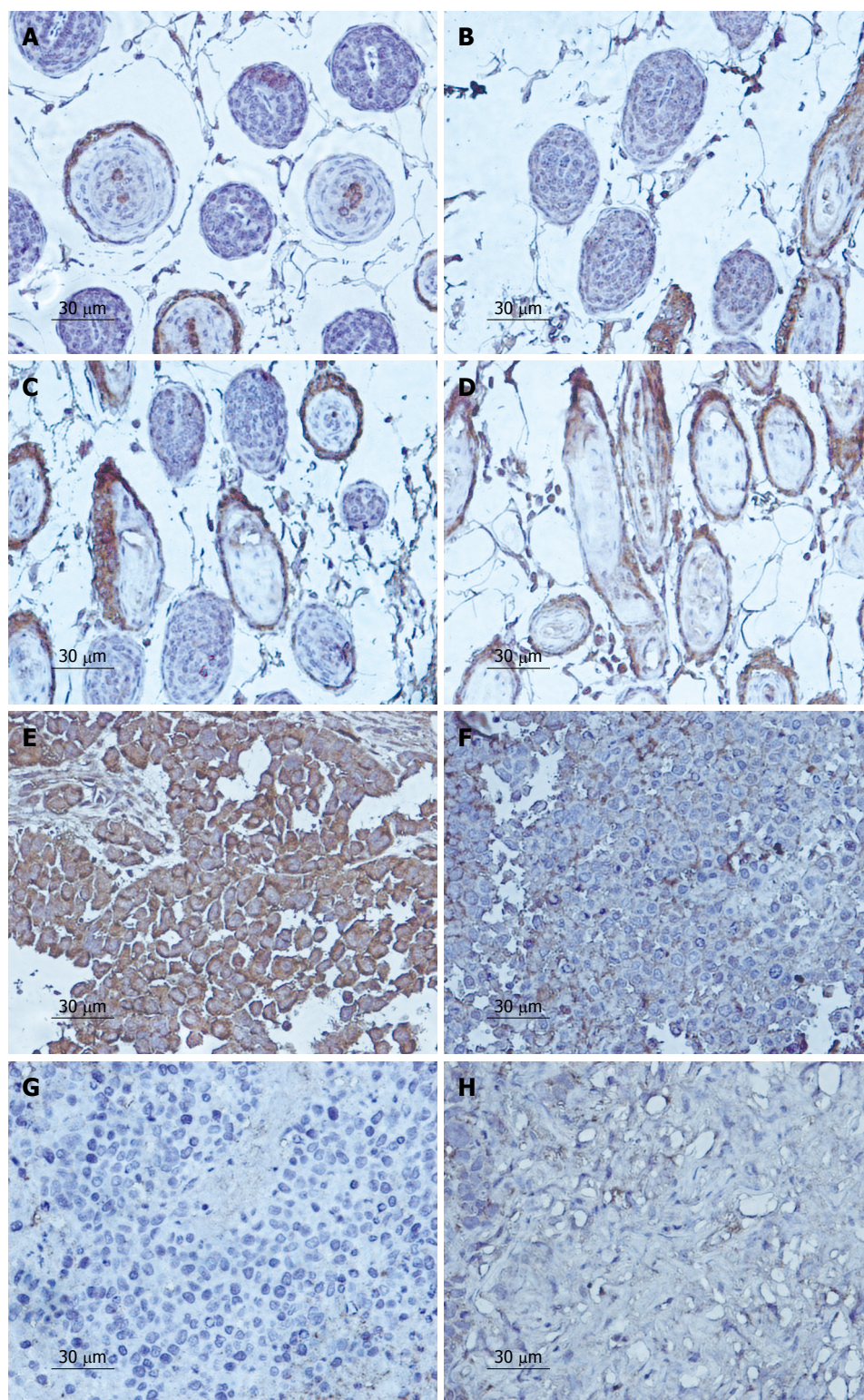


Figure 4 Pathological changes of the expression of MVD in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, $\times 200$) and tumor cells (E-H) (HE, $\times 100$); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

In the present study, there was a significant difference in the expression of MMP-2, MMP-9 and VEGF, and in MVD between the transplanted gastric tumor tissues treated by the class I PI3K inhibitor LY294002 and that of the control group ($P < 0.05$), indicating that LY294002 may inhibit the gene expression of MMP-2, MMP-9, MVD, and VEGF. We also showed that LY294002 reduced the viability and induced apoptosis in the implanted tumor of human SGC7901 cells, thus demonstrating the cytotoxic effects of LY294002. Both

the *in vitro* invasion assay and the *in vivo* nude mice assay suggested that LY294002 had the potential to inhibit the invasion and metastasis of gastric cancer. This may be the result of the decrease of the expression of MMP-2, MMP-9, MVD and VEGF together with the cytotoxicity towards the tumor cells, both induced by LY294002. Moreover, no gross adverse effects, i.e. loss of body weight, were observed during our experimental periods. These findings suggest that inhibition of the class I PI3K signaling pathway is a potent and safe strategy for

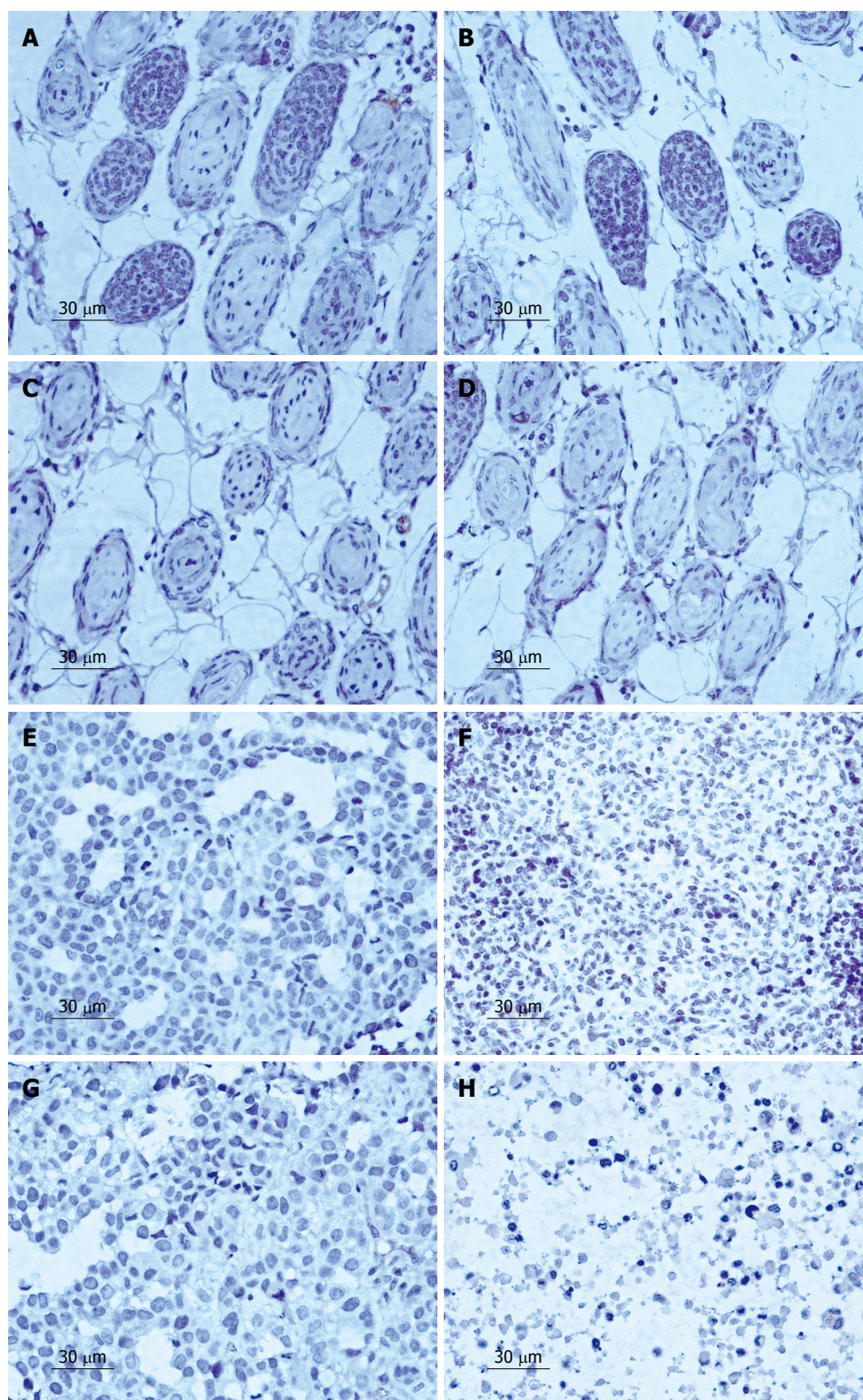


Figure 5 Pathological changes of the expression of VEGF in lymphatic vessels and SGC7901 tumor cells in the model control group (A, E) and treatment group (B-D, F-H). Lymphatic vessels (A-D) (HE, $\times 200$) and tumor cells (E-H) (HE, $\times 100$); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

managing gastric cancers, and further indicated that development of LY294002-based therapies may be an emerging approach for the next step in gastric cancer management.

COMMENTS

Background

The phosphatidylinositol 3-kinase (PI3K) pathway plays a central role in the regulation of cell proliferation, growth, differentiation, and survival. Dysregulation of

this pathway is frequently observed in a variety of tumors, including brain tumors and breast, ovarian and other carcinomas. Currently only a few chemotherapeutic drugs are effective for the treatment of human gastric carcinoma and there is an increasing interest in the use of drugs to prevent its occurrence or invasiveness.

Research frontiers

LY294002 is a specific inhibitor of class I PI3K. The antitumor activity of LY294002 may be related to the induction of apoptosis of tumor cells, but the precise mechanism of its antitumor activity is not well understood.

Applications

The research indicated that development of LY294002-based therapies may be an emerging approach for the next step in gastric cancer management.

Terminology

Matrix metalloproteinases (MMPs) are a family of structurally related zinc-endopeptidases. MMP-2 contributes to cell proliferation, migration, and matrix invasion in a number of cell types such as tumor cells and fibroblasts. MMP-9 is directly involved in tumor metastasis, and more recently MMP-9 activity has been linked with the process of tumor cell invasiveness. High MVD is an indirect measure of tumor aggressiveness. Vascular endothelial growth factor is a potent regulator of placental vascular function. Endothelial dysfunction is a key factor associated with preeclampsia.

Peer review

Overall, the experiment was well performed and the authors have presented interesting data to provide a mechanistic insight into the antitumor effect of LY294002.

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S-Editor Tian L L-Editor Cant MR E-Editor Zheng XM



Liver tumor infiltrating lymphocytes: Comparison of hepatocellular and cholangiolar carcinoma

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Supported by Centre of Molecular Medicine Cologne (CMMC), Köln, Germany

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Received: May 4, 2009 Revised: August 10, 2009

Accepted: August 17, 2009

Published online: October 28, 2009

predominance of CD8+ cells in the tumor tissue (52.6/10 HPF) and of CD4+ cells in the interface region (223.1/10 HPF). CD56+ cells of the innate immune system were scarce. There was no significant difference between hepatocellular or cholangiolar carcinoma. No correlation with the clinicopathological data was seen.

CONCLUSION: Liver TIL consists of intratumoral CD8+ T cells and peritumoral CD4+ T cells independent of histogenetic origin. Different functions of lymphocytes in these regions seem possible.

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Key words: Liver neoplasms; Hepatocellular carcinoma; Lymphocytes; Immunologic factors; Cholangiocarcinoma

Peer reviewer: Mark D Gorrell, Professor, Centenary Institute of Cancer Medicine and Cell Biology, Locked Bag No. 6, Newtown, NSW 2042, Australia

Kasper HU, Drebber U, Stippel DL, Dienes HP, Gillessen A. Liver tumor infiltrating lymphocytes: Comparison of hepatocellular and cholangiolar carcinoma. *World J Gastroenterol* 2009; 15(40): 5053-5057 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5053.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5053>

Abstract

AIM: To investigate the role of tumor infiltrating lymphocytes (TIL) in primary hepatocellular and cholangiolar carcinomas of the liver.

METHODS: Immunohistochemical analysis was performed including antibodies to CD3, CD4, CD8, CD20, CD56 and TIA-1 in formalin-fixed and paraffin-embedded tissue of 35 liver resection specimens of hepatocellular or cholangiocellular carcinomas. Semiquantitative evaluation was performed with emphasis on the area of the tumor itself and of the tumor/liver interface.

RESULTS: All hepatocellular carcinomas showed infiltration of lymphocytes predominantly around the tumor in the tumor/liver interface consisting mainly of CD3+ CD4+ T lymphocytes [164.3/10 high power fields (HPF)] and in the tumor itself of CD8+ cells (54.9/10 HPF). Cholangiocarcinomas contained a heterogeneous amount of TIL, composed mainly of CD3+ T cells with a

INTRODUCTION

Tumor infiltrating lymphocytes (TIL) are part of the tumor surveillance system^[1]. This immune response is thought to be a result of changes of surface components of tumor cells. The innate as well as the adaptive immune system is involved in tumor destruction with cell-mediated mechanisms playing the main role. They are frequently present in human solid tumors. Some CD8+ T cells are seen as final effector cells with the ability to induce apoptosis of cancer cells. Subpopulations of CD4+ T cells have a helper function, activating other immune cells *via* cytokine secretion or antigen processing. TIL are a target for immunotherapeutic strategies^[2].

The liver can be regarded as an immunological organ, specially equipped with liver-associated lymphocytes, mainly T lymphocytes and natural killer cells^[3]. They play an important role in the barrier function of the liver between the gastrointestinal tract and an organism. They

do not only function as part of the defense system but also as a regulator of immune tolerance.

Hepatocellular carcinoma is the leading cause of malignant cancer deaths worldwide and the morbidity is increasing year on year. It accounts for approximately 6% of all human cancers and up to 1 million deaths per year. The second most common primary malignancy of the liver, cholangiolar carcinoma also has a bad prognosis. Its resectability rate is very low, but surgical resection is the only treatment which can change outcome significantly^[4,5].

We studied the frequency and composition of TIL in primary liver cancers with special attention to the morphological distribution. The subtyping was performed to clarify their putative role in host response, immunotolerance and as a therapeutic target.

MATERIALS AND METHODS

Patients

Formalin-fixed and paraffin-embedded tissue of 35 liver resection specimens were investigated. The specimens were obtained from 8 women and 27 men with a median age of 60.5 years (38-82 years). Twenty seven of the cases were diagnosed as hepatocellular carcinoma (8 × T1, 3 × T2, 12 × T3 4 × T4; 6 × G1, 14 × G2, 7 × G3) and 8 as cholangiolar carcinoma (2 × T1, 1 × T2, 2 × T3, no T-stage available in 3; 6 × G2, 2 × G3) with a mean diameter of 7.9 cm and 8.3 cm, respectively.

Immunohistochemistry

A panel of immunohistochemical stains was performed including antibodies to CD3, CD4, CD8, CD20, CD56 and TIA-1. The specifications and titers are given in Table 1.

Routinely processed formalin-fixed and paraffin-embedded tissue sections of the tumor and the tumor/liver interface with a thickness of 4 μm were used. Sections were mounted onto capillary gap slides (DAKO, Glostrup, Denmark), dried overnight at 30°C, deparaffinized with xylene and rehydrated with ethanol in a graded series to distilled water. Staining was performed using an automated immunostainer (Techmate, DAKO, Glostrup, Denmark) with AEC (3-amino-9-ethylcarbazole) for visualization. The slides were counterstained with hemalaun and a coverslip placed on top. Appropriate positive and negative tissue control samples were used throughout. Tonsils served as positive controls.

Statistical analysis

Ten high power fields (HPF) of the tumor and the tumor-liver interface were randomly selected and the frequency of TIL was counted using an ocular grid. Results are reported as mean value and standard deviation per 10 HPF. Comparison of the groups was performed using the two-tailed Students *t*-test (SPSS for windows). The significance level was set as *P* < 0.05.

RESULTS

TIL in hepatocellular carcinoma

All hepatocellular carcinomas showed an infiltration of

Table 1 List of antibodies

Name	Clone	Pre-treatment	Titer	Manufacturer
CD3	PS1	10 min autoclave 120°C, citrate buffer pH 6	1:50	DAKO, Glostrup, Denmark
CD4	1F6	10 min autoclave 120°C, citrate buffer pH 9	1:10	Novocastra, Newcastle, UK
CD8	C8/144B	10 min autoclave 120°C, citrate buffer pH 6	1:100	DAKO, Glostrup, Denmark
CD20	L26	none	1:500	DAKO, Glostrup, Denmark
CD56	123C3	2 × 7 min microwave citrate buffer pH 6	1:500	Zymed, San Francisco, CA, USA
TIA-1	TIA-1	2 × 7 min microwave citrate buffer pH 6	1:500	Coulter Immunol., Hialeah, FL, USA

lymphocytes which was mainly localized around the tumor in the tumor/liver interface, with less among the tumor cells (Table 2, Figure 1). The TIL consisted mainly of CD3+ T lymphocytes. CD20+ cells and CD56+ cells were rarely found. In the tumor itself, the infiltration was dominated by CD8+ cells. In contrast, in the peritumoral area the amount of CD4+ cells was higher than the amount of CD8+ cells. TIA-1 containing cells were more frequent in the peritumoral region.

TIL in cholangiolar carcinoma

Cholangiocarcinomas contained a heterogeneous amount of TIL, composed mainly of CD3+ T cells. The relationship of the subpopulations was comparable to that of hepatocellular carcinoma, with a predominance of CD8+ cells in the tumor tissue and of CD4+ cells in the interface region. CD56+ and CD20+ cells were found only in a minor proportion. Cells containing the cytotoxic granula TIA-1 occurred often in the interface region. The details are summarized in Table 2 and Figure 2.

No statistical differences were found in the frequency and distribution according to age, sex, size or grade of the tumor.

DISCUSSION

Liver carcinoma evolves over a long period of time from precursor lesions to invasive cancer and metastases. The exact mechanisms of this pathway are not fully understood^[6]. It is known, however, that TIL have the potential to modulate this process. The extent of this modulation and the real effects of tumor growth and dissemination remain unclear.

Several methodical approaches have been used to measure TIL. Most often flow cytometry and immunohistochemistry is used. Flow cytometry is a rapid method, in which a higher amount of cells and a panel of markers can be investigated. Fresh tissue, however, is necessary. Therefore retrospective studies, especially with human tissue from pathological files, are not possible. An exact localization of the TIL cannot be determined. Immunohistochemistry can identify different cell populations and has the advantage of direct localization of the lymphocyte subsets. Manual counting and automated counting

Table 2 Frequency of TIL in hepatocellular and cholangiolar carcinoma

TIL	Hepatocellular carcinoma			Cholangiolar carcinoma		
	Intratumoral (/10HPF)	Peritumoral (/10HPF)	Level of significance	Intratumoral (/10HPF)	Peritumoral (/10HPF)	Level of significance
CD20	3.2 (± 9.7)	26.4 (± 49.5)	$P < 0.001$	0.1 (± 0.3)	11.1 (± 11.8)	$P = 0.035$
CD3	85.1 (± 78.2)	256.5 (± 90.5)	$P < 0.001$	52.6 (± 28.5)	310.4 (± 202)	$P = 0.008$
CD4	37.9 (± 42.5)	164.3 (± 26.4)	$P < 0.001$	18 (± 22.3)	223.1 (± 43.2)	$P = 0.043$
CD8	54.9 (± 57.9)	131.5 (± 86.8)	$P \leq 0.001$	40.7 (± 30.5)	118.7 (± 35.5)	$P \leq 0.001$
CD56	0.2 (± 0.6)	0.5 (± 1.1)	$P = 0.058$	0.4 (± 1.0)	1.9 (± 2.7)	$P = 0.088$
TIA-1	50.2 (± 40.5)	80 (± 64.5)	$P = 0.036$	41.1 (± 41.8)	72.5 (± 37.4)	$P = 0.071$

HPF: High power fields; TIL: Tumor infiltrating lymphocytes.

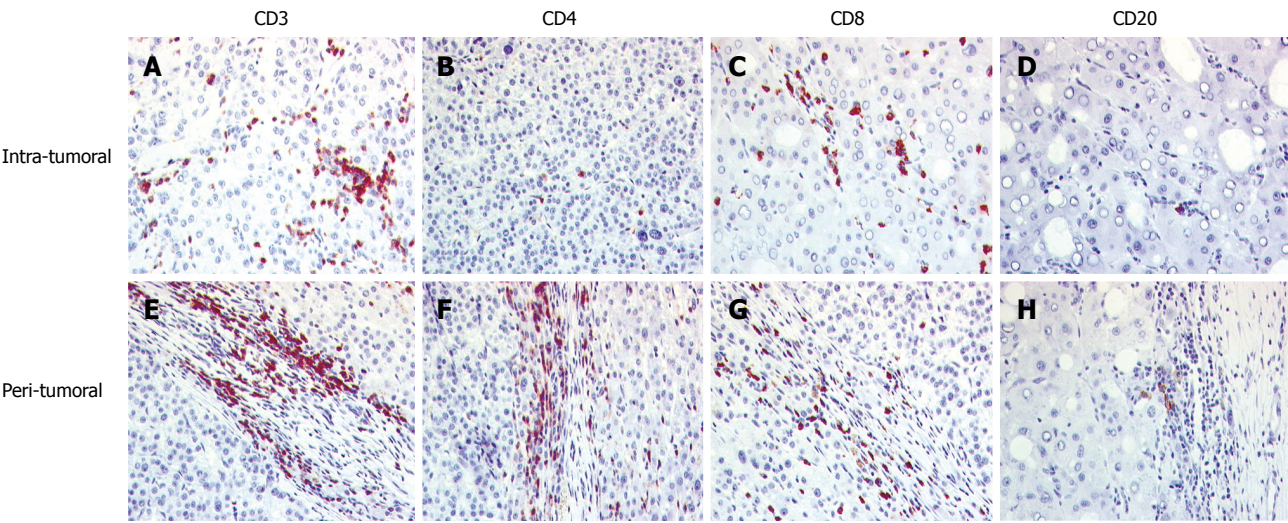


Figure 1 Lymphocytic infiltration in the tumor tissue of hepatocellular carcinoma. A-D: Intratumoral region; E-H: Tumor/liver interface (peritumoral); A and E: CD3+ T cells are the main infiltrate with a higher amount in the interface region; B and F: CD4+ cells were mainly located in the peritumoral area; C and G: In the tumor tissue, CD8+ cells were more often seen; D and H: CD20+ cells were scarce.

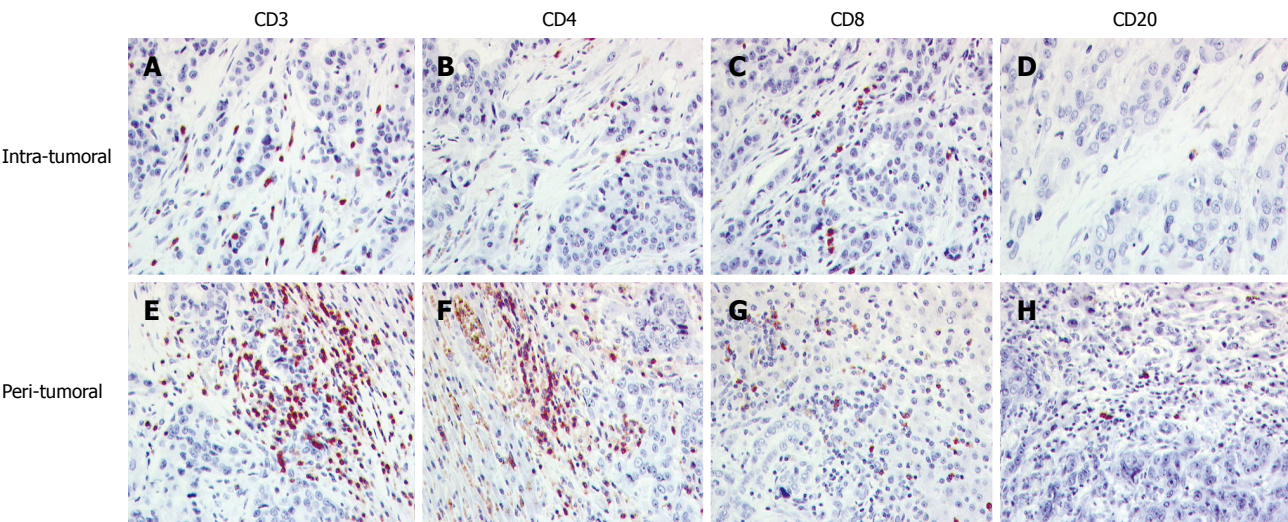


Figure 2 The distribution of tumor infiltrating lymphocytes in cholangiocellular carcinoma. A-D: Intratumoral region; E-H: Tumor/liver interface (peritumoral); A and E: CD3+ T cells were the dominant infiltrate; B and F: The quantity of CD4+ and CD8+ cells were opposite with a higher level of CD4 cells in the peritumoral region; C and G: CD8+ cells were more often found in the tumor tissue; D and H: CD20+ cells were scarce.

of digital images are possible^[7,8].

Using a histological approach we were able to localize TIL and could differentiate between lymphocytes in the tumor itself and in the environs of the tumor. In the

tumor, CD8+ cells were more frequent, showing a closer contact with the tumor cells. As these cells can have a cytotoxic function, a direct apoptotic effect *via* different pathways such as the FAS/FAS-ligand pathway seems

possible. Large numbers of CD8+ TILs are associated with a favorable prognosis in several solid carcinomas such as colorectal, ovarian, pancreatic and esophageal carcinoma^[9-14]. Other entities such as nasopharyngeal cancer, renal cell carcinoma and non-small cell lung cancer show opposite behavior^[15-17]. For hepatocellular carcinoma, further investigation is necessary.

In contrast, TIL in the interface are mostly of the CD4+ type. As CD4+ cells include helper T cells, their infiltration could activate cytotoxic killer cells by cytokine secretion or antigen presentation. Other authors, however, found an involvement of these cells, also known as regulatory cells, in immune tolerance^[18]. Tumor cells adapting to these mechanisms of the CD4+ lymphocytes could use these cells to induce a tumor-friendly environment. The infiltration of CD4+ T-cells could be a sign of tumor adaptation known as enhancement^[19].

Unitt *et al*^[20,21] indicated that lymphocytic infiltration of the tumor and a high CD4+/CD8+ T cell ratio were associated with a reduced risk of tumor recurrence after liver transplantation for hepatocellular carcinoma. This ratio was beneficial in hepatocellular carcinoma. A decreased ratio was shown to imply poor tumor response^[22]. CD4+ T lymphocytes can activate monocytes, macrophages and natural killer cells and can help CD8+ T lymphocytes to kill tumor cells. Thus, a positive effect of those infiltrations seems to exist. On the other hand, however, CD4+ T cells were also involved in tolerance mechanisms. As the liver is particularly involved in tolerance induction to food antigens, a supportive role of these infiltrates cannot be ruled out.

A lower quantity of CD8+ cells in the tumor and in the tumor periphery could signal a low level of T cell activation by tumor specific antigens in the liver. Primary reasons for this can be in the immune system itself such as development of self tolerance in the thymus or in the periphery^[23]. The tumor can also develop various evasion strategies like converting T helper cells or lowering the expression of tumor specific antigens to below the required threshold^[24-26]. These mechanisms would lead to a low quantity of TIL. Janicki *et al*^[27], however, could show that TIL can lose their effector function even after infiltrating the tumor. One reason could be a loss of adhesion capability^[28]. These results have critical implications for vaccination studies.

The liver normally contains a higher quantity of cells of the innate immune system such as natural killer cells. The lack of CD56+ cells in the tumor and in the interface to the liver is surprising. This cell type is thought to be involved in tumor defense. This is in contrast to a recent study investigating human glioblastomas^[29]. Half of all TIL in these tumor types were CD56+ cells, in particularly CD4+CD56+ T cells. Whether this cell type is unique for brain tumors has to be clarified by further studies.

In the liver, a primary defect or lack of cells of the innate immune systems could be regarded as an aid to tumor development. On the other hand, there is the possibility that a manifest tumor can suppress the innate immune system.

For cholangiolar carcinoma, only scarce data are

available regarding TIL. Takagi and coworkers found that patients with high numbers of TIL (CD8+ or CD4+) had significantly better prognosis^[30]. In our study, the amount of TILs was heterogeneous. In the tumor tissue itself, the CD8+ cells can act as cytotoxic cells. In this entity, regulatory cells are also found in the tumor environs. As in hepatocellular carcinoma, there is a lack of CD56+ cells of the innate immune system.

Earlier we investigated the lymphocytic reaction of the liver with liver metastases from different primary neoplasms^[31]. In this investigation, the results were similar to those demonstrated here. Also, in liver metastases, the frequency of TIL in the interface between the liver and tumor was higher than in the tumor itself. The infiltrate in the interface was also composed mainly of CD4+ T cells. Thus, a general rule can be seen in the reaction of the liver. A manifest tumor is accompanied by a CD4+ reaction in the liver. Whether this is a defense mechanism or more like an induced protection by the tumor cells needs further clarification.

In summary, we could demonstrate for hepatocellular carcinoma and cholangiolar carcinoma that TIL consists, in the tumor itself, of CD8+ T cells and, in the peritumoral region, of CD4+ T cells such as helper cells or regulatory cells. CD20+ cells and TIA-1+ cells were scarce. There was a lack of CD56+ cells of the innate immune system. The functional interaction of TIL in liver carcinomas needs further investigation especially if considered as a target for immunotherapeutic strategies.

COMMENTS

Background

Tumor development is based on an interaction between the tumor cells and the immune system of the body. Tumor infiltrating lymphocytes (TIL) are part of the tumor surveillance system. This immune response is thought to be a result of changes of surface components of tumor cells. TIL are a target for immunotherapeutic strategies in cancer treatment.

Research frontiers

Immune therapy is one of the newer strategies in cancer therapy. The main aim is vaccination against carcinomas. A prerequisite for the development of these vaccines is an understanding of the immunological tumor reaction.

Innovations and breakthroughs

Recent reports have shown different functions of lymphocyte subsets in tumor surveillance. These cells not only have antitumor potential but growth promotion by certain lymphocytes is also documented.

Applications

This study suggests that the immune reaction to liver cancer consists of different lymphocyte subtypes. Some can enhance tumor growth, others can destroy tumor cells. A complex strategy would be necessary for successful immune therapy of liver cancer.

Peer review

The authors investigated the role of TIL in primary hepatocellular and cholangiolar carcinomas of the liver. They found that liver TIL consist of intratumoral CD8+ T cells and peritumoral CD4+ T cells independent of histogenetic origin. This article is well written and deserves publication.

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S- Editor Li LF L- Editor Cant MR E- Editor Ma WH

BRIEF ARTICLE

UGT1A1 gene polymorphism: Impact on toxicity and efficacy of irinotecan-based regimens in metastatic colorectal cancer

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Received: March 26, 2009 Revised: September 27, 2009

Accepted: October 4, 2009

Published online: October 28, 2009

Abstract

AIM: To investigate the correlation between *uridine diphosphate glucuronosyl transferase 1A1 (UGT1A1)* gene polymorphisms and irinotecan-associated side effects and parameters of drug efficacy in patients with metastatic colorectal cancer (mCRC) receiving a low-dose weekly irinotecan chemotherapeutic regimen.

METHODS: Genotypes were retrospectively evaluated by gene scan analysis on the ABI 310 sequencer of the TATAA box in the promoter region of the *UGT1A1* gene in blood samples from 105 patients who had received 1st line irinotecan-based chemotherapy for mCRC.

RESULTS: The distribution of the genotypes was as follows: wild type genotype (WT) (6/6) 39.0%, heterozygous genotype (6/7) 49.5%, and homozygous genotype (7/7) 9.5%. The overall response rate (OR) was similar between patients carrying the (6/7, 7/7) or the WT genotype (6/6) (44.3% vs 43.2%, $P = 0.75$). Neither time to progression [(TTP) 8.1 vs 8.2 mo, $P = 0.97$] nor overall survival [(OS) 21.2 vs 18.9 mo, $P = 0.73$] differed significantly in patients who carried the

(6/6) when compared to the (6/7, 7/7) genotype. No significant differences in toxicity were observed: Grade 3 and 4 delayed diarrhoea [(6/7, 7/7) vs (6/6); 13.0% vs 6.2%, $P = 0.08$], treatment delays [(6/7, 7/7) vs (6/6); 25.1% vs 19.3%, $P = 0.24$] or dose reductions [(6/7, 7/7) vs (6/6); 21.5% vs 27.2%, $P = 0.07$].

CONCLUSION: This analysis demonstrates the non-significant influence of the *UGT1A1* gene polymorphism on efficacy and rate of irinotecan-associated toxicity in mCRC patients receiving low-dose irinotecan based chemotherapy.

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Key words: Irinotecan; Colorectal cancer; *UGT1A1*; Gene polymorphism; Toxicity; Efficacy; Delayed diarrhoea; Neutropenia

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Schulz C, Heinemann V, Schalhorn A, Moosmann N, Zwingers T, Boeck S, Giessen C, Stemmler HJ. *UGT1A1* gene polymorphism: Impact on toxicity and efficacy of irinotecan-based regimens in metastatic colorectal cancer. *World J Gastroenterol* 2009; 15(40): 5058-5066 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5058.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5058>

INTRODUCTION

Irinotecan (Camptosar®, CPT-11, Pfizer Oncology, New York, NY, US) is one of the most effective chemotherapeutic agents in the treatment of metastatic colorectal cancer (mCRC)^[1,2]. Data from phase III studies indicated an improved clinical outcome of patients who had received an irinotecan-based regimen when compared to those who had received 5-FU/LV alone. As reported by Saltz *et al*^[3], the irinotecan-based combination therapy not only improved the response rate (39% vs 21%), but also the progression free (PFS) (7.0 vs 4.3 mo; $P = 0.004$) and overall survival (OS) (14.8 vs 12.6 mo; $P = 0.04$).

Irinotecan is a semisynthetic derivative of camptothecin and acts as an inhibitor of intracellular topoisomerase- I^[4]. *In vivo*, the pro-drug is metabolized by carboxylesterase into its active metabolite SN-38. SN-38 is inactivated by uridine diphosphate glucuronosyl transferase 1 (UGT1A1) into SN-38G which is excreted with bile^[5].

The most common side effects of irinotecan include neutropenia, febrile neutropenia, nausea, alopecia and delayed diarrhoea which particularly often represents the main and dose-limiting toxicity. This major side effect is because betaglucuronidase in the bowel re-activates SN-38G into the active metabolite SN-38^[6-8].

UGT1A1, an essential enzyme for the inactivation of SN-38, is also involved in the metabolism of bilirubin. Changes in the metabolism of bilirubin result in several clinical disorders. They range from the clinically harmless condition of mild jaundice to the deadly disease Crigler-Najjar that may have a lethal outcome in adolescence^[9]. Patients with Gilbert's syndrome present with a mild unconjugated hyperbilirubinaemia without any structural liver disease or haemolysis. The activity of UGT1A1 is reduced in these individuals compared to those without a comparable defect^[10,11].

Molecular analyses have revealed that in the Caucasian population Gilbert's syndrome is most commonly caused by a polymorphism in the *UGT1A1* gene^[12]. It consists of a TA insertion in the TATAA element of the 5'-promotor region. Genotypes are defined (6/6), (6/7) and (7/7) according to the number of TA repeats. Therefore patients with the wild type (WT) genotype (6/6) (33% in the Caucasian population) are homozygous with 6 repeats of the TA insertion. Patients with the (7/7) genotype are homozygous with 7 TA repeats while the heterozygous genotype (6/7) consists of 1 allele with 6 TA repeats and of 1 with 7 TA repeats. Patients being heterozygous or homozygous for this variant allele (also named UGT1A1*28) show a reduced expression of the UGT1A1 enzyme resulting in lower rates of bilirubin and SN-38 glucuronidation^[13]. Compared to the wild type genotype (6/6) patients carrying the (7/7) genotype displayed a 70% reduction in transcriptional activity and are, by the attenuated expression of UGT1A1, theoretically predisposed to SN-38 associated side effects^[14]. Apart from that, there are rare genotypes with less than five or more than seven TA repeats leading to variable enzyme levels.

A number of trials have provided evidence of an association between UGT1A1 gene polymorphism and increased toxicity in patients who received irinotecan^[15-18]. Comparability and appliance of the results of these trials is difficult because of the various regimens and irinotecan dosages used. Due to the widespread use of irinotecan and the associated risk of severe side effects the question of defining subgroups of patients susceptible to irinotecan-related toxicities is of eminent clinical importance.

This retrospective analysis included a subgroup of 105 patients with mCRC who were treated with an irinotecan-based chemotherapy within a large prospective randomized multicenter phase III study which investigated the role

of a low-dose irinotecan-based chemotherapy in patients with metastatic or advanced colorectal cancer (FIRE-trial)^[19]. Patients underwent UGT1A1 genotyping in order to evaluate UGT1A1 as a predictor for drug efficacy and/or toxicity for patients with low-dose irinotecan-based chemotherapy and to provide a future tool for a patient tailored chemotherapy.

MATERIALS AND METHODS

Patient selection

One hundred and five Caucasian patients with mCRC were included in this analysis. The study population represents a subgroup of patients treated within the FIRE-trial. In the FIRE-trial a total of 492 patients from 56 centres were included, of which 478 patients could be evaluated for efficacy and toxicity. After an amendment UGT1A1 genotyping was offered and 105 patients were included in this subgroup analysis within the ongoing main study. The local ethics committees of the participating centres approved both the study protocol of the FIRE-trial and the amendment for UGT1A1 genotyping. Since the trial was a multicentre trial, monitoring for consistency was done by an external monitoring expert (ClinAssess GmbH, Leverkusen, Germany). Patients gave written informed consent prior to any study-specific procedures.

Patients with known Gilbert's syndrome or known DPD-deficiency were not allowed to enter the trial.

Treatment regimen

Patients within the FIRE-trial were randomly assigned to a modified FOLFIRI (mFOLFIRI) or modified IROX (mIROX) protocol. Randomisation was done by the external monitoring expert after an eligibility check. Inclusion criteria included serum bilirubin ≤ 1.25 or ≤ 1.5 of the upper institutional limits without or with hepatic metastasis. The modified FOLFIRI consisted of irinotecan 80 mg/m² i.v. over 30 min, folinic acid 500 mg/m² i.v. over 120 min, followed by 5-FU 2000 mg/m² i.v. over 24 h weekly for 6 wk. The modified IROX consisted of oxaliplatin 85 mg/m² i.v. over 120 min every 2 wk and irinotecan 80 mg/m² i.v. over 30 min weekly. For both arms, treatment cycles were repeated on day 50. Pre-medication with atropine (s.c.) was given routinely to prevent acute anti-cholinergic syndrome. Antiemetics were given according to good clinical practice and the local standards of the participating centres (generally 5-HT₃ antagonists). Treatment continuation was intended until progression, until unacceptable toxicity or confirmed complete response (CR).

Therapy was postponed for at least one week until bone marrow recovery or resolution of side effects (diarrhoea \geq grade 1, mucositis \geq grade 1, leucocytopenia \geq grade 2, thrombocytopenia \geq grade 1 or any other toxicity \geq grade 2). In case of toxicity, defined as diarrhoea \geq grade 3, mucositis \geq grade 3, leucocytopenia grade 4, thrombocytopenia \geq grade 3, severe obstipation \geq 96 h or hand-foot-syndrome \geq grade 3, a dose reduction of irinotecan and 5-FU to 80%

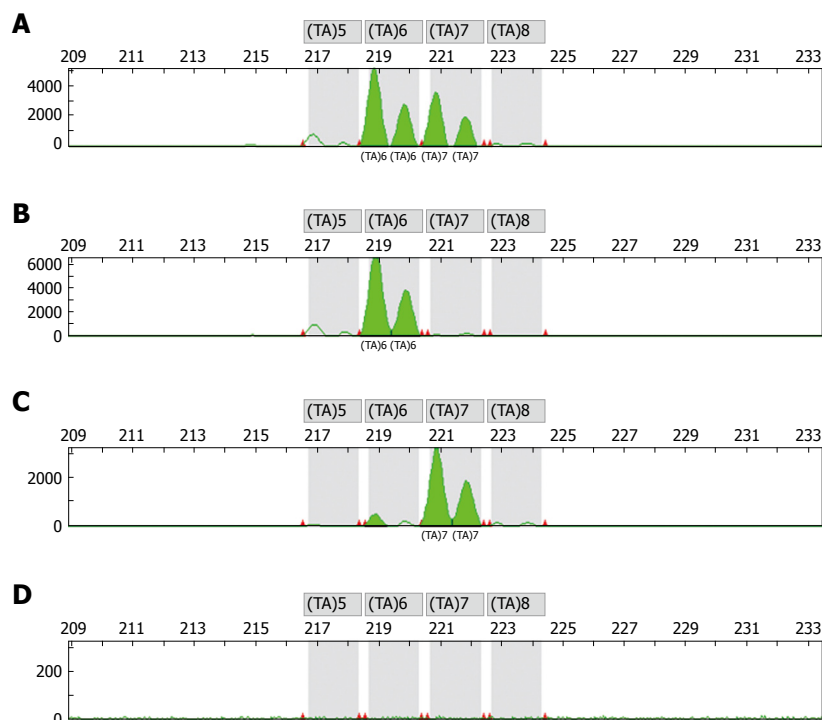


Figure 1 Electropherograms/gene scanning: Example results of TA promoter region of the *UGT1A1* gene. A: Control heterozygous genotype (6/7); B: Patient homozygous genotype (6/6); C: Patient homozygous genotype (7/7); D: Negative control.

was mandatory. Doses of irinotecan and oxaliplatin were reduced to 80% for obstipation \geq grade 3. Moreover, a dose reduction of oxaliplatin was required in cases of persistent paraesthesia (dose reduction 25%), painful paraesthesia with duration of > 7 d (dose reduction 25%) or paraesthesia with functional impairment with duration of > 7 d (dose reduction 50%).

In case of any toxicity as described above, following cycles were begun at a reduced dose level as defined by the scheme above.

***UGT1A1* genotyping**

Genotyping studies were performed by an independent laboratory (OncoScreen GmbH, Jena, Germany). Prior to any chemotherapy blood samples were collected for DNA isolation and determination of genotypes. DNA extraction, preparation and genotyping was performed using the methods as previously described^[12,14,16]. DNA was extracted from peripheral blood leucocytes using standard methods. A two-extra-nucleotide insertion (TA) within the TATA box resulting in the sequence (TA)₇TAA (-39 to 53) was researched. Genotypes were identified by gene scan analysis on the ABI 310 sequencer of the TATAA box in the promoter region of the *UGT1A1* gene. Figure 1 displays an electropherogram with typical overlapping peaks to distinguish the WT genotype from the heterozygous and homozygous genotype (Figure 1). The design of the primers to specifically amplify the TA expansion was done with support of GenBank AF297093.

Data collection

Standard evaluation by history, physical examination and routine laboratory tests (including complete blood count, chemical profile and electrolyte determination) were performed prior to the beginning of each cycle.

Drug administration, performance status and toxicity or adverse events were recorded after each cycle of chemotherapy. Toxicity was graded according to the NCI-CTC Classification (version 3.0). Imaging studies using computed tomography (CT) or magnetic resonance imaging (MRI) were performed prior to the beginning of each following cycle as well as for confirmation 4 wk after the end of chemotherapy. Data collection was monitored by an independent monitoring expert (ClinAssess GmbH, Leverkusen, Germany) and was done by an external data manager (Estimate GmbH, Augsburg, Germany).

Response evaluation

Patients' response was assessed by standard WHO criteria, as follows: complete response (CR) was defined as the disappearance of all known disease, documented by at least two observations not less than 4 wk apart, while partial response (PR) was defined as a decrease by at least 50% of the sum of the products of the largest perpendicular diameters of all measurable lesions, as determined by two observations not less than 4 wk apart. Stable disease (SD), lasting for at least 6 wk from the start of the study (i.e. first drug administration), was defined as a $< 50\%$ decrease and $< 25\%$ increase in the sum of the products of the largest perpendicular diameters of all measurable lesions. Progressive disease (PD) was defined as a $> 25\%$ increase in the size of at least one bidimensional or unidimensional measurable lesion, or the appearance of a new lesion.

Endpoints and statistical methods

The primary endpoint of the FIRE-trial was time to progression (TTP). Secondary endpoints included response rate (RR), overall survival (OS), resectability rate, toxicity and quality of life. TTP was defined as the interval

Table 1 Patient characteristics and UGT1A1 status (%)

	Total (<i>n</i> = 103)	WT (6/6) (<i>n</i> = 41)	(6/7) (<i>n</i> = 52)	(7/7) (<i>n</i> = 10)
Age (yr) median (range)	64 (41-79)	63 (41-76)	65 (42-79)	65 (52-74)
Gender (M/F)	70.5/29.5	70.7/29.3	67.3/32.7	80.0-20.0
Colon cancer	59.0	51.2	63.5	80.0
Rectal cancer	41.0	48.8	36.5	20.0
Adjuvant pre-treatment	35.2	43.9	30.8	20.0
KPS 70%-90%	43.8	29.3	51.9	70.0
KPS 100%	56.2	70.7	48.1	30.0
LDH ≤ 240 U/L	55.2	61.0	48.1	70.0
LDH > 240 U/L	44.8	39.0	51.9	30.0

KPS: Karnofsky performance status; LDH: Lactate dehydrogenase.

between the start of therapy and first documentation of disease progression. OS was measured from the date of starting treatment to the date of death from any cause (intent-to-treat). Probability of survival and TTP were estimated using the Kaplan-Meier method^[20]. Statistical comparisons between different genotypes were determined by using the χ^2 -test. Differences in OS and TTP were analyzed using the log-rank test. A difference of $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

The median age was 64 years with a gender distribution of 70.5% male and 29.5% female patients. 59% of the patients suffered from metastatic colon and 41% from metastatic rectal cancer. Performance status and adjuvant pre-treatment was similar between WT and heterozygous genotype patients. Detailed patient characteristics are provided in Table 1. Patients in the main trial were stratified according to performance status, lactate dehydrogenase (LDH) and adjuvant pre-treatment. These patients were well balanced between the two treatment arms.

Distribution of the UGT1A1 status

The distribution of the *UGT1A1* genotypes was analysed in 105 of 478 patients evaluated within the FIRE-trial. The majority of the patients (49.5%) had the heterozygous genotype (6/7), 39.0% showed the WT genotype (6/6) and the homozygous genotype (7/7) was found in 9.5% of the analyzed patients. There were also two single cases of the rare genotype (5/7) which were not included for further evaluation due to the low frequency. Results of the distribution of the genotypes are given in Table 2.

Response to chemotherapy with regard to UGT1A1 genotype

The overall response rate (ORR = CR + PR) was similar between the WT genotype (6/6) and the (6/7, 7/7) genotypes (43.2% *vs* 44.3% $P = 0.75$). However, the disease control rate (DCR = CR + PR + SD) appeared to be lower within patients carrying the WT genotype (6/6) as compared to those patients with the (6/7,

Table 2 Genotype distribution with regard to treatment *n* (%)

UGT1A1 status	FOLFIRI	IROX	All
WT (6/6)	19 (34.5)	22 (44.0)	41 (39.0)
(6/7)	28 (50.9)	24 (48.0)	52 (49.5)
(7/7)	7 (12.7)	3 (6.0)	10 (9.5)
(5/7)	1 (1.8)	1 (2.0)	2 (1.9)

FOLFIRI: 5-fluorouracil, leucovorin, irinotecan; IROX: Irinotecan, oxaliplatin.

Table 3 Response to treatment with regard to UGT1A1 status *n* (%)

	WT (6/6)	(6/7, 7/7)	All	χ^2 test [WT <i>vs</i> (6/7, 7/7)]
CR	3 (8.1)	7 (11.5)	10 (10.2)	
PR	13 (35.1)	20 (32.8)	33 (33.7)	
SD	11 (29.7)	29 (47.5)	40 (40.8)	
PD	7 (18.9)	5 (8.2)	12 (12.2)	
NA	3 (8.1)	-	3 (3.1)	
OR	16 (43.2)	27 (44.3)	43 (43.9)	$P > 0.05$
DCR	27 (73.0)	56 (91.8)	83 (84.7)	$P < 0.05$

CR: Complete remission; PR: Partial remission; SD: Stable disease; PD: Progressive disease; OR: Overall response rate (CR + PR); DCR: Disease control rate (CR + PR + SD); NA: Not available.

7/7) genotypes (73.0% *vs* 91.8%, $P = 0.008$). Detailed response data are presented in Table 3.

There are no efficacy data for the 2 patients carrying the rare genotype (5/7). One patient quit the trial while the other died early during therapy.

Toxicity with regard to UGT1A1 genotype

Overall toxicity according to genotype and treatment arm is given in Table 4. The incidence of grade 3-4 toxicity did not differ significantly between the treatment arms mFOLFIRI and mIROX both for patients with the WT (6/6) and those carrying the (6/7), (7/7) and (5/7) genotypes.

Haematological toxicity was generally mild (grade 3 and 4 < 5%). Comparing the incidence of grade 1-2 and grade 3-4 haematotoxicity in patients with the WT (6/6) genotype to those with the (6/7, 7/7) genotypes, there were no significant differences regarding leucocytopenia, anaemia or thrombocytopenia ($P > 0.05$) (Table 5).

Regarding non-haematological toxicity, the incidence of grade 3-4 delayed diarrhoea appeared higher in patients with the (6/7, 7/7) genotypes compared to the WT (6/6) genotype even though this difference did not reach the statistical level of significance (13.0% *vs* 6.2%, $P = 0.08$) (Table 6).

Both cases with the rare genotype (5/7) were excluded from the statistical evaluation. However, they experienced grade 3-4 toxicity in the course of the treatment (delayed diarrhoea and leucocytopenia, per cycle analysis).

There were no significant differences regarding toxicity-related treatment discontinuations or dose adjustments. Dose delays in patients with the WT (6/6) were observed in 27.2%, compared to 21.5% in patients with the (6/7, 7/7) genotypes ($P = 0.071$). Dose reductions

Table 4 Toxicity and UGT1A1 status according to treatment arm *n* (%)

Toxicity WHO	mFOLFIRI				mIROX				χ^2 test
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4	
WT (6/6)	2 (33.3)	10 (33.3)	6 (37.5)	1 (33.3)	-	17 (53.1)	3 (23.1)	2 (50.0)	$P > 0.05$
(6/7)	3 (50.0)	16 (53.3)	7 (43.8)	2 (66.7)	1 (100)	14 (43.8)	7 (53.8)	2 (50)	$P > 0.05$
(7/7)	1 (16.7)	4 (13.3)	2 (12.5)	-	-	1 (3.1)	2 (15.4)	-	$P > 0.05$
(5/7)	-	-	1 (6.3)	-	-	-	1 (7.7)	-	$P > 0.05$

Table 5 Haematological toxicity and UGT1A1 status (per cycle analysis) cycles (%)

	WT (6/6)			(6/7, 7/7)			χ^2 test
	Grade 0	Grade 1-2	Grade 3-4	Grade 0	Grade 1-2	Grade 3-4	
Leucocytes	69 (61.1)	43 (38.1)	1 (0.9)	102 (52.8)	85 (44.0)	6 (3.1)	$P > 0.05$
Neutropenic fever	113 (100.0)	-	-	191 (98.9)	1 (0.5)	1 (0.5)	$P > 0.05$
Anaemia	30 (26.5)	80 (70.8)	3 (2.7)	47 (24.3)	142 (73.6)	4 (2.1)	$P > 0.05$
Thrombocytes	89 (78.8)	23 (20.4)	1 (0.9)	157 (81.3)	36 (18.7)	-	$P > 0.05$

Table 6 Non-haematological toxicity and UGT1A1 status (per cycle analysis) cycles (%)

	WT (6/6)			(6/7, 7/7)			χ^2 test
	Grade 0	Grade 1-2	Grade 3-4	Grade 0	Grade 1-2	Grade 3-4	
Nausea	35 (30.9)	77 (68.1)	1 (0.9)	69 (35.8)	122 (63.2)	2 (1.0)	$P > 0.05$
Vomiting	77 (68.1)	35 (31.0)	1 (0.9)	123 (63.7)	69 (35.8)	1 (0.5)	$P > 0.05$
Diarrhoea early	81 (71.7)	30 (26.5)	2 (1.8)	149 (77.2)	42 (21.8)	2 (1.0)	$P > 0.05$
Diarrhoea delayed	51 (45.1)	55 (48.7)	7 (6.2)	78 (40.4)	90 (46.6)	25 (13.0)	$P > 0.05$
Mucositis	97 (85.8)	16 (14.2)	-	160 (82.9)	31 (16.1)	2 (1.0)	$P > 0.05$

Table 7 Dose reductions, treatment delays and UGT1A1 status (per cycle analysis) *n* (%)

	WT (6/6)	(6/7, 7/7)	χ^2 test
Dose reduction	22 (19.3)	48 (25.1)	$P > 0.05$
Dose delayed	31 (27.2)	41 (21.5)	$P > 0.05$

were more frequently observed in patients with the (6/7, 7/7) genotypes compared to patients who carried the WT (6/6) genotype (25.1% *vs* 19.3%, $P = 0.24$) (Table 7).

TTP and survival with regard to UGT1A1 genotype

TTP was similar between the WT (6/6) and the (6/7, 7/7) genotypes with 8.1 mo and 8.2 mo respectively ($P = 0.971$) (Figure 2). Moreover, there was no difference in survival between the WT compared to the genotypes [WT (6/6) *vs* (6/7, 7/7); 21.2 mo *vs* 18.9 mo, $P = 0.725$] (Figure 3).

DISCUSSION

More than 50 genetic alterations of the UGT1A1 gene locus have previously been described^[21]. Among the Caucasian population the UGT1A1*28 genotype (7/7) plays the most important role for the development of Gilbert's syndrome and an increased toxicity related to irinotecan-based chemotherapy.

The distribution of the UGT1A1 genotype in this retrospective analysis was comparable to that described by Iyer and co-workers^[15]. They reported a frequency of the WT (6/6) genotype in 45% of the patients (39% in the

present analysis), the (6/7) genotype in 35% (49.5% in the present analysis), and the (7/7) genotype in 20% (9.5% in the present analysis) respectively^[15]. Moreover, another analysis of 51 patients suffering from non-small cell lung cancer (NSCLC) showed a comparable distribution of the genotypes WT (6/6) 49%, (6/7) 36% and (7/7) 15%^[22].

Prior to discussion of an association of toxicity and UGT1A1 genotype the following preliminary remarks are inevitable: patients randomized within the FIRE-trial received different irinotecan-based regimes. The incidence of delayed diarrhoea, neurotoxicity, leucocytopenia and thrombocytopenia was more frequent in the IROX arm compared to the FOLFIRI arm^[19]. Differences in the frequency of adverse events might therefore partly influence the present analysis. Moreover, 7.3% of the patients (mainly with rectal cancer) had previously received locoregional radiotherapy prior to study entry. Local irradiation may also play a role in the development of severe diarrhoea or may at least worsen it, independent of the UGT1A1 genotype^[23]. Finally, in analogy to the study of Ando *et al*^[14], there is a certain bias by excluding patients with elevated pre-treatment bilirubin levels.

Among non-haematological toxicities, the incidence of grade 3 and 4 delayed diarrhoea was twice as high in our study population with a homozygous (7/7) or heterozygous (6/7) genotype compared to those carrying the wild type genotype WT (6/6), even though the difference did not reach the level of significance (13.0% *vs* 6.2%; $P = 0.08$). Nevertheless, this trend is supported by the data provided by Marcuello *et al*^[23] who found a significant correlation of the UGT1A1 genotype and

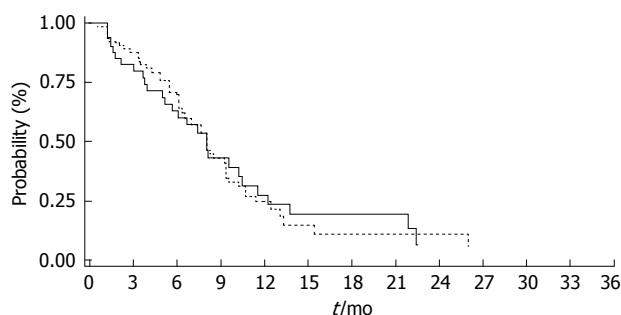


Figure 2 Time to progression and UGT1A1 status. WT (6/6) 8.1 mo (range = 5.7-10.5) vs (6/7, 7/7) 8.2 mo (range = 6.3-9.4), $P > 0.05$; WT: Solid line, (6/7, 7/7): Dashed line.

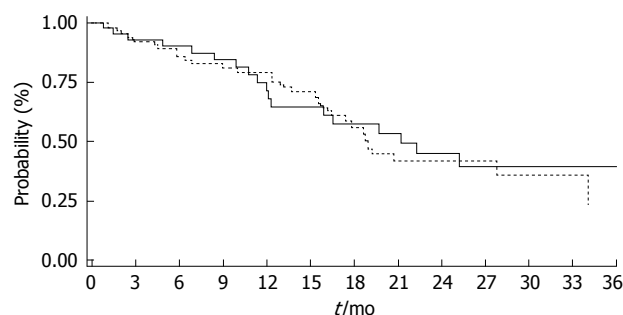


Figure 3 Overall survival and UGT1A1 status. WT (6/6) 21.2 mo (range = 12.3-41.0) vs (6/7, 7/7) 18.9 mo (range = 16.4-34.1), $P > 0.05$; WT: Solid line, (6/7, 7/7): Dashed line.

the frequency of severe delayed diarrhoea [WT (6/6) 17%, (6/7) 33%, (7/7) 70%; $P = 0.005$]. Moreover, Ando *et al.*^[14] reported on a 3.5-fold higher frequency of the UGT1A1*28 genotype in patients who suffered from severe diarrhoea and leucocytopenia during irinotecan-based chemotherapy. Taking the UGT1A1*28 genotype as a significant risk factor for the development of severe toxicity the authors suggest that UGT1A1 genotyping serves as a useful tool in predicting toxicity in patients receiving irinotecan. It is critical to state that the majority of these analyses were done retrospectively. In contrast, an exploratory analysis presented by Seymour *et al.*^[24] could not confirm such an association of UGT1A1 to irinotecan-related toxicity. Moreover comparability is hampered by the different doses of irinotecan applied within these trials. The interpretation of our data is limited by a slight imbalance with more patients with the (6/7) and (7/7) genotype being treated with the modified FOLFIRI protocol. Therefore, a tendency towards an increased rate of diarrhoea which might be associated with exposure to 5-FU can not be ruled out.

Comparing the incidence of severe haematotoxicity in patients with the WT (6/6) genotype to those with the (6/7, 7/7) genotype in our analysis, there were no significant differences regarding leucocytopenia, anaemia or thrombocytopenia ($P > 0.05$). The incidence of haematological toxicity was generally low, as expected from other trials with comparable doses of weekly scheduled irinotecan^[25,26]. Nevertheless, these findings are in contrast to previously published results of patients with mCRC treated with IRIFUFOL or FOLFIRI^[18]. In the study of Rouits *et al.*^[18] grade 3-4 neutropenia was significantly associated with the genotypes (6/7, 7/7). The results of a retrospective analysis of 128 Chinese patients with mCRC who received biweekly irinotecan indicate that the heterogenous and homogenous genotype UGT1A1 (6/7) and (7/7) predicts severe neutropenia and diarrhoea but not treatment efficacy^[27]. Necessity for dose reduction was significantly associated with the (6/7) or (7/7) genotype (42.3% vs 12.7%; $P < 0.01$).

Moreover, Innocenti *et al.*^[16] also observed no case of grade 4 neutropenia among patients with the wild type genotype WT (6/6), whereas 50% among patients with the homozygous genotype (7/7), and 12.5% of those with the heterozygous genotype (6/7) experienced grade 4 neutro-

penia. In their study irinotecan was administered at a dose of 350 mg/m² every 3 wk. Accordingly, data from Roth *et al.*^[28] indicated that the risk of severe neutropenia was higher among patients carrying the homogenous genotype (7/7). Interestingly, female sex was superior to UGT1A1 in predicting grade 4 neutropenia. Contrary, Marcuello *et al.*^[23] found an increased, but not significantly increased, haematological toxicity among patients with the genotypes (6/7, 7/7) during single-agent or combination chemotherapy with irinotecan given at a dosage of 350 mg/m² every 3 wk or 180 mg/m² every 2 wk.

The literature regarding UGT1A1 genotyping and irinotecan-related toxicity is heterogeneous and to some extent conflicting which may be partly explained by the retrospective character of most studies^[14,16,18,23,24]. Another aspect contributing to the heterogeneity of the results is due to the variety of irinotecan dosages and schedules which also applies for the present study. Patients within our trial received dose reduced and modified FOLFIRI and IROX. Patients in both treatment arms received weekly scheduled irinotecan (80 mg/m²) in combination with 5-FU 2000 mg/m² weekly or oxaliplatin 85 mg/m² biweekly. We assume that the mild toxicity and the absent impact of UGT1A1 genotype on toxicity in our study are a result of the low irinotecan dose. Stewart *et al.*^[29] concluded from their study of low-dose protracted irinotecan in pediatric patients that UGT1A1 genotyping is not a useful prognostic factor in predicting toxicity. Hoskins *et al.*^[30] advised a genotype-tailored therapy only for those patients who receive irinotecan at higher doses. Patients carrying the genotype UGT1A1*28 who receive irinotecan up to a dose of 150 mg/m² are at the same risk of experiencing severe neutropenia as any patients. Consequently, UGT1A1 genotyping is not generally recommended.

The response rate in the present analysis was similar between the WT (6/6) genotype and the (6/7, 7/7) genotypes (43.2% vs 44.3%, $P > 0.05$). Interestingly, the disease control rate appeared to be higher in patients with the (6/7, 7/7) genotypes compared to those with the WT (6/6) genotype (91.8% vs 73.0%, $P = 0.008$). However, this finding did not result in any advantage regarding TTP or OS.

Douillard *et al.*^[31] reported a TTP of 6.7 and an OS of 17.4 mo in previously untreated patients with mCRC who had received irinotecan and FU/FA. A large Italian study reported a comparable TTP of 7 mo and an OS of 14 mo

for the FOLFIRI regimen given as 1st line chemotherapy for mCRC^[32]. Ashley *et al*^[33] have found a TTP of 6.7 mo and an overall survival of 17.3 mo in previously untreated patients with mCRC who had received an IROX regimen consisted of oxaliplatin 85 mg/m² and irinotecan 200 mg/m² every 3 wk. These data are supported by the findings of Goldberg *et al*^[34] who found a similar TTP of 6.5 mo and an overall survival of 17.4 mo for patients who were treated with IROX. Comparing these data to those of our study a loss of activity due to the dose modifications with low-dose irinotecan dosage can be ruled out.

In a large prospective study conducted by Toffoli *et al*^[35], the response rate was higher in mCRC patients with the homozygous genotype (7/7) compared to the wild type genotype WT (6/6). These patients experienced a slightly improved survival of approximately 2 mo ($P > 0.05$). One may argue that in patients with the (6/7) or (7/7) genotype there is less detoxification of SN-38 resulting in higher blood levels of the active compound, more anti-tumor effect and therefore a better response rate. However, Marcuello *et al*^[23] observed no statistically significant impact of UGT1A1 gene polymorphism on response rate but a trend towards an improved OS in patients with the WT (6/6) genotype compared to patients with the (6/7) or (7/7) genotype (33 *vs* 21 mo; $P = 0.09$). This is partly explained by dose reductions in patients with homozygous or heterozygous genotypes (6/7, 7/7) because of severe diarrhoea. Another recent study reported on prospectively genotyped patients suffering from mCRC who had received either 1st line irinotecan/capecitabine or 2nd line single-agent irinotecan. Response rates, numbers of dose reductions and applied chemotherapy cycles were similar within the different genotypes^[36].

An important factor contributing to irinotecan metabolism is the existence of several UGT1A1 isoforms and their distribution among different patient populations^[37]. There exist an increasing number of reports on genetic variants of UGT1A1 as well as SNPs in the coding region of the gene locus potentially influencing drug metabolism. Moreover, other enzymes of the UGT1 family like UGT1A7 and UGT1A9 are also involved in the glucuronidation of SN-38^[38-40]. Patients of African, Caucasian and Asian descent show a different gene frequency of the UGT1A1*28 gene variant^[12]. When analysing UGT1A1 genotypes and irinotecan-related adverse events the different variants of the UGT1A1 gene locus among an ethnic population must be taken into consideration^[41]. Moreover, there is strong evidence that several other individual factors apart from ethnic affiliation may influence the irinotecan metabolism^[42].

In conclusion, UGT1A1 genotyping alone does not allow characterizing subgroups of patients who are at an increased risk of life threatening toxicity during low-dose irinotecan-based chemotherapy. Due to the incoherent findings of several small trials, a larger prospective phase III trial is warranted. The metabolism of irinotecan is highly complex with different UGT enzymes and drug transporters involved. Distinction between high-dose and low-dose irinotecan is of eminent importance when considering the use of UGT1A1 genotyping. There is

a need for a diagnostic panel including the testing of multiple gene polymorphisms that more reliably predicts toxicity. On the other hand, beside a comprehensive patient education, and the escalation of supportive therapy, dose modifications and alternative treatment schedules may help to provide patients who are at high risk with a safe irinotecan-based chemotherapy.

COMMENTS

Background

Irinotecan is one of the most effective chemotherapeutic agents in the treatment of colorectal cancer. Among other side effects irinotecan can lead to neutropenia and delayed diarrhoea. The active metabolite of irinotecan, SN-38, is inactivated by uridine disphosphate glucuronosyl transferase 1 (UGT1A1), the same enzyme by which bilirubin is metabolised. Genetic polymorphisms of the UGT1A1 gene result in variable levels of the enzyme. According to the number of TA repeats in the enhancer region of the gene a wild type genotype (6/6) can be differentiated from the heterozygous genotype (6/7) and the homozygous genotype (7/7).

Research frontiers

Preclinical and clinical studies have revealed a close dependency of the activity of UGT1A1 enzyme and the occurrence of irinotecan-associated side effects. Existing data are contradictory to some extent or are derived from heterogenous or small study populations. Therefore and against the background of other metabolic ways of toxification and detoxification the research hotspot is how to apply genetic testing of UGT1A1 gene polymorphisms to predict toxicity and efficacy of an irinotecan-based chemotherapy.

Innovations and breakthroughs

Recent studies have emphasized the impact of UGT1A1 genotyping to predict toxicity and outcome in patients undergoing an irinotecan-based chemotherapy. Distribution of the genotype in this study was well in line with data in the literature. No significant differences could be noticed between the homozygous and the heterozygous genotype compared to the wild type genotype in terms of efficacy, toxicity of higher grades, treatment delay or dose reduction. This is a retrospective study to report that UGT1A1 genotyping appeared not to be useful for predicting treatment efficacy and irinotecan-associated side effects in patients receiving low-dose irinotecan for mCRC. Furthermore, our analysis supports the idea that UGT1A1 genotyping should be considered when using irinotecan at a higher dosage.

Applications

By understanding the limitations of UGT1A1 genotyping and by understanding the complexity of irinotecan metabolism, this study may just add another piece of the puzzle for the future development of a patient-tailored chemotherapy with genotyping as one tool among others.

Terminology

The chemotherapeutic agent irinotecan is metabolised by the enzyme UGT1A1, the same enzyme by which bilirubin is metabolised. Genetic polymorphisms resulting in a decreased amount of the enzyme can lead to enhanced irinotecan-associated toxicity. Pre-existing data have partly shown genetic subgroups of patients with different toxicity and treatment efficacy under chemotherapy with irinotecan.

Peer review

The manuscript by Schulz *et al* describes the impact of UGT1A1 gene polymorphism on toxicity and efficacy of irinotecan-based regimens in metastatic colorectal cancer. The authors demonstrate that the genetic polymorphism of the UGT1A1 gene does not influence treatment efficacy. The manuscript is well written, the methods are adequately chosen.

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S- Editor Tian L L- Editor O'Neill M E- Editor Lin YP



Patient-reported outcomes in subjects with neuroendocrine tumors of the pancreas

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Received: July 28, 2009 Revised: August 29, 2009

Accepted: September 5, 2009

Published online: October 28, 2009

Abstract

AIM: To assess the patient-reported outcomes (PROs) of pancreatic neuroendocrine tumor (PNET) patients.

METHODS: Fifty-one consecutive patients (21 male, 30 female, 61.0 ± 10.3 years) with proven PNETs were studied. An SF-12 questionnaire capable of exploring the physical (PCS) and mental (MCS) aspects of daily life was used. Four questionnaires were also used [12 items General Health Questionnaire (GHQ-12) for non-psychotic psychiatric disorders, State Trait Anxiety Inventory (STAI) Y-1 and Y-2 for anxiety and BDI-II for depressive symptoms] to explore the psychological aspects of the disease. Forty-four sex- and age-matched Italian normative subjects were included and evaluated using the SF-12, STAI Y-1 and Y-2 questionnaires.

RESULTS: Seven patients refused to participate to the study; they were clinically similar to the 44 participants who agreed to complete the questionnaires. PNET patients had a PCS score (44.7 ± 11.0) were not significantly different from the norms (46.1 ± 9.9 , $P = 0.610$), whereas the MCS score was significantly lower in patients (42.4 ± 13.0) as compared to the norms (48.2 ± 9.8 , $P = 0.036$). GHQ-12 identified 11 patients (25.0%) as having non-psychotic psychiatric disorders.

The STAI scores were similar in the patients and in the normative population. Finally, BDI-II identified eight patients (18.2%) with moderate depression and 9 (20.5%) with mild depression whereas 27 patients (61.4%) had no depression.

CONCLUSION: The PNET patients had a good physical but an impaired mental component of their quality of life; in addition, mild or moderate depressive symptoms are present in about 40% of PNET patients.

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Key words: Endocrine gland neoplasms; Pancreatic neoplasms; Somatostatin; Quality of life; Quality indicators; Health care

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Pezzilli R, Campana D, Morselli-Labate AM, Fabbri MC, Brocchi E, Tomassetti P. Patient-reported outcomes in subjects with neuroendocrine tumors of the pancreas. *World J Gastroenterol* 2009; 15(40): 5067-5073 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5067.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5067>

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs) are a heterogeneous group of rare neoplasms, occurring in fewer than one in 100 000 people per year^[1]. These tumors have attracted considerable attention in recent years, both because they are relatively easy to palliate and because they demonstrate the chronic effects of the particular hormone whose level is elevated. In about 10%-50% of cases, the tumors are not associated with obvious signs or symptoms of hormone hypersecretion and are called non-functioning tumors^[2]. We have recently demonstrated that radical surgery continues to have a central role in the therapeutic approach to PNETs^[2], and

that medical treatment has a precise role in functioning neuroendocrine tumors. Even if the survival rate is good, especially in those patients who were resected, there is no extensive data available on the quality of life (QoL) in these patients compared to the general population. In addition, the majority of studies published on this topic until now examined a Scandinavian population^[3-10], and it is well known that some differences in perceiving well-being exist among different cultures^[11]. Finally, there are no studies specifically focusing on the localization site of neuroendocrine tumors such as PNETs. Therefore, we carried out this prospective study on a large series of subjects with PNETs in order to assess the patient-reported outcomes (PROs), using different questionnaires capable of exploring the physical and mental aspects of their QoL as well as various psychological factors.

MATERIALS AND METHODS

Patients

Fifty-one consecutive patients with pathological confirmation of PNET who were admitted as outpatients to our Department from January to April 2009 were enrolled in the study. The histological specimens were obtained at surgery in 41 patients and at percutaneous biopsy using computed tomography/ultrasonography/endoscopic ultrasonography in the remaining 10 patients. The epidemiological and clinical data of the 51 patients studied are reported in Table 1. There were 21 males (41.2%) and 30 females (58.8%). The mean age of the patients was 61.0 ± 10.3 years (range, 34-86 years) and the duration of the disease was 5.8 ± 5.2 years (range, 1-28 years). Forty-one patients (80.4%) were married and 10 (19.6%) were single. Thirteen patients had finished elementary school (25.5%), 18 had finished middle school (35.3%), 13 had a high school diploma (25.5%) and seven had a university degree (13.7%). The majority of the patients were still working (38, 74.5%) while 13 were retired (25.5%). Twenty patients in the present series were drinkers (39.2%) while 22 (43.1%) were smokers. Thirty-five patients (68.6%) had one or more comorbidities (arterial hypertension in 21 patients, cardiac diseases in nine patients, chronic obstructive pulmonary diseases in four patients, gastrointestinal diseases such as peptic ulcers, gallstones and/or colon polyposis in eight patients, neuroendocrine diseases such as thyroid or hypophysis diseases in five patients, urogenital diseases in three patients, and other diseases in the remaining four patients) and 41 (80.4%) received drugs for causes not related to the PNET. Forty patients (78.4%) had undergone surgery at least once for causes unrelated to the neuroendocrine neoplasia (cholecystectomy in eight patients, appendectomy in 22 patients, inguinal hernioplasty in seven patients, other abdominal surgery in three patients, tonsillectomy and other otorhinolaryngological procedures in 17 patients, urological surgery in seven patients, gynecologic procedures in 10 patients and other surgical procedures in three patients). Forty-one of the 51 patients (80.4%) underwent surgery for their neuroendocrine pancreatic

Table 1 Demographic and clinical characteristics of the 44 patients who completed the questionnaires (mean \pm SD)

	<i>n</i> (%)
Sex	
Males	18 (40.9)
Females	26 (59.1)
Age at interview (yr)	61.0 \pm 9.8
Disease duration (yr)	5.8 \pm 5.4
Body mass index (kg/m ²)	25.9 \pm 4.3
Marital status	
Single/Widowed/Divorced	10 (22.7)
Married	34 (77.3)
Diploma	
Elementary school	11 (25.0)
Middle school	18 (40.9)
High school	10 (22.7)
University degree	5 (11.4)
Job	
Current workers	32 (72.7)
Managers	6
Employees	16
Industrial workers	3
Housewives	7
Retired	12 (27.3)
Alcohol habit	
Alcohol drinkers	18 (40.9)
Current drinkers	14
Drinking duration (yr)	40.2 \pm 12.5
Smoking habit	
Smokers	20 (45.5)
Actual smokers	5
No. of cigarettes smoked per day	10.6 \pm 5.9
Smoking duration (yr)	28.7 \pm 13.3
Comorbidities	30 (68.2)
Non disease specific drugs	35 (79.5)
Previous surgery not due to neuroendocrine tumors	33 (77.0)
Surgery due to pancreatic neuroendocrine tumors	35 (79.5)
Pancreatic head resection	8
Distal pancreatectomy	20
Total pancreatectomy	2
Enucleation	5
Status of neuroendocrine tumors	
Disease free	25 (56.8)
Lymph node involvement/liver metastases	19 (43.2)
Specific drugs and treatment	
No drugs	30 (68.2)
Somatostatin analogues alone	9 (20.5)
Somatostatin analogues plus radiometabolic therapy	5 (11.4)
Pain in the month prior to complete the questionnaire	17 (38.6)
Dyspepsia	24 (54.5)
Diabetes	12 (27.3)

Data are reported as absolute and relative frequencies or mean \pm SD.

disease: resective surgery was performed in 36 patients and enucleation of the cancer in the remaining 5 patients. At the time of the study, 29 patients were disease-free (56.9%) and the remaining 22 had advanced disease (lymph node involvement or liver metastases). Seventeen patients (33.3%) were currently being treated medically: 11 (21.6%) with somatostatin analogues alone, and 6 (11.8%) with somatostatin analogues and radiometabolic treatment. Nineteen patients (37.3%) had experienced pain in the month prior to the interview whereas 30 patients (58.8%) had dyspeptic symptoms evaluated according to the Rome III criteria^[12]. Among the 30 patients with dyspeptic symptoms, the majority (25, 83.3%) had experienced post-

prandial distress syndrome and five had had heartburn (16.7%). Finally, 14 patients (27.5%) had diabetes secondary to pancreatic surgery.

The mean body mass index (BMI) was 25.7 ± 4.1 kg/m²; in particular, according to the WHO criteria^[13], 1 patient (2.0%) was underweight (BMI < 18.5 kg/m²), 22 (43.1%) had normal weight (BMI between 18.5 and 24.9 kg/m²), 21 (41.2%) were pre-obese (BMI between 25.0 and 30.0 kg/m²) and the remaining 7 (13.7%) were obese (BMI > 30 kg/m²).

Questionnaires

The Italian versions of the SF-12™ Health Survey (SF-12), State Trait Anxiety Inventory (STAI) Y-1 and Y-2, 12 items General Health Questionnaire (GHQ-12), and Beck Depression Inventory-II (BDI-II) were used for the purpose of the study.

The SF-12 questionnaires had previously been developed and tested on the Italian population in general^[14]. It consists of 12 items which generate two summary scales capable of exploring the physical and mental components. High scale scores of the SF-12 physical (PCS) and mental (MCS) component summaries represent a good QoL. The normative population tested with the SF-12 questionnaire included 61 434 Italian subjects; these subjects were randomly sampled from the electoral lists, regardless of their health status^[14]. The values of this group represent the average of the health-related QoL of the general Italian population. The stratified mean \pm SD values of the PCS and the MCS referring to 44 sex- and age-matched Italian subjects of this population were used as a normative group.

The GHQ-12 is a measure of current mental health and since its development^[15], it has been translated into the Italian language and has been used for the Italian population^[16,17]. The GHQ-12 has become a commonly used instrument for detecting psychiatric disorders^[18]. The scale asks whether the respondent has recently experienced a particular symptom or behavior. Each item is rated on a four-point scale (less than usual, no more than usual, slightly more than usual or much more than usual). The score ranges from 0 to 12^[19]; subjects with a score from 0 to 4 have a > 80% probability of having non-psychotic psychiatric disorders and they are generally considered cases (i.e. those subjects who needed psychological support) while those subjects with a score > 4 should be considered not affected by non-psychotic psychiatric disorders^[20].

Due to the fact that the GHQ-12 is a general questionnaire regarding the psychological aspects of daily life, we used the following two specific questionnaires: the STAI^[21] and the BDI-II^[22] to better evaluate anxiety disorders and depressive syndrome.

Regarding the assessment of anxiety, both state and trait anxiety can be assessed by using the 40 items of the STAI-Y-1 and Y-2^[21]. The state (Y-1) and the trait (Y-2) portions of the inventory each consist of 20 item Likert format statements. The STAI has been extensively validated and the Italian version has already been used^[23]. The scores of the two 20 item scales range from 20 to

80 and high scores represent a high level of anxiety^[21]. In brief, the state of anxiety can vary in intensity and fluctuate over time depending on the perceived threat. The trait of anxiety is a tendency to perceive a wide range of living conditions as threatening and to react to them with a high intensity; this trend remains latent until it is activated by stress associated with real or imagined dangers. The normative population tested with the STAI questionnaire included 2363 Italian working people sampled regardless of their health status^[23]. The stratified mean \pm SD values of the STAI Y-1 and the STAI Y-2 used with 44 sex- and age-matched Italian subjects of this population were used as a normative group.

The BDI-II^[22] is a 21-item self-report instrument which assesses the severity of depressive symptoms in adolescents and adults over the 2 wk prior to its use. Each item is rated on a 4-point scale (0-3) with total scores ranging from 0 to 63. For interpretation of the BDI-II, Beck *et al.*^[22] present a table of scores indicative of: severe (> 28); moderate (score ranging from 20 to 28) and mild (score ranging from 14 to 19) depression. Scores of ≤ 13 suggest an absence of depression. These scores observed in an American population having depressive symptoms can also be used for the respective Italian population^[24]. The Italian version of this questionnaire was used^[24].

All patients included in our study were fluent in the Italian language and the questionnaires were administered according to the recommendations suggested by the user manuals^[14,19,23,24].

Ethics

The study was approved by the Senior Staff Committee of the Department of Digestive Diseases and Internal Medicine of the University of Bologna and was carried out in accordance with the Helsinki Declaration of the World Medical Association. All study participants gave oral informed consent.

Statistical analysis

The descriptive statistics applied were: mean, SD and ranges as well as absolute and relative frequencies. Three-way ANOVA was applied in order to estimate the various effects related to the SF-12 and STAI scores by adjusting for age (increasing trend among the age categories) and gender (males *vs* females). The 95% confidence intervals (95% CIs) of the estimates were also calculated. One-way ANOVA, one-way linear term ANOVA, Pearson correlation, Fisher exact test, Pearson chi-squared and linear-by-linear association chi-square were also applied where appropriate.

All statistical evaluations were carried out by running the SPSS version 13.0 for Windows. Two-tailed *P* values less than 0.05 were considered statistically significant.

RESULTS

Forty-four (86.3%) of the 51 patients answered the questionnaires; the demographic and clinical characteristics of these patients are reported in Table 1. Seven patients

Table 2 Effects of neuroendocrine tumors of the pancreas on the SF-12 physical (PCS) and mental (MCS) component summaries estimated by means of three-way ANOVA adjusted for age (increasing trend among the age classes) and gender

	PCS		MCS	
	Effects (95% CI)	P value	Effects (95% CI)	P value
Overall effects of the disease (patients <i>vs</i> normative group)	-1.16 (-5.66 to 3.34)	0.610	-5.32 (-10.30 to -0.35)	0.036
Effects of the disease within males	-1.17 (-8.01 to 5.68)	0.735	-6.12 (-13.69 to 1.45)	0.112
Effects of the disease within females	-1.15 (-6.99 to 4.69)	0.697	-4.53 (-10.99 to 1.93)	0.167
Interaction between the effects of the disease and gender (males <i>vs</i> females)	-0.02 (-9.02 to 8.97)	0.996	-1.59 (-11.54 to 8.37)	0.752
Interaction between the effects of the disease and age	3.32 (-6.96 to 13.60)	0.522	9.54 (-1.84 to 20.92)	0.099
Interaction between the effects of the disease and age within males	4.10 (-11.88 to 20.09)	0.611	9.24 (-8.44 to 26.93)	0.301
Interaction between the effects of the disease and age within females	2.54 (-10.39 to 15.48)	0.697	9.84 (-4.47 to 24.15)	0.175
Interaction between the effects of the disease and age and gender	1.56 (-19.00 to 22.12)	0.880	-0.59 (-23.35 to 22.17)	0.959

95% CI: 95% confidence interval.

Table 3 Effects of neuroendocrine tumors of the pancreas on the STAI Y-1 (anxiety state) and Y-2 (anxiety trait) estimated by means of three-way ANOVA adjusted for age (increasing trend among the age classes) and gender

	STAI anxiety state (Y-1)		STAI anxiety trait (Y-2)	
	Effects (95% CI)	P value	Effects (95% CI)	P value
Overall effects of the disease (patients <i>vs</i> normative group)	-5.16 (-12.56 to 2.23)	0.169	0.77 (-5.84 to 7.39)	0.817
Effects of the disease within males	-4.66 (-16.98 to 7.67)	0.454	-0.48 (-11.50 to 10.54)	0.932
Effects of the disease within females	-5.66 (-13.84 to 2.51)	0.172	2.02 (-5.29 to 9.33)	0.584
Interaction between the effects of the disease and gender (males <i>vs</i> females)	1.00 (-13.79 to 15.79)	0.893	-2.50 (-15.72 to 10.73)	0.708
Interaction between the effects of the disease and age	7.69 (-7.10 to 22.48)	0.304	-2.58 (-15.81 to 10.64)	0.698
Interaction between the effects of the disease and age within males	5.30 (-19.35 to 29.95)	0.670	-0.05 (-22.09 to 21.99)	0.996
Interaction between the effects of the disease and age within females	10.07 (-6.28 to 26.42)	0.224	-5.12 (-19.74 to 9.51)	0.488
Interaction between the effects of the disease and age and gender	-4.77 (-34.35 to 24.81)	0.749	5.06 (-21.39 to 31.51)	0.704

(13.7%) refused to participate in the study: no significant differences among the demographic and clinical data were found between participants and those who refused to answer the questionnaire (data not shown for brevity).

Overall analysis of the SF-12 questionnaire in the 44 patients showed that the values of the PCS score are representative of a relatively good physical QoL and they were not significantly different from those of the normative population (PCS: 44.7 ± 11.0 *vs* 46.1 ± 9.9 , $P = 0.610$). The MCS score was significantly lower in patients (42.4 ± 13.0) as compared to the norms (48.2 ± 9.8 , $P = 0.036$). Moreover, a stratified analysis (Table 2) failed to show any significant interaction between sex and age and the effect of the disease on the MCS of PNET patients.

Regarding the GHQ-12 questionnaire, we identified 11 patients (25.0%) having non-psychotic psychiatric disorders. Shown in Table 3, the results of the STAI demonstrated that anxiety was similar in patients and the normative population.

Finally, in order to explore the depressive syndrome in detail, the BDI-II identified eight patients (18.2%) with moderate depression, nine patients (20.5%) with mild depression and 27 patients (61.4%) with no depression.

We also explored the relationships between the results of the various questionnaire scores. The MCS was highly related ($P < 0.001$) to both anxiety state and trait (STAI Y-1 and Y-2, respectively) whereas the PCS was only significantly related to anxiety trait ($P = 0.043$) but not to the anxiety state ($P = 0.222$). As shown in Table 4, only the STAI scores were significantly associated with the presence of non-psychotic psychiatric disorders as

evaluated by the GHQ-12 while both the SF-12 and the STAI were significantly related to the depressive symptoms as assessed by the BDI-II. In addition, a significant ($P = 0.011$) positive relationship was also found between the presence of non-psychotic psychiatric disorders and the depressive state (absence of depression: 4/27, 14.8%; mild depression: 2/9, 22.2%; moderate depression: 5/8, 62.5%).

Table 5 shows the possible relationships between the demographic and clinical characteristics of the PNET patients and the results of the questionnaires investigated. MCS significantly improved with age ($P = 0.042$), and anxiety state (STAI Y-1) significantly decreased with age ($P = 0.038$). Workers had an MCS (39.8 ± 13.1) significantly lower than retired people (49.2 ± 10.7 , $P = 0.032$). The patients that did not receive non-specific disease drugs had a PCS score (51.7 ± 8.6 , $P = 0.032$) significantly higher (42.9 ± 11.70) than those who were taking non-disease specific drugs; PCS was also significantly higher in patients who underwent surgery for PNET (46.4 ± 10.8) compared with those who were not operated on (38.3 ± 10.0 , $P = 0.049$). Pain worsened both the STAI Y-1 (patients with pain had a score of 48.9 ± 12.7 and those without 41.5 ± 11.0 , $P = 0.046$) and Y-2 (patients with pain had a score of 45.8 ± 12.0 and those without 39.1 ± 9.7 , $P = 0.049$) scores. Finally patients with dyspeptic symptoms had a worse MCS (patients with dyspepsia had a score of 38.4 ± 13.2 and those without 47.1 ± 11.4 , $P = 0.025$). The frequency of dyspepsia was 37.0% (10/27) in patients without depressive symptoms evaluated with the BDI-II, 88.9%

Table 4 Relationships between SF-12 and STAI scores, and GHQ-12 and BDI-II scores in the 44 patients with neuroendocrine tumors of the pancreas (mean \pm SD)

	SF-12		STAI	
	PCS	MCS	Anxiety state (Y-1)	Anxiety trait (Y-2)
GHQ-12 ¹				
Subjects without non-psychotic psychiatric disorders (score ≤ 4 , $n = 33$)	46.1 \pm 9.8	44.4 \pm 11.8	42.3 \pm 10.9	39.1 \pm 10.1
Subjects with psychotic psychiatric disorders (score > 4 , $n = 11$)	40.6 \pm 13.9	36.1 \pm 15.0	50.7 \pm 13.9	49.6 \pm 10.3
P value	0.156	0.066	0.043	0.005
BDI-II ²				
Absence of depression (score ≤ 13 , $n = 27$)	48.2 \pm 8.8	47.3 \pm 10.3	39.1 \pm 9.1	36.1 \pm 8.1
Mild depression (score 14-19, $n = 9$)	40.0 \pm 11.8	40.1 \pm 14.8	44.2 \pm 7.7	43.6 \pm 4.6
Moderate depression (score 20-28, $n = 8$)	38.4 \pm 13.3	28.2 \pm 8.3	62.3 \pm 7.2	58.8 \pm 4.3
Severe depression (score > 28 , $n = 0$)	-	-	-	-
P value	0.010	< 0.001	< 0.001	< 0.001

¹One-way ANOVA; ²ANOVA linear term.**Table 5** Relationship between demographic and clinical characteristics of the 44 patients who completed the questionnaires and the results of the questionnaires used (in bold the significant associations)

	P					
	SF-12 PCS	SF-12 MCS	GHQ-12	STAI Y-1	STAI Y-2	BDI-II
Gender (males <i>vs</i> females)	0.589 ¹	0.630 ¹	0.480 ²	0.317 ¹	0.214 ¹	0.387 ³
Age at interview	0.113 ⁴	0.042 ⁴ ($r = 0.309$)	0.351 ¹	0.038 ⁴ ($r = -0.314$)	0.309 ⁴	0.525 ⁵
Disease duration	0.962 ¹	0.751 ⁴	0.912 ¹	0.669 ⁴	0.752 ⁴	0.856 ⁵
BMI	0.766 ⁴	0.185 ⁴	0.125 ¹	0.241 ⁴	0.514 ⁴	0.723 ⁵
Marital status (single <i>vs</i> married)	0.181 ¹	0.093 ¹	0.237 ²	0.110 ¹	0.556 ¹	0.443 ³
Diploma (trend from elementary school to university degree)	0.185 ⁵	0.916 ⁵	0.648 ³	0.705 ⁵	0.373 ⁵	0.405 ³
Job (workers <i>vs</i> retired)	0.240 ¹	0.032 ¹ (-9.4 ± 4.23)	0.240 ²	0.054 ¹	0.057 ¹	0.227 ³
Alcohol habit (drinkers <i>vs</i> non-drinkers)	0.407 ¹	0.745 ¹	0.480 ²	0.653 ¹	0.724 ¹	0.930 ³
Smoking habit (smokers <i>vs</i> non-smokers)	0.349 ¹	0.465 ¹	0.294 ²	0.124 ¹	0.122 ¹	0.601 ³
Comorbidities (present <i>vs</i> absent)	0.651 ¹	0.923 ¹	1.000 ²	0.435 ¹	0.202 ¹	0.402 ³
Non-disease specific drugs (yes <i>vs</i> no)	0.032 ¹ (-8.7 ± 3.9)	0.322 ¹	0.085 ²	0.369 ¹	0.152 ¹	0.140 ³
Previous surgery not due to neuroendocrine tumors (yes <i>vs</i> no)	0.775 ¹	0.238 ¹	0.241 ²	0.439 ¹	0.437 ¹	0.321 ³
Surgery due to pancreatic neuroendocrine tumors (yes <i>vs</i> no)	0.049 ¹ (8.1 ± 4.0)	0.778 ¹	0.669 ²	0.987 ¹	0.880 ¹	0.957 ³
Disease status (disease free patients <i>vs</i> patients having lymph node involvement or liver metastases)	0.875 ¹	0.454 ¹	1.000 ²	0.300 ¹	0.169 ¹	0.105 ³
Specific treatment (yes <i>vs</i> no)	0.816 ¹	0.454 ¹	0.722 ²	0.925 ¹	0.733 ¹	0.985 ³
Pain in the last month (present <i>vs</i> absent)	0.152 ¹	0.371 ¹	0.075 ²	0.046 ¹ (7.4 ± 3.6)	0.049 ¹ (6.7 ± 3.3)	0.190 ³
Dyspepsia (present <i>vs</i> absent)	0.115 ¹	0.025 ¹ (-8.7 ± 3.8)	0.728 ²	0.199 ¹	0.195 ¹	0.015 ^{3,6}
Diabetes (present <i>vs</i> absent)	0.908 ¹	0.263 ¹	0.457 ²	0.883 ¹	0.581 ¹	0.938 ³

¹One-way ANOVA (effect estimates; mean \pm SE); ²Fisher exact test; ³Liner-by-linear association chi-square (frequencies); ⁴Pearson correlation (regression coefficient; r); ⁵One-way linear term ANOVA; ⁶Frequency of dyspepsia: according to BDI-II: absence of depression (score ≤ 13) 10/27 (37.0%); mild depression (score 14-19) 8/9 (88.9%); moderate depression (score 20-28) 6/8 (75.0%). In order to quantify the relationships, the various effect estimates - evaluated according to the statistical analysis applied - have been reported in parentheses.

(8/9) in those with mild depressive symptoms, and 75% (6/8) in those with moderate depressive symptoms ($P = 0.015$).

DISCUSSION

The correct management of neuroendocrine tumors of the pancreas includes diagnosis, management of the functional hormonal syndrome when present and management of the potentially malignant tumor. Control of the hormonal syndrome, when present, is achieved preoperatively in order to stabilize patient status for the operation^[25] whereas, in the case of recurrence and in the case of a non-surgical approach to these tumors,

medical treatment is the main option^[26].

In the present study, approximately 57% of the patients were disease-free at the time of the interview whereas, as previously reported^[2], patients with advanced disease were treated medically. Thus, especially in this latter group of subjects, PRO assessment seems to be important for evaluating the impact of this chronic disease^[27] in order to understand how biology interacts with cultural, social, interpersonal and psychological aspects. In fact, QoL also plays a central role in how the variety of symptoms and the medical management of disease are perceived by those affected. However, there are a limited number of studies evaluating the patient point of view regarding his own disease^[3-10,28]. All

these studies evaluated the QoL in patients with various neuroendocrine tumors of the gastrointestinal tract and almost all^[3-6,8-10,28] used the European Organisation for Research and Treatment of Cancer Quality of life Questionnaire-C30 (EORTC) questionnaire for quantifying the QoL of the patients; only one study explored the PROs using a different questionnaire such as the SF-36^[7]. Only two studies compared the EORTC results of neuroendocrine tumor patients with a normative population^[6,9], but the patients enrolled had carcinoid tumors which represent only a part of the neuroendocrine tumors of the gastrointestinal tract. Thus, we focused our attention on the PROs in a well defined group of patients, i.e. those with a diagnosis of PNET. For this purpose, we utilized the SF-12 questionnaire. The choice of this questionnaire in evaluating the PROs was based on the following assumptions: the simplicity of this questionnaire (it is based on 12 questions only whereas the EORTC contains 30 questions); the two SF-12 component summaries have a high level of reliability in evaluating the QoL similar to that of the domains/scores of the EORTC questionnaire^[29]; it is also possible to compare the data of patients with a nationwide normative population (there is no Italian normative population for EORTC) and, finally, SF-12 has been already tested in patients with neuroendocrine tumors of the ileum^[30]. Thus, from a practical point of view, the SF-12 questionnaire is more reliable and easier to use in routine clinical practice than the EORTC. Only a few studies have also explored psychological aspects of the disease, mainly using the Hospital Anxiety and Depression Scale (HADS) for evaluating this topic^[3,6,7,9,10]. Thus, we planned the present study in order to evaluate not only the presence of generic psychological distress by using the GHQ-12 questionnaire but also to determine whether the psychological distress eventually present is related to anxiety or depressive syndrome, and to compare these psychological aspects to those of the normative Italian population. For this purpose, we utilized two specific questionnaires, the STAI and the BDI-II, which are largely used in this setting.

The values of the SF-12 summary scores as compared to the norms showed that the 44 patients in the present study seemed to perceive their physical QoL as relatively good even if they had a tumor, and this finding agreed with previous reports^[4,5,9,10] whereas mental aspects were significantly impaired as compared to the norms. This seems to be due to the disease itself and is not related to the effects of gender and age. In fact, we carried out a stratified analysis which showed no significant interaction between age and sex, and the effect of the disease. We also attempted to identify the factors capable of modifying the mental component of QoL of patients with PNET. Surprisingly, we found that our patients were not affected by anxiety, but mild and moderate depressive symptoms were present in about 40% of the patients studied. Our data differed from those previously published which showed that patients

with neuroendocrine tumors had anxiety and depression in varying proportions from low, as reported by Larsson *et al*^[4], to high, as reported by Fröjd *et al*^[10]. These results may be due to the fact that we compared the data to the general population. In fact, when we analyzed the questionnaire score within the group of patients without comparison to the normative population, we found that the MCS and the anxiety state were better in older patients, and that workers had an MCS significantly lower than retired people. PCS was significantly higher in patients that did not receive non-disease specific drugs as compared to those who were taking non-disease specific drugs; this score was also significantly higher in patients who underwent surgery for PNET as compared to those who were not operated on. Furthermore, we also found that pain worsened both the anxiety state and trait (STAI Y-1 and Y-2) scores. Finally, patients with dyspeptic symptoms had worse MCS scores and they also presented with more depressive symptoms than those without. In clinical practice, these red flags should also be taken into consideration; even if the tumor does not affect physical condition (only 2% of patients were underweight), the workers were worried about their condition which could limit their daily activity and they probably needed psychological support.

Furthermore, these patients needed more intensive medical treatment for alleviating the pain flares and the dyspeptic symptoms.

Finally, the seven patients who refused to participate in the study were similar in demographic and clinical variables to the 44 subjects who completed the questionnaires; thus, we can assume that the results obtained can be extended to the entire Italian PNET population, at least under our experimental conditions.

In conclusion, knowledge of the patient's reported outcome in patients with PNET may help in decision-making providing important information about the long-term effects of depressive symptoms in cancer survivors, and help to identify potential adjustment problems. Current workers and especially those with dyspeptic symptoms are those patients who need specific and intensive medical and psychological support because of the presence of depressive symptoms.

COMMENTS

Background

Pancreatic neuroendocrine tumors (PNETs) are generally slow growing and patients may have prolonged survival. There are no studies specifically focusing on the localization site of neuroendocrine tumors such as PNETs.

Research frontiers

patient-reported outcome (PRO) assessment is important for evaluating the impact of this chronic disease in order to understand how biology interacts with the cultural, social, interpersonal and psychological aspects. In fact, quality of life (QoL) also plays a central role in how the variety of symptoms and the medical management of the disease are perceived by those affected.

Innovations and breakthroughs

Of the patients affected by PNETs, current workers and especially those with dyspeptic symptoms are those subjects who need a specific and intensive medical and psychological support because of the presence of depressive symptoms.

Applications

Knowledge of the patient's reported outcome in patients with PNETs may help in decision-making providing important information about the long-term effects of depressive symptoms in cancer survivors, and help to identify potential adjustment problems.

Terminology

Patient-reported outcomes provide a means of gaining an insight into the way patients perceive their health and the impact that treatments or adjustments to lifestyle have on their QoL.

Peer review

The paper by Pezzilli *et al* assessed the physical and mental status of patients with PNETs. They conclude that patients had a comparable physical but a lower mental score than these 44 individuals. The idea is rather innovative and the results are interesting.

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S- Editor Li LF L- Editor O'Neill M E- Editor Zheng XM



BRIEF ARTICLE

High circulating N-terminal pro-brain natriuretic peptide and tumor necrosis factor- α in mixed cryoglobulinemia

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Received: May 12, 2009 Revised: September 25, 2009

Accepted: October 2, 2009

Published online: October 28, 2009

patients of our study), 6% of MC+HCV and 0 controls had high NTproBNP (χ^2 , $P = 0.08$).

CONCLUSION: The study demonstrates high levels of circulating NTproBNP and TNF- α in MC+HCV patients. The increase of NTproBNP may indicate the presence of a subclinical cardiac dysfunction.

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Key words: NTProBNP; Tumor necrosis factor α ; Hepatitis C; Mixed cryoglobulinemia; Heart failure

Peer reviewer: Domenico Sansonno, Professor, Department of Internal Medicine and Clinical Oncology, University of Bari Medical School, Policlinico, Piazza Giulio Cesare 11, 70124 Bari, Italy

Antonelli A, Ferri C, Ferrari SM, Galetta F, Franzoni F, Santoro G, De Marco S, Ghiri E, Fallahi P. High circulating N-terminal pro-brain natriuretic peptide and tumor necrosis factor- α in mixed cryoglobulinemia. *World J Gastroenterol* 2009; 15(40): 5074-5079 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5074.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5074>

Abstract

AIM: To evaluate serum levels of N-terminal pro-brain natriuretic peptide (NTproBNP) and tumor necrosis factor α (TNF- α) in a large series of patients with hepatitis C associated with mixed cryoglobulinemia (MC+HCV).

METHODS: Serum NTproBNP and TNF- α levels were assayed in 50 patients with MC+HCV, and in 50 sex- and age-matched controls.

RESULTS: Cryoglobulinemic patients showed significantly higher mean NTproBNP and TNF- α levels than controls ($P < 0.001$; Mann-Whitney U test). By defining high NTproBNP level as a value higher than 125 pg/mL (the single cut-off point for outpatients under 75 years of age), 30% of MC+HCV and 6% of controls had high NTproBNP (χ^2 , $P < 0.01$). With a cut-off point of 300 pg/mL (used to rule out heart failure (HF) in patients under 75 years of age), 8% of MC+HCV and 0 controls had high NTproBNP (χ^2 , $P < 0.04$). With a cut-off point of 900 pg/mL (used for ruling in HF in patients aged 50-75 years; such as the

INTRODUCTION

The most common clinical features of hepatitis C associated with mixed cryoglobulinemia (MC+HCV) are correlated with vasculitis in the various organs and sometimes with increased viscosity of the plasma^[1,2]. Signs and symptoms include purpura, ulcers of the extremities, arthralgia, proteinuria, hepatic damage, abdominal pain, mental confusion, oligo-anuria, hemorrhagic diathesis, and sometimes congestive heart failure (HF)^[1-4]. Furthermore, HF has been described as heralding the clinical onset of essential mixed cryoglobulinemia (MC)^[5]. Moreover, many MC+HCV patients experience symptoms such as fatigue, dyspnea and reduced physical activity. However, in many patients, these symptoms are not proportional to the liver involvement and resemble symptoms of HF.

Several studies have shown that plasma levels of brain natriuretic peptide (BNP) and N-terminal proBNP (NTproBNP) are reliable diagnostic and prognostic

markers for cardiac disease^[6,7] that correlate with symptoms of HF and the severity of systolic and diastolic dysfunction^[8]. Some authors have recently stated that NTproBNP appears superior to BNP for the evaluation of suspected acute HF in patients with preserved left ventricular ejection fraction^[9,10]. In the study by O'Donoghue *et al*^[9], NTproBNP seems to correlate with HF severity better than BNP and is more sensitive.

Cytokines play an important role in chronic HF, and it has been shown that TNF- α and NTproBNP are independent predictors of long-term risk of death^[11,12] for HF. Circulating TNF- α has been recently shown to be high in patients with MC^[13].

However, to our knowledge, until now no study has evaluated circulating NTproBNP together with TNF- α levels, as possible markers of HF, in MC+HCV patients affected by cryoglobulinemic vasculitis.

The aim of this study was to evaluate serum levels of NTproBNP in a series of MC+HCV patients, and to correlate this parameter with the clinical features of the disease, and with the circulating levels of TNF- α .

MATERIALS AND METHODS

Patients

Fifty MC+HCV patients (37 females and 13 males; mean age 57 ± 9 years; mean disease duration 10 ± 11 years), consecutively referred to our Rheumatology Unit, were recruited for the study between 2001 and 2006. The diagnosis of MC+HCV was based on the presence of serum mixed (IgG-IgM) cryoglobulins and the classical clinical triad, purpura, weakness, arthralgias, and on the exclusion of other well-known systemic disorders, such as immuno-rheumatic and neoplastic diseases^[1,2,14-16].

The study included only patients with MC+HCV, without liver cirrhosis or hepatocellular carcinoma (assessed by histology, laboratory evidence of liver failure and/or ultrasound-proven portal hypertension)^[17,18]. None of the patients had evident signs of HF, organic renal disease (patients with serum creatinine > 1.2 mg/dL and/or proteinuria > 0.5 g/24 h were excluded), thyroid disease, diabetes, cancer or any other major diseases. All patients had normal cardiac physical examinations and normal blood pressure.

Thirty eight out of 55 (76%) MC+HCV patients underwent liver biopsy for diagnostic purposes; liver histology activity index (grade) or stage of liver fibrosis were evaluated according to Ishak *et al*^[19]. The mean activity index (grade) in MC+HCV patients was 5.0 ± 1.2 , and the mean stage was 2.0 ± 0.9 . Main demographic and clinico-serological features of MC+HCV patients are reported in Table 1.

Among the patients, 17 had been previously treated with interferon-alpha (IFN- α) for an average of 7 mo (range, 1-13 mo), at a mean dosage of 10.4 MU/wk; the time elapsed from the last course of IFN- α treatment ranged from 6 to 69 mo (mean 40 ± 22 mo). No statistically significant differences were observed in the main demographic and clinico-serological features of MC+HCV patients treated or untreated with IFN- α .

Table 1 Demographic and clinico-serological features of 50 MC+HCV patients

Age (yr)	57 \pm 9
Male/female	13/37
Disease duration with MC (yr)	10 \pm 11
Purpura	82%
Active vasculitis	31%
Weakness	91%
Arthralgias	83%
Arthritis	14%
Raynaud's phenomenon	51%
Sjogren's syndrome	45%
Peripheral neuropathy	59%
Aminotransferase elevation and/or histologic activity ¹	71%
Cryocrit (%)	4.2 \pm 8.9
CH50 (normal: 160-220 U)	111 \pm 36
C3 (normal: 60-130 mg/dL)	81 \pm 36
C4 (normal: 20-55 mg/dL)	14 \pm 18
Autoantibodies ²	25%

¹Increase of the liver enzyme (alanine aminotransferase) and/or liver histological alterations. ²Presence of anti-nuclear and/or anti-mitochondrial and/or anti-smooth muscle and/or anti-extractable nuclear antigen autoantibodies. MC+HCV: Hepatitis C associated with mixed cryoglobulinemia.

At the time of study, 35 MC+HCV patients were taking low doses of corticosteroids, 8 had previously been on corticosteroids and 7 had never been treated with corticosteroids. No MC+HCV patient had had plasma exchange treatment in the last year before the study. In both patients and controls, a careful medical history was collected, in particular with regard to family history of thyroid disease, smoking habits, and drugs. The presence of Raynaud's phenomenon, Sjogren's syndrome, skin ulcers, peripheral neuropathy, and renal and liver involvement in MC+HCV patients was evaluated as previously described^[16]. Routine blood chemistry was carried out by standard methods.

Controls

Each of the 50 MC+HCV patients eligible for the study was matched, by sex and age, one-to-one with a control group of healthy subjects of the general population from the same geographic area (North-West Tuscany). This control group was extracted from a larger sample of 1640 subjects taking part in a population-based survey of thyroid disorders; only HCV-negative subjects, without clinical and laboratory evidence of thyroid and liver disorders or autoimmune diseases and not treated with immunomodulators were included.

None of the controls had signs of HF, organic renal disease, thyroid disease, diabetes, cancer or any other major diseases. All patients had normal cardiac physical examination results and normal blood pressure.

Extraction of the control group from the original population was performed by finding the closest age match (± 2 years) to each case within either gender. When more than one age-match was available per case, the choice was made at random.

The study protocol was approved by the local Ethics Committee. All subjects gave their informed consent to enter the study.

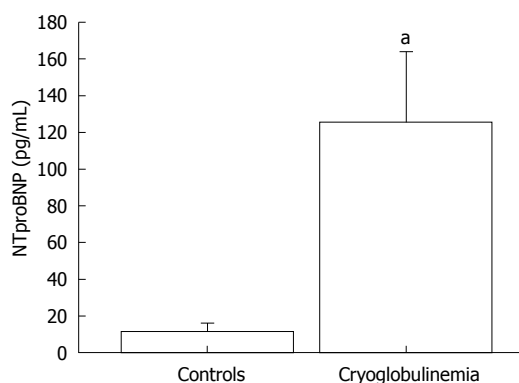


Figure 1 Plasma NTproBNP circulating levels (were significantly higher in MC+HCV patients than in controls; Mann Whitney *U* test). ^a*P* < 0.05 vs control.

Immunological studies

Cryocrit was measured as the percentage of packed cryoglobulins after cold centrifugation of the serum; cryoglobulin composition was determined by the incorporation in cryoprecipitates of monoclonal or polyclonal IgM-rheumatoid factor (i.e. MC type II or MC type III); hemolytic complement C3-C4 fractions were measured as previously described^[16], anti-nuclear, anti-smooth muscle, and anti-mitochondrial autoantibodies were detected by current techniques^[16]. Sera with a titre > 1:40 were considered positive. Anti-extractable nuclear antigen antibodies, including anti-Scl70, -Sm, -RNP, -SSA/SSB, -PCNA, -SL and -Jo1 specificities, were detected by counter-immuno-electrophoresis according to the methods described by Bunn *et al*^[20].

Virological studies

Antibodies against HCV (anti-HCV) and HCV-RNA were determined in serum clotted and centrifuged at 37°C and stored at -70°C. Anti-HCV and HCV-RNA levels [assayed by polymerase chain reaction (PCR) technique] in the serum were investigated as previously described^[21,22].

Cytokines, chemokines and analytical assays

Blood samples for analysis of plasma NTproBNP were collected, centrifuged and plasma was stored at -80°C until analysis. Plasma concentrations of NTproBNP were measured by a sandwich immunoassay on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany).

Serum TNF- α concentrations were measured using commercially available kits (R&D Systems, Minneapolis, MN). The mean minimum detectable dose was 0.12 pg/mL for TNF- α ; the intra- and inter-assay coefficients of variation were 5.8% and 10.2%. Samples were assayed in duplicate. Quality control pools of low, normal, or high concentration for all parameters were included in each assay. Alanine aminotransferase (ALT) was assayed by conventional methods^[18].

Statistical analysis

Values are given as mean \pm SD for normally distributed variables, or as median \pm IQR for not normally distributed variables (NTproBNP, TNF- α). Group values

were compared by univariate ANOVA, for normally distributed variables; or by Kruskal-Wallis (≥ 3 groups) or Mann-Whitney *U* (2 groups) tests. Proportions were compared by the chi-square test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test. Univariate analysis was performed by simple regression. A multivariate logistic regression analysis considering age, gender, ALT, and presence or absence of active vasculitis as independent variables and presence or absence of high levels of NTproBNP or TNF- α as dependent variables was performed in MC+HCV patients.

RESULTS

Plasma NTproBNP concentrations were significantly (*P* < 0.001; Mann-Whitney *U* test) higher in MC+HCV patients (mean 123 ± 112 pg/mL; median 36 pg/mL, range 8-1547 pg/mL) than in controls (mean 11 ± 12 pg/mL; median 3.1 pg/mL range 2-145 pg/mL) (Figure 1).

By defining high NTproBNP level as a value higher than 125 pg/mL (the single cut-off point for outpatients under 75 years of age^[23]) 15/35 MC+HCV and 3/47 controls had high NTproBNP (χ^2 , *P* < 0.002).

With a cut-off point of 300 pg/mL (used to rule out HF in patients under 75 years of age^[23]) 4/46 MC+HCV and 0/50 controls had high NTproBNP (χ^2 , *P* < 0.041).

With a cut-off point of 900 pg/mL (used for ruling in HF in patients with age 50-75; such as the patients of our study^[23]) 3/47 MC+HCV and 0/50 controls had high NTproBNP (χ^2 , *P* = 0.08).

In order to better define the role of increased serum NTproBNP in MC+HCV, mean levels of this chemokine were separately evaluated (by Mann Whitney *U* test) among MC+HCV patient subgroups defined according to main demographic and clinical features (age > 55 years; gender; disease duration > 10 years; presence or absence of purpura, active vasculitis, weakness, arthralgias, arthritis, Raynaud's phenomenon, Sjogren's syndrome, peripheral neuropathy, aminotransferase elevation and/or histologic activity in the liver), but no significance was found. No significant correlations were observed between NTproBNP levels and serological findings of MC+HCV (levels of cryocrit and complement, presence/absence of autoantibodies) or previous/ongoing treatments.

Serum TNF- α was detectable in 85% of controls and in all MC+HCV patients; mean levels were significantly (*P* < 0.01; Mann Whitney *U* test) higher in MC+HCV patients (mean 26 ± 65 pg/mL; median 8.3 pg/mL, range 1.3-269 pg/mL) than in controls (mean 1.6 ± 1.1 pg/mL; median 1.2 pg/mL, range 0.6-3.4 pg/mL). No correlation was found between serum TNF- α and any of the following; ALT level, liver histology activity index, stage of liver fibrosis, the presence of active vasculitis, or the other demographic, serological and clinical features of MC.

MC+HCV patients had, obviously, raised ALT enzymes (*P* < 0.0001), in comparison with controls.

No association was observed between NTproBNP or TNF- α levels and ALT levels in the MC+HCV patients.

DISCUSSION

Our study demonstrates the presence of significantly high serum levels of NTproBNP in patients with MC+HCV compared to healthy controls. Furthermore, our study confirms significantly high serum levels of TNF- α in patients with MC+HCV compared to healthy controls.

Some authors have recently stated that NTproBNP appears superior to BNP for the evaluation of suspected acute HF in patients with preserved left ventricular ejection fraction^[9,10]. Furthermore^[9], NTproBNP seems to correlate with HF severity better than BNP and appears more sensitive. The International Collaborative for NTproBNP Study (ICON) helped in defining appropriate cut-off points for NTproBNP in the emergency department^[23]: 300 pg/mL should be used to rule out HF, while 450 pg/mL, 900 pg/mL, or 1800 pg/mL, depending on age (< 50, 50-75, or > 75 years; respectively), should be applied for ruling in HF. The age stratifications do offer significant positive predictive value. For outpatient evaluation the manufacturer suggested a single cut-off point of 125 pg/mL for patients under 75 years of age and 450 pg/mL for patients above 75 years of age^[23].

The levels of NTproBNP found in MC+HCV are included in a gray zone (125-900 pg/mL) that is not necessarily associated with HF, however in 3/50 (6%) of MC+HCV patients the NTproBNP levels were higher than 900 pg/mL, which is the cut-off value for ruling in HF for patients of age 50-75 years, such as the patients of our study. Since NTproBNP level seems to correlate with HF severity, the values in the gray zone may be suggestive of a subclinical cardiac impairment. The exclusion of patients with renal failure from the study suggests that the NTproBNP increase is not related to any kidney involvement in the MC+HCV patients.

In a previous work, Matsumori *et al*^[24] showed there was a significantly higher NTproBNP level in HCV patients and observed that the presence of anti-HCV antibodies in sera was more prevalent in patients with myocarditis and HF than in the general population. Future studies comparing NTproBNP levels between HCV and MC+HCV patients will be needed to evaluate the specific influence of the cryoglobulinemic vasculitis.

The findings of the present study may have important implications for patients with MC+HCV. Most patients complain about fatigue, dyspnea and reduced physical capacity. The pathogenesis of these symptoms is not well understood and sometimes attributed to the liver injury. However, it seems possible that these patients experience cardiac impairment which could at least contribute to these symptoms. Furthermore, among signs and symptoms of MC+HCV, sometimes congestive HF may be found, which in some cases has been described as heralding the clinical onset of essential MC^[3-5].

Testing of NTproBNP level may serve as a screening marker for cardiac insufficiency in the differential diagnosis of fatigue and dyspnea and may aid the decision for further diagnostic testing of cardiac function as has been described for other groups of patients^[25-28]. Besides diagnostic consequences, evaluation of NTproBNP may have therapeutic consequences for patients with MC+HCV. In patients with known congestive HF, elevated plasma BNP concentrations could be reduced by treatment with ACE inhibitors^[29] or angiotensin II receptor antagonists^[30] as well as treatment with diuretics and vasodilators^[31]. As a consequence, plasma NTproBNP concentration may guide the intensity of pharmacotherapy as some interventional studies have suggested^[32,33].

Cytokines play an important role in chronic HF, and it has been shown that TNF- α and NTproBNP are independent predictors of long-term risk of death^[11,12] for patients with HF.

Our study confirms a high serum TNF- α level in HCV+ve patients, as previously demonstrated in other studies of HCV+ve patients^[34-36]. The increase of TNF- α in MC+HCV patients is unlikely to be due to a more aggressive liver disease; in fact, no correlation was found between TNF- α levels and ALT levels, or with degree of liver inflammation in our study^[13]. Other studies have shown an increased production of TNF- α by lymphocytes in MC+HCV patients^[37,38], suggesting that the increase of TNF- α may be due to activation of lymphoid cells.

The fact that TNF- α and NTproBNP are independent predictors of long-term risk^[11] is in agreement with the results of our study; in fact, no relationship has been observed between TNF- α and NTproBNP.

It has been shown that combining measurements of pro-inflammatory cytokine TNF- α and NTproBNP seems a promising tool in the prognostic assessment of HF patients^[12]. However, even if we have shown that NTproBNP and TNF- α are both high in the circulation of MC+HCV patients, we cannot exclude that different pathogenetic pathways may be differentially implicated in the increase of each of these factors.

The interest in finding reliable markers of cardiac dysfunction is intensified by studies that strongly suggest an association between HCV chronic infection and atherosclerotic disease in the carotid or coronary artery^[39-41].

In conclusion, our study shows elevated levels of NTproBNP in patients with MC+HCV, in association with TNF- α . This may indicate the presence of cardiac dysfunction and explain, at least in part, some of the clinical symptoms of patients with MC+HCV. Further larger, possibly multicenter prospective studies quantifying symptoms and correlating these with echocardiographic parameters are needed to confirm this association.

COMMENTS

Background

The most common clinical features of hepatitis C associated with mixed

cryoglobulinemia (MC+HCV) are correlated with vasculitis in various organs and heart failure (HF) has been described as heralding the clinical onset of essential mixed cryoglobulinemia (MC). To our knowledge, until now no study has evaluated circulating NTproBNP together with TNF- α levels as possible markers of HF, in MC+HCV patients affected by cryoglobulinemic vasculitis.

Innovations and breakthroughs

This study demonstrates elevated serum levels of NTproBNP in patients with MC+HCV, in association with high TNF- α levels. This may indicate the presence of cardiac dysfunction and explain, at least in part, some of the clinical symptoms of patients with MC+HCV who have no evident signs of HF. Further larger, possibly multicenter prospective studies quantifying symptoms and correlating these with echocardiographic parameters are needed to confirm this association.

Applications

NTproBNP evaluation may serve as a screening marker for cardiac insufficiency in the differential diagnosis of fatigue and dyspnea, may aid the decision for further diagnostic testing of cardiac function and may have therapeutic and diagnostic consequences for patients with MC+HCV. In patients with known congestive HF, elevated plasma BNP concentrations could be reduced by treatment with ACE inhibitors, angiotensin II receptor antagonists and treatment with diuretics and vasodilators. As a consequence, plasma NTproBNP concentration may guide the intensity of pharmacotherapy as some interventional studies have suggested.

Terminology

Plasma levels of BNP and NTproBNP are reliable diagnostic and prognostic markers for cardiac disease which correlate with symptoms of HF and the severity of systolic and diastolic dysfunction. TNF- α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. Circulating TNF- α has been recently shown to be high in patients with MC.

Peer review

The study by authors has evaluated the circulating levels of NTproBNP and TNF- α in patients with HCV-related cryoglobulinemia. They found higher levels of both proteins in these patients as compared with normal controls and conclude that they may have a potential role in cardiac dysfunction of cryoglobulinemic patients. Though crude, these data are interesting.

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S- Editor Tian L L- Editor Logan S E- Editor Yin DH



BRIEF ARTICLE

Hepatitis C virus genotype 3a infection and hepatocellular carcinoma: Pakistan experience

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Supported by Ministry of Science & Technology Government of Pakistan Scientific Project Grant

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Received: June 1, 2009 Revised: September 10, 2009

Accepted: September 17, 2009

Published online: October 28, 2009

the development of HCC. The time from HCV infection to appearance of cancer was 10-50 years. In the HCC patient population, broader distributions of genotypes were present with genotype 3a as the predominant genotype. Using the type-specific genotyping method, we found HCV genotype 3a in 40.96%, 3b in 15.66%, 1a in 9.63%, and 1b in 2.40% of HCC tissue samples. About 28% of cases were found with mixed genotypes. Two cases were unable to be genotyped because of low viral load. Sixty-six percent of treated patients with cirrhosis had an end of treatment response, but unfortunately they relapsed quickly when the treatment was discontinued, and HCC developed during a median 3.8 years.

CONCLUSION: There was a strong association between chronic HCV infection and HCC in Pakistan, and between HCV genotype 3a and HCC.

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Key words: Hepatocellular carcinoma; Hepatitis C; Genotyping; Etiology; Prevalence

Peer reviewers: Chifumi Sato, Professor, Department of Analytical Health Science, Tokyo Medical and Dental University, Graduate School of Health Sciences, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan; Henry LY Chan, MD, Associate Professor, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, China

Idrees M, Rafique S, Rehman I, Akbar H, Yousaf MZ, Butt S, Awan Z, Manzoor S, Akram M, Aftab M, Khubaib B, Riazuddin S. Hepatitis C virus genotype 3a infection and hepatocellular carcinoma: Pakistan experience. *World J Gastroenterol* 2009; 15(40): 5080-5085 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5080.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5080>

Abstract

AIM: To assess the association between chronic hepatitis C virus (HCV) infection and hepatocellular carcinoma (HCC) in Pakistan, and the genotype distribution among these HCC patients.

METHODS: One hundred and sixty-one subjects with HCC were included in this study. Liver biopsy was performed on 145 of the patients; sixteen were excluded because they failed to fulfill the inclusion criteria. Qualitative polymerase chain reaction (PCR) was performed for hepatitis B virus and HCV. Samples positive for HCV RNA were genotyped using genotype-specific PCR and confirmed by HCV 5' noncoding region sequencing analysis.

RESULTS: Chronic HCV infection was identified a major risk factor (63.44% of tested HCC patients) for

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the commonest cancers in the world, with an estimated incidence of 5000000 to 10000000 new cases every year^[1]. Hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, alcoholic liver disease, and non-alcoholic fatty liver disease are the major causes of cirrhosis in patients with HCC^[2,3].

Chronic HCV infection frequently leads to liver cirrhosis and is associated with an elevated risk for progression into HCC^[4,5]. Epidemiological surveys have identified HCV in 10%-80% of HCC patients reported in different populations^[5,6]. HCV has also been reported to be the major cause of HCC in Japan^[7], Italy^[6] and Spain^[8], but is less important in South Africa^[9] and Taiwan^[10]. Association of HCV infection with HCC has also been well documented in the United States^[11].

Etiology, clinical features, and survival of HCC vary considerably in different populations^[12]. In Pakistan, HCC is a leading cause of death and accounts for 60%-90% of all primary liver malignancies^[13]. Some studies have shown hepatitis B surface antigen (HBsAg) positivity in 60% of patients with HCC^[14,15]. However, some other studies have reported positivity for HCV infection in up to 80% of patients with HCC^[16]. It is believed that HCV infection is a major etiological factor for HCC^[17], however, not all patients with HCV infection develop HCC. A number of host factors such as male sex, older age at infection, long disease duration, excessive alcohol consumption, and high liver iron overload have been reported to influence disease progression^[18,19]. Several additional studies have noted variables such as chronic co-infection with HBV and human immunodeficiency virus (HIV)^[20], obesity and steatosis^[21], type 2 diabetes^[22], and asymptomatic cryoglobulinemia^[17,18,23]. In addition to these host factors, several viral factors such as genotype and peripheral viral load have also been reported to influence disease progression^[24]. Some studies have identified that cirrhotic patients infected with HCV type 1b carry a significantly higher risk of developing HCC compared to those infected with other HCV types^[25,26]. However, the results of other studies^[27,28] are in disagreement with these studies, and demonstrate no association of a particular HCV genotype with the development of HCC.

No such studies on the association or otherwise of HCV genotype with the development of HCC are available from Pakistan. Therefore, this study was performed to: (1) study various risk factors for the development of HCC; (2) investigate the prevalence of HCV in patients with HCC; and (3) evaluate if there is any association between particular HCV genotypes and HCC.

MATERIALS AND METHODS

Patients

For initial examination, 161 subjects with chronic hepatitis managed as end-stage liver disease patients at various hospitals of Punjab and North West Frontier Province of Pakistan were enrolled. All these patients underwent ultrasound-guided liver biopsy. Of these 161 subjects, 145 satisfied the inclusion criteria such as: HCC was confirmed by liver imaging (ultrasonography and computed tomography); histologically confirmed HCC; chronic liver disease of any etiology, with ascites and encephalopathy. Sixteen patients were excluded from the study because they failed to fulfill the study

criteria (14 subjects) or were unwilling to participate in the study (two subjects). The study was started in March 2001 and ended in April 2009. The clinical records of these patients were examined to identify the etiology of HCC. Documentation of the histology of liver tissue surrounding the cancer, together with possible sources of transmission and duration of blood-borne infectious hepatitis, was made. The time of transmission of HCV infection was calculated from the time of first major/minor surgery or first blood transfusion; only these patients were used to calculate the range/median duration of infection. Serum samples were collected and stored at -20°C, at the time of diagnosis of HCC. All the liver biopsies were transported in liquid nitrogen and stored at -70°C. Liver function tests such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and bilirubin levels of all the samples were estimated using an auto-analyzer (Hitachi, Tokyo, Japan). Serum α -fetoprotein (AFP) concentration was determined by solid-phase, two-site chemiluminescent immunometric commercial diagnostic assay, using an Immulite-100 automated immunoassay system (Diagnostic Products, Los Angeles, CA, USA). From all the subjects, written informed consent was obtained. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee and Institutional Review Board of the Centre.

ELISAs for HBsAg, anti-HBsAg, anti-hepatitis B core antigen (HBc), anti-HCV and anti-HIV 1 and 2

All the patients were screened for HBsAg, anti-HBsAg, anti-HBc, anti-HCV and anti-HIV 1 and 2 using third-generation ELISA kits (DRG Instruments, Germany) as described by the manufacturer.

HBV qualitative polymerase chain reaction (PCR)

Hepatitis B viral DNA was extracted from 200 μ L of stored serum and 2-5 mg of liver tissue of each of the patients using Gentra DNA Isolation Kit (PUREGENE, USA). Qualitative detection of serum HBV DNA was done by PCR amplification of the surface antigen gene using specific forward (5'AGAACATCGCATCAGGAC TC-3'; nt: 159-178) and reverse (5'CATAGGTATCTTG CGAAAGC-3'; 642-623) primers. One microliter of the first-round products was re-amplified using nested PCR with internal forward (5'AGGACCCCTGCTCGTGTT AC-3'; 181-200) and reverse (5'AGATGATGGGATG-GGAATAC-3'; nt: 619-600) primers. The amplified products were detected on agarose gel electrophoresis after staining with ethidium bromide and visualization on a UV transilluminator.

Qualitative and quantitative detection of HCV RNA

HCV qualitative RT-PCR was carried out as described previously^[29]. HCV RNA was quantified using a SmartCycler II Real-time PCR (Cepheid, USA), using HCV RNA quantitative kits (Sacace Biotechnologies, Italy) according to the kit protocol.

Table 1 Characteristics, biochemistry and etiology of HCC patients (*n* = 145) *n* (%)

Risk factor	Value
Age \pm SD (yr)	58 \pm 11
Male	107 (73.79)
ALT	61 (42.1)
AST	61 (42.1)
Alkaline phosphatase	145 (100)
Bilirubin	145 (100)
AFP elevation	125 (86.2)
Cirrhosis present	98 (67.58)
HBsAg-positive (alone)	18 (12.41)
Anti-HBc-positive (alone)	2 (1.37)
Anti-HBs-positive	10 (6.89)
HBV-DNA PCR-positive (alone)	26 (17.93)
Anti-HCV-positive (alone)	92 (63.44)
HCV-RNA PCR-positive	83 (57.24)
HBV- and HCV-positive	19 (13.10)
No known etiology	6 (4.13)

HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aminotransferase; AFP: α -fetoprotein; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

HCV genotyping

Core HCV genotyping were performed as described previously^[30] for all HCV-RNA-positive sera and tissues. Genotypes were confirmed by HCV 5' noncoding region (5' NCR) sequencing using ABI PRISM 3100 Genetic Analyzer (Applied Biosystem Inc., Foster City, CA, USA) in both directions. Sequences of isolates were aligned with representative sequences for each major genotype and subtype selected from the GenBank database with the help of the Multalign program. The phylogenetic analysis of HCV isolates was performed with MEGA 3.0 software^[31], and phylogenetic trees were constructed by the neighbor-joining method, using the bootstrap-resampling test from the MEGA program (1000 bootstrap replications).

HCV treatment

The medical records of the HCV-related HCC patients showed that a total 21 patients had been treated previously for HCV infection. These treated patients had received 3 MU recombinant interferon- α three times weekly, subcutaneously, and ribavirin (10 mg/kg per day) for a total of 24 wk.

Statistical analysis

The data were analyzed and summary statistical analysis was carried out using SPSS for Windows version 10.0. The results for all variables were given in the form of averages (SD). The χ^2 /Fisher's exact test and independent sample *t* test were used for categorical/continuous variables.

RESULTS

Characteristics and biochemistry of HCC patients

Patient demographics and biochemical and clinical data are shown in Table 1. HCC patients were older (58 \pm 11 years), were predominantly male (73.8%), and had no history of chronic alcoholism. Data collection was

incomplete for one aspect, namely, exact duration of illness. The time of HCV transmission was calculated from the time of first major/minor surgery or first blood transfusion, which might not have been the exact date of virus acquisition. All the patients with HCC had raised levels of serum bilirubin (> 1.0 mg/dL) and alkaline phosphatase (> 300 U/L). ALT and AST levels were abnormal (ALT > 40 IU/mL, AST > 35 IU/mL) only in 42% of patients. AFP level was elevated (> 15 IU/mL) in 86.2% of patients with HCC. Cirrhosis was present in 67.6% of HCC patients. All the patients were found to be negative for anti-HIV.

Etiology of HCC

Out of the 145 patients with HCC, HCV antibodies were present in 92 (63.4%) serum samples. Two patients were found to be tissue-positive by PCR but no anti-HCV antibodies were present. Eighty-one patients were found to be tissue- positive by PCR out of 92 anti-HCV-positive patients (88.04%). Of these patients with HCC caused by HCV, 68 were male and 13 were female. The mean age was 55 \pm 10 years for HCV-related HCC. HCV RNA was detected in the serum of all these 81 tissue-positive patients. All the patients with HCV-related HCC had a history of chronic HCV infection. The peripheral HCV RNA loads were as low as 10000 copies to as high as 3.7×10^8 copies/mL. No significance difference was found between the viral loads in serum and tissues of the same patients. Twenty-eight cases were caused by HBV, of whom 18 (19.31%) also had markers for current HBV infection (HBsAg-positive), and two patients (1.37%) had markers for past infection (HBsAg-negative; anti-HBsAg-positive; anti-HBc positive). The age was 65 \pm 12 years for HBV-associated HCC patients. Nineteen (13.1%) of the HCC cases had markers for HCV and HBV. Out of these 19 cases with dual infection, two were HBV-DNA-positive and HBsAg-negative. In 6 (4.13%) cases, the etiology of liver cancer could not be determined from the medical records or serology. All these six HCC patients with unknown etiology were younger than the HCV-related HCC patients (45-50 years).

Distribution of HCV genotypes in HCC patients

Table 2 shows the results of HCV genotyping. A total of 83 tissue samples (81 positive for HCV RNA and anti-HCV, and two positive for HCV RNA and negative for anti-HCV) were used for HCV genotyping. Using the type-specific genotyping method, we found HCV genotype 3a in 40.96%, 3b in 15.66%, 1a in 9.63%, and 1b in 2.40% of HCC tissue samples. Twenty-four tissues (28.91%) were found with mixed genotypes. Of the 24 mixed genotypes, 10 were infected with genotypes 3a and 3b, eight with 1a and 3a, and six with 1a and 3b. Two tissue samples were found to be untypable as no genotype was detected. Both of the untypable patients had no cirrhosis and had a low viral load (< 10^4 IU/mL). The genotyping results for all single genotypes were confirmed by sequencing. The sequence data of the sequences were submitted to GenBank. The Accession

Table 2 Results of HCV genotype determination in HCC patients (*n* = 83)¹

HCV genotype	No. of HCC cases	Percentage
1a	8	9.63
1b	2	2.40
3a	34	40.96
3b	13	15.66
3a + 3b	10	12.48
1a + 3a	8	9.63
1a + 3b	6	7.22
NT	2	2.40

¹Eighty-one were positive for tissue/serum RNA by PCR and positive for serum anti-HCV, and two were positive for tissue/serum RNA by PCR but negative for serum anti-HCV. Eleven patients with HCC caused by HCV were not genotyped, as these were anti-HCV-positive by ELISA, but were HCV-RNA-negative, thus they could not be genotyped utilizing the molecular genotyping method. NT: Not typed.

Numbers provided for our nucleotide sequences by GenBank are EF173955-EF174011.

Anti-viral treatment history

The medical records of the patients showed that 21 of 94 patients with HCV-associated HCC had received previous standard interferon therapy for a total of 24 wk. Of these treated patients, 13 were male and eight were female. Cirrhosis was present in all of these 21 treated patients. Twenty of these patients had genotype 3a (12 male and eight female) and one 3b (male). Fourteen (66.7%) of these patients (eight male and six female; all with genotype 3a) had an end of treatment response but relapsed after discontinuation of treatment, with no sustained viral response.

DISCUSSION

Several viral and host factors have been studied extensively since the identification of HCV infection as a major risk factor for the development of HCC^[4,9,17]. Among the viral factors, the presence of some HCV genotypes adds to the list of risk factors for HCC. In the present study, the etiology of 145 patients with HCC was assessed with special emphasis on HCV genotype. More than 73% of the enrolled patients with HCC were male. It has been reported already that men have a higher liver cancer rate than women, with a ratio between 2:1 and 4:1^[32]. The reasons for the higher proportion of male patients with HCC might be the possibility that more men are infected with HBV and HCV, consume alcohol, smoke, have increased iron stores, higher body mass index, and a possible involvement of male sex hormones in the onset of HCC^[33]. Most patients (96.5%) in the current study were elderly and their ages ranged from 58 to 68 years. They were possibly infected on receiving injections or major/minor surgery at a median time of 20 years previously. Our observation of late onset of HCC is in agreement with earlier reports from other parts of the world where the transition from acute infection to cirrhosis and detection of HCC took 20-30 years^[34]. It is important to mention here that, in

recent years, with the increasing incidence of HCC, the age of patients with HCC has been decreasing among persons aged 45-60 years^[2]. AFP elevation was observed in the present study in about 86% of patients with HCC. Cirrhosis was present in > 67% of HCC cases studied. Previous studies have shown that cirrhosis underlies HCC in > 80% of affected individuals^[35,36]. Therefore, any agent that leads to cirrhosis should be seen as a risk factor for the development of HCC. It has also been reported that the risk among those with cirrhosis increases in parallel with the impairment of liver function, and in subjects with increased AFP concentration^[35].

Thirty-two percent of our patients with HCC were without liver cirrhosis, which showed that infection with HCV and HBV could be correlated with the emergence of HCC, even in the absence of liver cirrhosis. It has been established that the mechanism for the development of HCC in HBV-related cases is associated with the integration of HBV DNA into hepatocytes^[37]. However, such a mechanism has not been established for HCV, because to date, integration of HCV RNA into cellular DNA has not been reported, even when there has been evidence for the direct involvement of HCV in oncogenicity. According to recent reports, the possible risks are involvement of various viral proteins such as core, NS3 and NS4 in the induction of liver cell proliferation, by interfering directly with the major cellular transduction networks^[38,39].

Several major findings have emerged from the current study. The first finding is the identification of chronic HCV infection as a major risk factor for the development of HCC in Pakistan, because anti-HCV was observed in > 63% of patients with HCC. The overall anti-HCV prevalence rate is 14%-15% and HBV carrier rate is 2%-3% in the general population of Pakistan^[29,40]. Overall, our data are consistent with the results of studies already reported from high-risk areas for HCC such as Japan, Italy and Spain, where majority of reported HCC cases are HCV-related^[6-8]. It is clear from the present study and from others that the greatest proportional increases have occurred recently in HCV-related HCC worldwide, and that HBV-related HCC has been stable and at its lowest rate^[16]. The rate of HCV-related HCC is likely to continue to increase, and it is estimated that this increase will peak around the year 2010, not only in North America and Europe^[41], but also in the rest of the world including Pakistan. Presently, the annual incidence of HCV-related HCC ranges between 2% and 8%^[38].

The second major finding of the current study is the evidence that links HBV with HCC, with about 19% of cases caused by HBV. This link was expected and is unquestionable, as has been reported previously^[40]. Co-infection with HBV was also found as an additional etiological factor for HCC in the current study, which supports other published studies^[42,43]. In two HCV-RNA-positive patients, HBV DNA was detected even in the absence of serological markers for HBV in serum. Previously, it has also been reported that the rate of

occult infection in such patients can be as high as 63%^[44]. It has been reported that the implementation of HBV vaccination has resulted in a significant decrease in the incidence of HBV-related HCC^[45]. In 4.13% cases in the present study, the etiology of liver cancer could not be determined from medical records or from serology and molecular biology. All these patients were non-drinking males, but were chain smokers.

Another more interesting and somewhat surprising finding in the present study was the observation that HCV genotype 3a was the predominant genotype in 41% of HCC cases. This suggests that genotype 3a is a major risk factor associated with the development of HCC compared with other HCV genotypes. However, the question whether HCV genotype plays a role in the development of liver cirrhosis and HCC is still debatable. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for HCC^[25,26]. However, some other studies have revealed no preferential role of individual HCV genotypes in HCC^[27,28]. Although in our study HCV genotype 3a was predominant in HCV-related HCC, this genotype has been reported previously to induce a high sustained response, and has been less responsible for severe disease as compared to genotypes 1a, 1b and 4^[46]. It seems that the high percentage of HCC in patients with HCV genotype 3a might result from the fact that genotype 3a can equally cause increased oncogenicity, as can other genotypes such as 1a and 1b. Finally, patients with cirrhosis had no sustained response rates and treatment did not reduce the incidence of HCC. However, more studies with a large number of cirrhotic patients, along with adequate controls, are required to confirm this observation of the current study.

In conclusion, HCC was found mostly in patients with chronic HCV infection and with liver cirrhosis in Pakistan. There also seemed to be a strong association between chronic HCV infection with genotype 3a and HCC, as the high prevalence of genotype 3a in the HCC population reflected increased oncogenicity. Treatment did not stop the development of HCC. However, studies with larger numbers of patients could confirm that HCV genotypes vary in their propensity to produce clinically significant liver disease.

ACKNOWLEDGMENTS

We thank all the clinicians and patients for their cooperation in the study. We thank Dr. Saleem Kamili, Chief, Reference Laboratory, Division of Viral Hepatitis CDC for critically reading the manuscript. This project was partially supported by the Ministry of Science & Technology, Government of Pakistan.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is currently one of the fastest growing causes of cancer-related deaths worldwide. The geographical prevalence varies considerably from country to country and Pakistan is a high-risk area for the

disease. A strong association has been established between chronic hepatitis C virus (HCV) infection and hepatocarcinogenesis. A specific HCV genotype could play a role in the development of HCC.

Research frontiers

In Pakistan, HCC is a leading cause of death and accounts for 60%-90% of all primary liver malignancies. Positivity for HCV infection is up to 80% in HCC. Are HCV genotypes playing a role in the development of liver cirrhosis and HCC? This question remains debatable. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for the development of HCC. However, some other studies have revealed no preferential role of individual HCV genotypes in HCC. Therefore, the current study was carried out to assess if there was any association between chronic hepatitis infection with various HCV genotypes and HCC.

Innovations and breakthroughs

In the current study, the authors identified various risk factors for the development of HCC and particularly investigated the prevalence of HCV in patients with HCC. They further assessed the association between chronic hepatitis infection with various HCV genotypes and HCC, and found a strong association between chronic HCV infection with genotype 3a and HCC. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for HCC.

Peer review

This paper describes the relationship between HCV genotype 3a infection and HCC development. Although there have been many studies on the difference among HCV genotypes in hepatocarcinogenesis, there has not been a sufficient number of reports on genotype 3a. Therefore, this paper deals with an interesting issue.

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BRIEF ARTICLE

Gastric leptomeningeal carcinomatosis: Multi-center retrospective analysis of 54 cases

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Supported by The Dong-A University Research Fund and the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST; R13-2002-044-05001-0)

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Received: July 13, 2009 Revised: September 18, 2009

Accepted: September 25, 2009

Published online: October 28, 2009

malignancy to the diagnosis of LMC was 6.3 mo, ranging between 0 and 73.1 mo. Of the initial endoscopic findings for the 45 available patients, 23 (51%) of the patients were Bormann type III and 15 (33%) patients were Bormann type IV. Pathologically, 94% of cases proved to be poorly differentiated adenocarcinomas. Signet ring cell component was also observed in 40% of patients. Headache (85%) and nausea/vomiting (58%) were the most common presenting symptoms of LMC. A gadolinium-enhanced magnetic resonance imaging was conducted in 51 patients. Leptomeningeal enhancement was noted in 45 cases (82%). Intrathecal (IT) chemotherapy was administered to 36 patients—primarily methotrexate alone (61%), but also in combination with hydrocortisone/± Ara-C (39%). The median number of IT treatments was 7 (range, 1-18). Concomitant radiotherapy was administered to 18 patients, and concomitant chemotherapy to seven patients. Seventeen patients (46%) achieved cytological negative conversion. Median overall survival duration from the diagnosis of LMC was 6.7 wk (95% CI: 4.3-9.1 wk). In the univariate analysis of survival duration, hemoglobin, IT chemotherapy, and cytological negative conversion showed superior survival duration ($P = 0.038$, $P = 0.010$, and $P = 0.002$, respectively). However, in our multivariate analysis, only cytological negative conversion was predictive of relatively longer survival duration (3.6, 6.7 and 14.6 wk, $P = 0.030$, RR: 0.415, 95% CI: 0.188-0.918).

CONCLUSION: Although these patients had a fatal clinical course, cytologic negative conversion by IT chemotherapy may improve survival.

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Key words: Carcinomatosis; Gastric cancer; Intrathecal chemotherapy; Leptomeningeal

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Oh SY, Lee SJ, Lee J, Lee S, Kim SH, Kwon HC, Lee GW, Kang JH, Hwang IG, Jang JS, Lim HY, Park YS, Kang WK, Kim HJ. Gastric leptomeningeal carcinomatosis: Multi-center retrospective analysis of 54 cases. *World J Gastroenterol* 2009;

Abstract

AIM: To identify the clinical features and outcomes of infrequently reported leptomeningeal carcinomatosis (LMC) of gastric cancer.

METHODS: We analyzed 54 cases of cytologically confirmed gastric LMC at four institutions from 1994 to 2007.

RESULTS: The male-to-female ratio was 32:22, and the patients ranged in age from 28 to 78 years (median, 48.5 years). The majority of patients had advanced disease at initial diagnosis of gastric cancer. The clinical or pathologic tumor, node and metastasis stage of the primary gastric cancer was IV in 38 patients (70%). The median interval from diagnosis of the primary

15(40): 5086-5090 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5086.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5086>

INTRODUCTION

Leptomeningeal carcinomatosis (LMC) is defined as malignant infiltration of the pia mater and the arachnoid membrane. LMC is one of the most serious complications that can occur in cancer patients^[1]. According to the results of a large autopsy study, the incidence of LMC was 5%-8% in cancer patients^[2]. As a significant proportion of these patients have asymptomatic microscopic disease, the clinical diagnosis of LMC has been established in 2%-4% of patients during the course of their malignancy^[1]. LMC is frequently detected in patients with leukemia, breast cancer, lymphoma, and lung cancer^[3]. Among solid tumors, LMC is observed more frequently in cases of disseminated and progressive disease. Although a subset of patients, particularly those with lymphoma or breast cancer, may survive for more than 12 mo with a reasonable quality of life, leptomeningeal metastasis from solid tumors is associated with a poor overall prognosis. The treatment of LMC is palliative and unsatisfactory. No evidence demonstrating the superiority of intrathecal (IT) treatment compared to best palliative care is currently available from clinical trials.

Furthermore, the development of LMC from a gastric cancer is a very rare occurrence. Some articles have reported that the incidence of LMC in patients with gastric cancer was responsible for 0.16% of all cases of gastric cancer^[4]. Due to its rarity, the clinical features and prognostic factors of LMC as a metastasis from gastric carcinoma have yet to be clearly characterized. The benefits of IT chemotherapy are also currently a matter of some contention.

Gastric cancer is the most common malignancy in Korea^[5]. Because of the high prevalence of gastric cancer in Korea, we took the opportunity to study gastric cancer patients with LMC. The principal objective of this study was to review our experience with LMC associated with gastric cancer, and to evaluate its clinical features and the efficacy of a variety of treatment modalities in terms of neurological status and overall survival (OS).

MATERIALS AND METHODS

Patients

From 1995 to 2007, 22154 patients were diagnosed with gastric cancer at four independent institutions. Among them, 54 patients who were diagnosed with leptomeningeal seeding metastasis of gastric cancer were analyzed. Although it is not representative of the cohort of patients, the prevalence of LMC was 0.24%.

Eligibility for this study included: (1) patients with histologically confirmed adenocarcinoma of the stomach; (2) cytologically confirmed malignancy on cerebrospinal fluid (CSF) analysis, patients with

suspected LMC by magnetic resonance imaging (MRI) and negative cytology were excluded; (3) no history of any other malignancies.

We retrospectively analyzed the patients' medical records including the patients' characteristics, clinical symptoms, laboratory and radiologic findings, treatment modality and outcomes, final follow-up, and survival duration.

Statistical analysis

Comparisons of categorical variables among groups were conducted using the chi-square test and Fisher's exact test. OS was calculated from the cytological confirmation of LMC and plotted *via* the Kaplan-Meier method. Comparison of survival according to prognostic factors was evaluated *via* a log-rank test, and forward stepwise Cox proportional hazard models were employed to evaluate the joint effect of predictive variables. $P < 0.05$ was considered significant. Analysis of the data was conducted using SPSS for Windows V. 15.0 (SPSS Inc., Chicago, IL, USA) statistical software.

RESULTS

Patients' characteristics

We analyzed 54 cases of cytologically confirmed gastric LMC at four institutions from 1994 to 2007. The clinical characteristics of these patients are summarized in Table 1. The male-to-female ratio was 32:22, and patients ranged in age from 28 to 78 years (median, 48.5 years). The majority of patients had advanced disease at initial diagnosis of gastric cancer. The clinical or pathologic tumor, node and metastasis stage of the primary gastric cancer was IV in 47 patients (87%). Stage I - III patients received curative operation. Among the stage IV patients, 13 patients had T4N1-2 or N3 (No. of nodes > 15) by pathologic features through curative operation. M1 node positive patients were counted as palliative surgery. Of the initial endoscopic findings in the available 45 patients, Bormann type III and IV were reported for 23 (51%) and 15 (33%) patients, respectively. Pathologically, 94% of cases proved to be poorly differentiated adenocarcinomas. Signet ring cell component was also observed in 40% of patients.

LMC patterns

The median interval from diagnosis of the primary gastric cancer to the diagnosis of LMC was 6.3 mo, ranging from 0 to 73.1 mo. Five patients presented with initial LMC. The majority of patients (59.3%) initially presented with metastatic gastric cancer without LMC, and then progressed to LMC. One-third of the patients presented with curable disease at the initial diagnosis of gastric cancer (Table 2).

Clinical symptoms

The most frequently observed presenting symptoms of LMC were nonspecific symptoms such as headache (85%) and nausea/vomiting (58%). In addition, various neurological clinical signs and symptoms were noted

Table 1 Patients' characteristics (*n* = 54)

No. of patients	<i>n</i> (%)
Gender	
Male/female	32 (59.3)/22 (40.7)
Age (yr)	
Median (range)	48 (28-78)
≥ 60/< 60	15 (27.8)/39 (72.2)
Initial stage	
I - II	2 (3.7)
III	2 (3.7)
IV	47 (87.0)
Not available	3 (5.6)
Operation	
Curative	17 (31.5)
Palliative	15 (27.8)
Inoperable	18 (33.3)
Not available	4 (7.5)
Initial endoscopic finding (<i>n</i> = 47)	
Site	
Cardia	1 (1.9)
Fundus	1 (1.9)
Body	20 (37.0)
Antrum, pylorus	16 (29.7)
Diffuse whole stomach	9 (19.1)
Borrmann type (<i>n</i> = 45)	
Early gastric cancer	2 (4.4)
I (polypoid)	1 (2.2)
II (ulcerative)	4 (8.9)
III (ucero-infiltrative)	23 (51.2)
IV (diffuse infiltrative)	15 (33.3)
Differentiation (<i>n</i> = 47)	
Moderate	3 (6.4)
Poor	25 (53.2)
Poor with signet ring cell	19 (40.4)

Table 2 Patterns of leptomeningeal carcinomatosis (*n* = 54)

	<i>n</i> (%)
Time to LMC (mo)	
Median (range)	6.3 (0-73.1)
LMC presentation	
Curative/recurred/progression	7 (13.0)
Curative/recurred LMC	10 (18.5)
Metastatic/progression	32 (59.3)
Initially LMC	5 (9.3)

LMC: Leptomeningeal carcinomatosis.

including altered mental status, seizure, motor weakness, sensory change, diplopia, hearing loss, and facial palsy (Table 3).

CSF analysis and image findings

Lumbar puncture and analysis of CSF is a crucial laboratory test in the diagnosis of LMC. All the patients presented with malignant cells on cytological analysis *via* the inclusion criteria. An elevated opening pressure on lumbar puncture was noted in 58% of the subjects. The mean CSF pressure in the patients was 222.1 mm CSF. 78.8% and 53.8% of patients had elevated white blood cells and protein in CSF, respectively (Table 4).

Brain computed tomography was assessed in eight patients and leptomeningeal enhancement was observed only in one patient. A gadolinium-enhanced MRI was

Table 3 Symptoms of leptomeningeal carcinomatosis (*n* = 54)

	<i>n</i> (%)
Cerebral symptom	
Headache	46 (85.1)
Nausea & vomiting	32 (59.2)
Dizziness	13 (24.0)
Mental change	12 (22.2)
Seizure	10 (18.5)
Gait difficulty	2 (3.7)
Dysarthria	2 (3.7)
Psychosis	1 (1.9)
Cranial symptom	
Diplopia	3 (5.6)
Hearing loss	2 (3.7)
Facial palsy	1 (1.9)
Ptosis	1 (1.9)
Spinal symptom	
Weakness	5 (11.1)
Paresthesia	2 (3.7)
Back pain	1 (1.9)

Table 4 CSF finding of leptomeningeal carcinomatosis

CSF	No. of > WNL ¹	mean ± SD
Pressure (mm CSF)	29/50 (58.0%)	222.1 ± 158.4
WBC (<i>n</i> /mm ³)	41/52 (78.8%)	36.7 ± 59.0
Protein (mg/dL)	28/52 (53.8%)	129.5 ± 250.8

¹CSF pressure > 160 mm; CSF protein > 50 mg/dL; Cell count > 5/mm³. CSF: Cerebrospinal fluid; WNL: Within normal limit; WBC: White blood cell.

conducted in 51 patients. Leptomeningeal enhancement was noted in 45 cases (82%).

Treatment modalities and outcomes

IT chemotherapy was administered to 36 patients, principally with methotrexate (MTx) alone (61%) or in combination with hydrocortisone/± Ara-C (41%). The median number of IT treatments was 7 (range, 1-18). Seventeen patients (46%) achieved cytological negative conversion (Table 5).

Thirteen patients were treated with whole brain irradiation coupled with IT chemotherapy. Six patients received radiation treatment alone.

Additional systemic chemotherapy was given to 10 patients. Three patients were treated with the orally available 5-fluorouracil (5-FU) drugs - capecitabine, S-1, and tegafur-uracil. Irinotecan/leucovorin/5-FU, 5-FU/cisplatin, and paclitaxel/cisplatin were administered to four, two and one patients, respectively. Seven patients were treated with chemotherapy plus IT chemotherapy. Three patients received chemotherapy alone. The median number of cycles administered was 2 (range, 1-6). Among the treated patients, only one exhibited a detectable response to treatment.

Survival and prognostic factors

Median OS duration from diagnosis of LMC was 6.7 wk (95% CI: 4.3-9.1 wk) (Figure 1). In the univariate analysis of survival duration, hemoglobin, IT chemotherapy, and cytologic negative conversion showed superior

Table 5 Treatment with intrathecal chemotherapy (*n* = 36)

	<i>n</i> (%)
Regimen	
MTx	22 (61.1)
MTx + steroid	4 (11.1)
MTx + Ara-C + steroid	10 (27.8)
Concurrent/sequential	
+ alone	16 (44.4)
+ radiotherapy	13 (36.1)
+ chemotherapy	2 (5.6)
+ chemoradiotherapy	5 (13.9)
No. of cycles	
Median (range)	7 (1-18)
Cytological response	
(-) conversion	17 (47.2)

MTx: Methotrexate; Ara-C: Cytarabine.

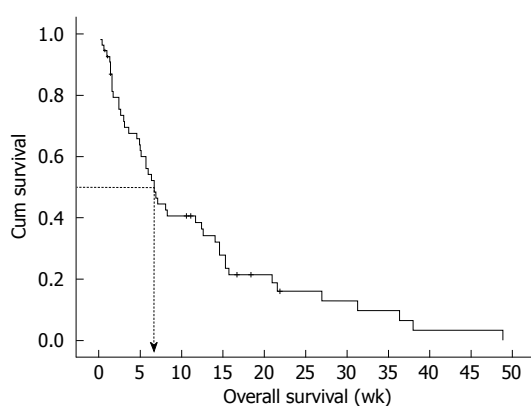


Figure 1 Median overall survival (OS) duration from diagnosis of leptomeningeal carcinomatosis. Median OS was 6.7 wk (95% CI: 4.3-9.1 wk).

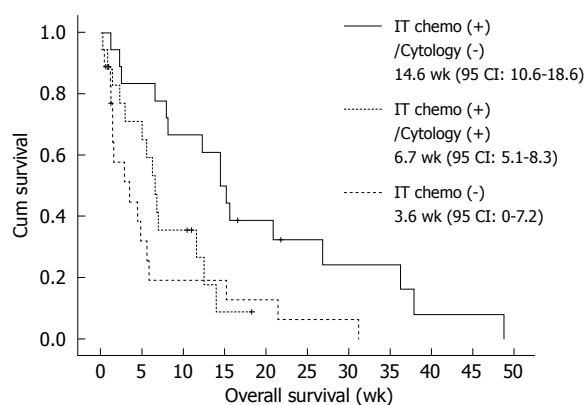


Figure 2 Cytologically negative conversion proved predictive of relatively longer survival duration ($P = 0.030$, RR: 0.415, 95% CI: 0.188-0.918).

survival duration ($P = 0.038$, $P = 0.010$, and $P = 0.002$, respectively). However, in the multivariate analysis, only cytologic negative conversion was predictive of relatively longer survival duration (3.6, 6.7 and 14.6 wk, $P = 0.030$, RR: 0.415, 95% CI: 0.188-0.918) (Table 6, Figure 2).

DISCUSSION

Adenocarcinoma is the predominant histological type in LMC of solid tumors^[6]. Among patients diagnosed with

Table 6 Survival duration and prognostic factors

Factors	Median OS (mo)	Univariate	Multivariate
Gender			
Male	7.1	0.491	-
Female	6.7		
Age (yr)			
> 60	12.4	0.214	-
≤ 60	6.4		
PS (LMC)			
0, 1	11.7	0.975	-
≥ 2	6.4		
Hb (LMC)			
> 11	12.4	0.038	NS
≤ 11	5.7		
MRI enhance			
Negative	5.7	0.316	-
Positive	7.1		
CSF pressure			
> 120	6.4	0.163	NS
≤ 120	8.1		
CSF protein			
> 40	6.0	0.539	-
≤ 40	11.7		
IT chemotherapy			
No	3.6	0.010	NS
Yes	11.7		
Radiotherapy			
No	4.6	0.516	-
Yes	6.9		
Cytologic response			
No	5.1	0.002	0.030
Yes	14.6		(HR: 0.415, 95% CI: 0.188-0.918)

OS: Overall survival; PS (LMC): Performance status scale by Eastern Cooperative Oncology Group at leptomeningeal carcinomatosis; Hb (LMC): Hemoglobin at leptomeningeal carcinomatosis; MRI: Magnetic resonance imaging; CSF: Cerebrospinal fluid; IT: Intrathecal; HR: Hazard ratio; CI: Confidence interval.

LMC, the most frequently encountered solid tumors are breast (12%-34%), lung (14%-29%), and melanoma (17%-25%)^[3]. Unlike Western reports, gastric cancer is the principal etiology of LMC in solid tumors in Korea^[7].

CNS metastasis is a very rare complication of gastric cancer, and occurs in 0.16%-0.69% of gastric cancer patients in general, including Korean reports^[4,8,9]. Although all the included patients demonstrated CSF cytologically confirmed malignancy, the prevalence of LMC in this study was 0.24% in all gastric cancer patients.

Consistent with other studies, the majority of patients had Bormann type III or IV advanced gastric cancer of poorly differentiated or signet-ring cell histopathology, which increased the tendency for distant metastasis and poor prognosis^[4,10,11]. Similar to the results of a previous study, LMC patients presented with an advanced stage and Bormann type III or IV advanced gastric cancer of poorly differentiated or signet-ring cell histopathology.

LMC is an ultimately fatal disease^[12-15]. A minority of patients, usually those with breast cancer or lymphoid malignancies, may achieve disease-free survival of a year or more, however, the median OS for patients with LMC is only 4-6 wk if untreated and 2-4 mo with therapy^[6,12,15]. In our study, the median survival duration was just

6.7 wk. Although LMC patients tend to have a poor performance status, approximately two-thirds of patients who receive IT chemotherapy and 47.2% patients who responded to therapy achieved longer survival duration. The independent prognostic factor for survival was cytologic negative conversion by IT chemotherapy. Although the small sample size and inherent selection bias of the retrospective design of this study makes any conclusions regarding the outcomes of treatment somewhat difficult, the findings of our study indicate that cytologic negative conversion by IT chemotherapy may improve survival by arresting neurologic progression in selected patients.

MTx remains the most frequently utilized drug for IT administration, despite its limited success and serious complications^[16,17]. Combination IT chemotherapy with MTx, arabinoside and hydrocortisone has been reported to be more effective than MTx alone in solid tumor LMC^[18]. However, approximately 10% of gastric cancer patients were enrolled in this study, and the efficacy of arabinoside against gastric cancer is questionable.

Craniospinal irradiation may be a one-treatment modality. However, the additional or sequential role of radiation has been controversial^[19]. In our study, additional effects of radiotherapy were not observed. As response to radiation is associated with the sensitivity or resistance of primary tumors and malignant cells circulating in the CSF space, radiation is occasionally not feasible for palliative treatment.

Systemic chemotherapy was also administered to a limited number of patients who had better performance status^[6,15,20]. In our study, patients who were treated with systemic chemotherapy showed the best median OS duration (21.6 wk, 95% CI: 3.2-40 wk). However, all the treatments were administered sequentially after IT chemotherapy and the patients responded to treatment.

COMMENTS

Background

Leptomeningeal carcinomatosis (LMC) occurs in approximately 5% of cancer patients. The most common cancers involving the leptomeninges are breast and lung cancer. However, gastric adenocarcinoma has been infrequently reported in conjunction with LMC. This retrospective analysis was performed to identify the clinical features and outcomes of infrequently reported LMC of gastric cancer.

Research frontiers

This is the first large scale study on gastric LMC. LMC is a rare component in the clinical manifestation of gastric cancer. LMC usually presents at a relatively young age, at an advanced stage, and is of a poorly differentiated pathologic type.

Innovations and breakthroughs

Although gastric LMC had a fatal clinical course, the findings of our study suggest that cytologic negative conversion by intrathecal (IT) chemotherapy may improve survival by arresting neurologic progression in selected patients.

Applications

These results could provide basic clinical data on gastric LMC for physicians and demonstrate the role of IT chemotherapy.

Terminology

Leptomeninges (literally thin meninges) is a term referring to the pia mater and arachnoid mater. LMC is a condition in which a tumor diffusely spreads to the leptomeninges. Intrathecal chemotherapy involves anticancer drugs injected

into the fluid-filled space between the thin layers of tissue that cover the brain and spinal cord.

Peer review

The results are interesting and suggest that cytologic negative conversion by IT chemotherapy may improve survival by arresting neurologic progression in selected patients.

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Mitigation of indomethacin-induced gastric mucosal lesions by a potent specific type V phosphodiesterase inhibitor

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Received: June 15, 2009 Revised: August 4, 2009

Accepted: August 11, 2009

Published online: October 28, 2009

Abstract

AIM: To investigate the gastroprotective effect of vardenafil against indomethacin-induced gastric damage.

METHODS: Forty-eight female Wistar albino rats were randomly divided into 6 groups. Group 1 received saline only. Group 2 (indomethacin) received indomethacin. Rats in group 3 and 4 were pretreated with different doses of famotidine. Group 5 and 6 were pretreated with different doses of vardenafil. Rats in groups 3 to 6 received 25 mg/kg indomethacin 30 min after pretreatment. The animals were sacrificed 6 h later and their stomachs were opened. Gastric lesions were counted and measured. The stomach of each animal was divided in two parts for histopathological examinations and nitric oxide (NO) and malondialdehyde (MDA) assays, respectively.

RESULTS: There were no gastric mucosal lesion in the saline group but all rats in the indomethacin group had gastric mucosal ulcerations (ulcer count; 6.25 ± 3.49 , and mean ulcer area; 21.00 ± 12.35). Ulcer counts were

diminished with famotidine 5 mg/kg (4.12 ± 2.47 , $P > 0.05$), 20 mg/kg (2.37 ± 4.43 , $P < 0.05$), vardenafil 2 mg/kg (4.37 ± 3.06), and vardenafil 10 mg/kg (1.25 ± 1.38 , $P < 0.05$) compared to the indomethacin group. Gastric mucosal lesion areas were diminished with famotidine 5 mg/kg (8.62 ± 2.97 , $P < 0.001$), famotidine 20 mg/kg (0.94 ± 2.06 , $P < 0.001$), vardenafil 2 mg/kg (6.62 ± 5.87 , $P < 0.001$), and vardenafil 10 mg/kg (0.75 ± 0.88 , $P < 0.001$) compared to the indomethacin group. MDA levels were significantly higher in indomethacin group (28.48 ± 14.51), compared to the famotidine 5 mg/kg (6.21 ± 1.88 , $P < 0.05$), famotidine 20 mg/kg (5.88 ± 1.60 , $P < 0.05$), vardenafil 2 mg/kg (15.87 ± 3.93 , $P < 0.05$), and vardenafil 10 mg/kg (10.97 ± 4.50 , $P < 0.05$). NO concentration in gastric tissues of the famotidine groups were significantly increased ($P < 0.05$), but the NO increases in the vardenafil groups were not statistically significant. Histopathology revealed diminished gastric damage for pretreatment groups compared to the indomethacin group ($P < 0.05$).

CONCLUSION: Vardenafil affords a significant dose-dependent protection against indomethacin induced gastric mucosal lesions in rats.

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Key words: Vardenafil; Gastric ulceration; Indomethacin; Gastroprotection; Rats

Peer reviewer: Atsushi Mizoguchi, Assistant Professor, Experimental Pathology, Massachusetts General Hospital, Simches 8234, 185 Cambridge Street, Boston, MA 02114, United States

Karakaya K, Hanci V, Bektas S, Can M, Ucan HB, Emre AU, Tascilar O, Ozkocak Turan I, Comert M, Irkorucu O, Karadeniz Cakmak G. Mitigation of indomethacin-induced gastric mucosal lesions by a potent specific type V phosphodiesterase inhibitor. *World J Gastroenterol* 2009; 15(40): 5091-5096 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5091.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5091>

INTRODUCTION

Gastric mucosal damage is a common disorder of the gastrointestinal system. The pathogenesis of gastric ulcers is based on complex interactions between aggressive and protective factors. Nonsteroidal anti-inflammatory drugs

(NSAID) are known to be aggressive agents for gastric ulcer development. People of advancing age need many drugs, including NSAIDs, for the treatment of pain and inflammation due to rheumatological disturbances.

Vardenafil is a phosphodiesterase (PDE) V inhibitor that has been used for the treatment of erectile dysfunction^[1] and, more recently, for pulmonary hypertension^[2,3]. Recent laboratory studies demonstrated successful effects of PDE V inhibitors on cardioprotection after ischemia reperfusion injury^[4,5], as well as in ischemic injury of other organs, such as the colon, liver, and brain^[6,7]. Deibert *et al*^[8] revealed that vardenafil had increased portal flow and lowered portal pressure in patients with cirrhotic livers.

Diminished mucosal circulation has been blamed as one of the etiological factors in gastric ulcer formation. Like prostaglandins, the L-Arginine/nitric oxide (NO) pathway is a major protective system in gastric mucosa^[9] via relaxation of the arterial smooth muscles. Inhibition of nitric oxide synthase aggravates the injury in animal models of gastric ulcers^[10].

In this study, we have studied the effects of vardenafil on the acute gastric injury caused by administration of indomethacin.

MATERIALS AND METHODS

The study was approved by the Zonguldak Karaelmas University (ZKU) Animal Experiments Local Ethic Committee. The study was carried out on 48 female Wistar albino rats weighing 200-250 g, obtained from the Experimental Animal Laboratory of Medical Faculty of ZKU. The rats were kept under standard conditions (temperature; 22-24°C, and 12:12 h light/dark). The experimental procedures were carried out in accordance with international guidelines for the use and care of laboratory animals. All animals were fed with pellet food produced especially for experimental animals. Water was available ad libitum. All experiments were performed at the same time of the day to avoid diurnal variations of putative regulators of gastric functions.

Famotidine and vardenafil were dissolved in distilled water. All drug solutions and suspensions were freshly prepared. Gastric ulcers were inflicted by oral administration of indomethacin 16-18 h after starvation.

Animals were randomly divided into six groups. In Group 1 (n:8) rats received only 8 mL/kg of saline by gavage. Rats in Group 2 (n:8) received 25 mg/kg indomethacin in a volume of 8 mL/kg of saline. The rats in group 3 and 4 were pretreated with famotidine, 5 mg/kg and 20 mg/kg, respectively. Rats in groups 5 and 6 were pretreated with 2 mg/kg and 10 mg/kg vardenafil, respectively. After 30 min, 25 mg/kg indomethacin in a volume of 8 mL/kg of saline were administered by gavage. Six hours after oral administration of indomethacin, all rat groups were anesthetized with an intramuscular injection of 100 mg/kg Ketamine (Ketalar®, Parke Davis-Eczacıbaşı, Istanbul, Turkey). A midline abdominal incision was then performed. All rat groups were sacrificed *via* cardiac puncture, and their stomachs rapidly removed, opened by an incision along the lesser curvature, and rinsed in ice-cold distilled water^[11]. Gastric tissues were pinned out on a

wax platform. Macroscopic damage to the gastric mucosa was assessed. Hemorrhagic and ulcerative lesions were counted and their lengths were measured on square millimeter paper. Gastric mucosal lesions were expressed as the sum of the lengths (mm) of all lesions for each stomach, which was used as the ulcer index (UI)^[12,13]. Gastric lesions were judged by two independent researchers who were blinded to the protocol. The average score of the two independent observers were taken into account, and the sum of the total scores was divided by the number of animals to obtain the mean UI for each group.

The stomach of each animal was divided into two parts. One part of the stomach was excised, immersed in saline, and immediately stored at -40°C for measurement of NO and MDA levels.

Gastric tissues were homogenized in ten volumes of 150 mmol/L ice-cold KCl using a glass teflon homogenizer (Ultra Turrax IKA T18 Basic) after cutting the tissues into small pieces with scissors (for 2 min at 5000 r/min). The homogenate was then centrifuged at 5000 × *g* for 15 min. The supernatant was used for analysis. High-performance liquid chromatographic analysis was performed using a Shimadzu HPLC system (Kyoto, Japan) with an MDA kit (Immundiagnostik AG, Bensheim, Germany). Spectrophotometric measurements of total antioxidant status (TAS) (Randox, Crumlin, UK) was performed using a Shimadzu UV-1601 (Kyoto, Japan) spectrophotometer. Serum nitric oxide levels (nitrite + nitrate) were measured, after conversion of nitrate to nitrite by copperized cadmium granules, by a spectrophotometer at 545 nm (Shimadzu, Tokyo, Japan)^[14]. Protein assays were measured on an Advia 2400 chemistry analyzer (Bayer Healthcare Instruments, Tarrytown, NY, USA). Results were expressed as μmol/g protein for NO and nmol/g protein for MDA.

The other part of the stomach was fixed in 10% neutral formalin, embedded in paraffin, and cut into 5 μm sections. The sections were stained with hematoxylin eosin (HE) and examined under the light microscope by a blinded pathologist for histological changes.

The results obtained from vardenafil groups were evaluated by comparing them with those of sham, indomethacin, and famotidine groups.

Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows. All data are expressed as the mean ± SD. Mann-Whitney *U* and χ^2 tests were used for statistical analysis of data among all groups. *P* < 0.05 was considered as statistically significant.

RESULTS

Macroscopic analysis showed that there was no gastric mucosal lesion in the sham group. There were gastric mucosal lesions in all stomachs of the indomethacin 25 mg/kg treated group. The mean ulcer area was 21.00 ± 12.35 in the indomethacin group. Gastric mucosal damage was significantly reduced by famotidine 20 mg/kg and vardenafil 10 mg/kg pretreatments. In both groups, the mean count of ulceration and the mean count of

Table 1 Macroscopic evaluation of gastric mucosa

Groups	n	Weight (gr)	GML count	GML area mm ²
Sham	8	223.50 ± 18.40 (200-249)	0	0
Indomethacin	8	225.00 ± 13.77 (201-248)	6.25 ± 3.49 (1-11) ^a	21.00 ± 12.35 (1-36) ^b
Famotidine 5 (F5)	8	223.25 ± 13.13 (203-236)	4.12 ± 2.47 (2-8)	8.62 ± 2.97 (3-12)
Famotidine 20 (F20)	8	224.75 ± 14.78 (200-247)	2.37 ± 4.43 (0-13)	0.94 ± 2.06 (0-6) ^d
Vardenafil 2 (V2)	8	221.12 ± 13.27 (204-242)	4.37 ± 3.06 (0-8)	6.62 ± 5.87 (0-16) ^e
Vardenafil 10 (V10)	8	224.12 ± 15.16 (202-250)	1.25 ± 1.38 (0-3) ^{c,g}	0.75 ± 0.88 (0-2) ^{d,g}

GML: Gastric mucosal lesion. The values are presented as mean ± SD, (min-max). ^a*P* < 0.05, ^b*P* < 0.001 *vs* all other groups; ^c*P* < 0.05, ^d*P* < 0.001 *vs* group F5; ^e*P* < 0.05 *vs* group F20; ^g*P* < 0.05 *vs* group V2.

Table 2 MDA and NO levels in gastric tissues in each group

Group	n	MDA (nmol/g protein)	NO (μmol/g protein)
Sham	8	9.4 ± 4.47 (3.6-14.3)	35.67 ± 5.69 (30.21-46.63)
Indomethacin	8	28.48 ± 14.51 (7.1-45) ^a	27.20 ± 6.25 (20.0-38.78)
Famotidine 5 (F5)	8	6.21 ± 1.88 (3.4-9.5)	40.82 ± 9.42 (31.08-59.92) ^c
Famotidine20 (F20)	8	5.88 ± 1.60 (3.3-7.7)	51.22 ± 15.27 (34.24-77.50) ^c
Vardenafil 2 (V2)	8	15.87 ± 3.93 (11.6-23.8) ^e	31.01 ± 20.27 (21-55.15) ^e
Vardenafil 10 (V10)	8	10.97 ± 4.50 (5.4-19.9) ^{g,h}	33.55 ± 9.29 (22.16-48.51) ^g

The values are presented as mean ± SD, (min-max). ^a*P* < 0.05 *vs* the other group; ^c*P* < 0.05 *vs* indomethacin group; ^e*P* < 0.05 *vs* famotidine groups (F5 and F20); ^g*P* < 0.05 *vs* group F20; ^h*P* < 0.01 *vs* group F20.

ulcer area were significantly lower than the control group. Gastric mucosal lesion areas were significantly diminished in rats pretreated with famotidine 5 mg/kg and vardenafil 2 mg/kg, when compared with the control group, but this did not reach statistical significance in respect to ulcer count. The mean ulcer area in the vardenafil 2 mg/kg group and vardenafil 10 mg/kg group were 6.62 ± 5.87 and 0.75 ± 0.88 , respectively. Macroscopic evaluation of gastric mucosal lesion counts and gastric mucosal lesion areas for each group are presented in Table 1. In damaged stomachs, mucosal lesions of various sizes and forms were dispersed to all stomach surfaces. Those lesions consisted of elongated bands parallel to the long axis of the stomach. Lesions of the gastric mucosa in each group are shown in Figure 1. Tissue MDA and NO levels are presented in Table 2 for each group.

On histopathological examination, erosion, inflammation, hemorrhage, and necrosis were abundant in the indomethacin group. Those lesions were encountered with increasing frequency in the Famotidine 20 mg/kg, vardenafil 10 mg/kg, famotidine 5 mg/kg, and vardenafil 2 mg/kg groups. There were statistically significant (*P* < 0.05) differences between the indomethacin group and pretreatment groups. Famotidine 20 mg/kg pretreatment had the most efficient protective effect against indomethacin-induced gastric mucosal lesions. Minimal hemorrhage, minimal focal necrosis, and superficial erosions were observed in 25% of the rats given 2 mg/kg vardenafil. A high dose (10 mg/kg) of vardenafil had a potent protective effect against indomethacin-induced gastric mucosal lesions, but vardenafil in low doses (2 mg/kg) did protect the gastric mucosa against the harmful effects of indomethacin, similarly famotidine 5 mg/kg did. Microscopic views of the normal and damaged gastric mucosa are shown in Figure 2. In our

study, vardenafil has gastroprotective effects against indomethacin-induced gastric mucosal damage. The potency of this effect is stronger in high doses than low doses.

DISCUSSION

Despite the great progress in the treatment protocols, peptic ulcers are still a major ongoing health problem. The gastric barrier protects the mucosa against damage of its deeper structures by noxious substances. Mucosal microcirculation of the stomach has an important role in gastric mucosal protection^[15]. Prostaglandins and NO are the main factors that regulate gastric blood flow. NSAIDs cause gastric mucosal damage by inhibiting endogenous prostaglandins due to inhibition of COX-1 and COX-2^[16,17]. Prospective data from the Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS) states that 13 of every 100 patients with rheumatoid arthritis treated with NSAID for one year suffer from serious gastrointestinal complications related to the NSAIDs^[18]. Indomethacin administration increases aggressive factors but decreases protective factors^[19,20].

There is no doubt on the protective effect of H₂ blockers. The gastroprotective effects of H₂ blockers are significantly greater when given in high doses than in low doses. The results in our experiment, either in low or in high doses of H₂ blockers, were in accordance with the literature. Widespread use of H₂ blockers could not prevent peptic ulcer related disorders. Therefore, the search for new alternatives with novel mechanisms of action is ongoing.

PDE type-5 inhibitors, which were developed as cardioprotective drugs, are commonly used in the treatment of erectile dysfunction. Sildenafil citrate

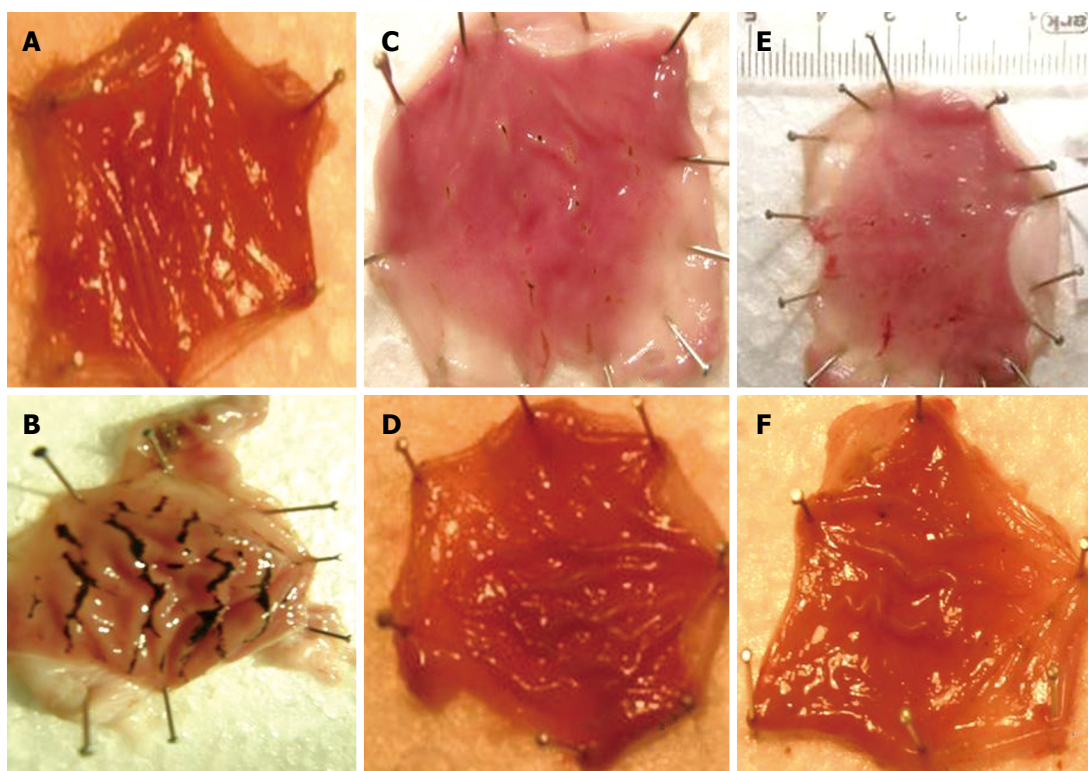


Figure 1 Gross appearance of the opened stomachs in the experimental groups. A: Appearance of normal mucosa of the stomach (Saline); B: Severe mucosal injury (Indomethacin); C: Diminished mucosal injury (Group F5); D: Gastric mucosa without any lesion (Group F20); E: Partially protected gastric mucosa against the harmful effect of indomethacin (Group V2); F: Lesion free gastric mucosa (Group V10).

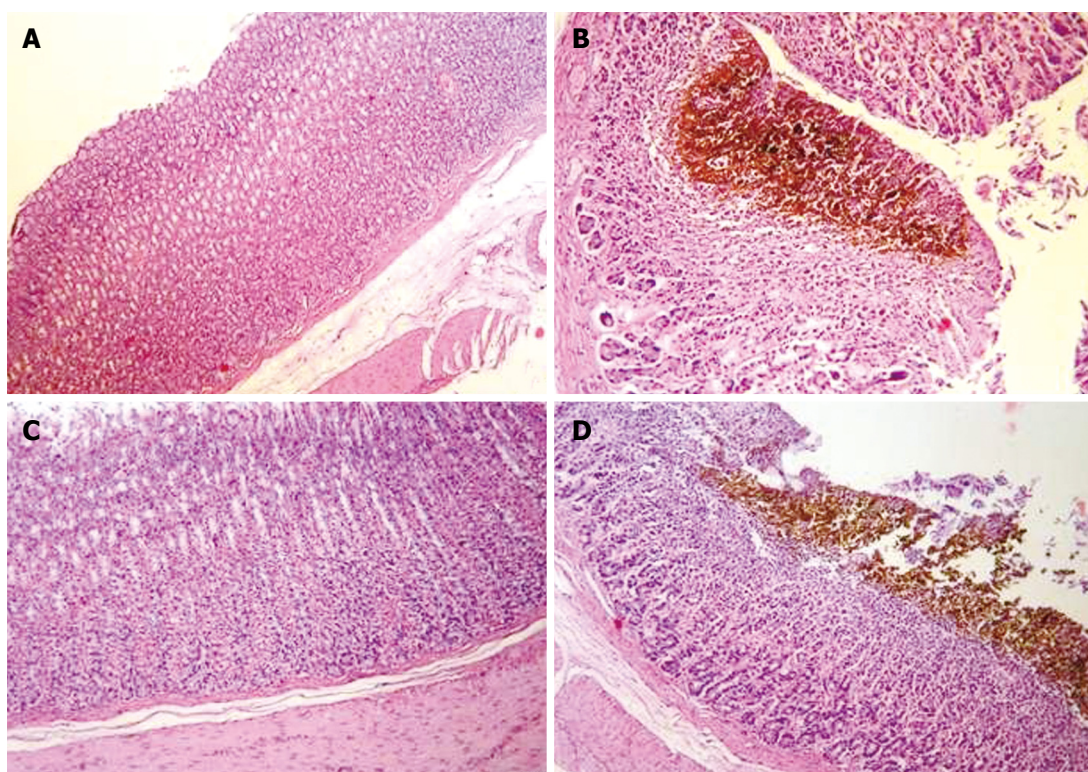


Figure 2 Normal rat gastric mucosa in the saline group and Group V10 (A and C, HE; × 100); gastric mucosal hemorrhage and necrosis in indomethacin group and Group V2 (B and D, HE; × 200, × 100) are shown.

has shown gastroprotective effects in experimental studies^[21-23], and its gastroprotective effect was dose-

dependent. Vardenafil is more potent than sildenafil. The gastroprotective effect of vardenafil has not yet been

studied. In our study, the antiulcer activity of vardenafil was investigated against indomethacin-induced gastric mucosal damage. Vardenafil decreased indomethacin-induced gastric mucosal lesions significantly at high doses (10 mg/kg). Macroscopically, vardenafil at a dose of 2 mg/kg has a protective effect on the gastric mucosa similar to famotidine at 5 mg/kg.

Macroscopic evaluations of gastric tissues revealed that vardenafil given in 2 mg/kg protects gastric mucosa better than famotidine at a dose of 5 mg/kg. Vardenafil has clinically important gastroprotective effects at high doses (10 mg/kg). Thus, the gastroprotective effect of vardenafil was dose dependent. In the stomach tissue of rats given indomethacin, the level of the lipid peroxidation product, MDA, increased significantly compared to the sham operated group. Tissues exposed to oxidative stress include large amounts of toxic oxygen radicals, which induce lipid peroxidation leading to MDA formation^[24,25]. The lowest MDA values were detected in the famotidine groups. The mean MDA values in the vardenafil groups were similar to the sham group (Table 2). Thus, vardenafil pretreatment has inhibited MDA production in indomethacin treated rats.

Possible mechanisms of gastroprotection of PDE V inhibitors are increased production of tissue NO^[23,26-28] or increased tissue cGMP level without modifying NO content^[22,25,29,30]. The NO levels are slightly elevated in vardenafil pretreated rats in our study; however, the level of NO in either of vardenafil groups did not surpass the level of NO determined in the sham group. Some studies revealed gastroprotective effects of some agents without significant alterations in NO or MDA levels^[31]. Determination of tissue cGMP level was not included in our study design. This is a short armcoming of our study design. PDE V inhibitors might prevent indomethacin-induced gastric mucosal damage in either mechanism.

In conclusion, vardenafil reduced gastric mucosal damage significantly at a high dose. Patients treated with PDE type-5 inhibitors might benefit from the additional gastroprotective advantages of these drugs, especially in high doses.

COMMENTS

Background

Peptic ulcer is a common disorder of the gastrointestinal system. The increase in non-steroid anti inflammatory drug (NSAID) ingestion in the treatment of inflammation, fever, and pain is one of the major etiological factors for peptic ulcers. Despite the many drug treatment protocols used to date, peptic ulcer still remains a major public health problem.

Research frontiers

Vardenafil has being used in the treatment of functional impotence; however, its effects on gastric mucosa have not yet been investigated. The research's aim was to determine its effectiveness in gastroprotection against NSAID-induced gastric lesions in comparison with famotidine.

Innovations and breakthroughs

Multiple agents have been used to prevent NSAID-induced peptic ulcer. This work is the first experimental study that show the beneficial effects of Vardenafil (a phosphodiesterase type V inhibitor) on NSAID-induced gastric ulcer. The gastroprotective effect of vardenafil against NSAID-induced peptic ulcer is dose dependent.

Applications

The study results suggest that vardenafil might be used as a potential therapeutic drug to prevent NSAID-induced gastric ulcer formation.

Peer review

This work might provide the first experimental data that directly shows the beneficial effect of a phosphodiesterase V inhibitor on gastric ulcers. In this manuscript, Karakaya et al report that administration of a phosphodiesterase V inhibitor (Vardenafil), dose-dependently suppresses indomethacin-induced gastric ulcers in rats. For comparison purposes, famotidine was used. There is only limited information suggesting the potential beneficial effect of vardenafil on ulcer healing, the data presented would be considered to provide attractive clinical information, although a clear mechanistic insight is not provided.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Ma WH



Efficacy and safety of rabeprazole in non-steroidal anti-inflammatory drug-induced ulcer in Japan

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Received: March 31, 2009 Revised: September 27, 2009

Accepted: October 4, 2009

Published online: October 28, 2009

Key words: Rabeprazole; Non-steroidal anti-inflammatory drug; Non-steroidal anti-inflammatory drug-induced ulcer; Endoscopic findings; Continuous non-steroidal anti-inflammatory drug administration

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

Mizokami Y. Efficacy and safety of rabeprazole in non-steroidal anti-inflammatory drug-induced ulcer in Japan. *World J Gastroenterol* 2009; 15(40): 5097-5102 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5097.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5097>

Abstract

AIM: To investigate the efficacy and safety of rabeprazole under continuous non-steroidal anti-inflammatory drug (NSAID) administration for NSAID-induced ulcer in Japan.

METHODS: Subjects comprised patients undergoing NSAID treatment in whom upper gastrointestinal endoscopy revealed an ulcerous lesion (open ulcer) with diameter ≥ 3 mm, who required continuous NSAID treatment. Endoscopies were performed at the start of treatment, during the treatment period, and at the conclusion (or discontinuation) of treatment. Findings were evaluated as size (maximum diameter) and stage based on the Sakita-Miwa classification. An ulcer was regarded as cured when the "white coating" was seen to have disappeared under endoscopy. As criteria for evaluating safety, all medically untoward symptoms and signs (adverse events, laboratory abnormalities, accidental symptoms, *etc.*) occurring after the start of rabeprazole treatment were handled as adverse events.

RESULTS: Endoscopic cure rate in 38 patients in the efficacy analysis (endoscopic evaluation) was 71.1% (27/38). Among those 38 patients, 35 had gastric ulcer with a cure rate of 71.4% (25/35), and 3 had duodenal ulcer with a cure rate of 66.7% (2/3). Three adverse drug reactions were reported from 64 patients in the safety analysis (interstitial pneumonia, low white blood cell count and pruritus); thus, the incidence rate for adverse drug reactions was 4.7% (3/64).

CONCLUSION: The treatment efficacy of rabeprazole for NSAID-induced ulcer under continuous NSAID administration was confirmed.

INTRODUCTION

In clinical practice, non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for arthralgia and rheumatoid arthritis (RA)^[1,2]. NSAIDs exert potent anti-inflammatory and analgesic effects but are clinically problematic in that gastric mucosal injury can be induced as an adverse reaction^[3,4]. For example, up to 25% of patients using NSAIDs develop peptic ulcer^[5,6]. A United States study reported that the number of people taking NSAIDs has reached 13 million per year, with approximately 100 000 requiring hospital treatment for upper gastrointestinal injury and 17 000 reported deaths annually^[7]. The medical cost exceeds \$4 billion per year in the USA^[8].

In Japan, the Japan Rheumatism Foundation has reported the results of an epidemiological survey of the incidence of NSAID-induced gastric mucosal lesions in arthritis patients^[9]. According to that report, of the 1008 patients with arthritis who took NSAIDs for ≥ 3 mo, upper gastrointestinal tract lesions were observed in 62.2%, including 15.5% with gastric ulcer and 1.9% with duodenal ulcer. This suggests that NSAID-induced gastric ulcers occur at a higher rate than the incidence of gastric ulcer found by physical examination (2.2%-4.1%). Moreover, more than 40% of patients were asymptomatic despite the presence of a lesion. In Japan, which faces an increasingly aging society, chronic diseases requiring long-term treatment with NSAIDs (e.g. RA, low back pain and arthralgia) are expected to increase. Devising methods for coping with the expected associated increases in gastric mucosal lesions will thus become increasingly important.

Preparations like proton pump inhibitors (PPIs) and misoprostol are known for curing NSAID-induced gastrointestinal mucosal damage^[10-13]. However, misoprostol often induces some adverse reactions like diarrhea^[14].

Given this background, the “Evidence-Based Guidelines for Gastric Ulcer” were issued in 2003 (2nd edition in 2007) proposing policies for the treatment and prevention of NSAID-induced ulcers^[15]. The guidelines recommend the following therapies: (1) if possible, discontinuation of NSAIDs and initiation of conventional ulcer treatment^[16]; and (2) if NSAID treatment cannot be discontinued, initiation of treatment with a PPI or prostaglandin preparation. However, the evidence (clinical results) put forth in the guidelines was based entirely on foreign reports, with no evidence of use from Japanese studies^[15].

Rabeprazole, a newer PPI, provides reliable control of gastric acid secretion, with more potent antisecretory activity than that of other PPIs such as omeprazole and lansoprazole, a more rapid rise in intragastric pH, and less effect of CYP2C19 on its metabolism^[17-20].

Based on our belief in the importance of investigating the efficacy and safety of the PPI rabeprazole on NSAID-induced ulcers in Japanese individuals, we conducted this survey of 38 medical departments (primarily departments of gastroenterology and internal medicine) at 38 facilities nationwide. This survey was conducted between 1 August 2004 and 31 January 2006, in accordance with good post-marketing surveillance practice.

MATERIALS AND METHODS

Subjects

Subjects included in this survey were patients undergoing NSAID treatment in whom upper gastrointestinal endoscopy revealed an ulcerous lesion (open ulcer) with diameter ≥ 3 mm, who required continuous NSAID treatment. Patients taking low-dose aspirin to prevent clot/thrombus formation were excluded as subjects. Patients meeting any of the following criteria were also excluded: (1) history of hypersensitivity to rabeprazole components; (2) exposed blood vessels in the ulcer base; or (3) judged by an investigator as unsuitable for participation in this survey.

Methods

A central registration system was adopted. The registration form was mailed within 1 wk of the registration date. Rabeprazole were to be administered in accordance with the following dose: once daily oral administration of 10 mg of rabeprazole sodium, which can be increased to once daily oral administration of 20 mg, depending on the symptoms (Standard dosage for adults): Standard treatment duration is 8 wk in the case of gastric or anastomotic ulcer, and 6 wk in the case of duodenal ulcer. The Japanese standard dosages of the three most used NSAIDs in this study are as follows; diclofenac sodium, 25-100 mg/d; loxoprofen, 60-180 mg/d; lornoxicam, 12-18 mg/d.

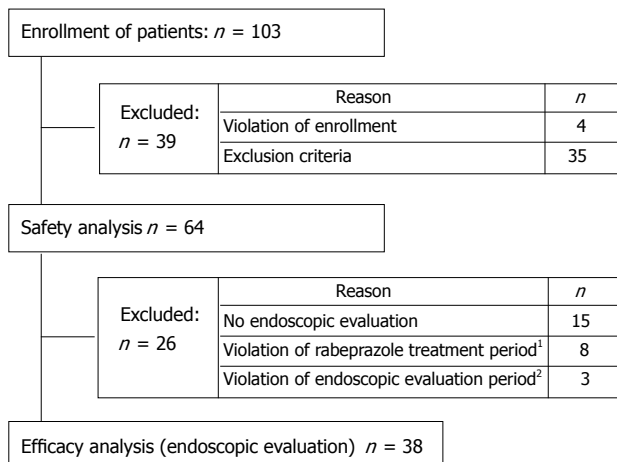


Figure 1 Patient disposition. ¹Patients treated for longer than the allowed treatment period. The allowed treatment period was defined in the protocol as 8 wk for patients with gastric ulcer and 6 wk for patients with duodenal ulcer, + 2 wk for each (i.e. 10 wk for gastric ulcer and 8 wk for duodenal ulcer); ²Patients for whom endoscopic evaluation was performed prior to 31 d before the start of rabeprazole treatment or more than 31 d after the conclusion of rabeprazole treatment.

Evaluation

Endoscopic findings: Endoscopies were performed at the start of treatment, during the treatment period, and at the conclusion (or discontinuation) of treatment. Findings were evaluated as size (maximum diameter) and stage based on the Sakita-Miwa classification. When multiple ulcers existed in a single subject, only the largest one was recorded. An ulcer was regarded as cured when the “white coating” was seen to have disappeared under endoscopy.

Adverse events: As criteria for evaluating safety, all medically untoward symptoms and signs (adverse events, laboratory abnormalities, accidental symptoms, etc.) occurring after the start of rabeprazole treatment were handled as adverse events.

RESULTS

Patient disposition

One hundred three patients were enrolled in the present study. Due to violation of enrollment and patients meeting exclusion criteria like no concomitant use of NSAIDs, 38 patients were put in the efficacy analysis and 64 patients in the safety analysis (Figure 1).

Efficacy

Demographic and baseline characteristics: Demographic and baseline characteristics of the 38 patients in the efficacy analysis (endoscopic evaluation) are shown in Table 1. Well-used NSAIDs by patients were diclofenac sodium in 36.8% (14/38), loxoprofen 36.8% in (14/38) and lornoxicam in 13.2% (5/38). The main reasons for use of NSAIDs observed were RA in 39.5% (15/38) and low back pain in 36.8% (14/38).

Among 25 subjects undergoing examination for *Helicobacter pylori* (*H. pylori*) infection, 64.0% (16/25) were positive, and 36.0% (9/25) were negative.

Table 1 Demographic & baseline characteristics [Efficacy analysis (endoscopic evaluation: $n = 38$)]

Item		Subjects	
		<i>n</i>	%
Age at baseline (yr)	20-39	0	0.0
	40-64	16	42.1
	≥ 65	22	57.9
Sex	Male	14	36.8
	Female	24	63.2
Diagnosis	Gastric ulcer	33	86.8
	Duodenal ulcer	3	7.9
	Gastric/duodenal ulcer	2	5.3
Ulcer history	Initial occurrence	15	39.5
	Reoccurrence	13	34.2
	Unknown	10	26.3
Prior history	No	28	73.7
	Yes	10	26.3
History of allergies	No	31	81.6
	Yes	5	13.2
	Unknown	2	5.3
Type of NSAID (duplicates included)	Diclofenac sodium	14	36.8
	Loxoprofen sodium	14	36.8
	Other	15	39.5
Anti-ulcer treatment before rabeprazole treatment (within 1 mo)	No	12	31.6
	Yes	26	68.4
Concomitant medication (not including NSAIDs)	No	5	13.2
	Yes	33	86.8
Endoscopic findings before start of treatment (ulcer size)	3 ≤ size < 10 mm	21	55.3
	10 ≤ size < 20 mm	13	34.2
	≥ 20 mm	4	10.5
Rabeprazole treatment duration	≤ 56 d	20	52.6
	> 56 d	18	47.4
Rabeprazole dosage	10 mg	24	63.2
	20 mg	12	31.6
	10 mg→20 mg	1	2.6
	20 mg→10 mg	1	2.6

Table 2 Endoscopic cure rate

	10 mg group	20 mg group	Modified dose group ²	Total
Gastric ulcer ¹	71.4% (15/21)	75.0% (9/12)	50.0% (1/2)	71.4% (25/35)
Duodenal ulcer	66.7% (2/3)	-	-	66.7% (2/3)
Total	70.8% (17/24)	75.0% (9/12)	50.0% (1/2)	71.1% (27/38)

¹The 2 patients with “gastric/duodenal ulcer” were included in tabulations for gastric ulcer; ²Modified dose group consisted of 1 patient changing from 10 mg to 20 mg and 1 patient changing from 20 mg to 10 mg.

Endoscopic cure rate: Endoscopic cure rate for the 38 patients in the efficacy analysis (endoscopic evaluation) was 71.1% (27/38) (Table 2). According to diagnosis, the cure rate was 71.4% (25/35) for gastric ulcer and 66.7% (2/3) for duodenal ulcer. In addition, the cure rate was lower in the gastric antrum (55.6%; 10/18) than in the gastric corpus (91.7%; 11/12).

Safety

Demographic and baseline characteristics: Demo-

Table 3 Demographic & baseline characteristics (Safety analysis: $n = 64$)

Item		Subjects	
		<i>n</i>	%
Age at baseline (yr)	20-39	1	1.6
	40-64	34	53.1
	≥ 65	29	45.3
Sex	Male	27	42.2
	Female	37	57.8
Diagnosis	Gastric ulcer	57	89.1
	Duodenal ulcer	4	6.3
	Gastric/duodenal ulcer	2	3.1
	Anastomotic ulcer	1	1.6
Ulcer history	Initial occurrence	29	45.3
	Reoccurrence	20	31.3
	Unknown	15	23.4
Prior history	No	47	73.4
	Yes	17	26.6
History of allergies	No	55	85.9
	Yes	6	9.4
	Unknown	3	4.7
Type of NSAID (duplicates included)	Diclofenac sodium	34	53.1
	Loxoprofen sodium	20	31.3
	Other	27	42.2
Anti-ulcer treatment before rabeprazole treatment (within 1 mo)	No	19	29.7
	Yes	45	70.3
Concomitant medication (not including NSAIDs)	No	7	10.9
	Yes	57	89.1
Endoscopic findings before start of treatment (ulcer size)	3 ≤ size < 10 mm	31	48.4
	10 ≤ size < 20 mm	25	39.1
	≥ 20 mm	5	7.8
Rabeprazole treatment duration	≤ 56 d	3	4.7
	> 56 d	27	42.2
(gastric ulcer, gastric/duodenal ulcer, duodenal ulcer, anastomotic ulcer)	10 mg	39	60.9
	20 mg	19	29.7
	10 mg→20 mg	1	1.6
	20 mg→10 mg	5	7.8

graphic and baseline characteristics of 64 patients in the safety analysis are shown in Table 3. Well-used NSAIDs by patients were diclofenac sodium in 53.1% (34/64), loxoprofen in 31.3% (20/64) and lornoxicam in 10.9% (7/64). The main reasons for using NSAIDs were RA in 40.6% (26/64), low back pain in 28.1% (18/64) and osteoarthritis in 15.6% (10/64). Among 42 subjects undergoing examination for *H. pylori* infection, 59.5% (25/42) were positive, and 40.5% (17/42) were negative.

Incidence of adverse drug reactions: Among the 64 patients in the safety analysis, 3 adverse drug reactions were observed in 3 patients. The incidence rate for adverse drug reactions was thus 4.7% (3/64). Adverse reactions comprised 1 case of “interstitial pneumonia” (serious; outcome: recovering) (1.6%); 1 case of “low white blood cell count” (non-serious; outcome: recovered) (1.6%); and 1 case of “pruritus” (non-serious; outcome: recovered) (1.6%).

DISCUSSION

Endoscopic cure rate was 71.1% (27/38). By disease, the endoscopic cure rate in gastric ulcer patients after 8 wk of treatment was 71.4% (25/35) and the endoscopic cure rate in duodenal ulcer patients after 6 wk of treatment was 66.7% (2/3). In other clinical studies of rabeprazole, reported cure rates have been 93.5% (72/77 patients)^[21] and 96.4% (27/28) for gastric ulcer, and 100% (23/23) for duodenal ulcer^[22]. Endoscopic cure rate in this survey, at 71.1%, did not reach the general cure rates obtained for chronic gastric and duodenal ulcers. These results strongly suggest that continuous administration of NSAIDs may act to delay ulcer healing.

Conversely, in studies similar to this study relating to the healing of gastric and duodenal ulcers in the presence of NSAID treatment, according to the results of Shiokawa *et al*^[23] in the first such study in Japan, cure rates for gastric and duodenal ulcer were 70% (35/50) and 83.3% (5/6), respectively, when a prostaglandin was used at 800 µg/d. With regard to the healing effect of PPI use, only results from foreign studies are available^[24-27]. Hawkey *et al*^[26] conducted a comparative examination of the results of studies on the effects of 20 mg and 40 mg of omeprazole, and 800 µg of misoprostol. After 8 wk of administration, the gastric ulcer cure rate was 87.2% (102/117) with 20 mg of omeprazole and 79.5% (105/132) with 40 mg. The cure rate in the group administered 20 mg of omeprazole was significantly higher than the 72.8% (91/125 patients) in the control group administered 800 µg of misoprostol. In a study of lansoprazole, Agrawal *et al*^[27] compared the results of 15 mg and 30 mg of lansoprazole versus 300 mg of ranitidine hydrochloride. Gastric ulcer cure rates after 8 wk of treatment were 68.6% (81/118) with 15 mg lansoprazole and 72.6% (85/117) with 30 mg. Cure rates in both lansoprazole groups were significantly higher than the 53.0% (61/115 patients) obtained using ranitidine hydrochloride. The present survey found no significant difference between rabeprazole doses with respect to cure rates for NSAID-induced ulcer, with a cure rate of 70.8% (17/24) using a dose of 10 mg and 75.0% (9/12) at 20 mg. The cure rates observed here did not differ significantly from those obtained using the other PPIs described above.

Conversely, how *H pylori* infection affects healing of NSAID-induced ulcers remains controversial and is an important topic that should be considered in future studies, and a recently published guideline addressed this point^[28]. This survey investigated the dependency of cure rate on *H pylori* infection, but no significant difference was observed. The cure rate in *H pylori*-positive patients was 81.3% (13/16), compared to 77.8% (7/9) in *H pylori*-negative patients.

Symptoms and signs, and the conditions of occurrence of these presentations, are commonly considered to differ between NSAID-induced ulcer patients and patients with ordinary ulcers. Saigenji *et al* reported that the pretreatment incidence of epigastralgia was 100% (72/72 patients) in patients with gastric ulcer^[21]. However, the

Table 4 Clinical characteristics of NSAIDs-induced ulcer (safety analysis: *n* = 64)

Site of ulcer	<i>n</i>	%	Ulcer form	<i>n</i>	%	No. of ulcers	<i>n</i>	%
Ulcer condition								
Corpus	19	29.7	Round	27	42.2	Single	42	65.6
Angle	7	10.9	Elliptical	23	35.9	Multiple	21	32.8
Antrum	31	48.4	Irregular	11	17.2			
Bulb	4	6.3	Other	2	3.1			
Efferent loop	1	1.6						

pretreatment incidence rate for epigastralgia was 70.2% (33/41) in the present survey, which was comparatively lower than the incidence observed with ordinary gastric ulcer. In general, incidences for symptoms and signs are lower with NSAID-induced ulcers^[29], and the present results support this.

Data on the site, shape and number of NSAID-induced ulcers were also tabulated in this survey. For the 90 patients with NSAID-induced ulcer (not including the 9 patients in the dataset of patients analyzed who did not display NSAID-induced ulcer), the original ulcer site was most frequent at the gastric antrum (43.3%) and the most common shapes were round (35.6%) and elliptical (37.8%), although irregularly shaped ulcers were also observed (23.3%). In terms of the number of ulcers, multiple ulcers were present in 33.3% (30/90) of cases. The clinical characteristics of NSAID-induced ulcers that were confirmed in the 64 patients in the safety analysis were also tabulated. The gastric antrum was the site of the ulcer in 48.4% (31/64) of patients; the shape was round in 42.2% (27/64) and elliptical in 35.9% (23/64) while 32.8% (21/64) of cases displayed multiple ulcers (Table 4).

NSAID-induced ulcer in gastric antrum, the most common in the present study, seemed to be harder to cure compared to NSAID-induced ulcers in the gastric corpus or gastric angle, as reported by Mizokami *et al*^[30].

Measures for responding to NSAID-induced lesions found in normal medical examinations will become increasingly important as society continues to age. This survey, conducted in accordance with the guidelines, proposes one direction for treatment.

ACKNOWLEDGMENTS

This survey was conducted in 38 medical facilities nationwide under the direction of the late Professor Shinichi Ota. We wish to express sincere gratitude to all the participating physicians as follows: Hiroyuki Hisai, Date Red Cross Hospital; Toshimi Chiba, Iwate Medical University Hospital; Harufumi Oizumi, Oizumi Gastrointestinal and Medical Clinic; Yuji Mizokami, Tokyo Medical University Kasumigaura Hospital; Hideharu Miyabayashi, National Hospital Organization Matsumoto National Hospital; Naoshi Nakamura, Marunouchi Hospital; Akihiko Suzuki, Japanese Red Cross Azumino Hospital; Yoshihiro Matsukawa, Nihon University Itabashi Hospital; Kazuo Kashiwazaki, Kyosai Tachikawa Hospital; Norihiko Tono-

zuka, Akishima Hospital; Toru Segawa, National Hospital Organization, Murayama Medical Center; Toshifumi Kita, Fujimura Hospital; Koji Yakabi, Saitama Medical Center, Saitama Medical University; Shinichi Ota, Saitama Medical University Hospital; Yoshihiro Matsukawa, Kamifukuoka Kyodo Clinic; Akira Tokunaga, Nippon Medical School, Musashikosugi Hospital; Masakazu Tomiyama, Yokohama Minami Kyosai Hospital; Naoto Mitsugi, Yokohama City University Medical Center; Tetsuya Mine, Tokai University Hospital; Yoshifumi Yokoyama, Higashi Municipal Hospital, City of Nagoya; Hiroaki Iwase, National Hospital Organization Nagoya Medical Center; Masahiro Mitake, Suzuki-Mitake Clinic; Shin Yamamoto, Kansai Medical University Takii Hospital; Junichi Yamao, Nara Medical University Hospital; Nobuo Aoyama, Kobe University Hospital; Norimasa Yoshida, University Hospital, Kyoto Prefectural University of Medicine; Atsushi Sugahara, Kawasaki Hospital, Kawasaki Medical School; Hironao Komatsu, JA Hiroshima Koseiren Hiroshima General Hospital; Tomohiko Shimatani, Shinji Tanaka, Hiroshima University Hospital; Seizo Yamana, Higashi-Hiroshima Memorial Hospital; Hitoshi Hasegawa, Ehime University Hospital; Takahiro Horie, Tokushima Prefectural Miyoshi Hospital; Hiroaki Kubo, Fukuoka Prefectural Kaho Hospital; Tetsuya Ishida, Oita Red Cross Hospital; Aoi Yoshiwa, Oita University Hospital; Masahiro Yoshinaga, National Hospital Organization Beppu Medical Center; Shinji Nasu, Yamaga Hospital; Shinsuke Nagai, Kagoshima Red Cross Hospital.

COMMENTS

Background

A non-steroidal anti-inflammatory drug (NSAID) is widely prescribed for patients with rheumatoid arthritis, low back pain, etc. However, NSAID damages gastrointestinal mucosa and induces peptic ulcers. Use of a proton pump inhibitor (PPI) is recommended to cure those peptic ulcers.

Research frontiers

This multicenter study all over Japan revealed the safety profile and efficacy of rabeprazole, a type of PPI, for NSAID-induced ulcer.

Innovations and breakthroughs

This is one of the studies to investigate the safety profile and efficacy of rabeprazole for NSAID-induced ulcer.

Applications

Safety and efficacy of rabeprazole was comparable to other types of PPIs, which means more diversity of treatment options for patients suffering from NSAID-induced ulcer, and this is important since the number of patients using NSAIDs is expected to increase in the aging society in Japan.

Peer review

The manuscript by Mizokami provides the results of studies on the efficacy and safety of rabeprazole for ulcerous lesions in patients undergoing NSAID treatment. This is a reasonable study and the data are well presented.

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S- Editor Tian L L- Editor O'Neill M E- Editor Ma WH



Unexplained liver laceration after metastasis radiofrequency ablation

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Author contributions: Uña E, Trueba J and Montes JM contributed in the literature review; Trueba J assisted in reporting of the case; Montes JM contributed in revising the manuscript; Uña E drafted and edited the manuscript and contributed in its final revision and its approval.

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Received: July 30, 2009

Revised: August 26, 2009

Accepted: September 2, 2009

Published online: October 28, 2009

Abstract

Many studies have established the role of radiofrequency (RF) ablation as a minimally invasive treatment for liver metastases. Although relatively safe, several complications have been reported with the increased use of RF ablation. We describe here a case of unexplained liver laceration after a RF procedure. A woman who presented a solitary metachronous liver metastasis underwent RF ablation treatment for this lesion. Six hours later the patient displayed fatigue and pallor. Emergency blood tests showed a haemoglobin level of < 7 g/dL and markedly elevated transaminase levels. A computed tomography examination revealed two areas of liver laceration with haematoma, one of them following the path of the needle and the other leading away from the first. Following a blood transfusion, the patient was haemodynamically stable and completely recovered 24 h later. The patient remained in bed for 1 wk. No surgical intervention was required, and she was discharged 1 wk later.

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Key words: Colon cancer; Liver haemorrhage; Liver laceration; Liver metastases; Radiofrequency ablation

Peer reviewer: Dr. Yogesh K Chawla, Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Uña E, Trueba J, Montes JM. Unexplained liver laceration after

metastasis radiofrequency ablation. *World J Gastroenterol* 2009; 15(40): 5103-5105 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5103.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5103>

INTRODUCTION

Many studies have demonstrated the benefits of radiofrequency (RF) ablation as a minimally invasive treatment for hepatic metastases from colon carcinoma^[1-3]. Good outcomes with low morbidity and mortality have been identified. Therefore, the use of RF ablation is increasing in the field of oncology^[4,5]. Although a wide spectrum of complications has been described, all of these complications occur at low frequencies. Haemorrhage is the most common complication, mainly related to mechanical injury of the blood vessels in patients with cirrhosis, and it is probably exacerbated by a coagulation deficit and the rich blood supply of the tumour^[6].

We present here a case of multiple liver haematomas occurring as a secondary response to RF ablation of a solitary metastasis.

CASE REPORT

A 72-year-old woman underwent RF ablation for the treatment of a metachronous solitary liver metastasis from rectal adenocarcinoma, which had been treated 18 mo before with preoperative chemoradiotherapy and anterior resection. The lesion was located in segment VIII.

The procedure went well and the patient was initially haemodynamically stable. One hour later her condition suddenly worsened. She was clinically dizzy and had fatigue and pallor. We detected hypotension and tachycardia. Emergency blood tests showed a haemoglobin level of < 7 g/dL and markedly elevated transaminase levels. A computed tomography examination revealed an accumulation of intraperitoneal fluid in the pelvis and two liver haematomas. The images showed two liver lacerations, one following the path of the needle (Figure 1A-C) and another, unexplained laceration leading away from the first (Figure 2). The patient had no serious coughing or hiccupping after the RF treatment, or any other complications that might have caused increased abdominal pressure and tumour rupture.

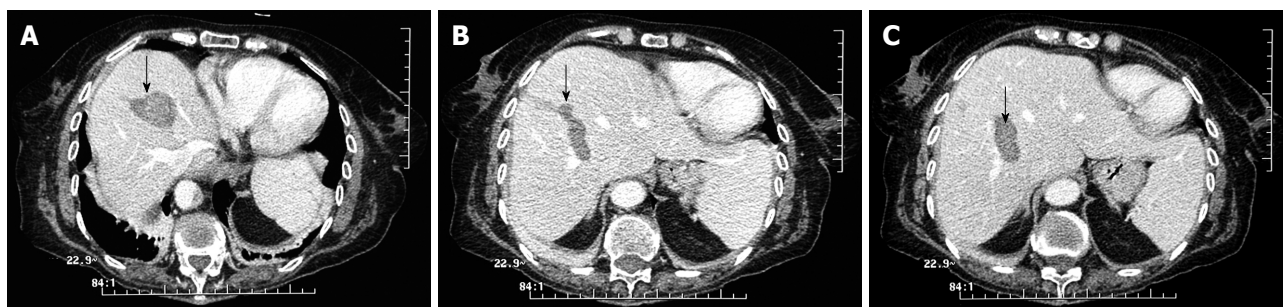


Figure 1 Secondary lacerations resulting from a radiofrequency ablation procedure to treat a liver metastasis from colorectal adenocarcinoma. A: Image of liver laceration grade III in a central location (arrow); B: Image showing the first laceration following the path of the RF needle (arrow); C: Image of the first laceration in a lower liver tomography cut (arrow).



Figure 2 The second and unexplained liver laceration located in segments V-VI (arrow).

Subsequently, the patient received a blood transfusion and close monitoring. She was haemodynamically stable after the second blood transfusion and completely recovered 24 h later. The patient remained in bed for 1 wk. No surgical intervention was required.

DISCUSSION

RF ablation is a minimally invasive treatment of hepatic metastases from colon carcinoma, and can achieve good outcomes with low morbidity and mortality rates. Therefore, the use of RF ablation is increasing in the field of oncology^[1,2]. Although some studies which included large numbers of patients have found low rates of complications after RF ablation of hepatic tumors (ranging from 2.4% to 8.9%), the rate of intraperitoneal haemorrhage is low (0.46%-1.6%) but relevant because this technique is increasingly used with few selection criteria for patients^[6]. The etiology of this potentially grave complication is variable.

The reported reasons for haemorrhage are usually related to mechanical injuries to the liver blood vessels and occur most often in patients with cirrhosis, and are probably due to a coagulation deficit and the rich blood supply of the tumour^[4,6]. Other cases have been attributed to serious coughing or hiccups after the RF treatment^[7]. These complications might cause increased abdominal pressure and tumour rupture, particularly if the tumour location is near the capsule^[6].

Liver laceration has rarely been described as a cause of haemorrhage. Although this latter complication is very infrequent, it has been reported, and has been associated with inappropriate electrode positioning or mechanical injury of the soft liver during the procedure^[8]. Such mechanical injury can be induced by coughing or position changes, causing increasing abdominal pressure, and possibly displacing the electrode slightly^[8].

In our case the procedure went well, without incident, and the patient did not present any of the complications described above, such as coughing or hiccups. The tumour was not located near the capsule but at a depth within segment VIII.

We suggest that these two lacerations might have resulted from direct mechanical injury due to penetration by the electrode into liver tissues that were soft and damaged as a result of prior treatment with chemotherapy. The second laceration may have occurred due to difficulty in positioning the electrode to access such a profound lesion, causing liver disruption away from the first laceration.

Most haemorrhages require blood transfusions or surgical intervention^[6]. In our case the patient recovered spontaneously after blood transfusion and bed rest without the necessity of surgical intervention.

It is absolutely essential to minimise complications associated with RF ablation treatments, and to correctly deal with complications which do arise^[6]. The close observation of patients after RF ablation treatments, and early intervention to minimise the damage and severity of complications, are warranted.

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S- Editor Li LF L- Editor Webster JR E- Editor Zheng XM



CASE REPORT

Gastric choriocarcinoma admixed with an α -fetoprotein-producing adenocarcinoma and separated adenocarcinoma

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Received: May 27, 2009 Revised: September 10, 2009

Accepted: September 17, 2009

Published online: October 28, 2009

producing adenocarcinoma and separated adenocarcinoma. *World J Gastroenterol* 2009; 15(40): 5106-5108 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5106.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5106>

INTRODUCTION

Choriocarcinoma is a rapidly growing, highly invasive, widely metastasizing neoplasm that derives from either trophoblastic or totipotential germ cells. It often arises in the uterus in association with pregnancy, and its most common extragonadal sites are the mediastinum, ovary and testis^[1].

Primary gastric choriocarcinoma (PGC) is a rare neoplasm that constitutes less than 1% of all gastric cancers^[2]. It was first described by Davidsohn in 1905 and more than 140 cases have been reported in the international medical literature. Several studies have indicated that the pathogenesis of PGC can be explained by the dedifferentiation of malignant adenocarcinoma tissue to the level of the embryonal ectoderm, and the retention of an ability to form trophoblasts^[3]. However, the clinicopathological and prognostic factors of PGC are unreliable because of the small numbers of PGC cases reported.

α -fetoprotein (AFP) is a fetal serum protein that is produced by fetal liver and yolk sac cells, and some fetal gastrointestinal cells. AFP levels decrease gradually after birth and reach adult levels at 8-12 mo. However, AFP levels are elevated in patients with hepatocellular carcinoma and in those with non-cancerous liver disease associated with liver regeneration. AFP-producing tumors have been reported in several different organs, and commonly in the stomach^[4-8]. The proportion of gastric cancers that secrete AFP has been reported to be 2.7%-8.0%^[9]. AFP-producing gastric carcinomas have high proliferative activity and are associated with low levels of apoptosis and rich neovascularization. They are divided into three subtypes: hepatoid, yolk sac tumor-like, and fetal gastrointestinal^[10]. Here, we report the case of a 70-year-old man with gastric choriocarcinoma admixed with AFP-producing adenocarcinoma.

Abstract

We report a case of gastric choriocarcinoma admixed with an α -fetoprotein (AFP)-producing adenocarcinoma. A 70-year-old man was hospitalized for gastric cancer that was detected during screening by esophagogastroduodenoscopy (EGD). Initial laboratory data showed the increased serum level of AFP and EGD revealed a 5-cm ulcerofungating mass in the greater curvature of the gastric antrum. The patient underwent radical subtotal gastrectomy with D2 lymph node dissection and Billroth II gastrojejunostomy. Histopathological evaluation confirmed double primary gastric cancer: gastric choriocarcinoma admixed with an AFP-producing adenocarcinoma and separated adenocarcinoma. At 2 wk postoperatively, his human chorionic gonadotropin and AFP levels had reduced and six cycles of adjuvant chemotherapy were initiated. No recurrence or distant metastasis was observed at 4 years postoperatively.

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Key words: α -fetoproteins; Adenocarcinoma; Choriocarcinoma; Stomach neoplasms

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Eom BW, Jung SY, Yoon H, Kook MC, Ryu KW, Lee JH, Kim YW. Gastric choriocarcinoma admixed with an α -fetoprotein-

CASE REPORT

A 70-year-old man was referred to our hospital for



Figure 1 Gross pathology showed a 5.8 cm × 3.2 cm ulcerofungating mass in the antrum, with extensive hemorrhage and light gray fibrosis, and a 2.5 cm × 2.0 cm ulcerative lesion.

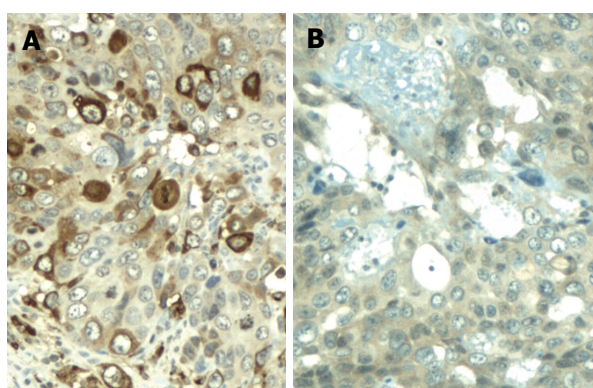


Figure 3 Immunohistochemical staining showed positive immunoreactivity for β-human chorionic gonadotropin (A) and focal positivity for α-fetoprotein (B).

gastric cancer that was detected during screening by esophagogastroduodenoscopy (EGD). No significant medical history was identified, except dysuria caused by bladder contraction. Initial laboratory data showed a serum level of AFP of 32.3 ng/mL (normal range: 0-15 ng/mL), but no other abnormality, which included other tumor markers, such as, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). EGD revealed a 5-cm ulcerofungating mass that was comprised of three septate ulcers in the greater curvature of the gastric antrum. A pathological examination of endoscopic biopsy tissues confirmed the presence of moderately differentiated tubular adenocarcinoma. Subsequent abdominopelvic computed tomography visualized a gastric mass with deep ulceration in the gastric antrum with perigastric lymph node enlargement. No metastatic lesions were observed in the liver, lung or peritoneum, and chest radiography showed no significant findings.

Radical subtotal gastrectomy with D2 lymph node dissection and Billroth II gastrojejunostomy were performed. Grossly, the resected specimen contained double lesions: the first was a 5.8 cm × 3.2 cm ulcerofungating mass in the antrum, with extensive hemorrhage and light gray fibrosis; and the second was a nearby 2.5 cm × 2.0 cm ulcerative lesion (Figure 1).

Microscopically, massive numbers of pleomorphic,

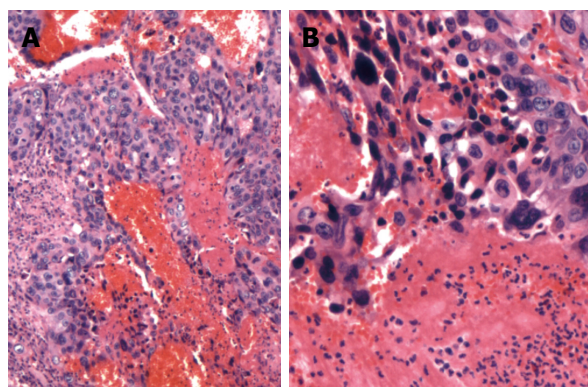


Figure 2 Microscopically, massive numbers of pleomorphic, bizarre tumor cells with hemorrhage were revealed: syncytiotrophoblasts and cytotrophoblasts (HE, A × 40, B × 100).

bizarre tumor cells with hemorrhage (syncytiotrophoblasts and cytotrophoblasts) were observed in the first lesion. Hematoxylin and eosin (HE)-stained tissues revealed a bubbly purple cytoplasm and giant nuclei at a magnification of 40 × (Figure 2A) and 100 × (Figure 2B). The tumor involved the proper muscle layer (T2a) and metastasis was found in four of 56 regional lymph nodes (N1). Immunohistochemical staining showed positive immunoreactivity for β-human chorionic gonadotropin (HCG) (Figure 3A) and focal positivity for AFP (Figure 3B). These findings confirmed the presence of gastric choriocarcinoma that contained small foci of an AFP-producing adenocarcinoma. The second lesion was moderately differentiated tubular adenocarcinoma, which extended to the submucosal layer (T1b). It was close to, but distinct from the first lesion, which was negative by immunohistochemical staining for β-HCG and AFP.

The patient had an uneventful postoperative course and was discharged on postoperative day 9. Two weeks later, his HCG level was 176 mIU/mL (normal range: 0-10 mIU/mL) and his AFP level was 10.0 ng/mL. Six cycles of adjuvant chemotherapy with capecitabine (Xeloda; Hoffmann-La Roche Inc., Nutley, NJ, USA) was started at 2500 mg/m² per day for 14 d/cycle. After two cycles, his β-HCG level had declined to < 3 mIU/mL, and has since remained at this level. No recurrence or distant metastasis had occurred at his 4-year postoperative follow-up.

DISCUSSION

Several theories have been proposed to explain the histopathogenesis of primary choriocarcinoma of the stomach. These hypotheses include an origin from a gonadal angle displaced in the abdomen^[1], a histological resemblance to choriocarcinoma^[1], an origin from an underlying gastric teratoma^[12], and the dedifferentiation or opisthoplatia of carcinoma cells to the level of the embryonal ectoderm with an ability to form trophoblasts^[13]. Of these, the dedifferentiation theory, proposed by Pick in 1926, is accepted most widely. Based on the observation that many cases of PGC have been found with coexistent adenocarcinoma, Pick proposed that choriocarcinoma could arise by overgrowth

and elimination of the original adenocarcinoma^[4]. Furthermore, the findings of a comparative genomic hybridization and fluorescence *in situ* hybridization study by Liu *et al*^[14] support this theory; they have concluded that PGC possesses genetic characteristics of adenocarcinoma and gestational choriocarcinoma.

Applying the dedifferentiation theory proposed by Pick to the present case, we are led to consider that β -HCG-producing choriocarcinomas is dedifferentiated from AFP-producing adenocarcinomas. However, this is supposition and further studies on the pathogenesis of choriocarcinoma are required.

Of the three subtypes of AFP-producing gastric cancers, the described case was of the fetal gastrointestinal type that appeared to develop as a result of fetal gastrointestinal epithelium recapitulation in a tubular adenocarcinoma. The clinical implications of the three subtypes have not been evaluated, although the best-characterized hepatoid type, which is the most common, is known to have a high malignant potential and to be associated with poor survival^[15].

The prognosis of PGC is poor because of its high metastatic potential, especially to the liver, lung and regional lymph nodes, and most PGC patients succumb within a year of operation. Therefore, the treatment of choice for PGC is controversial, especially palliative gastric resection. However, in several cases, curative resection and adjuvant chemotherapy (such as 5-fluorouracil and cisplatin combination) have been found to promote long-term survival^[1].

Furthermore, survival for AFP-producing gastric cancer is also dismal because of the high risk of liver metastasis^[16]. A high proportion of patients with AFP-producing early gastric cancer that underwent curable resection died from liver metastasis^[17]. In the described case, the patient survived recurrence free without distant metastasis for more than 3 years after surgery and adjuvant chemotherapy.

In conclusion, the described case suggests that gastric choriocarcinoma admixed with an AFP-producing adenocarcinoma has a good prognosis. Curative resection, appropriate chemotherapy, and the absence of synchronous liver metastasis are considered favorable prognostic factors in PGC. Further evaluations of its pathogenesis and of the cause of its good prognosis are necessary.

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S- Editor Li LF L- Editor Kerr C E- Editor Yin DH



A case of intussuscepted Meckel's diverticulum

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Received: August 10, 2009 Revised: September 18, 2009

Accepted: September 25, 2009

Published online: October 28, 2009

Key words: Colonoscopy; Intussusception; Meckel's diverticulum

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INTRODUCTION

Abdominal computed tomography (CT) is currently considered the most sensitive radiological method for confirming intussusception^[1,2]. However, it is quite possible for colonoscopy to be selected as the initial diagnostic method when intussusception presents primarily as hematochezia. Colonoscopy may be useful for confirming the presence of intussusception, localizing the disease, and demonstrating the underlying organic lesion serving as the lead point. Intussusception might be misinterpreted as another lesion, such as a hematoma or polyp, on a colonoscopic examination, especially by an inexperienced endoscopist. Here, we report a colonoscopic feature of an intussuscepted Meckel's diverticulum, presenting with hematochezia, which was initially misinterpreted as a hematoma on colonoscopic examination.

CASE REPORT

A 35-year-old woman presented to the emergency room with acute onset hematochezia that occurred over a weekend. She first noticed the hematochezia accompanied by transient, sharp, severe epigastric pain 6 h earlier. On physical examination, she was a lean young woman with stable vital signs. Her abdomen was soft with no tenderness or muscle guarding. Bowel sounds were hypoactive with a normal pitch. Digital rectal examination showed bright red stools on the glove. Laboratory tests revealed normal peripheral blood cell counts and blood chemistry, except for leukocytosis with a white blood count of $145\,000/\text{mm}^3$. Because she was admitted at a weekend, endoscopy was performed by an inexperienced endoscopist who had trained at

Abstract

We report colonoscopic features of an intussuscepted Meckel's diverticulum, presenting with hematochezia. A 35-year-old woman presented to the emergency room with acute onset, transient, sharp, severe epigastric pain that began 6 h earlier. Colonoscopy revealed a reddish, soft, fist-sized polypoid lesion in the terminal ileum. The lesion was misinterpreted as a hematoma by an inexperienced endoscopist. The patient began to complain of intermittent, severe periumbilical pain following the colonoscopic examination. Subsequent computed tomography showed an enteric intussusception. An exploratory laparotomy revealed an intussuscepted Meckel's diverticulum, with transmural infarction. Colonoscopy was of little use in assessing the intussusception. However, colonoscopic examination may be performed initially, especially in an intussuscepted Meckel's diverticulum presenting with hematochezia. Endoscopists should note the endoscopic features of an intussuscepted Meckel's diverticulum.



Figure 1 A polypoid lesion in the terminal ileum.



Figure 2 Contrast-enhanced abdominal CT showed massive invagination of long segmental ileal loops.

our endoscopic center for 2 years. Gastroscopy was negative. Colonoscopy revealed blood and mucus throughout the colon. At colonoscopy, a reddish, soft, fist-sized polypoid was found 30 cm into the terminal ileum (Figure 1). Forcing forceps against the lesion depressed the lesion. The endoscopist tried to remove the lesion using various endoscopic accessories, because it was misdiagnosed as a hematoma. The patient began to complain of intermittent, severe periumbilical pain following the colonoscopic examination. Subsequent CT showed a typical inhomogeneous target-shaped soft-tissue mass with a layering effect, suggestive of enteric intussusception (Figure 2). An exploratory laparotomy revealed enteric intussusception, with gangrene. Segmental resection of the small intestine was performed. The pathology revealed an intussuscepted Meckel's diverticulum with transmural infarction along the antimesenteric border (Figure 3). Microscopic examination disclosed an ectopic gastric mucosa and a complete proper muscle layer in the diverticular wall (Figure 4). The patient had an uneventful postoperative course and was discharged on the ninth post-operative day.

DISCUSSION

Adult intussusception constitutes 5% of all cases of intussusception and accounts for only 1%-5% of intestinal obstructions in adults^[3]. Almost 90% of the cases of intussusception in adults are secondary to a pathological

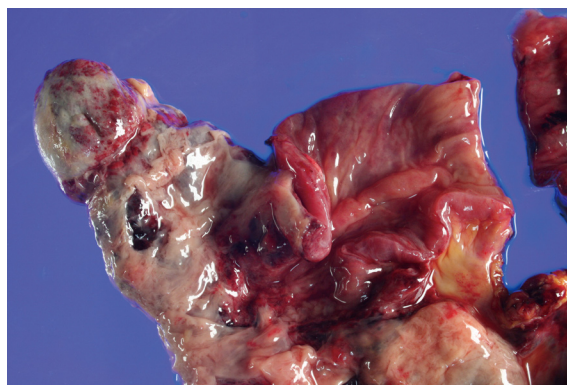


Figure 3 Macroscopically, a diverticular sac was noted along the antimesenteric border. The entire wall of both the diverticulum and intestine was affected by hemorrhagic infarction and the luminal surfaces were covered with a necrotic exudate.

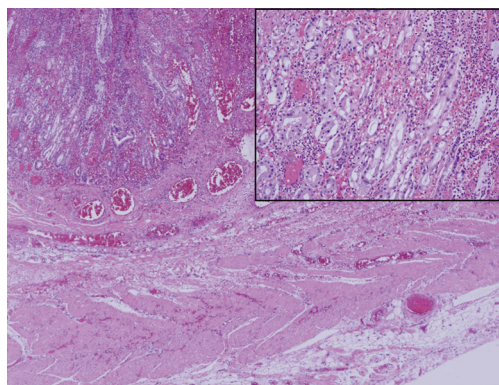


Figure 4 Microscopically, the diverticular sac was totally involved in transmural infarction. Its wall had a continuous proper muscle layer and heterotopic gastric mucosa (Inset). (HE, × 40; inset × 200).

condition that serves as a lead point, such as carcinomas, polyps, Meckel's diverticulum, colonic diverticulum, and strictures or benign tumors, which are usually discovered intraoperatively^[4-6]. A Meckel's diverticulum is a remnant of the omphalomesenteric duct, which is normally obliterated by the 5th-8th wk of gestation. It is seen in 2% of the population. The lifetime risk of complications in patients with a Meckel's diverticulum is only 4%^[7]. Thus, an intussuscepted Meckel's diverticulum is very rare, despite the well-known association of enteric intussusception and Meckel's diverticulum.

Abdominal CT can help to confirm the presence of intussusception and distinguish between lead point and non-lead point intussusception and can potentially eliminate unnecessary surgery^[7]. Colonoscopy is of little use for assessing intussusception. There are few reports on the colonoscopic features of an intussuscepted Meckel's diverticulum^[8,9]. An inexperienced endoscopist might be unfamiliar with these features, which appear to depend on the severity, chronicity, and etiology of the intussusception. A spring-shaped polypoid mass with surface erosion or a large number of blood vessels has been seen at colonoscopy in intussuscepted Meckel's diverticulum without complications^[8,9]. Incidental snare polypectomy might be performed in patients with

chronic intussusception that presents as a polypoid mass on a barium or endoscopic examination^[10]. This poses a high risk of perforation in a background of chronic tissue ischemia and possible necrosis of the intussuscepted bowel segment wall. An intussuscepted Meckel's diverticulum, with gangrene, such as in our case, might be misdiagnosed as a hematoma at colonoscopy, because of the severe hyperemic and edematous mucosal changes. In conclusion, endoscopists should be aware of the endoscopic features of an intussuscepted Meckel's diverticulum, because a colonoscopic examination can be performed initially, especially in an intussuscepted Meckel's diverticulum presenting with hematochezia.

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S- Editor Tian L L- Editor O'Neill M E- Editor Zheng XM



CASE REPORT

Liver transplantation for polycystic liver with massive hepatomegaly: A case report

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Supported by A grant from Jiangsu Health Department of China, No. RC2007058

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Received: July 11, 2009

Revised: September 7, 2009

Accepted: September 14, 2009

Published online: October 28, 2009

Peer reviewer: Dr. Shoji Kubo, Department of Hepato-Biliary-Pancreatic Surgery, Osaka City University Graduate School of Medicine, Osaka, 545-8585, Japan

Jiang WW, Zhang F, Pu LY, Wang XH, Kong LB. Liver transplantation for polycystic liver with massive hepatomegaly: A case report. *World J Gastroenterol* 2009; 15(40): 5112-5113
Available from: URL: <http://www.wjgnet.com/1007-9327/15/5112.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5112>

INTRODUCTION

A previous study has shown that liver or combined liver-kidney transplantation can be a valuable surgical technique for the treatment of polycystic liver disease^[1]. Here, we present the case of a 35-year-old woman with polycystic liver disease, who underwent orthotopic liver transplantation (OLT) on November 11, 2008.

CASE REPORT

In October 2008, a 35-year-old woman with a body weight of 52 kg was admitted to the Liver Transplantation Center, the First Affiliated Hospital of Nanjing Medical University to undergo liver transplantation. This patient had a significant family history of polycystic disease, and her mother had died of dyscrasia that resulted from polycystic liver and kidney diseases. She had normal liver function [alanine aminotransferase (ALT) 35.7 U/L, aspartate aminotransferase (AST) 21.7 U/L, total bilirubin 16.5 μ mol/L, and prothrombin time 13.8 s], and normal renal function (urea 4.33 mmol/L, creatinine 77 μ mol/L, glomerular filtration rate 62.4 mL/min), but had dyspnea, anorexia, abdominal pain, hypertension, increased abdominal girth, mild ascites, and a liver span of 20 cm below the left costal margin and 25 cm below the right costal margin before transplantation. OLT was performed on November 11, 2008, and the whole-size graft was taken from a 51-year-old man who had died of a heart attack. The blood phenotypes of the donor and recipient were type A, RH (+). OLT was performed, but resection of the massive hepatomegaly was very difficult, because the enlarged polycystic liver occupied nearly the whole epigastric zone and mid abdomen (Figure 1A), which resulted in a small surgical space, especially for

Abstract

A previous study has shown that liver or combined liver-kidney transplantation can be a valuable surgical technique for the treatment of polycystic liver disease. Herein, we present the case of a 35-year-old woman with polycystic liver disease, who underwent orthotopic liver transplantation (OLT) on November 11, 2008. The whole-size graft was taken from a deceased donor (a 51-year-old man who died of a heart attack). Resection in a patient with massive hepatomegaly is very difficult. Thus, after intercepting the portal hepatic vein, left hepatectomy was performed, then the vena cava was intercepted, the second and third porta hepatic isolated, and finally, right hepatectomy was performed. OLT was performed successfully. The recipient did well after transplantation. This case suggested that OLT is an effective therapeutic option for polycystic liver disease and left hepatectomy can be performed first during OLT if the liver is over enlarged.

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Key words: Hepatectomy; Liver transplantation; Polycystic liver

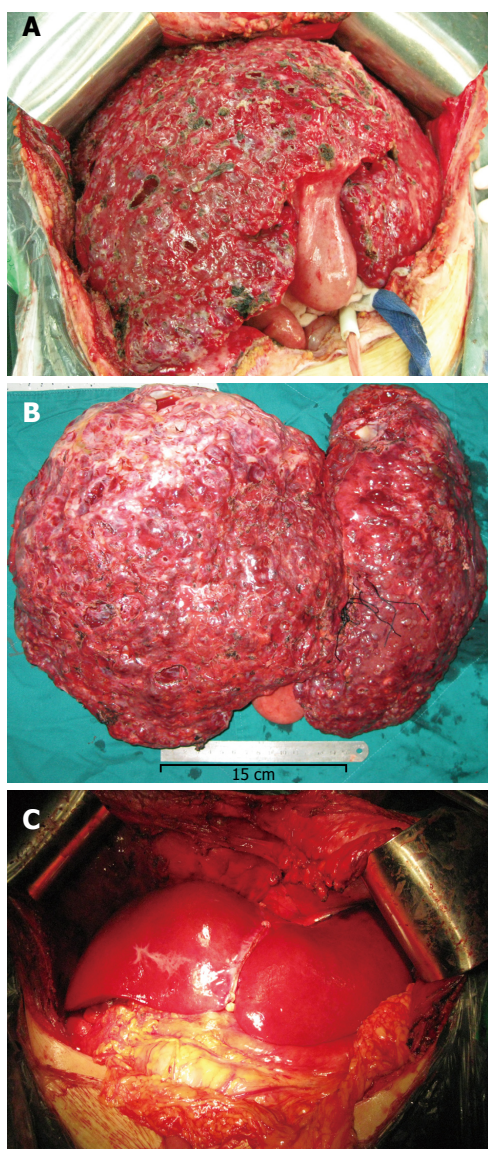


Figure 1 The liver before and after liver transplantation. A: Polycystic liver after cyst fenestration. The liver was still very large; B: The excised polycystic liver contained innumerable cysts; C: After OLT, the donor liver was much smaller than the recipient liver.

manipulation of hepatic blood vessels behind the liver. Thus, after intercepting the portal vein and vena cava, left hepatectomy was performed, followed by isolation of the hepatic veins and short hepatic veins at the posterior of the liver, and finally, right hepatectomy. OLT was then performed successfully (Figure 1C). The excised liver weighed 10.2 kg (approximate 20% of the body weight), which contained innumerable cysts and a large amount of cystic fluid (Figure 1B). The recipient did well after transplantation, and the hospitalization period was 21 d.

DISCUSSION

Polycystic liver disease is a rare, benign disorder^[2].

Symptoms of polycystic liver disease are related mainly to the size of the liver. Patients with massive hepatomegaly may suffer from abdominal pain, vena caval obstruction, hypertension, hemorrhage, cyst infections, dyspnea, increased abdominal girth, and poor quality of life^[3-6]. Cyst fenestration can be used to ameliorate the symptoms of massive hepatomegaly, but symptom relief is only temporary. Previous studies have shown that cyst aspiration, liver or combined liver-kidney transplantation may be a valuable surgical technique for the treatment of polycystic liver disease^[1,7]. The first patient to undergo liver transplantation for polycystic liver-kidney disease was in 1988, however, the patient died intraoperatively from intractable bleeding^[1].

In the present case, resection of massive hepatomegaly was very difficult, because the enlarged polycystic liver occupied nearly the whole epigastric zone and mid abdomen, therefore, the surgical space was very small, especially for manipulation of the hepatic veins and short hepatic veins at the posterior of the liver. During transplantation, cyst fenestration was used, but the volume of the liver could not be reduced significantly. Thus, after intercepting the portal vein and the vena cava, left hepatectomy was performed, followed by isolation of the hepatic veins and short hepatic veins, and finally, right hepatectomy. OLT was performed successfully. Left liver resection may be an effective treatment of choice, if the space is too small for manipulation of liver blood vessels behind the liver.

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LETTERS TO THE EDITOR

Association of hepatitis C virus infection and diabetes

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Received: July 16, 2009 Revised: August 24, 2009

Accepted: August 31, 2009

Published online: October 28, 2009

Abstract

Epidemiologic studies have suggested a relation between hepatitis C virus (HCV) infection and diabetes mellitus. HCV infection is emerging as a metabolic disease, and diabetes mellitus as a risk factor for HCV infection. However, some data on the prevalence of antibodies to HCV in patients with diabetes are conflicting. These seroprevalence data should be interpreted with caution. Some potential bias may occur in those clinic-based studies that target a specific disease group. In this letter we explain some reasons for these conflicting studies.

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Key words: Prevalence; Hepatitis C; Diabetes mellitus

Peer reviewer: Dr. Fumio Imazeki, Department of Medicine and Clinical Oncology, Chiba University, Chiba 260-8670, Japan

Rouabhia S, Malek R, Bounecer H. Association of hepatitis C virus infection and diabetes. *World J Gastroenterol* 2009; 15(40): 5114-5115 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5114.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5114>

TO THE EDITOR

We read with great interest the article of Kaabia and collaborators^[1] and we congratulate them for the originality

of this interesting study in the Maghreb area. In this study, Kaabia and colleagues performed hepatitis C virus (HCV) screening in 1269 diabetic patients and 1315 non-diabetic patients, attending in the same health centers in Sousse, Tunisia. Authors found that the frequency of HCV antibodies was low in diabetic patients and in the control group, with no significant difference between the groups (1.3% *vs* 0.6%, $P = 0.057$).

The results of this study do not match those of several studies performed in numerous areas in the world, which found a higher prevalence of hepatitis C in diabetic patients^[2,3]. Moreover, in Kaabia's study, anti-HCV seroprevalence was significantly higher in type 2 diabetes sub-group than in the control group (1.4% *vs* 0.6%, $P = 0.04$). Anti-HCV seropositivity was detected only in one patient of 121 patients with type 1 diabetes, which was lower than in type 2 diabetes group, but the difference was not statistically significant (0.82% *vs* 1.4%, $P = 0.50$).

Kaabia's study has several deficiencies; their results must be interpreted with precaution. The diabetic patients were more aged than non-diabetic patients, it will be more interesting if the authors have compared the HCV seroprevalence between the two groups with age adjustment. Another bias of selection was introduced in this study by using the prevalence and not the incidence of HCV infection. Moreover, diabetes is an independent co-factor of fibrosis in chronic hepatitis C^[4], and in patients with cirrhosis the survival rate is reduced in case of associated diabetes^[5]. The hepatitis C prevalence is underestimated than in diabetic patients. Classification errors of diabetes could be made in the group of non-diabetics. Indeed the manner in which the diagnosis of diabetes was eliminated in this group was not specified in the article. Starting from the assumption that 50% of diabetics are not diagnosed, we wonder in which group the new cases of screening diabetes were classified? It is also impossible to establish a chronological relation between the diabetes and hepatitis C in this transversal study and it is possible that the infection by virus C precedes the occurring of diabetics. The authors have not compared the risk factors of hepatitis C in infected diabetic patients and in non-infected diabetic patients. Several studies found that diabetic patients infected with HCV had the same frequency in drug-addiction past and in blood transfusion than diabetic patients not infected with HCV. And these factors when they exist are present before the occurring of diabetes^[6].

Our group presented in 2006 in the ALFEDIAM Congress in Paris a study suggesting that the prevalence of hepatitis C is higher in Algerian diabetic patients^[7]. In

this retrospective study, we investigated hepatitis C virus markers in 739 patients attending internal medicine department of university hospital center of Batna (Algeria) from January to December 2005. One hundred and fifty nine patients (73 men and 86 women) with diabetes mellitus diagnosed by conventional criteria^[8] were studied. Their mean age was 60 years. Type 2 diabetes was present in 90% of patients. The control group consisted of 580 non-diabetic patients (229 men and 351 women). Their mean age was 50 years. Anti-HCV serology was determined in both groups using the third-generation micro-particle enzyme immunoassay. Anti-HCV seropositivity was 17.5% in diabetic patients and 8.4% in non-diabetic patients ($P < 0.01$). Despite our small size sample, we found a statistically significant higher prevalence of hepatitis C among diabetic patients. However, after adjustment for age, this difference is statistically significant only in patients aged between 40 and 65 years (22.2% *vs* 9.3%, $P = 0.024$).

Is diabetes mellitus a risk factor for HCV infection; or is this later a risk factor for type 2 diabetes mellitus? That is the question. Further studies are required to elucidate the mechanism of this interesting association.

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ACKNOWLEDGMENTS

Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health
Disparities in Racial/Ethnic Minorities
and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A
Vital Partnership in Cancer Drug
Discovery and Development

February 13-16, 2009
Hong Kong Convention and
Exhibition Centre, Hong Kong, China
19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training
Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic:
Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills
Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on
Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology
(BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest
Group Meeting

April 18-22, 2009
Colorado Convention Center,
Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the
European Association for the Study
of the Liver (EASL)
<http://www.easl.ch/>

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
<http://www.ddw.org>

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent
Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research
(Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World
Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention
Center (BICC), Beijing, China
World Conference on Interventional
Oncology
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward
A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical
Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail,
CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center,
Seattle, Washington, United States
Practical Solutions for Successful
Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
Beijing International Convention
Center (BICC), Beijing, China
19th World Congress of the Interna-
tional Association of Surgeons,
Gastroenterologists and Oncologists
(IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009
Boston Park Plaza Hotel and Towers,
Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and
Marina, San Diego, CA,
United States
Advances in Breast Cancer Research:
Genetics, Biology, and Clinical
Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on
Tumor Microenvironment: Progre-
ssion, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial
Convention Center, Boston, MA,
United States
AACR-NCI-EORTC Molecular
Targets and Cancer Therapeutics

November 21-25, 2009
London, UK
Gastro 2009 UEGW/World Congress
of Gastroenterology
www.gastro2009.org



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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua 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 PMID: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 1274 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]

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Personal author(s)

- 10 **Sherlock S,** Dooley J. Diseases of the liver and biliary 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. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S,** Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious 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/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (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 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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